A Fast Replica Plating Technique for the Isolation of Post-integration Mutants of the Moloney Strain of Murine Leukaemia Virus

By R. Rude, Gary E. Gallick and P. K. Y. Wong

Department of Microbiology and School of Basic Medical Sciences, University of Illinois, Urbana, Illinois 61801, U.S.A.

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SUMMARY

Seven temperature-sensitive (ts) mutants of Moloney murine leukaemia virus (Mo-MuLV) were isolated using a rapid, non-selective replica plating technique designed to select for post-integration mutants. Thymus-bone marrow (TB) cells, infected with mutagenized virus, were cloned and incubated at the non-permissive temperature (39 °C) for 10 days. The resulting colonies were screened for production of virus by replica plating supernatant from the 'master' tray on to a second tray pre-seeded with fu-i (a cell line derived from L8 myoblasts) indicator cells. The 'master' tray was shifted to the permissive temperature (34 °C) for 48 h, then re-screened for virus production. Any colony on the 'master' tray which produced syncytia-inducing virus at 34 °C but not at 39 °C was potentially producing a ts mutant. Preliminary characterization by shift-down experiments and scanning electron microscopy of three of the ts mutants isolated by this technique revealed a mutant blocked before budding, one blocked at an early stage in the budding process and one with a defect after release of the virus.

INTRODUCTION

Temperature-sensitive (ts) mutants are ideally suited to investigate the complex sequence of events which occurs during the replication of leukaemia viruses because they allow comparative studies of a defective function at a permissive and non-permissive temperature, thus permitting the determination of the role of a virus protein in the replicative cycle. However, ts mutants of animal viruses have proven relatively difficult to obtain and thus few are being currently studied. In the murine leukaemia/sarcoma virus system, while mutants have been isolated in the Kirsten (Scolnick et al. 1972), Moloney (Wong et al. 1973) and Rauscher (Stephenson et al. 1972) systems, they are relatively free. A major problem in the isolation of ts mutants stems from the necessity of screening large numbers of clones and the time required to test each clone. The methods of Stephenson et al. (1972) and Wong et al. (1973) offer a means of screening for a wide variety of mutants, but require 30 days or longer. In this paper, we report a microtitre replica plating system for the isolation of post-integration ts mutants which permits screening large numbers of colonies and subsequent isolation of ts mutants in only 12 days. The speed of this technique derives from the sensitivity and rapid syncytia formation of fu-i indicator cells when directly infected with Moloney leukaemia virus. With the methodology described in this paper, we have isolated seven ts mutants which are blocked before and at various stages during the budding process.
METHODS

Cell cultures. Virus was propagated in TB cells, a thymus–bone marrow cell line derived from CFW/D mice (Ball et al. 1964). Cell lines used for the assay of virus were: 15F cells, a non-transformed, sarcoma-positive, leukaemia-negative cell derived from the infection of TB cells with Moloney sarcoma virus (Ball et al. 1973), fu-1 cells, a non-fusing line from rat L8 myoblast cells (Kaufman, 1975), and XC cells, a rat cell line harbouring a Rous sarcoma virus genome (Klement et al. 1969). All cell lines were grown in Eagle’s minimal essential medium supplemented with 10% foetal calf serum (Grand Island Biological Co., Grand Island, N.Y., U.S.A.), 50 international units (iu)/ml penicillin and 50 µg/ml streptomycin. Cultures were maintained in 75 cm² tissue culture flasks at a seeding density of 1 × 10⁶ cells/flask in 12 ml of complete medium.

Virus assays. The 15F assay used for virus titre is a modification of the focus-forming assay described by McCarter (1977). 15F cells were seeded in 60 mm Petri dishes with 1 × 10⁶ 15F cells/plate 18 h prior to infection. Virus samples to be assayed were diluted in 28 µg/ml DEAE-dextran in base medium. Then 0·4 ml of the virus dilutions were allowed to adsorb for 30 min at 34 °C after which the medium in the plate was replenished. Maximum focus formation required 5 days incubation at 34 °C. Titre was expressed in focus-forming units/ml (f.f.u./ml).

Procedures for the monolayer fu-1 and XC syncytial assays have been previously described in detail (Wong et al. 1977). Briefly, overnight cultures of either XC or fu-1 cells were prepared by seeding 35 mm Petri dishes with 1 × 10⁶ cells/plate. The virus samples were diluted 1:2 in 28 µg/ml DEAE-dextran in base medium. Adsorption was allowed to proceed for 40 min at 37 °C. Unadsorbed virus was aspirated and 2 ml of complete medium was added to the culture plates. Cells were fixed and stained after 6 h incubation at 34 °C. Multinucleated cells (containing three or more nuclei) were counted in four randomly selected fields. Fields from triplicate plates were counted and titre is expressed as number of syncytia/cm².

Mutagenization of virus stock. Bromodeoxyuridine (BrdUrd; Calbiochem, Los Angeles, Calif., U.S.A.) was used to mutagenize murine leukaemia virus (MuLV) in a procedure modified from the method of Stephenson et al. (1972). Infected cultures of MuLV were treated with 50 µg/ml of BrdUrd (a concentration of mutagen which reduced the yield of infectious virus 100-fold) for 12 h, after which supernatant was harvested. The mutagenized virus stock was used for isolation of ts mutants.

Preparation of cell cultures for scanning electron microscopic (SEM) examination. TB cells were infected in suspension with either wild-type (wt) or mutant virus at an m.o.i. of 2. Uninfected control and infected cells were seeded on to glass coverslips (18 mm diam.) contained within 35 mm Petri dishes. At designated times, the coverslips were washed with phosphate-buffered saline (PBS) and fixed in 4% glutaraldehyde. After fixation, the coverslips were washed with PBS, dehydrated through a graded ethanol series, then critical point dried. The specimens were coated with gold (2 min sputter) and were examined in a JSM-U3 electron microscope (30° specimen tilt, 25 kV).

RESULTS

Determination of the optimal conditions for maximal syncytia formation

To develop an efficient system which would allow mass screening of large numbers of infected clones for ts mutants, a rapid and sensitive method for determination of virus production was required. MuLV-induced syncytia formation in fu-1 cells offered such a method. Syncytia formation in these cells is near maximal in 4 to 6 h and is two to four
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Table 1. Comparison of titres of ts mutants when assayed at the permissive temperature (34 °C) and at the non-permissive temperature (39 °C)

<table>
<thead>
<tr>
<th>Mutant</th>
<th>34 °C* (f.f.u./ml)</th>
<th>39 °C (f.f.u./ml)</th>
<th>Ratio 34/39 °C titres</th>
</tr>
</thead>
<tbody>
<tr>
<td>wt Mo-MuLV</td>
<td>2.47 × 10⁶</td>
<td>1.75 × 10⁶</td>
<td>1.41</td>
</tr>
<tr>
<td>ts 10</td>
<td>9.85 × 10⁶</td>
<td>2.5 × 10⁵</td>
<td>1.97 × 10⁵</td>
</tr>
<tr>
<td>ts 11</td>
<td>3.15 × 10⁶</td>
<td>5.0 × 10⁵</td>
<td>6.3 × 10⁵</td>
</tr>
<tr>
<td>ts 12</td>
<td>1.13 × 10⁶</td>
<td>5.0 × 10⁵</td>
<td>4.22 × 10⁵</td>
</tr>
<tr>
<td>ts 13</td>
<td>4.0 × 10⁵</td>
<td>1.0 × 10⁴</td>
<td>8.0 × 10⁴</td>
</tr>
<tr>
<td>ts 14</td>
<td>9.5 × 10⁵</td>
<td>5.0 × 10⁴</td>
<td>1.9 × 10⁵</td>
</tr>
<tr>
<td>ts 15</td>
<td>7.73 × 10⁴</td>
<td>5.0 × 10³</td>
<td>1.55 × 10³</td>
</tr>
<tr>
<td>ts 16</td>
<td>3.0 × 10⁴</td>
<td>5.0 × 10²</td>
<td>6.0 × 10³</td>
</tr>
</tbody>
</table>

* Supernatants from infected clones grown at 34 °C were assayed for infectivity at the temperature indicated by the 15F f.f.u. assay as described in the text.

Times as sensitive as syncytia formation in XC cells (Wong et al. 1977). The optimal number of fu-1 cells for maximum syncytia formation in microtitre wells was established as 6 × 10³ fu-1 cells/well. Optimum syncytia production from infected TB clones occurred at 9 days p.i. By 12 days, cell death in the colonies of the ‘master’ plates became significant and yields of virus were reduced.

General procedure for the isolation of ts mutants using microtitre replica plating

Cells infected with BrdUrd-mutagenized MuLV at an m.o.i. of 0.8 were seeded into 96-well microtitre trays at a density of one cell/well. The low m.o.i. and cell density were needed to ensure that each colony was likely to be derived from a single cell infected with only one infectious virus particle. These microtitre ‘master’ trays were incubated for 10 days at the non-permissive temperature (39 °C). Each resulting colony was then screened for virus production by replicate plating of 0.025 ml supernatant from each well of the ‘master’ tray into another microtitre tray (‘indicator’ tray) pre-seeded overnight with 6 × 10³ fu-1 cells/well. The indicator tray was scored for syncytia production after 6 h incubation at 34 °C. The medium in the ‘master’ tray was replenished and that tray was then transferred to the permissive temperature (34 °C).

Following 48 h incubation at 34 °C, 0.025 ml from each well of the ‘master’ tray was again transferred to another microtitre ‘indicator’ tray seeded with fu-1 cells. This ‘indicator’ tray was also scored for syncytia after 6 h incubation at 34 °C. Any well (corresponding to a colony on the ‘master’ tray) which produced syncytia in the corresponding well in the indicator tray after 48 h incubation at 34 °C, but had failed to do so at 39 °C, represented a colony producing a putative ts mutant. Supernatants from such colonies in the ‘master’ tray were collected and the colonies themselves trypsinized and transferred to 35 mm tissue culture plates. The presumptive mutant-infected cells were then grown and virus-containing supernate harvested and titred at 34 and 39 °C to confirm virus temperature-sensitivity further.

Temperature-sensitivity of mutants isolated

Using the above procedures, seven ts mutants were isolated from 518 clones scored as positive for virus production by the fu-1 syncytia assay. Each of the seven mutants was recloned to ensure its stability and the recloned virus was assayed by the 15F direct focus assay at 34 and 39 °C. Results of these assays are shown in Table 1.

The results presented in Table 1 indicate that most of the mutants appear to be extremely
Fig. 1. Temperature shift-down of ts 10. Ts 10-infected cultures were grown for 2 days at 39 °C (the non-permissive temperature) in 75 cm² tissue culture flasks for infectivity assays or on coverslips for scanning electron microscopy examination, then shifted to 34 °C (the permissive temperature). (a) SEM of the infected cell surface at the time of shift. (b) SEM of the infected cell surface 30 min after shift. Representative virus is indicated by arrows. (c) Release of infectious virus after shift-down from the non-permissive temperature (39 °C) to the permissive temperature (34 °C) (●—●). Infectivity was measured by the 15F f.f.u. assay as described in Methods. Unshifted controls were maintained at 39 °C (□—□).
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Fig. 2. Temperature shift-down of ts 11. (a) SEM of the cell surface of ts 11-infected cultures at 39 °C at the time of shift. Early budding virus (small protuberances) is indicated by black arrows, late budding virus (larger, more 'completed' particles) by white outlined arrows. (b) SEM of the cell surface of ts 11-infected cultures 10 min after shift to 34 °C. (c) Release of infectious virus after shift-down from 39 °C to 34 °C (○—○). Infectivity was measured by the 15F f.f.u. assay as described in Methods. Unshifted controls were maintained at 39 °C (□—□).

'non-leaky', with 34/39 °C infectivity ratios in excess of 100, compared to the wild-type MuLV 34/39 °C infectivity ratio of 1.4.

Time of expression of the ts defect

To examine the stage of the replicative cycle at which the mutants were defective, virus release following shift-down of three of the ts mutants was monitored by both SEM and the 15F f.f.u. assay. TB cells were infected with either ts 10, ts 11 or ts 16 at an m.o.i. of 2
as described in Methods. Infected cells were grown on coverslips for SEM examination, or on 75 cm² tissue culture flasks for infectivity assays. Two days p.i. the cultures were transferred to 34 °C. At the time of the shift and at various times post-shift, coverslips were fixed and processed for SEM examination and samples from the flasks were filtered and assayed for infectivity as described in Methods.

The results of the temperature shift-down for ts 10 appear in Fig. 1. No virus particles were observed by SEM on the cell surface prior to the time of the shift (Fig. 1a). However, 1 h after shift to the permissive temperature, virus particles were observed on the cell surface (Fig. 1b). This appearance of virus particles on the cell surface corresponded to a rapid increase in infectious particles in the supernatant 1 h after shift-down as measured by 15F f.f.u. assay (Fig. 1c). No infectious particles were detected in ts 10 control cultures maintained at 39 °C. These results suggest that the ts 10 defect occurs shortly before the budding process.

The results of the shift-down experiment for ts 11 appear in Fig. 2. By SEM, primarily 'early budding' particles with a few 'late budding', (i.e. more mature particles) were observed on the cell surface of 39 °C cultures (Fig. 2a). These early budding particles became late budding particles within 10 min after shift to the permissive temperature (Fig. 2b). By 20 min post-shift, most of the virus particles were released from the cell surface. The release of the virus from the cell surface upon shift-down was accompanied by a rapid increase of infectious virus appearing in the supernatant (Fig. 2c). The rapid conversion of the early budding particles to late budding particles after shift to the permissive temperature suggests that the ts 11 defect occurs at an early stage in the budding process.

Results of temperature shift-down experiments with ts 16 appear in Fig. 3. SEM revealed
the presence of budding virus on infected cell surfaces at 39 °C (Fig. 3a). Similar numbers of virus particles were observed on the cell surface after shift (data not shown). Infectivity assays demonstrated that ts 16 cultures maintained at 39 °C released some infectious virus; however, 2 h after shift, the level of infectious virus released in the shifted cultures was 80-fold more than in the unshifted control cultures (Fig. 3b). Although ts 16 is released at the non-permissive temperature, the virus produced at that temperature is extremely heat labile, with a half-life at 39 °C of 10 min (unpublished data). Therefore, while infectivity was detected by the 15F f.f.u. assay in 39 °C cultures of ts 16, the accumulation of virus at that temperature was insufficient to form syncytia in fu-1 cells.

DISCUSSION

The isolation of ts mutants of leukaemia and sarcoma viruses is time consuming and difficult. To alleviate these problems, we have devised a method which allows for the selection and isolation of post-integration ts mutants of Moloney leukaemia virus within 12 days. The technique is limited to post-integration mutants because infected clones are incubated at the non-permissive temperature; thus synthesis and/or integration into the cellular genome of a DNA copy of a putative early ts mutant would not occur. Despite this limitation, the ts mutants which were selected by our technique represent a broad class, encompassing events in the virus replicative cycle from before budding to after release of virus progeny. Furthermore, while the assembly, budding, maturation and release processes comprise a majority of the events in the life cycles of RNA tumour viruses, very few mutants are currently available to assist in understanding these events.

The only ts mutants of mammalian RNA tumour viruses which have been partially characterized occur in the Moloney (Wong & McCarter, 1974) and Rauscher (Stephenson et al. 1972) systems. The post-integration mutants in the Moloney system are ts 3 (Wong & McCarter, 1974), which is blocked at a late stage in the budding process (Wong & McCleod, 1975) and ts 7 (Wong & Gallick, 1978) which is blocked in an event after the release of virus (G. E. Gallick & P. K. Y. Wong, unpublished data). Among the partially characterized Rauscher mutants, ts 26 is blocked in the synthesis of envelope glycoprotein message (Ruta et al. 1979), ts 24 is blocked in an early budding event (Yeger et al. 1976) and ts 29 is blocked in a late budding stage (Yeger et al. 1976). The usefulness of ts mutants in delineating features of the leukaemia virus replicative cycle has been demonstrated by studies on ts 3, which has been employed in examining both the extracellular processing of pr 65 gag (Witte & Baltimore, 1978), and other maturation events associated with the conversion of 'immature' to 'mature' virions (Lu et al. 1979). Nevertheless, the budding, release and maturation events of RNA tumour viruses are extremely complex and the isolation and characterization of many more ts mutants will be helpful in the elucidation of the mechanisms and virus gene products involved in the processes. The method for the isolation of ts mutants presented in this paper has resulted in the successful isolation of mutants blocked in different stages of the replicative cycle and offers the potential for obtaining additional mutants with a variety of defects.

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REFERENCES


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