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Interaction of Minute Virus of Mice with Differentiated Cells: Strain-Dependent Target Cell Specificity Is Mediated by Intracellular Factors

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The prototype strain of minute virus of mice and the immunosuppressive strain are unable to grow lytically in each other's murine host cell type. To characterize these strain-dependent virus-host cell interactions further, we have compared the early events of both productive and restrictive infections. Each virus binds to specific receptors on the surface of both productive and restrictive cell types. Competition experiments show that both viruses recognize the same receptor on each cell type. Penetration and uncoating are presumed to be similar in both productive and restrictive infections, since incoming viral genomes are converted to parental replicative form DNA independent of the final outcome of the virus-host cell interaction. In contrast to the majority of other systems studied to date, these differences in minute virus of mice target cell specificity are not mediated at the cell surface, but by the interaction of a strain-specific viral determinant with intracellular host factors that are expressed in particular cell types as a function of differentiation. These cellular factors catalyze a step in viral replication which occurs after the initiation of viral DNA synthesis, but before the detectable expression of the viral capsid polypeptide genes.

The autonomous parvoviruses are a group of small, icosahedral animal viruses that contain a linear, single-stranded DNA genome (31). The majority of these agents are known to be teratogenic agents, causing a variety of fetal and neonatal abnormalities in both natural and experimental infections by destroying specific proliferating cell populations (10, 15, 17). Early in vitro studies showed that these viruses required a host factor, expressed transiently during the S-phase of the cell cycle, for their own replication (23, 25, 27, 32). In addition, the viruses were found to be incapable of stimulating resting cells to enter the mitotic cycle (27), explaining their dependence upon cell proliferation both in vitro and in vivo (15). Subsequent studies have shown that cell cycling is a necessary, but not sufficient, requirement for parvovirus replication and that the susceptibility of many dividing cell populations depends upon their differentiated state (19, 20, 28). Thus, the expression of particular developmentally regulated host factors also plays a major role in the determination of viral susceptibility. For a number of parvoviruses, different strains of the same serotype have been found to show different pathological effects in vivo (9, 16) and host cell specificities in vitro (4–6, 18, 29), suggesting that an interaction between these host factors and a mutable viral component is necessary for lytic infection (29). Studies of the strain-specific tissue tropism exhibited by members of other virus groups have generally shown that each strain recognizes a different specific cell surface receptor and that this receptor is present on the target cell for that strain, but not on the surface of other potential host cells (7, 8, 21, 22, 26, 34, 35).

In an accompanying paper (29), we have described the different virus-host cell interactions of two closely related strains of minute virus of mice (MVM) (33), which we have called allotropic variants since each replicates in dissimilar differentiated mouse cells. The prototype virus, MVM(p), grows productively in cells of fibroblast origin, whereas the immunosuppressive strain, MVM(i), grows in T lymphocytes and suppresses a number of T cell-mediated functions in vitro (2, 5, 18, 29). The two virus variants are indistinguishable by antiserum neutralization kinetics (29) and have very similar structural proteins and DNA sequence (18), yet each is restricted for lytic growth in the other's host cell type (29). In the restrictive interaction, viral capsid antigens are not synthesized in the great majority of the infected cells, which remain viable and continue to grow (29). The

discrimination between host cell types has been shown to be a stable property of the virus strain, specified by a genetic locus we call the allotropic determinant (29).

In this paper we investigate early events in the productive and restrictive infections of these two allotropic variants, to define the point in the viral life cycle at which this viral determinant interacts with the developmentally regulated host cell factor(s). We show that, in contrast to the majority of viruses, target cell specificity is mediated intracellularly, rather than by differential receptor recognition.

MATERIALS AND METHODS

Cell lines and virus stocks. A9 ouab^{r}11 cells were derived from the original HGPRT$^-$ L cell line A9 by selection for clones resistant to 10^{-3} M ouabain after nitrosoguanidine mutagenesis (29). A9 clone 8E is an MVM(p)-resistant derivative of A9 which does not carry the receptor for MVM(p) on its surface. S49 1TB2 is a thymidine kinase-negative mutant of the T-cell lymphoma line S49, and RPC-5.4 is an immunoglobulin G2a-secreting myeloma line. 324K cells are a simian virus 40-transformed human newborn kidney fibroblast line. The origins of these cell lines and conditions for their culture are described in the accompanying paper (29).

The original cloned stock of the prototype strain MVM(p) has been described elsewhere (27, 29). The immunosuppressive strain, MVM(i), was cloned by terminal dilution in lymphocyte cultures, and both viruses were assayed by plaque titration on NB324K cell monolayers as described in the accompanying paper (29).

Virus production and purification. Unlabeled virus stocks were prepared by low-multiplicity infection of the appropriate host cell, A9 ouab^{r}11 for MVM(p) and S49 1TB2 for MVM(i), as described elsewhere (29, 30). Such infections generate DNA-containing, infectious, "full" virions that band between 1.41 and 1.46 g/ml in cesium chloride, and "empty" capsids, devoid of DNA, that band at 1.32 g/ml (30). Full virions used in infections were separated from empty capsids on glycerol gradients (29), and empty capsids were further purified on cesium chloride gradients.

The empty capsids used in the infectious center competition experiments were subjected to three cycles of cesium gradient purification. The final band was collected from the gradient directly by side puncture of the tube and dialyzed into 50 mM Tris–0.5 mM EDTA pH 8.7 (TE 8.7) (30). This preparation contained less than 1 PFU/10^{10} particles. Full virions labeled to high specific activity with ^{32}P were produced in 324K monolayers, infected at 10 PFU per cell, and incubated for 48 h in monolayer medium containing 20% of the normal phosphate concentration and 100 μCi of carrier-free $^{32}P_i$ per ml. The infected cells were harvested by scraping the monolayer into the medium, pelleting the cells, washing in phosphate-buffered saline (PBS), and freeze-thawing in TE 8.7 for three cycles. After clearing by centrifugation, the virus suspension was digested with RNase (10 μg/ml) for 1 h at 37°C and purified by sedimentation through a glycerol gradient as before.

Purified empty capsids were radioiodinated by the procedure of Bray and Brownlee (3). Purified capsids (75 μg) in 100 μl of 10 mM Tris (pH 7.5) were mixed with 10 μl of chloramine T (5 mg/ml in water) and then added to 5 mCi of sodium [^{125}I]iodide (New England Nuclear Corp.; carrier free, 0.5 to 1 mCi/μl). After 30 min, the reaction was quenched with excess dithiothreitol, and the iodinated empty particles were separated from unreacted iodide by chromatography on Sephadex G-50 in 10 mM Tris–1 mM EDTA (pH 8.7). Before use in binding studies, the iodinated capsids were further purified on sucrose gradients (30).

Binding assay. Binding of virus to cells was carried out as described by Linser et al. (13, 14), with the following changes. The cells for assay were fixed with 0.5% glutaraldehyde (Sigma Chemical Co.; EM grade) in PBS for 30 min at 4°C at a concentration of 10^6 cells per ml. After fixation, these cells were washed in PBS containing 0.1 M glycine and were stored in this buffer at 10^7 cells per ml for up to a week at 0 to 4°C.

Virus and cells were incubated in PBS containing 1.0 mM $CaCl_2$ and 0.5 mM $MgCl_2$ at 4°C for 1 h, filtered on polycarbonate filters (Nuclepore Corp.), and then washed extensively with PBS. The dried filters were counted in a Packard γ counter.

Analysis of viral parental DNA fate after infection. Asynchronous suspension cultures of either A9 ouab^{r}11 or S49 1TB2 were infected with ^{32}P-labeled MVM(i) or MVM(p) at a multiplicity of 0.5 PFU per cell. Samples of infected cells (5×10^5 cells) were collected at 0, 3, 6, 9, and 15 h postinfection by pelleting the cells and washing them in cold PBS. Cell pellets were then lysed in 400 μl of 1% Sarkosyl in 10 mM Tris-hydrochloride–10 mM EDTA (pH 8.0) and digested sequentially with RNase (100 μg/ml) and proteinase K (200 μg/ml) for 5 h at 37°C. The DNA was sheared through a 27-gauge syringe needle and then phenol extracted and precipitated with 3 volumes of ethanol. The DNA was suspended in 10 mM Tris-hydrochloride (pH 7.5)–1 mM EDTA and electrophoresed on a 1.4% agarose gel (as described by Sharp et al. [24]) run horizontally. The gels were usually run for 12 h at 50 V, dried under vacuum onto 3MM Whatman filter paper, and fluorographed using the procedures of Laskey and Mills (11).

RESULTS

Competition of MVM empty capsids with MVM full virion for receptor sites. The earliest step in viral infection involves binding of the virus to the cell surface before entry. We therefore first sought to study the interaction of the viral capsids of each allotropic variant with the cell surface of both hosts.

It has been previously reported that MVM empty capsid particles compete with full virions for cell surface receptors on A9 cells (14). However, in the case of polyoma virus, a papovavirus, it has been shown that two types of receptor exist on the surface of mouse kidney cells. Bolen and Consigli (1) have shown that one class of receptor binds full virions only and leads to the productive infection, whereas the other class of receptor binds both full and empty particles, but

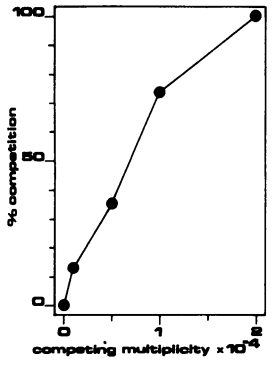

FIG. 1. Competition of MVM capsids with virions for productive binding sites. Exponentially growing A9 ouab^{r}11 cells were harvested and suspended in ice-cold medium at 2×10^5 cells per ml. Samples (0.5 ml) of this cell suspension were incubated on ice with 5 PFU of glycerol gradient-purified MVM(p) full virions per cell and various amounts of highly purified MVM(p) empty capsids. After 60 min the cells were extensively washed in ice-cold medium to remove unbound virus, diluted, and mixed with an equal volume of agarose overlay medium. Samples (1 ml) of these cell suspensions were plated on indicator monolayers of 324K cells in 60-mm dishes, allowed to set, and overlaid with a further 7 ml of overlay medium. Plaques due to A9 ouab^{r}11 infectious centers were visualized by neutral red staining after 7 days of incubation at 37°C.

leads only to their degradation in lysosomes. We have performed competition experiments between infectious MVM virions and highly purified empty capsids by using an infectious center assay to monitor productive infection, to determine whether a similar situation pertains for MVM-cell binding interactions.

Figure 1 shows the results of infectious center assays in which increasing quantities of purified empty particles competed with full virions for those MVM-specific binding sites, on the surface of A9 ouab^{r}11 cells, which lead to productive infection. The number of plaques arising from infected cells can be seen to decrease by almost 100-fold in the presence of 20,000 competing empty particles per cell (a 20:1 empty/full ratio). This drop clearly indicates that empty capsids compete efficiently with full virions for sites leading to infection; in this respect, therefore, the MVM-cell surface interaction is quite different from the polyomavirus-receptor interaction (1).

Characterization of MVM binding to cell surface receptors. Figure 2A compares the binding curves of MVM(p) on A9 ouab^{r}11 cells, a productive host for MVM(p) but restrictive for MVM(i) growth, and on S49 1TB2 cells, a productive host for MVM(i) but restrictive for MVM(p) growth. It also compares the binding of MVM(p) to A9-8E cells, an A9 clone selected for resistance to MVM(p) infection (13) and to RPC-5.4 cells, a B-cell tumor line resistant to infection by both viruses (29). Similarly, Fig. 2B compares the binding curves of MVM(i) with these same cell lines.

These comparisons show that both MVM(p) and MVM(i) bind to both A9 ouab^{r}11 and S49 1TB2 cells with similar biphasic kinetics and to similar levels of saturation. There appear to be somewhat more receptors for both viruses on S49 1TB2 cells than on A9 ouab^{r}11 cells. In both cases, binding to the other two cell lines tested showed nonspecific, unsaturable binding at a level comparable to that in cell-free controls.

Competition of MVM(p) and MVM(i) for cell surface receptors. The above results indicate that both viruses bind to the same extent to each cell type and may indeed bind to the same receptor on each individual cell line. To test this possibility further, we performed competition experiments (Fig. 3). The input multiplicity of labeled particles was chosen to be 2×10^6/cell, the point of saturation on the binding curves. Above this multiplicity, as unlabeled competing particles are added, the total percentage of particles bound per cell does not change significantly. Therefore, if competition between the two types of particles is complete, the addition of unlabeled particles simply dilutes the specific activity of the input virus and reduces the counts

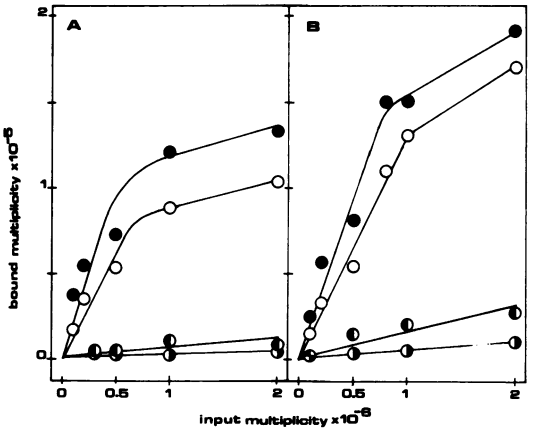

FIG. 2. MVM(p) and MVM(i) binding to cells: 1×10^6 fixed cells were incubated with (A) iodinated MVM(p) and (B) iodinated MVM(i). Each virion preparation was adjusted to 1 cpm = 4×10^6 virus particles. Input multiplicities ranging from 1×10^5 to 2×10^6 particles per cell were examined. After incubation, the bound multiplicity was determined from the counts retained on a Nuclepore filter as described in the text. In cell-free virus control samples less than 0.2% of input virus was retained on the filter. Symbols: ●, S49 1TB2 cells; ○, A9 ouab^{r}11 cells; ◐, A9-8E cells; ◑, RPC5.4 cells.

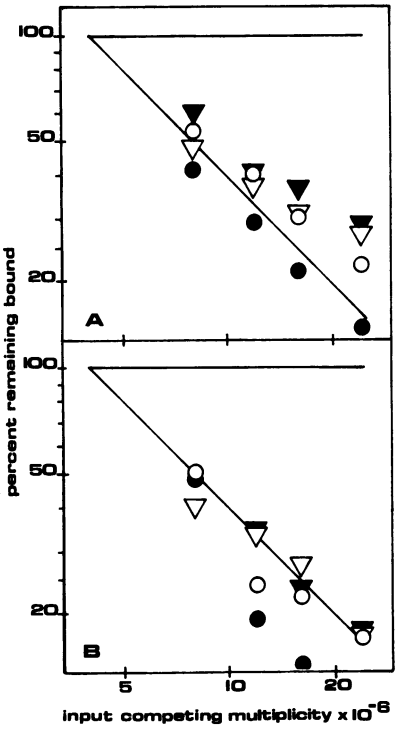

FIG. 3. Competition between MVM(p) and MVM(i) capsids for binding sites on A9 ouab^{r}11 and S49 1TB2 cells: 2×10^5 fixed cells were incubated with iodinated MVM(p) or MVM(i) at a multiplicity of 2×10^6 particles per cell. Competing incubations contained purified, unlabeled MVM empty particles at multiplicities ranging from 8×10^6 to 24×10^6 particles per cell. Incubation and filtering were performed as described for the binding assay; 100% represents the counts retained on the filter in the absence of competing capsids. Competition is expressed as the percentage of this control sample and is plotted logarithmically against input competing virus. Symbols: ▼, [^{125}I]MVM(p) in competition with MVM(p); ▽, [^{125}I]MVM(p) in competition with MVM(i); ●, [^{125}I]MVM(i) in competition with MVM(i); ○, [^{125}I]MVM(i) in competition with MVM(p). The horizontal line indicates the theoretical curve for noncompetition; the diagonal line indicates the theoretical curve for complete equivalence between labeled and unlabeled particles.

bound per cell. A theoretical line for complete competition can then be drawn which parallels the dilution of input specific activity. As controls, [^{125}I]MVM(p) competed with unlabeled MVM(p) and [^{125}I]MVM(i) competed with unlabeled MVM(i) on both cell lines. Points for the competition bindings on both cell lines clearly fall along the line for complete competition, indicating that viral particles of both MVM(i) and MVM(p) recognize the same receptor on the surface of each cell type.

Fate of parental viral DNA during infection. Figure 4A shows a comparison of the fate of parentally ^{32}P-labeled MVM(i) and MVM(p) in infections of S49 cells from the initiation of the infection until 15 h after infection. Although in the restrictive infection the appearance of monomer replicative form (RF) arising from single-stranded viral DNA is delayed by about 3 h compared with the productive infection, both infections reach the same level of input viral DNA converted to monomer RF by 15 h after infection.

Figure 4B shows the reciprocal infections in A9 cells. Again, the appearance of monomer RF is delayed for about 3 h in the restrictive infection, but both infections reach the same levels of conversion by 15 h. The increase in mobility of single-stranded viral DNA bands with time in this gel system is not presently understood, nor is the appearance of label running with high-molecular-weight DNA in the A9 infections. Nevertheless, it is clear from these results that the defect in both restrictive infections occurs at a step later than the synthesis of parental monomer RF.

DISCUSSION

It is of central importance to the understanding of parvovirus tissue tropism to determine the biochemical nature of the developmentally regulated host cell factors involved and the steps in virus replication at which they act. Strain-dependent target cell specificities for many viruses, notably, poliovirus (8), reovirus (7, 34, 35), Sindbis virus (26), and the cardioviruses (21, 22), have been shown to be mediated by the presence or absence of specific receptors for the virus, displayed on the cell surface as a function of differentiation. We therefore started our analysis of the restriction of MVM growth in differentiated cells by quantitating receptors, specific for each MVM strain, on the surfaces of various cells of lymphocyte and fibroblast origin.

We have shown here that MVM(p) empty capsids will effectively compete with full MVM(p) virions for sites, on the surface of A9 cells, which lead to the establishment of lytic infection. This finding has allowed us to use empty viral capsids to explore the distribution of potentially productive MVM receptors on mouse cells of dissimilar differentiated phenotype. The relevance of this result to the study of MVM receptor distribution is that cell culture-derived MVM preparations contain many more capsids than they do virions (30), and abundance of available ligand was of greater importance than infectivity in the competition experiments described here.

By using the empty capsid binding assay, the binding curves for MVM(p) and MVM(i) were

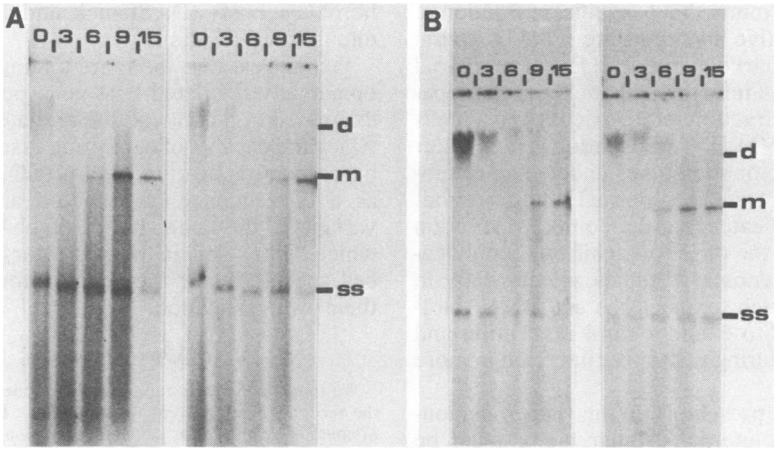

FIG. 4 Agarose gel electrophoresis of [^{32}P]MVM infections. (A) Infection of S49 1TB2 cells with (left) MVM(i) and (right) MVM(p). (B) Similar infections of A9 ouab^{r}11 with (left) MVM(i) and (right) MVM(p). Cells were extracted at various times as indicated (hours after infection). Samples were prepared as described in the text and run on 1.4% agarose gels. Each lane was matched in terms of cell equivalents. The position of dimer RF (d), monomer RF (m), and single-stranded viral DNA (ss) were determined from independently prepared markers run on the same gel.

determined for the reciprocally restrictive hosts A9 ouab^{r}11 and S49 1TB2 and for A9-8E and RPC5.4, two cell lines that are resistant to both virus strains (29). The similarity of the binding curves for the two viruses on either host cell line suggests that on a given host cell both viruses recognize the same receptor. This suggestion is further supported by the binding data for the viruses on A9-8E cells and RPC5.4 cells. A9-8E cells were selected for resistance to MVM(p) infection by Linser et al. (14), who showed that this resistance is due to the loss of the MVM(p) cell surface receptor. The binding data presented here show that the A9-8E cells have also lost the receptor for MVM(i). Similarly, RPC5.4, which is naturally resistant to infection by both MVM variants (29), does not bind either virus. Taken together, these data further support the suggestion that MVM(p) and MVM(i) bind to the same cell surface receptor on a given host cell. This was confirmed by competition experiments in which both viruses competed fully with each other for the receptor binding sites on both host cells. This competition does not show that the same virus receptor exists on A9 ouab^{r}11 and S49 1TB2, but it does show that the productive viral receptor on a host cell is recognized equally well by the restricted virus. As there appears to be only one type of receptor (i.e., that capable of leading to productive infection), these results demonstrate that the reciprocal restriction of these viruses is not mediated at the level of the cell surface receptor.

As restriction in these MVM infections obviously does not occur at the level of the cell surface receptor, we proceeded to investigate early events in the replication of viral DNA. The first step in this process involves the synthesis of the complementary strand of the input single-stranded parental DNA to produce a double-stranded replicative form of the DNA. Since input DNA is distributed uniformly among all cells, any processing of this DNA must reflect a phenomenon occurring in the general cell population. The experiment presented in Fig. 4 examines input viral DNA at time points early after infection and clearly shows that the processing of viral DNA to monomer RF is nearly the same in both the restrictive and productive infections. The kinetics of conversion show a time lag in conversion of approximately 3 h, but the final level of conversion by 15 h is identical in both sets of reciprocal infections. These results suggest that penetration and uncoating of the virion also occur normally in both types of infections with similar kinetics.

The production of viral capsid antigen occurs late in the lytic cycle of MVM. As we have shown in the accompanying paper (29), viral capsid antigen cannot be detected in the majority of cells in a restrictive infection, yet we have demonstrated here that the initial events of viral replication occur whether the infection is productive or restrictive. The amplification of the viral DNA species within the infected population is the next step leading to production of progeny virions. The method of in situ hybridization has been employed to examine the distribution of cells in productive and restrictive infections which are producing viral DNA spe-

cies. We have found that a significant fraction of cells in restrictive infections are producing viral DNA, but at markedly reduced levels compared with productive infections (manuscript in preparation). This fraction of restrictive host cells involved in MVM DNA replication is far greater than the small, or sometimes undetectable, subset of cells that undergo normal lytic infection. This result indicates that the point of restriction occurs before the onset of significant amplification of viral genomes. Studies are currently in progress in which we use DNA and RNA "blotting" analysis to examine viral replication and transcription during restrictive infection in more detail.

The results presented in this paper demonstrate that an internal cellular factor must be regulating the reciprocal restrictions of MVM(p) and MVM(i) in T lymphocytes and fibroblasts, respectively. Further studies (29) have shown that this factor is not an inhibitor produced by the host cell, as A9 ouab^{r}11 × T cell hybrids can support lytic infection by either virus. It therefore follows that each host cell provides some unique intracellular component that specifically interacts with only one of the MVM variants, allowing it to proceed with productive infection. Whether this factor interacts with the virus at the level of progeny RF DNA synthesis, transcription, or translation remains to be determined

The target cell specificity of MVM may therefore be mediated at two levels. We have shown that at least one B cell line does not carry the MVM specific receptor on its surface, and we have preliminary evidence suggesting that a number of differentiated cell types are naturally resistant to MVM infection through nonexpression of the receptor. However, in contrast to the situation with many other virus types (7, 8, 21, 22, 26, 34, 35), the strain-specific tropism examined here is mediated intracellularly. In this respect it resembles the dependence of *hrt*-like mutants of polyoma virus on factors expressed in mouse embryo cells, but not in 3T3 cells (6), and the interaction of wild-type polyoma virus with teratocarcinoma stem cells, where virus mutants can be obtained which overcome the intracellular defect in these cells and are able to grow lytically (reviewed in reference 12). It has been shown that MVM(p) also requires a host cell factor that is not expressed in teratocarcinoma stem cells (19, 28), but is expressed as a function of differentiation down the pathway to fibroblast (28). It is not known exactly at what point this factor acts in the virus life cycle, but our preliminary data indicate that it operates before the onset of viral DNA replication, and thus earlier than the factors that discriminate between MVM(p) and MVM(i) in T cell and fibroblast hosts (D. Stanick and P. Tattersall, unpublished results).

We suggest that there are a number of developmentally regulated host gene products, each expressed in individual differentiated cell types, which are capable of catalyzing essential steps in parvovirus replication. We further suggest that, as a consequence of mutation and selection, variants of the same viral serotype have evolved which differ solely in the particular differentiated cell specific factor they exploit for this step in their own replication.

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