In Vitro System for Production of Mouse Mammary Tumor Virus

D. L. FINE, L. O. ARTHUR, J. K. PLOWMAN, E. A. HILLMAN, AND F. KLEIN
Frederick Cancer Research Center, Frederick, Maryland 21701

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An in vitro system for production, purification, and concentration of mouse mammary tumor virus is described. Monolayer cultures of C3H mouse mammary tumor cells propagated at 34 C in roller bottles in the presence of dexamethasone, a glucocorticoid hormone, release B-type particles which possess ribonucleic acid and a ribonucleic acid-dependent deoxyribonucleic acid polymerase. One thousandfold concentration by ultracentrifugation with subsequent gradient fractionation yielded > 7 x 10^14 particles per ml in the 1.16- to 1.18-g/ml region. Mouse mammary tumor virus produced in this system was free of detectable C-type virus.

As a virus reagent, mouse mammary tumor virus (MMTV) is obtained from milk, mammary tumors, or from explants or continuous cell lines derived from mammary tumors of inbred strains of mice. Although mouse milk and tumors yield large numbers of virus particles, these materials are generally contaminated with lipid or protein, whereas MMTV produced from tissue culture is relatively free of impurities. Over the past 21 years, reports from a number of laboratories have described systems for in vitro production of MMTV (2, 5-8, 17, 18, 21). In general, each has been unsuccessful, due to contaminating type-C virus (5, 7), finite life span of the cultures (2, 6, 17) or decreased virus production (8, 18, 21). Utilizing the continuous cell line derived from a C3H mouse mammary tumor, described by Owens and Hackett (14), we have developed an in vitro system for intermediate or large-scale production of MMTV. In this system, MMTV production is enhanced by dexamethasone (DXM), a synthetic glucocorticoid, as demonstrated previously by our laboratory (3) and that of Parks et al. (15).

MATERIALS AND METHODS

Cell cultures. The C3H mouse mammary tumor cell line (Mm5mt/c1) obtained from A. Hackett, Naval Biomedical Research Laboratories, Oakland, Calif., was maintained on Dulbecco's modified essential medium-high glucose containing 10% heat-inactivated fetal calf serum, insulin (10 µg/ml), tylocline (60 µg/ml), penicillin (100 U/ml), and streptomycin (100 µg/ml). Cells, at passages 21 through 34, were passed at confluence by trypsinization. The cells were found to be negative for mycoplasma by bioassay (Flow Laboratories, Rockville, Md.) and by the [3H]thymidine incorporation assay of Todaro et al. (22).

Reagents. 5-Iodo-2'-deoxyuridine (IUDR) (Sigma Chemical Co., St. Louis, Mo.) was sterilized by filtration and added to growth medium before use. DXM (Sigma Chemical Co., St. Louis, Mo.) was included in growth medium before sterilization, at a concentration of 10^-4 M as previously described (3). Isotopes ([3H]thymidine triphosphate, [3H]guanosine triphosphate, 100-150 counts/min per pmol) and templates (oligo dT-poly rA, oligo dG-poly rC) used were obtained from New England Nuclear and Collaborative Research, respectively.

Assays. The membrane immunofluorescence assay used to detect cell-associated MMTV-specific antigens was described previously (3). Immunoprecipitin tests used double diffusion in 2% agar gel plates (Hyland Laboratories, Costa Mesa, Calif.).

Culture supernatants were concentrated by centrifugation for 1 h at 100,000 x g after intermediate clarification centrifugations at 4,000 x g and 10,000 x g for 15 and 20 min, respectively.

The virus pellets were resuspended in 0.05 M sodium citrate, pH 7.2. Negative stain preparations (2% phosphotungstic acid, pH 4.2) were examined in a Hitachi HU-12 or RCA-3G electron microscope. Virus particle counts were performed following the method of Monroe and Brandt (12).

Ribonucleic acid-dependent deoxyribonucleic acid-polymerase (RDDP) assays were performed by resuspending 50-alter virus samples in TNE buffer [10 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.4) containing 10 mM NaCl and 1 mM ethylenediaminetetraacetic acid] and lysing with 1% Triton X-100 in the presence of 10 mM dithiothreitol at 25 C for 15 min. The virus lysate was added to a reaction mixture (pH 8.4) consisting of 25 mM glycine, 12 mM MgCl2, 75 mM NaCl, 10 mM dithiothreitol, 1 µg of oligo dT-poly rA or 2 µg of oligo dG-poly rC, and 1.25 µM [3H]thymidine triphosphate.
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(150 counts/min per pmol) or 1.0 μM [3H]deoxyguanosine triphosphate (150 counts/min per pmol). The mixture was incubated for 15 and 30 min at 37 C, and the reaction was terminated by absorbing the template on Whatman DE-31 filters. The filters were washed in 5% Na2HPO4, dried, placed in 10 ml of Liquifluor-toluene, and radioactive activity was determined in a liquid scintillation counter.

Proteins were determined by the procedure of Lowry et al. (9) with bovine serum albumin as a standard.

Virus was isotopically labeled by growing Mm5mt/c1 cells for 48 h in the presence of [3H]thymidine (5 μCi/ml). Culture fluids were processed as above, and the virus pellets were resuspended in TNE buffer then layered over 20 to 55% continuous sucrose gradients. The virus was sedimented to buoyant density equilibrium by centrifugation at 80,000 g in a Beckman SW41 rotor. The gradients were fractionated by puncture of the tube bottom and the radioactivity was determined by assaying 0.4 ml of each fraction.

Scale-up and purification of MMTV. Scale-up of Mm5mt/c1 cells from seed ampoules is shown in Fig. 1. For production, the Mm5mt/c1 cells were propagated in roller bottles (Bellco, Vineland, N.J.). Small (10 by 25 cm) and large (10 by 50 cm) bottles were seeded with 1.5 × 10^6 cells and 3.0 × 10^6 cells, respectively. Bottles were incubated at 34 C on roller racks (0.5 rpm) and medium was changed every 3 days (125 ml/small roller bottle and 250 ml/large roller bottle). Spent medium was stored at −70 C until used. The medium was thawed, clarified at 8000 × g (100 ml/min) in a continuous-flow Sorvall SS-4 centrifuge with the Szent-Gyorgi and Blum system, and banded on a 20 to 60% continuous sucrose gradient with an RK ultracentrifuge using an RK-3 rotor (Electronucleonics, Inc., Rockville, Md.) at 90,000 × g. Equivalent results were obtained when clarification and concentration were performed concurrently using an RK-6 rotor at 90,000 × g. The gradient was unloaded, monitored at 260 and 280 nm, and the refractive index of each 50 ml fraction was determined. Those fractions which absorbed at 260 and 280 nm and had a density of 1.15 to 1.19 g/ml were pooled, diluted to 2% sucrose with TNE, and pelleted at 100,000 × g for 1 h in a Beckman L2-75B centrifuge using a type 35 rotor. The pellets were resuspended at either 100 or 1,000 in TNE and frozen at −70 C.

RESULTS

Stimulation of MMTV production by DXM versus IUdR plus dexamethasone. Subsequent to our observation (3) that DXM enhanced production of MMTV in IUdR-stimulated Mm5mt/c1 cultures, the requirement for IUdR in MMTV stimulation was determined. Replicate 75-cm² Falcon flasks were treated with DXM 48 h postseeding. Half of these cultures were then treated with IUdR (20 μg/ml) for 48 h after addition of DXM. Twenty-four hours later the medium from both the DXM-treated and IUdR/DXM-treated cultures was replaced with fresh DXM-treated medium. Control cultures were untreated except for medium change concurrent with the treated cultures. Supernatant fluids (approximately 100 ml) were collected on days 3 and 5 after IUdR treatment and assayed for numbers of virus particles and presence of RDDP as described in Materials and Methods. The results of those assays are presented in Table 1. On the basis of these results, it was concluded that IUdR is not required for production of MMTV in DXM-treated cultures. Furthermore, no enhancement was obtained with IUdR over that of DXM alone. These findings agree with those of Parks et al. (15).

Stability of DXM in growth medium. Medium containing DXM was stored at 4 C and checked for virus stimulatory activity over a 2-month period. Medium with freshly added DXM served as a control (Fig. 2). No significant differences in membrane immunofluorescence, particulate RDDP activity was noted with fresh DXM as compared to aged DXM. Similarly, while no difference was observed in virus particle count in fresh or 6-week-aged DXM, a slightly higher particle count (0.7 log₁₀) was observed in fresh as compared to 9-week-aged DXM. Although this difference might be attributed to breakdown of DXM due to storage, it is just as likely accounted for by experimental error in sampling and electron microscope preparation and counting. These data indicate that, in an MMTV production scheme, growth medium containing DXM could be prepared in advance and stored at least 9 weeks before use.

Effect of cell seeding in the presence of

Fig. 1. Flow diagram illustrating scale-up of Mm5mt/c1 cells from seed ampoule to roller bottle cultures.
Table 1. Comparison of DXM and IUdR/DXM treatment on stimulation of virus particles and virus-related RDDP in Mm5mt/c1 cell cultures

| Treatment* | RDDP* activity (pmol/ml/h) | Virus particles/ml³ |
|------------|-----------------------------|----------------------|
|            | Day 3 | Day 5 | Day 3 | Day 5 |
| DXM        | 55.2  | 196.5 | 1.9 × 10⁵ | 4.4 × 10⁵ |
| IUdR plus DXM | 70.0 | 148.5 | 9.3 × 10⁵ | 4.3 × 10⁵ |
| Control    | 0.5   | 0.9   | 1.4 × 10⁴ | Not done |

* DXM (10⁻⁴ M), added 48 h postseeding; IUdR (20 μg/ml, 24 h), added 48 h after DXM; control-untreated.

- RDDP activity using exogenous incorporation with oligo dG·poly rC template.
- Particle counts performed on ×100 concentration of culture fluid.
- Days after addition of IUdR.

![Fig. 2. Stability of DXM in growth medium: effect on stimulation of MMTV production in Mm5mt/c1 cell cultures.](http://aem.asm.org/)

**DXM.** Cells were seeded in the presence and absence of DXM and allowed to attach for 24 h at 37 C. The spent medium was removed and replaced with medium containing DXM. Replicate flasks were assayed at 2-day intervals post-seeding. As demonstrated in Fig. 3, the presence of DXM at the time of cell seeding had no effect on either cell growth, particulate RDDP activity, or virus particle production in culture fluids. Higher RDDP activities and virus particle counts were observed on days 5 and 7 in cultures seeded in the presence of DXM than in those seeded in the absence of DXM, suggesting an earlier response of MMTV production in the former cultures. These results indicated that, in a production scheme, the DXM could be included in the growth medium at the time of cell seeding, thus eliminating a procedural step of adding DXM to the cultures after cell attachment.

**Scale-up of MMTV in roller bottles.** Mm5mt/c1, cells from 75-cm² flasks were seeded in small roller bottles and incubated in the presence of DXM until confluent. During this time cell numbers increased from 1.5 × 10⁷ to 1.8 × 10⁸ per bottle. Supernatant fluids were harvested and processed for virus particles and particulate RDDP (data not shown). Cells were trypsinized and a portion was assayed for cell-associated MMTV-specific antigens by membrane immunofluorescence. The remaining cells were then transferred, in scale-up, to 20 large roller bottles and incubated in medium containing DXM. Medium was changed at 3-day intervals and pooled before concentration and purification as described in Materials and Methods. The results of this production run are presented in Table 2.

![Fig. 3. Presence of DXM in growth medium at time of cell seeding: effect on cell growth, RDDP and MMTV production.](http://aem.asm.org/)
A major peak with a 260-nm absorbance was found in the sucrose gradient with a density of 1.156 to 1.189 g/ml (Fig. 4). This region, when fractionated, contained appreciable particulate RDDP activity (Table 2). Significantly high incorporation was observed with the dG·rC template, considered more specific for virus-related RDDP (20), whereas low incorporation was observed with the oligo dT template, indicating low terminal transferase activity (11). The ×1000 concentrated material contained 0.7 mg of protein per ml.

A duplicate sample of this material was found to contain 7.6 × 10^10 virus particles per ml. Comparable particle counts were obtained in another production run of 6.5 liters (Table 2). Examination of negative-stained preparations (Fig. 5) revealed pleomorphic-tailed particles, having surface spikes, measuring 105 nm in average diameter, and typical of the B-type particles described in mouse milk (10, 13, 19).

**Density of MMTV produced in vitro.** Material collected from supernatant fluids from 7-day DXM-treated Mm5mt/c1 cells incubated in the presence of [3H]uridine was found to contain a peak of radioactivity at the 1.16-g/ml region of a sucrose gradient (Fig. 6). Material contained in this fraction was sensitive to digestion with ribonuclease, indicating the presence of ribonucleic acid. No significant radioactivity was observed in the 1.16-g/ml region of the gradient in supernatant material from untreated Mm5mt/c1 cells.

**Immunological reactions of MMTV produced in vitro.** To determine whether MMTV produced from Mm5mt/c1 cells contained antigens common to MMTV from mouse milk or the major group-specific antigen of murine leukemia virus, immunodiffusion tests were performed. Antisera to MMTV and a 27,000-molecular-weight peptide (p27) isolated from MMTV virus obtained from R111 mouse milk (supplied by Wade Parks) and antisera against murine (Rauscher) leukemia virus (RLV) p30 (gs) (supplied by D. Fish) were tested against lysed MMTV produced from Mm5mt/c1 cells. A single precipitin line (Fig. 7) was observed between the antiserum to MMTV p27 and the MMTV from Mm5mt/c1 cells. Whereas a precipitin line formed between RLV p30 and the corresponding antiserum to RLV p30, no line of precipitation was observed between the tissue culture-produced MMTV and the antiserum to RLV p30.

Virus samples were sent to D. Bolognesi for estimation, by radioimmunoassays, of possible murine leukemia virus proteins (p30 and gp 71). Bolognesi reported the results of the assay for the presence of gp 71 as negative and only a slight blocking (2 ng/10 μlifiers of sample) was noted for the presence of the p30 protein. Discrepancies between the p30 and gp 71 radioimmunoassays may be based on the degree of specificity of the respective proteins.

**DISCUSSION**

The results presented here form the basis for a protocol which can be used in other laboratories for intermediate- or large-scale tissue culture production of MMTV. Monolayer cultures of mammary tumor cells, e.g., the Mm5mt/c1 culture, are easily propagated in roller bottles. In addition, ease of implementation of the protocol is based on the fact that the MMTV-stimulating component DXM can be added to

![Image](https://example.com/image.png)

**Fig. 4. Isopycnic banding of MMTV produced from Mm5mt/c1 cell cultures measured by optical density at 260 nm.**

| Production lot | Culture volume (liters) | Particle counts/ml | Total protein (mg/ml) | RDDP activity (pmol/ml per h)* |
|---------------|------------------------|-------------------|----------------------|-----------------------------|
| 1             | 6.5                    | 1.9 × 10^11       | 0.8                  | dG·rC 15,250  dT·rA 1,760  |
| 2             | 22                     | 7.6 × 10^10       | 0.7                  |                                           |

* Data expressed per milliliter of ×1,000 concentrates.
growth medium at the time of cell seeding, without effecting a change in either cell growth or virus production. Similarly, DXM is stable in refrigerated growth medium, and can be prepared in advance of virus production. Supernatant culture fluids are clarified by centrifugation and subsequently concentrated and purified by isopycnic centrifugation. The resultant virus preparations are relatively free of extraneous cellular debris and membraneous material normally found in mouse milk preparations (1). In addition, the system growth medium at the time of cell seeding, without effecting a change in either cell growth or virus production. Similarly, DXM is stable in refrigerated growth medium, and can be prepared in advance of virus production. Supernatant culture fluids are clarified by centrifugation and subsequently concentrated and purified by isopycnic centrifugation. The resultant virus preparations are relatively free of extraneous cellular debris and membraneous material normally found in mouse milk preparations (1). In addition, the system growth medium at the time of cell seeding, without effecting a change in either cell growth or virus production. Similarly, DXM is stable in refrigerated growth medium, and can be prepared in advance of virus production. Supernatant culture fluids are clarified by centrifugation and subsequently concentrated and purified by isopycnic centrifugation. The resultant virus preparations are relatively free of extraneous cellular debris and membraneous material normally found in mouse milk preparations (1). In addition, the system
described here has one major advantage over that of milk-borne virus, namely absence of detectable type-C virus particles. The problem of contaminating type-C virus was noted in a report by Howk et al. (4) where milk from R111 mice contained two distinct RDDPs, one of which corresponded immunologically as well as biochemically to the enzyme from type-C virus. Similarly, in a recent study in which MMTV antigens were purified from R111 mouse milk, Parks et al. (16) state, "The goat antiserum prepared against MMTV p27 showed significant cross-reaction with gp 52 (SI) and a low level reaction with MuLV p30. This was most likely due to contamination of MMTV preparations with murine type-C virus." These studies show the need for MMTV, free of type-C virus, for viral-component antisera preparation. In the initial characterization of the Mm5mt/c1 culture, immunological as well as electron microscope examinations failed to reveal any type-C virus in the cells (14). Data presented here, in addition to electron microscope examination, fail to demonstrate the presence of type-C virus in either the Mm5mt/c1 cells or virus product.

Quantitatively, Mm5mt/c1-produced MMTV compares morphologically, immunologically, and biochemically with type-B particle preparations from MMTV bearing mouse milk. Furthermore, type-B RDDP purified from Mm5mt/c1-produced virus exhibits biochemical characteristics (L. O. Arthur, D. L. Fine, and A. Bandyopadhyay, Abstr. Annu. Meet. Amer. Soc. Microbiol., 1974, V280, p. 247) common to type-B RDDP purified from R111 mouse milk (4). Quantitatively, virus particle counts performed on the tissue culture-produced MMTV when concentrated at $1,000$ from 22 liters of culture fluid were $7.6 \times 10^{14}$ per ml. Moore et al. (13) reported $7.5 \times 10^{11}$ virus particles per ml from pooled R111 mouse milk purified on gradients prepared from 60% sucrose. BALB/c$\times$C3H mouse milk contains $10^{11}$ virus particles per ml, based on recoverable protein after sequential rate zonal and isopycnic sucrose banding (Aldridge, personal communication). Thus, it appears that 1 liter of supernatant fluid from DXM-stimulated Mm5mt/c1 cell cultures contains approximately the same number of MMTV particles as 1 ml of mouse milk.

Although the system described here was developed using a C3H mouse-derived mammary tumor culture, it should be feasible for use with continuous mammary tumor lines derived from other strains of mice. Parks et al. (15) demonstrated DXM enhancement of MMTV production in L8A Cl 11 cells cloned from CCL-51 cultures originally derived from a (C3HBL X A)F1 mammary tumor (21). Continuous tumor cell lines have the distinct advantage over tumor explant cultures in that cloning can be accomplished to derive high virus-producing lines.

Studies are in progress to determine whether the virus produced from Mm5mt/c1 cells is infectious for either mice or murine cell cultures. In preliminary experiments in which weanling female BALB/c mice were inoculated with serial dilutions ($10^{-1}$ to $10^{-4}$) of Mm5mt/c1-derived virus, 5 of 10 mice demonstrated mammary tumors within 9 months postinoculation. The average incidence of spontaneous tumors in the BALB/c colony is 3% over a period of 15 months.

![Fig. 6. Incorporation of $[^H]$uridine into particles banding in sucrose in Mm5mt/c1 cell cultures treated with DXM, day 7 post-treatment.](http://aem.asm.org/)

![Fig. 7. Agar-gel double diffusion demonstrating precipitin reactions of MMTV. The center well (A) contains lysed MMTV; well 1 contains anti-MMTV p27; well 2 contains RLV p30; well 3 contains anti-RLV p30; wells 4 and 5 are empty.](http://aem.asm.org/)
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