Differential Host Ranges for \textit{in vitro} Infectivity of Mouse Mammary Tumour Viruses

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\textbf{SUMMARY}

Host-range variants of mouse mammary tumour viruses (MMTVs) have previously been shown to productively infect cells of several species \textit{in vitro} (Howard \& Schlom, 1978). We report here that cell lines have been identified which exhibit differential restriction for replication of different MMTV variants. In addition, a cell line has been identified that changes as a function of passage in culture from being permissive to being restrictive to infection with MMTVs. MMTVs propagated in both murine and non-murine cells retained their antigenic reactivities in a group-specific radioimmunoassay for MMTVs and demonstrated no evidence for the presence of type-C viruses as determined by a variety of techniques. These studies thus establish \textit{in vitro} cell substrate tropisms that can be used to differentiate between MMTVs.

\textbf{INTRODUCTION}

An understanding of the mechanism by which mouse mammary tumour viruses (MMTVs) induce mammary carcinoma and of the relationship of MMTVs to cellular control mechanisms has been hampered by the lack of suitable \textit{in vitro} systems in which to study these processes. Lasfargues and his associates (1976) and Howard \textit{et al.} (1977) have demonstrated the ability of the highly oncogenic MMTVs of C3H, RIII and GR mice to infect both feline and mink cells \textit{in vitro} productively. Vaidya \textit{et al.} (1978) also demonstrated the ability of MMTV from RIII mouse milk to infect murine epithelial cells.

We have recently reported (Howard \& Schлом, 1978) the isolation of host-range variants of MMTVs that are highly infectious for feline cells, with \textit{de novo} virus detected in as short a latent period as 4 days post inoculation compared with latent periods of up to 3 months using wild-type MMTVs (Lasfargues \textit{et al.} 1976; Howard \textit{et al.} 1977). In addition, productive infections with MMTV host-range variants could be obtained with an m.o.i. of less than or equal to one, whereas establishment of productive infections with wild-type MMTVs usually required an m.o.i. as high as $5 \times 10^5$ (Lasfargues \textit{et al.} 1976; Vaidya \textit{et al.} 1976; Howard \textit{et al.} 1977; Howard \& Schлом, 1978). We have shown previously that MMTV host-range variants retained their antigenic reactivities in group-specific radioimmunoassays for MMTV virion proteins, had a polypeptide profile similar to that of wild-type MMTVs and contained a virion-associated DNA polymerase which utilized magnesium preferentially over manganese (Howard \& Schлом, 1978). Stocks of host-range variants also showed no evidence of either murine or feline type-C viruses as determined by radioimmunoassay for p30, electron microscopy, SDS-polyacrylamide gel electrophoresis of

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virion proteins and assays for virion associated DNA polymerase activity (Howard & Schlom, 1978).

We report here that MMTV host-range variants can productively infect numerous cell types from a wide variety of species including feline, murine, canine, rat, bat and human cells. MMTV variants grown in these cells retained their antigenic reactivities in a group-specific radioimmunoassay for the major external glycoprotein of MMTVs (gp52) and showed no evidence of contamination with type-C viruses. Two cell lines have been identified which exhibited differential restriction of growth of the host-range variants. In addition, a third cell line, FeF, changed, with passage in culture, from being permissive to being restrictive to virus infection.

METHODS

Cells. The feline embryo kidney cell line, CrFK (Crandell et al. 1973), and the cell line NMuMG, derived from mammary glands of an adult Namru mouse (Owens et al. 1974), were obtained from Dr A. Hackett, Naval Biomedical Research Laboratories, Oakland, Calif., U.S.A. The feline embryo fibroblast cell line, FeF (at passage 16), was obtained from Dr P. Fischinger, National Cancer Institute, Bethesda, Md., U.S.A. The embryonic mouse cell lines, NIH-3T3 and SC-1, the cloned bat lung cell line, TbiLu (ATCC CCL88), the mink lung cell line, Mv1Lu (ATCC CCL64), the human rhabdomyosarcoma cell line, A204, and the human lymphoblastic cell line, NC-37, were obtained from Dr G. Todaro, National Cancer Institute, Bethesda, Md. The canine thymus cell, Cf2Th (Nelson-Rees et al. 1976) was obtained from Dr R. Heberling, Southwest Foundation for Research and Education, San Antonio, Tex., U.S.A. The C57MG cell line, derived from the mammary glands of a C57BL/6 mouse (Vaidya et al. 1978), was obtained from Dr E. Lasfargues, Institute for Medical Research, Camden, N.J., U.S.A. The Fischer rat embryo cell line, FRE, was supplied by Dr E. Scolnick, National Cancer Institute, Bethesda, Md. Primary cultures of feline and rat mammary gland cells were established as described (Wicha et al. 1979; D. Janss, personal communication). The human placenta cell line, HP574, and primary rat embryo cultures were prepared by trypsin digestion of finely minced tissues as described previously (Young et al. 1975).

All cell lines were routinely grown in Eagle’s minimal essential medium containing 10% (v/v) heat-inactivated foetal calf serum, 10 μg/ml bovine insulin, 2 μg/ml dexamethasone, 100 units/ml penicillin and 100 μg/ml streptomycin. Coded samples of cell lines were tested for species identification both before and 2 to 4 months after infection by isoenzyme analyses, karyotyping and immunofluorescence analyses, through the Office of Resources and Logistics, Biological Carcinogenesis Branch, NCI. All cell lines tested were demonstrated to be of proper species identity. Cells were monitored for the presence of mycoplasma and were found to be negative.

Viruses. MMTV(C3H) was obtained from culture fluids of the C3H mammary tumour cell line, Mm5mt/c, and concentrated 1000-fold by isopycnic centrifugation in sucrose gradients as previously described (Drohan et al. 1977). MMTV(RIII) was isolated from freshly collected milk from RIII mice at the third or higher parity. Alternatively, MMTV-(RIII) was obtained from culture fluids of primary cultures of spontaneous mammary tumours from RIII mice and concentrated as described for MMTV(C3H). The host-range variants, designated MMTV(RIII)vp4 and MMTV(C3H)vp4, were isolated by four serial virus passages in CrFK cells as previously described (Howard & Schlom, 1978). Virions obtained from collections at 24 h of supernatant culture fluids of infected CrFK cells were concentrated by isopycnic centrifugation in sucrose gradients as previously described (Drohan et al. 1977). CrFK cells were infected with MMTV preparations derived from
Differential host ranges for MMVs

pools of several preparations of virus. The same pools of MMTV(RIII)vp4 and MMTV-(C3H)vp4 were used throughout these studies. Rauscher murine leukaemia virus was isolated from the plasma of leukaemic BALB/c mice. Feline leukaemia virus was obtained from the Rickard-422 leukaemic feline thymus cell line, and the endogenous feline virus, RD-114, was obtained from culture fluids of the RD-114 cell line. The latter three viruses were obtained through the Office of Resources and Logistics, Biological Carcinogenesis Branch, National Cancer Institute. Type-C virus from C3H mice was obtained from culture fluids of the C3HT101 clone 8 cell line.

Infection of cells with MMVs. Infection of CrFK cells with MMVs has been described in detail by Howard et al. (1977). Briefly, the procedure for infecting cells was as follows: cells were seeded at a density of $1 \times 10^4$ or $1 \times 10^6$ cells per 75 cm$^2$ flask and virus was added 24 h later in culture medium containing 4 $\mu$g/ml polybrene. All infections were done at an m.o.i. of $1 \times 10^3$ particles per cell unless otherwise stated. Virus stocks used for infection were filtered through a 0.45 $\mu$m Millipore membrane, pre-coated with polyvinylpyrrolidone (Lasfargues et al. 1976), to remove any contaminating bacteria or cells. Virus titres were determined using group-specific radioimmunoassays for both the 52,000 mol. wt. major external glycoprotein (gp52) and the 28,000 mol. wt. internal protein (p28) of MMTV. Calculation of the number of virus particles from the determined amount of virion protein was performed as described previously (Vogt, 1965; Kimball et al. 1976; Howard et al. 1977). The titres obtained by radioimmunoassay approximated to those obtained by electron microscopy (Howard & Schlom, 1978). These results indicated that the method of quantification was measuring whole virus and not free antigen. Twenty-four hours after the addition of viruses to the cells, the medium was changed to culture medium without polybrene. At regular intervals thereafter, 24 h collections of culture medium were assayed for the presence of progeny MMTV using a group-specific radioimmunoassay for MMTV gp52.

Radioimmunoassay. MMVs were routinely quantified using a minor modification of the group-specific radioimmunoassay for gp52 of p28 or MMVs as described previously (Teramoto et al. 1977; Teramoto & Schom, 1978). Briefly, 20 $\mu$l of 3% Nonidet P-40 (NP40) was added to competing antigen in 100 $\mu$l of TNE (10 mM-tris-HCl, pH 8.3, 150 mM-NaCl, 2 mM-EDTA) containing 0.2% bovine serum albumin and 0.1% NP40, and incubated at 37 °C for 15 min to disrupt any virions present. At the end of this incubation, 10 $\mu$l of a 1 in 4000 dilution of goat anti-MMTV gp52 sera were added and the incubation continued for an additional hour at 37 °C. Iodinated MMTV gp52 (20,000 ct/min) in 20 $\mu$l of TNE was then added and the sample was incubated for 1 h at 37 °C. Finally, 50 $\mu$l of porcine anti-goat immunoglobulin G were added and the incubation was continued for an additional hour at 37 °C and overnight at 4 °C. The radioimmunoassay for MMTV p28 was performed in the same manner except that the antisera used were rabbit anti-MMTV-(C3H)p28 and goat anti-rabbit IgG and the iodinated antigen was $^{125}$I-MMTV(C3H)p28. After centrifugation at 10,000 g for 1.5 min, the supernatant was removed and the residual pellet assayed for radioactivity. The percentage of control binding was calculated by comparing the ct/min in the pellet in the presence of competitor to that in the absence of competitor. Binding in the absence of competitor was routinely between 30 and 40%. Non-specific binding, i.e. binding in the absence of immune sera (0 to 5%), was subtracted from all results. These assays are capable of detecting less than 1 ng of purified MMTV. MMVs from GR, RIII and C3H mice all competed with identical kinetics in these assays as previously detailed (Teramoto et al. 1977; Teramoto & Schom, 1978).

DNA polymerase assays. Twenty-four hour collections of culture fluids were clarified of cells and debris by centrifugation at 3000 g and 8000 g, each for 10 min. The supernatants were then removed and underlaid with 2.5 ml of 20% (v/v) glycerol in TNE and centrifuged.
at 195000 g for 60 min in a Beckman SW41 rotor. Pellets were resuspended in 50 μl of 10 mM-tris-HCl (pH 8.3) and DNA polymerase activity was determined using the synthetic primer-template oligo(dG)_{12-18}.poly(rC) (Bethesda Research Laboratories, Bethesda, Maryland) in samples containing either 20 mM-Mg^{2+} or 0.2 mM-Mn^{2+} as previously described (Michalides et al. 1975).

RESULTS

Propagation of MMTV host-range variants

We have recently demonstrated that serial passage of MMTVs from RIII, C3H and GR mice in feline CrFK cells resulted in the isolation of variants of those MMTVs that efficiently infect feline cells in culture (Howard & Schlom, 1978). To determine if a reliable supply of these host-range variants could be maintained, MMTV-infected CrFK cells were propagated in roller bottles over an extended period. Cultures continuously released virus during an observation period of greater than 6 months; both the C3H variant and the RIII variant were produced at an average level of 80 to 160 ng of virus (or 2 to 4 × 10^8 virions—see Methods for calculation of virus particle numbers) per ml per 24 h, as determined by radioimmunoassay (see Methods). This corresponds to the production of 4 to 8 μg of virus per roller bottle (containing 50 ml) per 24 h. Since each roller bottle contained approx. 2 × 10^7 cells, this level of production corresponds to the synthesis of approx. 500 to 1000 virus particles per cell per 24 h. Titres of as high as 3000 virus particles per cell per 24 h have been obtained.

Differential host-range restriction of MMTVs

Preliminary studies using RIII and C3H variants indicated that these viruses were able to infect some cell lines other than CrFK (Howard & Schlom, 1978). In this preliminary report, however, we did not analyse effects due to differences in cell substrate passage level, virus passage level, or m.o.i. We have now used the MMTV(C3H)vp4 and MMTV(RIII)vp4 variants to infect a wide variety of cell lines at identical m.o.i. (see Methods). As shown in Table 1, twelve cell lines from six species including feline, murine, canine, rat, bat and human cells were susceptible to productive infection. As expected, variability between cell lines in latent period to the appearance of de novo virus was observed. Latent periods ranged from as little as 5 days in CrFK cells to as much as 75 days in primary cultures of normal feline mammary glands (Table 1). In general, the RIII variant was more infectious than the C3H variant. For example, using identical m.o.i. with MMTV(RIII)vp4 and MMTV(C3H)vp4, progeny virus appeared 24 days after infection of FeF cells with the RIII variant and 63 days after infection with the C3H variant (Table 1). These infections were performed at the same time on duplicate cultures of FeF cells. Increasing the titre of the inoculum of the C3H variant by a factor of ten resulted in a shortening of the latent period by several days but progeny virus still did not appear as soon after infection as did progeny of the RIII variant. Both the RIII and the C3H variants were infectious for a variety of primary cell cultures or established cell lines of normal mammary glands of murine, feline and rat origin (Table 1).

The kinetics of infection of cell lines with the two MMTV variants is shown in Fig. 1. Canine thymus cells (Fig. 1a) and murine NIH-3T3 cells (Fig. 1b) could readily be infected with both viruses with latent periods up to the detection of de novo virus varying from 15 to 20 days. Some cell lines, however, exhibited restriction of growth of one variant while being permissive to replication of the other variant. Secondary rat embryo cultures, for example, were permissive to infection with the RIII variant but restrictive to the growth of the C3H variant (Fig. 1c). Conversely, the bat lung cell line, Tb1Lu, was permissive to infection by
### Table 1. Host range and titres of MMTV variants

<table>
<thead>
<tr>
<th>Species</th>
<th>Cell line designation</th>
<th>Tissue of origin</th>
<th>Days to initial virus production*</th>
<th>Maximum titre of MMTV* (particles/ml/24 h)</th>
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<tr>
<td></td>
<td></td>
<td></td>
<td>MMTV(RIII)vP4</td>
<td>MMTV(C3H)vP4</td>
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<tr>
<td>Cat</td>
<td>CrFK</td>
<td>Embryo kidney</td>
<td>5</td>
<td>6</td>
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<tr>
<td></td>
<td>FeF (passage 17)</td>
<td>Embryo fibroblast</td>
<td>24</td>
<td>63</td>
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<td></td>
<td>Normal mammary gland</td>
<td>Mammary gland</td>
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<td>75</td>
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<tr>
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<td>NIH-3T3</td>
<td>Embryo</td>
<td>39</td>
<td>53</td>
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<tr>
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<td>Mammary gland† of Namru mouse</td>
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<td>16</td>
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<tr>
<td></td>
<td>C37MG</td>
<td>Mammary gland of C57BL mouse</td>
<td>36</td>
<td>30</td>
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<td></td>
<td>SC-1</td>
<td>Feral mouse embryo</td>
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<td>&gt; 71</td>
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<td>FRE</td>
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<td>50</td>
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<tr>
<td></td>
<td>Secondary embryo</td>
<td>Embryo</td>
<td>33</td>
<td>&gt; 62</td>
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<tr>
<td></td>
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<td>Mammary gland</td>
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<td>25</td>
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<td>TbI Lu</td>
<td>Lung</td>
<td>&gt; 83</td>
<td>47</td>
</tr>
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</table>

* 24 h collections of supernatant fluids from confluent 75 cm² flasks were assayed at 7 to 10 day intervals by radioimmunoassay for gp52.

† These cells were infected at an m.o.i. of 5 × 10⁴. All other cells listed were infected at an m.o.i. of 1 × 10⁸.

‡ NT = not tested.
Fig. 1. Host-range restriction of infectivity of MMTV variants: MMTV(RIII)vP4 (●—●) and MMTV(C3H)vP4 (○—○) were used as inocula at an m.o.i. of 10^2. Supernatant fluids were harvested at the times indicated and the virus content was quantified in a group-specific radio-immunoassay for MMTV gp52 as described in Methods. (a) Cf2Th canine cells; (b) NIH-3T3 murine cells; (c) secondary rat embryo cells; (d) TbiLu bat cells.

the C3H variant but totally restricted the growth of the RIII variant (Fig. 1 d). This result has been confirmed in several separate experiments. Raising the m.o.i. of the RIII variant 40-fold to 4 x 10^4 still did not result in a productive infection of this cell line during the 53-day observation period. The Mv1Lu mink cell line, previously shown to be permissive to infection with a MMTV(RIII) variant (Howard & Schlom, 1978) subsequently showed variation in its susceptibility to infection; in some experiments this cell line could be productively infected with MMTV(RIII) variants while at other times growth of MMTVs was restricted. Karyotype analyses of this cell line revealed the presence of both male and female karyotypes (W. D. Peterson & C. S. Stulberg, personal communication). One explanation for the variation in susceptibility to infection could therefore be the modulation of different cell types in this uncloned cell line.

Effect of cell passage on susceptibility to MMTV infection

Fischinger and his co-workers (1975) have reported that the feline embryo fibroblast cell line, FeF, undergoes a crisis at approximately cell passage level 25; whereas cells are susceptible to infection with murine sarcoma virus pseudotypes before this crisis, the titre of progeny virus decreased 100- to 1000-fold when cells were infected after undergoing the crisis. We have conducted experiments on the relative susceptibility to infection by MMTV of both CrFK cells and FeF cells with prolonged passage in culture. In a period of continuous culture extending more than one year, we have not seen any change in the susceptibility of CrFK cells to infection by MMTV host-range variants. As can be seen in Fig. 2(a), CrFK cells at passage levels of 160 and 212 were equally susceptible to infection by the RIII host-range variant. We have obtained similar results using the C3H variant. A variety of cell lines including NIH-3T3, Cf2Th, FRE and TbiLu similarly did not exhibit
any change in their susceptibility to infection with MMTV variants in a period of extended culture lasting more than one year. On the other hand, FeF cells at passage 17 were readily susceptible to infection, but the same cells at passage 152 were refractive to productive infection (Fig. 2b). We have obtained similar results with both the RIII and the C3H MMTV variants. FeF cells (early v. late passage) therefore provide an excellent system to analyse those factors that may influence susceptibility to MMTV infection.

**Titres of de novo virus**

The maximum titres of progeny virus obtained after infection of cells with the two MMTV variants varied over almost a 1000-fold range (Table 1). In any one cell line permissive to the growth of both viruses, however, the titres rarely differed by more than twofold. Infection of murine NMuMG and feline CrFK cells with the RIII variant resulted in the highest maximum titres while the C3H variant gave the highest titre in the CrFK cell line (Table 1).

**Characterization of de novo MMTV**

As seen in Fig. 3, the MMTV(RIII) variant grown in canine, murine, rat and human cells retained its antigenic reactivity in a group-specific radioimmunoassay for MMTV gp52. The displacements of curves are most likely the result of the differences in virus titres in these high speed pellets from clarified culture fluids. Similar results were obtained with the MMTV(C3H) variant grown in canine, murine, rat, bat and human cells. Preparations of the RIII and C3H MMTV variants grown in murine, rat and canine cells were tested by competitive radioimmunoassays (kindly performed by Dr S. Aaronson, National Cancer Institute, Bethesda, Md.) for antigenic determinants related to type-C viruses. The assays used detect p30 of the RD-114-baboon virus group and of the murine-feline leukaemia virus group (Barbacid et al. 1977). No reactivity in these assays was detected using RIII and C3H preparations, indicating that less than 1% and, in most cases, less than 0.1%, of the virus-associated protein of these MMTV host-range variants was related to the p30 of known murine or feline type-C viruses. Preparations of wild-type MMTVs isolated from mouse milk and from culture fluids of the C3H mammary tumour cell line, Mm5mt/c1, however,
Fig. 3. Analysis of MMTV host-range variants in group-specific radioimmunoassay for MMTV gp52. Anti-MMTV gp52 at an input dilution of 1:4000 was used to precipitate $^{125}$I-labelled MMTV gp52. MMTV(RIII)vp4 grown in C2Th cells (○), NIH-3T3 cells (▲), FRE cells (△) and HP574 cells (■), as well as purified MMTV(C3H) (□), were used as competitors. Other competitors were tested at multiple input of protein (only two points are depicted for clarity): high-speed pellets from culture fluids of each of the uninfected cells (●); foetal calf serum proteins (▽); whey from C57BL mice (◇); C57BL lactating mammary gland extract (♦); C3H type-C virus (+); uninfected CrFK cell extract (×).

did contain some type-C virus determinants as expected. It has been shown previously (Dion et al. 1974; Michalides et al. 1975; Howard et al. 1977) that MMTVs grown in mouse cells have a DNA polymerase with a strict magnesium divalent cation preference, while murine and feline type-C viruses will utilize both magnesium and manganese, with a preference for manganese. MMTV host-range variants grown in a variety of cell lines contained a virion-associated DNA polymerase with a strict divalent cation preference for magnesium over manganese. Very little or no activity was seen when manganese was used as divalent cation and the ratios of activities with magnesium to those with manganese were greater than 50:1 and in most cases greater than 100:1.

We have previously performed tryptic peptide analyses on the major structural glycoproteins and non-glycosylated proteins of wild-type MMTVs and MMTVs passaged once in CrFK cells (Gautsch et al. 1978). We were able to identify strain-specific markers for several MMTV gene products and these differences were retained in MMTVs passaged once in CrFK cells. Drs R. Lerner and J. Gautsch (Scripps Clinic and Research Foundation, La Jolla, California) kindly performed tryptic and chymotryptic peptide analyses of the major structural 52000 mol. wt. glycoprotein and the non-glycosylated 28000 and 14000 mol. wt. proteins of the MMTV host-range variants, MMTV(RIII)vp4 and MMTV(C3H)vp4. The peptide maps obtained were similar to those previously published for MMTV(C3H), MMTV(RIII) and MMTVs passaged once in CrFK cells (Gautsch et al. 1978).
DISCUSSION

MMTV host-range variants, isolated by serial virus passage in CrFK cells, have been shown to possess wide host-ranges \textit{in vitro}. These viruses productively infected a total of twelve cell lines from six different species. Two cell lines have been identified which exhibit differential restriction of replication of MMTVs. The difference in the ability of the viruses to propagate in these cells is unlikely to be a property of the physical condition of the virions themselves since both viruses were able to infect eleven other cell lines productively. The restriction of growth of the RIII variant by TbILu cells appeared to be substantial since increasing the m.o.i. by a factor of 40 still did not result in a productive infection in these cells during a 53-day observation period. The use of TbILu cells and secondary cultures of Fischer rat embryos should prove useful in determining which viral and/or cellular functions are involved in the replication, or restriction of replication, of MMTVs. These studies also establish cell substrate tropism as a means of distinguishing between MMTVs.

The feline embryo fibroblast cell line, FeF, has been shown to change its susceptibility to infection by MMTV host-range variants with passage in culture. This change correlated with a period of crisis which these cells undergo at the 25th passage. A similar change in permissiveness of this cell line with respect to replication of type-C viruses has been reported previously (Fischinger \textit{et al.} 1975). This cell line should also prove particularly useful in determining which cellular function or functions are involved in the restriction of growth of both type-B and type-C Retroviridae.

While the reason for the long latent period up to the appearance of \textit{de novo} progeny virus in some cell lines (e.g. normal feline mammary gland cells, NIH-3T3 cells) is unclear, one possible explanation is that only a very small percentage of cells in the culture are initially infected. Vacquier & Cardiff (1979) have shown that in some cases 1 infectious unit of MMTV is represented by more than $10^6$ virus particles. Thus, at an m.o.i. of $1 \times 10^8$ per cell, less than 1% of the cell population would be infected by the original virus inoculum and the long latent period would result from the expression of progeny virus by these cells and subsequent cycles of infection of other cells in the population.

The ability of MMTV host-range variants to infect normal mammary gland cells of a variety of species now makes possible an understanding of the interaction of MMTVs with their natural target cell and should facilitate an elucidation of the interaction of MMTVs with various hormones, carcinogens and tumour promoters in the initiation and promotion of transformation of mammary gland cells.

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