Isolation of Temperature-Sensitive Mutants of Murine Sarcoma Virus

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Three separate murine sarcoma virus nonproducer cell lines have been isolated which are temperature sensitive for the maintenance of transformation. In each case, a viral rather than a cellular genetic mutation is the reason for the temperature-sensitive effect. Superinfection of one of the mutants with murine leukemia virus overcomes the temperature-sensitive change in the transformed state.

Of the type C ribonucleic acid (RNA) tumor viruses of mammalian origin, murine sarcoma virus (MSV) and murine leukemia virus (MuLV) have been most extensively studied. MuLV causes leukemias in vivo (10) and replicates in tissue culture without causing morphologic alteration of cells. MSV is known to induce solid tumors in animals (17) and to produce foci of transformed cells in tissue culture (11). Recent studies indicate that MSV lacks certain function(s) required for its replication; MSV-transformed cells have been isolated which lack any evidence of virus replication (1, 12). The MSV genome can remain associated with such transformed cells through many hundreds of cell generations (21) and can be rescued by addition of MuLV (1, 12).

Although several avian sarcoma temperature-sensitive (ts) mutants have been reported (5, 7, 8, 13–16, 22), ts mutants of MSV have not yet been obtained. In addition to the usefulness of obtaining ts mutants in transforming functions of a mammalian sarcoma virus, the ability to obtain nonproducer transfromants containing ts sarcoma genomes would afford the opportunity to study solely those viral functions affecting transformation in the absence of virus production. The present report describes the isolation of conditional mutants of this nature.

MATERIALS AND METHODS

Cells and virus. Cells were grown in Dulbecco's modification of Eagle's medium supplemented with 10% calf serum (Colorado Serum Co.). The clonal cell lines used included BALB/3T3, a normal rat kidney cell line (NRK), and a Kirsten MSV (KiMSV)-transformed nonproducer NRK cell line, K-NRK (3). Clonal isolates of KiMSV(KiMuLV) and KiMuLV were used in all studies.

Mutagenized virus stocks. Stocks of mutagenized KiMSV were prepared by either of two methods. For mutagenesis with bromodeoxyuridine (BrdU), NRK cells were infected at 32°C with KiMSV(KiMuLV) at a multiplicity of 0.1 to 1.0, followed by the addition within 1 hr of BrdU at 100 μg/ml. After 24 hr the medium was changed, and supernatant fluids were harvested 3 days later. For mutagenesis with azacytidine, chronically infected cultures were exposed to azacytidine (5 μg/ml) for 48 hr. In each case, at the indicated concentrations, drug treatment resulted in approximately a 100-fold reduction in the sarcoma virus titer. These mutagenized stocks were used in the experiments described below.

Virus assays. Sarcoma virus assays were performed as previously described (1, 11). Assays at 37 or 40°C were scored at 7 days, whereas assays at the permissive temperature (32°C) generally were scored at 12 to 14 days. The XC plaque test was performed as previously described (19).

RNA-DNA hybridization. The 3H-deoxyribonucleic acid (DNA) product from a virus preparation containing KiMSV(KiMuLV) was prepared from an endogenous viral reverse transcriptase reaction. Reaction mixtures of from 2.0 to 4.0 ml were incubated at 37°C for 1.0 hr and contained 0.04 M tris(hydroxymethyl)aminomethane (Tris)-hydrochloride, pH 7.8; 0.06 M potassium chloride; 2×10⁻⁴ M dithiothreitol; 6×10⁻³ M magnesium chloride; 0.014% (v/v) Triton X-100; 10⁻⁴ M deoxyctydine, deoxyguanosine, and deoxyadenosine triphosphates; and 10⁻⁴ M 3H-thymidine triphosphate (18 Ci/mmol). The DNA product was deproteinized by phenol extraction (9), and the RNA was removed by hydrolysis at 41°C with 0.5 N NaOH for 16 hr. After dialysis against 0.01 M Tris-hydrochloride (pH 7.2) and 10⁻³ M ethylenediaminetetraacetic acid (EDTA), the product was stored at −20°C until use.

Hybridization was carried out with approximately 2,000 trichloroacetic acid-precipitable counts of DNA at 41°C for 24 hr in 0.20-ml reactions containing: 0.02 M Tris-hydrochloride, pH 7.2; 38% (v/v) formamide; 0.15 M sodium chloride; 10⁻³ M EDTA; and RNA as indicated in Table 3. Hybridization was
measured by the use of nuclease S1 (4), an enzyme which degrades single-stranded DNA but not RNA-DNA hybrids or double-stranded DNA. Further details will be described elsewhere (R. Benveniste and E. M. Scolnick, manuscript in preparation).

RESULTS

Isolation of ts MSV-transformed nonproducer cells. The methods employed for obtaining MSV mutants were based on a microtiter procedure recently developed for the isolation of MuLV mutants (20). Petri dishes containing 10^6 NRK cells were infected with approximately 50 focus-forming units (FFU) of mutagenized virus. After 6 hr of incubation at 32 C, the cells were trypsinized and transferred to Falcon microtest II plates at 500 cells per well. This resulted in approximately 25% of the wells receiving one sarcoma virus-infected cell. Cultures were incubated at 32 C with weekly change of medium. At 14 to 21 days, when foci of transformed cells became visible, the medium was removed from each well by means of a multiple aspirator device (20), and 0.05 ml of 0.1% trypsin was added to each well. Plates were incubated at 37 C for 30 min, and the cells were then replated to pairs of recipient microtest plates. One plate of each pair was incubated at 32 C and the other at 39 to 40 C.

After 5 to 7 days of incubation at 39 to 40 C, or 10 to 14 days at 32 C, the recipient plates were scored for transformed foci. Where corresponding wells contained morphologically transformed cells at 32 C but not at 39 to 40 C, the cells from the 32 C wells were trypsinized and cloned. The morphological appearance of these clonal lines was subsequently reexamined at the permissive and nonpermissive temperatures. Out of approximately 3,000 individual transformants screened by this procedure, three nonproducer lines were isolated and cloned which were transformed in appearance at 32 C but which reverted to normal morphology when grown at 39 to 40 C. The first two of these lines, ts 1 and ts 2, were isolated from stocks of BrdU-mutagenized virus; ts 3 was obtained from an azacytidine-mutagenized stock.

As was previously found with other KiMSV-transformed nonproducer clones, each of the three potential mutant virus-transformed clones was found to lack any evidence for spontaneous virus release. Furthermore, none showed detectable murine or rat viral group-specific antigens.

Rescue of ts sarcoma viral genome from nonproducer cells. Studies were carried out to determine whether the temperature sensitivity of the three ts MSV-transformed nonproducer clones was due to mutation of viral rather than of cellular gene(s) required for maintenance of transformation. Examples of the latter type have been described with DNA virus (6, 18) as well as RNA virus-transformed cells (21). To resolve these possibilities, each potential mutant and a wild-type (wt) nonproducer clone line were infected at 32 C with KiMuLV at a multiplicity of 1; 25 days later, the supernatant fluids were harvested, filtered, and titrated for focus-forming ability on NRK cells at 32 C and 39 to 40 C. As shown in Table 1, MSV rescued from the wt MSV nonproducer line titered 10^{2.1} FFU/ml at 32 C and 10^{2.6} FFU/ml at 39 to 40 C. The sarcoma viruses rescued from ts 1 and ts 3 cloned transformed lines produced relatively low levels of focus-forming virus at 32 C (10^{2.4} to 10^{2.8} FFU/ml). Under the same conditions, these rescued viruses did not produce any foci at the nonpermissive temperature. MSV rescued from ts 2 was approximately 30-fold less efficient at focus formation at 39 to 40 C than at 32 C. The results are consistent with the assumption that the temperature sensitivity of the transformed morphology of the three ts lines was due to temperature sensitivity of a viral rather than cellular gene product.

The genetic stability of two of the ts sarcoma viruses was tested by transmission of MSV rescued from each of the clonal lines, selection of new focus-derived lines from foci produced at 32 C, and cloning of these to establish new lines.

The cell morphology of the new transformants was observed at 31 C and 39 to 40 C. Three transformed lines each of NRK cells produced

<table>
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<tr>
<th>Table 1. Titration of MSV rescued from wild-type (wt) and temperature-sensitive (ts) MSV-transformed nonproducer cellsa</th>
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<td>Nonproducer cells superinfected</td>
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<tr>
<td></td>
</tr>
<tr>
<td>wt</td>
</tr>
<tr>
<td>ts 1</td>
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<tr>
<td>ts 2</td>
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<tr>
<td>ts 3</td>
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*a Each nonproducer cell line was superinfected with the Kirsten strain of MuLV at a multiplicity of 1. After 3 weeks of incubation at 32 C with weekly subculture (1:15), the supernatant fluids were harvested and titrated for focus formation on NRK cells at 32 C and 39 to 40 C. All stocks had equal quantities of XC plaque-forming units (10^6) of KiMuLV at this time.
by transmission of ts 1 and ts 3, even though virus producing, were as temperature sensitive (see below) for expression of transformation as were their respective original ts nonproducer lines. In marked contrast, virus-producing trans- 

formsants examined which contained ts 2 were not found to be temperature sensitive.

Quantitation of temperature sensitivity of transformed clones by growth on contact-inhibited monolayers. A characteristic property of MSV-transformed cells is a lack of contact inhibition of cell division. One method of quantitating the degree of contact inhibition is the measurement of the ability of cells to form colonies on monolayers of contact-inhibited fibroblasts (2). The colony-forming efficiency (CFE) of NRK cells and of wt and ts mutant MSV-transformed NRK clones on confluent monolayers of either contact-inhibited BALB/3T3 or NRK cells was therefore studied. As shown in Fig. 1, control NRK cells failed to form piled-up foci on BALB/3T3 monolayers at either 32 or 37 C. In marked contrast, wt MSV-transformed nonproducer cells formed colonies at high-efficiency and equally well at both temperatures. Although ts 1 and ts 3 MSV-transformed nonproducer cells each formed densely piled-up colonies at 32 C, their ability to pile up as colonies at 37 C was markedly reduced. Ts 2, however, showed only a slight reduction in CFE at 37 C.

Experiments were next performed to determine whether MuLV superinfection of the ts MSV-nonproducer lines would influence their CFE on contact-inhibited monolayers at the nonpermissive temperature. Since these studies were done not only at 32 and 37 C, but also at 39 to 40 C, it was necessary to use monolayers of NRK which survived well at 39 to 40 C, rather than BALB/3T3. To control for minor differences in plating efficiencies, results are expressed as the ratio of CFE on monolayers divided by CFE in empty petri dishes. NRK cells failed to form colonies on monolayers at any temperature tested whether or not they were preinfected with KiMuLV (data not shown). As shown in Table 2, wt MSV-transformed cells, both infected and noninfected, formed foci at greater than 95% efficiency at each temperature. The CFE ratios for ts 1 and ts 3 on NRK mono-

TABLE 2. Colony-forming efficiency (CFE) of ts and wt MSV-transformed nonproducer cells

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<tr>
<th>Cell line</th>
<th>Ratio of CFE on monolayers/empty petri dishes*</th>
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<tbody>
<tr>
<td></td>
<td>32 C</td>
</tr>
<tr>
<td>wt +KiMuLV</td>
<td>1.00</td>
</tr>
<tr>
<td>ts 1 +KiMuLV</td>
<td>0.75</td>
</tr>
<tr>
<td>ts 2 +KiMuLV</td>
<td>0.75</td>
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<tr>
<td>ts 3 +KiMuLV</td>
<td>0.80</td>
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<td></td>
<td>0.60</td>
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* Cell suspensions from cultures grown at 32 C were plated on either empty 50-mm petri dishes or petri dishes containing confluent monolayers of NRK cells at 10^4, 10^5, or 10^6 cells per petri. Foci on monolayers or colonies on empty dishes were scored after 7 to 10 days at 37 C and 39 to 40 C, or 16 to 20 days at 32 C. Results are expressed as the ratio of the number of colonies on monolayers divided by the number of colonies on empty petri dishes. Similar results were obtained with cells grown at 39 to 40 C prior to the experiment. All studies represent each point in triplicate with 10% variation from plate to plate.

+ KiMuLV infection of nonproducer cells was carried out at a multiplicity of 1.0, and cells were incubated at 32 C for 5 days before testing for CFE.

Fig. 1. Colony-forming efficiency of ts and wt MSV-transformed nonproducer cells. 10^4 cells from 32 C cultures of each of the respective cell lines were plated on confluent monolayers of BALB/3T3 at 37 C (upper plates) and 32 C (lower plates). Plates were stained with hematoxylin and photographed after 7 days of incubation at 37 C and 16 days at 32 C. A, NRK; B, wt; C, ts 1; D, ts 2; E, ts 3.
layers were 0.75 and 0.60, respectively, at 32 C, but less than 0.01 for each at 37 C and 39 to 40 C. The CFE was not influenced by prior superinfection of either mutant lines with Ki-MuLV. Similar results were obtained with the foci derived from transmission of ts 1 and ts 3. With ts 2 nonproducer cells, there was only a threefold reduction in CFE ratio at 37 C. However, at 39 to 40 C these cells failed to form any detectable colonies on the NRK monolayers. In contrast, after superinfection with Ki-MuLV, approximately 10% of the cells plated developed into colonies at 39 to 40 C. It is clear from these results that the ts nonproducer clones will form colonies on contact-inhibited monolayers at 32 C but not at 39 to 40 C. In the case of ts 2, but not ts 1 or ts 3, the impairment to colony formation at 39 to 40 C can be partially overcome by superinfection with Ki-MuLV.

**Viral-specific RNA in ts mutant MSV-transformed clonal lines.** The gene products of the MSV genome in nonproducer cells are unknown. However, by RNA-DNA hybridization techniques, virus-specific RNA can be measured in such cells (9). To determine whether the temperature sensitivity of any of the ts mutants was associated with a transcriptional block at the nonpermissive temperature, the amount of virus-specific RNA in each of the ts mutants and of the wt MSV-transformed nonproducer cells at both 32 C and 39 to 40 C was quantitated. The data obtained for ts 1 is summarized in Table 3. At both 32 C and 39 to 40 C, approximately 240 to 360 μg of cellular RNA was required to achieve saturation for hybridization. Similar results were obtained with ts 2, ts 3, and the wt clones. These findings indicate that the amount of viral-specific RNA in the ts 1 nonproducer clone is the same at both temperatures. It appears, therefore, that the block to expression of transformation at the nonpermissive temperature is not related to an impairment in total viral-specific RNA production.

**DISCUSSION**

There have been several reports of ts mutants of avian RNA sarcoma viruses which were defective in their ability to maintain the transformed state (5, 7, 8, 14, 16, 22). In these cases, the sarcoma virus strains used to isolate mutants were able both to replicate and transform cells. Thus, in general, individual ts viruses could be obtained by titrating clonal isolates of mutagenized virus at a permissive and nonpermissive temperature and scoring foci obtained at the two temperatures. Appropriate foci were then shifted to the nonpermissive temperature and screened for foci which subsequently became morphologically normal. Since MSV cannot replicate without added helper MuLV, a different approach to isolation of MSV mutants has been used. After mutagenesis of MSV, MuLV-free nonproducer foci were obtained, and ts foci were detected by replica-plating these cells to a permissive and nonpermissive temperature. By using this approach, three separate ts nonproducer clones have been isolated. Ts 1 and ts 3 are similar in several respects. Although transformed at 32 C, both became morphologically normal at 37 C. Although MuLV grows as well in these two lines as in the wt nonproducer cell, the ts lines yield much less infectious sarcoma virus even at the permissive temperature. The reason for the poor efficiency of transmission is unknown. This might be due to some link between the mutation affecting the maintenance of the transformed state and the ability of MSV to be rescued by MuLV. Another possibility is that these mutants resulted from more than one lesion, thereby affecting both transforming functions and the ability to be rescued. Alternatively, the defective gene product may simply be inefficient compared to wt even at the permissive temperature.

Nonproducer line ts 2 behaves differently from ts 1 or ts 3. It fails to revert to a normal morphology until 39 to 40 C. In contrast to ts 1 and ts 3, superinfection of ts 2 with KiMuLV is able to overcome its defect at the nonpermissive temperature. Although the reason for the MuLV effect is not known, possible explana-

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**Table 3. Kirsten sarcoma virus-specific RNA in ts 1 nonproducer NRK cells at 32 C and 39 to 40 C.**

<table>
<thead>
<tr>
<th>Total cellular RNA added (μg)</th>
<th>Percent of viral DNA product hybridized</th>
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<tr>
<td></td>
<td>32 C</td>
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<tr>
<td>20</td>
<td>2.5</td>
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<tr>
<td>60</td>
<td>10.0</td>
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<tr>
<td>120</td>
<td>20.0</td>
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<tr>
<td>240</td>
<td>34.0</td>
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<tr>
<td>360</td>
<td>32.0</td>
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* Logarithmically growing ts 1 cells were grown for 48 hr at 32 C or 39 to 40 C; the medium was changed; the cells were harvested 24 hr later, and total cellular RNA were extracted by the hot sodium dodecyl sulfate-phenol procedure (9). Hybridization was carried out as described in Materials and Methods. With no cellular RNA added, 1.0% of the 3H-DNA was resistant to degradation by nuclease S1. All points are the average of duplicate determination; reproducibility of each point was within 10%.
tions include a high reversion rate during replication of MuLV and MSV, an increased gene dosage after MuLV superinfection, or complementation by MuLV of the defect in ts 2.

As with ts 1 and ts 3, the titer of ts 2 is reduced compared to wt MSV even at the permissive temperature. Since MuLV is in 100-fold excess of ts 2 MSV under these conditions, it has not been possible to transmit ts 2 to new nonproducer cells (which lack MuLV). Since the normal morphology of ts 2 at 39 to 40°C tends to revert to a transformed morphology in producer clones in the presence of replicating MuLV, it has thus not yet been possible to isolate transmitted foci of ts 2 MSV which also show the temperature-sensitive effect. Since MuLV superinfection of ts 1 and ts 3 does not produce phenotypic reversion to transformed morphology, it has been possible to transmit them to new producer lines which do show the temperature-sensitive effect. Thus, the evidence that ts 2 is a viral rather than cellular mutation is less rigorous than the evidence for ts 1 or ts 3. However, the fact that the virus, rather than the cell, was mutagenized and the fact that a 30-fold reduction in ts 2 MSV titers could be demonstrated at 39 to 40°C versus 32°C both argue that the ts effect on the maintenance of the transformed state in ts 2 is due to a mutation in a viral rather than a cellular gene.

Since MSV cannot replicate in the absence of MuLV, a direct comparison is difficult to make between the MSV mutants and the avian sarcoma mutants. However, a unique result of avian leukosis superinfection on an avian ts sarcoma mutant (15) has been reported. In that case, however, superinfection by leukosis virus results in a lytic event rather than transformation.

The ability to obtain nonproducer lines temperature-sensitive in the maintenance of the transformed state should provide a useful model system for studying the expression of transformation in the absence of virus replication. Further studies are in progress to isolate additional mutants and to determine the number of complementation groups involved in the maintenance of the transformed state in mammalian cells.

ACKNOWLEDGMENTS

We would like to thank Wade Parks for assaying the nonproducer clones for mouse and rat group-specific antigen by radiolmmunossay. This work was supported by a contract from the Special Virus Cancer Program of The National Cancer Institute. J.R.S. is the recipient of a special fellowship from The Medical Research Council of Canada.

LITERATURE CITED