Isolation of host-range variants of mouse mammary tumor viruses that efficiently infect cells *in vitro*

(mammary neoplasia/RNA tumor viruses/type B viruses/viral variants/mouse mammary tumor virus infectivity)

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ABSTRACT Host-range variants of mouse mammary tumor virus (MMTV) have been isolated that have the ability to productively infect cells *in vitro* with high efficiency (at multiplicities of infection ≤ 1) and with extremely short latent periods to the production of *de novo* virus (as short as 4 days after infection). These variants of the highly oncogenic MMTV of RIII, C3H, and GR mice were obtained by serial virus passage in feline cells. The resultant variant stocks react in group-specific radioimmunoassays for the MMTV major external glycoprotein (gp52) and major internal protein (p28), possess a protein profile similar to that of wild-type MMTV, and contain a virion-associated DNA polymerase with a magnesium cation preference. Addition of dexamethasone and insulin to culture media enhances the titer of *de novo* MMTV to levels of approximately 10^{10} particles per 75-cm² flask (containing 5×10^6 cells) per 24 hr. Variant stocks exhibit no evidence of contamination with either murine or feline type C retroviruses, as assayed by various techniques. The variants of MMTV derived from C3H and RIII mice exhibit differential host ranges that include the ability to productively infect feline, canine, bat, mink, murine, and human cells. Use of these MMTV host-range variants now facilitates the study of the complete replicative cycle of MMTV as well as an elucidation of the interaction of MMTV with various hormones, physical or chemical carcinogens, and tumor promoters in the initiation and promotion of mammary neoplasia.

A major obstacle in the study of mouse mammary tumor viruses (MMTV) and their relationship to cellular control mechanisms has been the inability of MMTV to productively infect both murine and heterologous cells *in vitro*. Lasfargues and his associates (1) and Howard *et al.* (2) have demonstrated that the highly oncogenic MMTV of C3H, RIII, and GR mice could infect both feline and mink cells. Establishment of these productive infections, however, required extremely high multiplicities of infection (moi) (as high as 5×10^{11} particles were required to infect 10^6 cells) and long latent periods for the synthesis of MMTV *de novo* (up to 10 weeks) (2).

We report here the isolation of host-range variants of MMTV of RIII, C3H, and GR mice. Each can readily infect heterologous cells at extremely low moi; under appropriate conditions of infection virus appears *de novo* within 4 days after infection. Initial MMTV variants were obtained from supernatant fluids of feline cells that had been infected with MMTV. Passage of these MMTV variants up to three more times in feline cells resulted in new variants that could infect feline cells even more efficiently. These MMTV variants also productively infect a broad host range of cells, including feline, mink, canine, murine, bat, and human cells.

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MATERIALS AND METHODS

Cells. The CrFK feline embryo kidney cell line and the Mm5mt/c₁ C3H mouse mammary tumor cell lines were grown as described (2-4). The FeF feline embryo cell line (at passage 16) was kindly supplied by P. Fischinger (National Cancer Institute). A human placenta cell strain (HP574) was originated in this laboratory and was infected with MMTV at passage 4. Coded samples of cell lines and cell strains were tested both before and after MMTV infection for species identification. Isoenzyme analyses, karyotyping, and immunofluorescence analyses demonstrated proper species identity of all cell lines and cell strains. All cell lines and strains were monitored for the presence of mycoplasma and found to be negative.

Viruses. MMTV(C3H) was purified from the supernatant fluids of cultures of the C3H mammary tumor cell line, Mm5mt/c₁, as described (5). MMTV(RIII) and MMTV(GR) were obtained from milk of mice at third parity and above.

Infection of Cells with MMTV. Infection of CrFK cells with MMTV(C3H), MMTV(RIII), and MMTV(GR) has been described in detail (2). The viruses produced by these cells are designated MMTV(C3H)vp1, MMTV(RIII)vp1, and MMTV(GR)vp1, respectively (vp1 denotes virus passaged one time in feline cells). All inocula used to infect feline cells or cells of other species were first filtered through a 0.45- μ m Millipore membrane filter precoated with polyvinylpyrrolidone (1). Sterility tests showed filtered samples to be free of bacteria and fungi. An aliquot of each filtered inoculum was taken for determination of MMTV content by radioimmunoassay for MMTV gp52 as described below. Cells, plated 24 hr previously at a concentration of 1×10^6 cells per 75-cm² plastic tissue culture flask, were then infected with filtered virus-containing medium containing 4 μ g of polybrene per ml as described (2). All such infected cells were routinely grown in Eagle's minimal essential medium containing 10% (vol/vol) heat-inactivated fetal calf serum, 10 μ g of bovine insulin per ml, 2 μ g of dexamethasone per ml, 10 units of penicillin per ml, and 10 μ g of streptomycin per ml.

When CrFK cells were infected with MMTV(C3H)vp1, the virus subsequently produced in the supernatant fluid was designated MMTV(C3H)vp2. This latter virus was then used to infect additional CrFK cells, and the virus subsequently produced by these cells was designated MMTV(C3H)vp3. MMTV(C3H)vp4 was obtained from supernatant fluids of CrFK cells infected with MMTV(C3H)vp3. In a similar manner, we obtained MMTV(RIII)vp1, vp2, and vp3 and MMTV(GR) vp1, vp2, and vp3.

Radioimmunoassays. MMTV, obtained from $195,000 \times g$

Abbreviations: MMTV, mouse mammary tumor virus; moi, multiplicity of infection.

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FIG. 1. Relationship of moi to kinetics of virus production for MMTVvp2 host-range variants. Feline CrFK cultures, containing 1×10^6 cells per 75-cm² flask, were infected with (A) MMTV(C3H)vp2, (B) MMTV(RIII)vp2, and (C) MMTV(GR)vp2. At the times indicated, *de novo* MMTV in supernatant fluids was quantitated by group-specific MMTV gp52 radioimmunoassay. (A) Titers of infecting MMTV particles used were: ●, 1×10^8 ; ○, 7×10^6 ; ▲, 3×10^6 ; △, 2×10^6 ; ■, 1×10^5 ; □, 1×10^4 ; ×, 1×10^3 ; ○, 1×10^2 . ▼, Uninfected cells; ■, CrFK cells infected with 3×10^9 murine-grown MMTV(C3H) particles. (B) Titers were: ●, 3×10^8 ; ○, 3×10^7 ; ▲, 3×10^6 ; △, 3×10^5 ; ■, 3×10^4 ; □, 3×10^3 ; ×, 3×10^2 . ▼, Uninfected cells. (C) Titers were: ●, 3×10^8 ; ○, 3×10^7 ; ▲, 3×10^6 ; △, 3×10^5 ; ■, 3×10^4 ; □, 3×10^3 . ▼, Uninfected cells.

pellets of clarified supernatant fluids, were routinely quantitated in group-specific radioimmunoassays for the 52,000-dalton major external glycoprotein (gp52) and the 28,000-dalton major internal protein (p28) of MMTV as described (6, 7). All calculations of virus titers, in particles/ml, were obtained from radioimmunoassay results as described in detail (8, 9). These titers approximated particle counts by electron microscopy.

RESULTS

Infectivity of MMTV Obtained from Murine and Feline Cells. We have recently demonstrated that MMTV from GR, RIII, and C3H mice can productively infect feline and mink cells in culture, but progeny viruses were not released until up to 10 weeks after infection (2). We have used the MMTV released by feline CrFK cells, designated MMTV(C3H)vp1, MMTV(RIII)vp1, and MMTV(GR)vp1, to infect other CrFK cells. As can be seen from Table 1, a dramatic reduction in the moi required to infect feline cells is observed with MMTVvp1 preparations as compared to MMTV obtained from murine cells. With mouse milk-derived virus, i.e., MMTV(RIII), a minimum of 5×10^9 particles was necessary for productive infection in CrFK cells (10^6 cells/culture), whereas as little as 4×10^4 particles of MMTV(RIII)vp1 elicited a productive in-

fection in these same cells. Virus particle titers were determined by radioimmunoassays for the 52,000-dalton major envelope glycoprotein (gp52) of purified MMTV.

With MMTV(C3H) (from supernatant fluids of mouse mammary tumor cells), as many as 3×10^9 particles did not establish a productive infection within the 52-day observation period (Table 1); 3×10^{12} particles were required to establish a productive infection. This high moi required the use of 1000-fold virus concentrates prepared in zonal rotors; these preparations contained the same level of intact gp52 as freshly harvested supernatant fluids. MMTV(C3H)vp1, on the other hand, productively infected CrFK cells with 6×10^4 particles (Table 1).

These results demonstrate that the MMTV(C3H)vp1 host-range variant can establish a productive infection at moi of at least 1/50,000th that required with wild-type MMTV(C3H) virions. This difference is unlikely due to physical damage of MMTV(C3H) virions since both virus preparations were obtained from 24-hr supernatant fluids that were filtered and added to identical aliquots of indicator cells at the same time.

The viruses produced by CrFK cells infected with MMTV (C3H)vp1, MMTV(RIII)vp1, and MMTV(GR)vp1 were

Table 1. Multiplicity of infection required to establish a productive infection in feline CrFK cells

Virus	Source of virus*	MMTV particles required to establish productive infection	moi†
MMTV(C3H)	C3H mammary tumor cell line	$>3 \times 10^9$ ‡	$>3 \times 10^3$
MMTV(C3H)	C3H mammary tumor cell line, 1000-fold concentrate	3×10^{12}	3×10^6
MMTV(C3H)vp1	MMTV(C3H)-infected CrFK cells	6×10^4	<1
MMTV(C3H)vp2	MMTV(C3H)vp1-infected CrFK cells	$\leq 1 \times 10^2$	<1
MMTV(RIII)	RIII mouse milk	5×10^9	5×10^3
MMTV(RIII)vp1	MMTV(RIII)-infected CrFK cells	$\leq 4 \times 10^4$	<1
MMTV(RIII)vp2	MMTV(RIII)vp1-infected CrFK cells	$\leq 3 \times 10^2$	<1
MMTV(GR)vp1	MMTV(GR)-infected CrFK cells	4×10^7	4×10^1
MMTV(GR)vp2	MMTV(GR)vp1-infected CrFK cells	$\leq 3 \times 10^3$	<1

* Unless otherwise stated, the source of virus was 24-hr harvests of filtered supernatant culture fluids. CrFK cells (10^6) in a 75-cm² flask were infected. Virus particle titers were obtained from radioimmunoassays for MMTV gp52.

† Minimum moi required to establish a productive infection.

‡ No evidence of productive infection at particle inputs up to 3×10^9 during the 52-day observation period.

designated MMTV(C3H)vp2, MMTV(RIII)vp2, and MMTV(GR)vp2, respectively. These latter three viruses were then used to infect CrFK cells. As seen in Table 1, as few as 10^2 MMTV(C3H)vp2 particles added to 10^6 CrFK cells resulted in a productive infection. Similarly, infecting with as few as 3×10^2 MMTV(RIII)vp2 particles also resulted in a productive infection (Table 1).

The kinetics of productive infection obtained with MMTV(C3H)vp2 are shown in Fig. 1A. When 1×10^8 particles were used per 10^6 cells, *de novo* MMTV was observed in supernatant fluids at approximately 10 days after infection. Lowering the moi resulted in longer latent periods to time of appearance of *de novo* virus. Infecting with as little as 10^2 particles resulted in the appearance of *de novo* virus in supernatant fluids at approximately 55 days after infection. Similar kinetics of virus production were also observed with MMTV(RIII)vp2 (Fig. 1B) and MMTV(GR)vp2 (Fig. 1C).

In these and all other studies reported here, media were changed 24 h after infection. When supernatant fluid was assayed by radioimmunoassay for MMTV 2 days after infection, all results were negative; these results rule out the possibility that supernatant fluids obtained on subsequent days contained residual infecting virus.

The final titer of *de novo* MMTV in supernatant fluids was independent of the dose of infecting virus (Fig. 1A). Similar results were obtained with MMTV(RIII)vp2 and MMTV(GR)vp2 stocks (Fig. 1B and C). By calculations detailed previously (8, 9), the 400 ng of MMTV synthesized per ml per 24 hr represents approximately 10^9 MMTV virions. Since the 75-cm² flasks contained 5×10^6 CrFK cells (greater than 95% of which are positive for MMTV antigens by immunofluorescence) and 10 ml of medium at the time of assay, approximately 2×10^8 particles of *de novo* MMTV are being synthesized per cell per 24 hr.

The virus produced by cells infected with MMTV(C3H)vp2 was designated MMTV(C3H)vp3. Infection of feline cells with MMTV(C3H)vp3 resulted in the synthesis of *de novo* MMTV in as short a latent period as 4 days (Fig. 2A). This figure clearly demonstrates the shorter latent period to the synthesis of *de novo* virus, for any given moi, as a result of passage of MMTV in feline cells. A similar pattern is observed with MMTV from

the GR strain (Fig. 2B). The latent period to *de novo* virus production was directly related to titer of infecting virus in all cases.

To determine the effect of freezing on the infectivity of MMTV host-range variants, MMTV(C3H)vp2 was tested at various moi using the same stock of freshly harvested and frozen virus. There appears to be only a slight reduction (by approximately half) in infectivity after freezing and thawing. Virus stored for up to 14 weeks at -196°C showed no further reduction in infectivity.

Effect of Hormones on MMTV Synthesis. It has previously been shown that both insulin and dexamethasone stimulate the synthesis of MMTV in mouse mammary tumor cells (10–13) and CrFK cells (14). As shown in Table 2, a 3-fold stimulation in the synthesis of MMTV host-range variants is observed when insulin is added to cell culture medium. When dexamethasone is added, however, either with or without the presence of insulin, a 15-fold stimulation of virus production is observed.

Characterization of MMTV Variants. As seen in Fig. 3, MMTV(C3H), passed four times in feline cells, retained its antigenic reactivities in group-specific radioimmunoassays for both MMTV gp52 and MMTV p28. MMTV of GR and RIII, passed several times in feline cells, displayed similar reactivities. Antisera prepared against disrupted MMTV (in rabbits) and against purified MMTV gp52 (in goats) both neutralized the infectivity of MMTV(C3H)vp4. The following antisera, however, had no effect on the infectivity of these same virus preparations: preimmune bleeds of the above sera, anti-murine leukemia virus (Rauscher), anti-RD114, anti-fetal calf serum proteins, and anti-MMTV p28.

The polypeptide profiles of MMTV variants were examined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. As shown in Fig. 4, the major structural proteins of MMTV(C3H), obtained from supernatant fluids of a C3H mammary tumor cell line, are retained in MMTV(C3H)vp4 virions. These include gp52, gp36–38, p28, p14, and p10. The only major difference is the appearance of a 46,000-dalton peak in the MMTV host-range variant. This has been examined by tryptic peptide map analysis and shown to be actin (16). Similar profiles were obtained with all feline-grown MMTV examined. Purified virion preparations of MMTV host-range variants had a density of approximately 1.17 g/ml in sucrose and contained a 60–70S high molecular weight RNA, as detailed for MMTVvp1 preparations (2).

All MMTV host-range variants contained a virion-associated DNA polymerase with a divalent cation preference of magnesium over manganese, as determined with the synthetic primer-template oligo(dG)_{12–18}·poly(rC) (17). This high magnesium to manganese ratio, along with the typical MMTV protein profile, are indicative of the lack of contamination of preparations of MMTV host-range variants with either murine or feline type C viruses (2, 17, 18). Electron microscopic studies

FIG. 2. Effect of multiple passage of MMTV in feline cells on efficiency of infection. Feline CrFK cells, plated 24 hr previously at 1×10^6 cells per 75-cm² flask, were infected with (A) MMTV(C3H) variants and (B) MMTV(GR) variants at various moi. The appearance of progeny virus was monitored by group-specific radioimmunoassay for MMTV gp52. (A) Infectivity of (Δ) MMTV(C3H)vp1; ($\circ$) MMTV(C3H)vp2; ($\bullet$) MMTV(C3H)vp3. (B) Infectivity of ($\circ$) MMTV(GR)vp1; ($\bullet$) MMTV(GR)vp2.

Table 2. Effect of dexamethasone and insulin on synthesis of MMTV(C3H)vp4 variant in feline CrFK cells

Insulin (10 $\mu\text{g/ml}$)	Dexamethasone (2 $\mu\text{g/ml}$)	ng MMTV/ 24 hr*	Stimulation of virus synthesis, -fold
—	—	37	1.0
+	—	122	3.3
—	+	565	15.3
+	+	550	14.9

* Measured by radioimmunoassay for MMTV gp52 from 24-hr supernatant fluid harvests from 490-cm² roller bottles containing approximately 5×10^7 cells.

FIG. 3. Analysis of MMTV host-range variant in MMTV radioimmunoassays. Group-specific competition radioimmunoassays for gp52 and p28 MMTV polypeptides. (A) Anti-MMTV gp52 at an input dilution of 1:4000 was used to precipitate ^{125}I -labeled MMTV gp52. Purified MMTV(C3H) gp52 (●) and MMTV(C3H)vp4 (□) were used as competitors. (B) Anti-MMTV p28 at an input dilution of 1:5000 was used to precipitate ^{125}I -labeled MMTV p28. Purified MMTV(C3H) p28 (○) and MMTV(C3H)vp4 (□) were used as competitors. Other competitors were tested at multiple input of protein; however, only two points are depicted for clarity. Δ, High-speed pellets from culture fluids on uninfected CrFK cells; ◇, fetal calf serum proteins; ♢, whey from C57Bl mice; +, C57Bl lactating mammary gland extract; ×, C3H type C virus; ▽, feline kidney cell (CrFK) extract.

also revealed intracellular and extracellular particles typical of MMTV in MMTV(C3H)vp4-infected cells as described for MMTVvp1-infected cells (2).

MMTV(C3H), MMTV(RIII), and MMTV(GR) host-range variants (vp3–4) were tested by competitive radioimmunoassays (kindly performed by S. Aaronson, National Cancer Institute) for antigenic determinants related to type C viruses. The various assays used detect p30 and gp70 of the RD-114–baboon virus group and p30 and gp70 of the murine–feline leukemia virus group (19). All results with MMTV host-range variants were negative; i.e., less than 0.05% of the viral associated protein of MMTV host-range variants is related to p30 or gp70 of known murine or feline type C viruses.

Host-Range Studies. MMTV(C3H)vp4 was used to infect cell lines from several species. As seen in Table 3, this MMTV host-range variant can productively infect feline, canine, bat, and murine cells. The cell lines listed in Table 3 were infected at moi of 1000. With moi of as high as 100,000, bat and mink cells were negative when infected with wild-type MMTV obtained from murine cells.

The MMTV(RIII)vp3 host-range variant displayed the ability to rapidly infect a variety of heterologous cells with a host range somewhat different from that of the MMTV(C3H) variant. As shown in Fig. 5, the synthesis of *de novo* MMTV was observed in feline CrFK cells, feline FeF cells, mink Mv1Lu cells, and human placenta cells in as short a latent period as 6–16 days after infection.

FIG. 4. Polyacrylamide gel electrophoresis of polypeptides of MMTV derived from murine and feline cells. Cultures of C3H mouse mammary tumor cells, and feline CrFK cells infected with MMTV(C3H)vp3, were labeled with 100 μCi of ^3H -labeled mixed amino acids per ml and the labeled viruses in supernatant fluids were concentrated and purified as described (15). Labeled viruses were disrupted and subjected to polyacrylamide gel electrophoresis as described (15). Migration is from left to right. ●, MMTV(C3H)vp4; ○, MMTV(C3H).

DISCUSSION

MMTV host-range variants, derived from MMTV of C3H, RIII, and GR mice, have been isolated as a result of serial passage in feline cells. Just one passage in feline cells decreased the moi required to establish a productive infection by at least 1/50,000th. Further passage of these virus stocks in feline cells increased their efficiency of replication in cells *in vitro*.

Table 3. Host range of MMTV(C3H)vp4 variant

Species	Cell line	Day first positive after infection*	ng MMTV/flask per 24 hr†
Cat	CrFK	9	357
Dog	Cf2Th	45	100
Bat	Tb1Lu	46	83
Mouse	NIH-3T3	45	333
Rabbit	SIRC	—‡	<3
Mink	Mv1Lu	—‡	<3
Rhesus monkey	DBS-FRHL-1	—‡	<3

* Supernatant fluids from confluent 75-cm² flasks were assayed at weekly intervals by radioimmunoassay for MMTV gp52.

† Levels of MMTV synthesis at day 73 after infection as measured by radioimmunoassay for MMTV gp52.

‡ Negative through the 73rd day after infection.

FIG. 5. Host range of MMTV(RIII)vp3 variant. MMTV(RIII)vp3 was used to infect a variety of cell lines (10^6 cells per culture) at a moi of 1000. Supernatant fluids at the times indicated were harvested, concentrated, and assayed by group-specific MMTV gp52 radioimmunoassay. ●, CrFK feline cells; ○, FeF feline cells; ▲, Mv1Lu mink cells; ■, human placenta HP574; ○, Cf2Th canine cells; □, SIRC rabbit cells, SC-1 feral mouse cells, A204 human rhabdomyosarcoma cells, and NC-37 human lymphoblastoid cells.

The variants derived from MMTV(RIII) and MMTV(C3H) appear to differ in their ability and/or efficiency to productively replicate in cells of different species. Host-range studies may therefore be used as another parameter to distinguish one MMTV strain from another. The term "host-range variants" was selected due to the ability of these virus preparations to efficiently infect cells from a variety of species. Perhaps the term "virulence variants" could also be applied to some of these virus preparations, since they gradually increased in their efficiency to infect a given cell line (i.e., CrFK) with increasing virus passage.

At least three mechanisms exist for the derivation of host-range variants: (i) the selection, as a result of passage in feline cells, of a preexisting variant in the wild-type MMTV population; this could have been masked by the possible existence of auto-interfering virus in mouse-cell-derived stocks; (ii) a phenotypic mixing, pseudotyping, or genomic masking (20) with an undetected feline or murine virus; and/or (iii) the recombination of MMTV genetic information with normal feline cellular DNA or with proviral DNA from feline cells, resulting in a recombinant virus. Preliminary nucleic acid hybridization experiments, however, indicate little if any hybridization between radioactive 70S RNA of MMTV(C3H)vp4 and normal feline (CrFK) cellular DNA.

The use of host-range variants of MMTV described here now makes possible the study of the complete replicative cycle of MMTV in homologous and a variety of heterologous cells. Human cells infected with MMTV can now be used to search for cell-mediated reactivities between human lymphocytes and MMTV-coded determinants. The efficient infection and rapid synthesis of *de novo* MMTV *in vitro* now also makes possible a better understanding of the interaction of mammary tumor viruses with host-cell regulatory functions, including interactions with hormones, physical and chemical carcinogens, and tumor promoters, in the initiation and/or progression of mammary carcinoma.

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