Murine Sarcoma Virus Gene Expression: Transformants Which Express Viral Envelope Glycoprotein In The Absence Of The Major Internal Protein And Infectious Particles
(Kirsten murine sarcoma virus)

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ABSTRACT Expression of the major internal protein and the envelope glycoprotein of murine C-type viruses in focus-derived lines of normal rat kidney cells infected with Kirsten murine sarcoma virus was measured by radioimmunoassay. Of the clones selected, which do not produce virus particles or the major viral structural protein, approximately half express the viral envelope glycoprotein at concentrations found in productively infected cells. Expression of the envelope glycoprotein did not appear to alter significantly the properties of the transformed cells in culture.

All strains of mammalian sarcoma viruses isolated to date, with one possible exception (1), are defective for replication; synthesis of infectious progeny by sarcoma virus transformants has been found only upon concomitant infection with a helper leukemia virus. The nature of the defectiveness of sarcoma viruses, and thus of the mechanism of helper virus rescue, may differ among the isolates studied. At least two distinct classes of cells transformed by murine sarcoma virus (MuSV) in the absence of murine leukemia virus (MuLV) have been described. Some transformed cell lines, S^+L^- (2), HTG-1 (3), WSV-NRK (4), contain "viral gs antigen," indicating the presence of one or more of the viral structural proteins. In some cases viral reverse transcriptase and noninfectious viral particles can be detected. Another class, cells transformed by Kirsten, Moloney, or Harvey MuSV, lack any evidence of virus particle formation; these cells, designated as nonproducers, have not been found to synthesize a detectable level of any viral protein (4–7) although they possess a rescuable sarcoma virus genome and viral specific mRNA (8, 9). The genetic differences between these two classes are stable, as reversions from one class to another have not been observed, and Aaronson et al. (10) have demonstrated that the MuSV rescued or induced from nonproducer or S^+L^- cells reproduced their respective phenotypes upon transfer to an identical cell type. It is not yet entirely clear whether the nonproducer phenotype reflects the lack of genetic information for the synthesis of viral structural proteins, or if the control is at the level of viral structural proteins, or if the control is at the level of expression; however, the bulk of evidence from studies of sarcoma virus infection and analysis of viral DNA by hybridization techniques (11) suggest that the control is at the level of viral structural proteins.

Abbreviations: NRK, Normal rat kidney; MuSV, murine sarcoma virus; MuLV, murine leukemia virus; Ki, Kirsten strain of MuSV or MuLV; R, Rauscher strain of MuLV; CFE, colony forming efficiency; TEN, buffer composed of 20 mM Tris-HCl at pH 7.6, 1 mM ethylenediaminetetraacetate, 100 mM NaCl.

The present studies were undertaken to define further the nature of defective sarcoma viruses. In this report we describe the characterization of focus-derived lines of NRK cells nonproductively transformed by Kirsten MuSV and the analysis by radioimmunoassay of the synthesis of two of the major molecular components of MuLV particles, the major internal protein (p30) and the envelope glycoprotein (gp69/71). It has been found that a nonmutagenized stock of the murine sarcoma and murine leukemia virus complex gives rise with equal frequency to two classes of nonproductive transformed cells. One class does not direct the synthesis of either the p30 or gp69/71 proteins. A second class shows a cellular concentration of the gp69/71 equal to that of the productively infected cell, but contains no detectable p30.

MATERIALS AND METHODS

Cells and Virus. All cells were propagated in Dulbecco's modified Eagle's medium supplemented with 10% heat-inactivated fetal bovine serum (Flow Laboratories, Rockville, Md.) and penicillin (5 μg/ml), streptomycin (5 μg/ml), and neomycin (5 μg/ml). Virus-free NRK-Clone 3 and the previously described V-NRK line (12) which spontaneously produced rat type-C virus were the gift of Dr. E. Scolnick, NIH. The Kirsten transformed nonproducer cell line 1255-B7 (13), as well as the KIMoSV/KiMuLV producer line no. 38967' (14) and the NRK-9 subclone were kindly provided by Dr. V. Klement, Children's Hospital, Los Angeles. The media from the virus producing cells was replaced every other day until the cells reached confluence; thereafter, virus was harvested daily.

Virus Infection and Assay. Virus suspensions were clarified by centrifugation at 2000 × g for 10 min and passed through a 0.45-μm membrane filter immediately prior to use. One day prior to infection, 10^6 NRK cells in growth medium containing 2 μg/ml of polybrene (Aldrich Chemicals, Cedar Knolls, N.J.) were seeded in 60-mm petri dishes. Cultures were infected with 0.5 ml of appropriately diluted virus and were incubated for 1 hr at 37°C. Medium was changed on day 5 and foci were scored on days 7 or 9. Reverse transcriptase activity present in the medium was measured as described by Scolnick and Parks (4).

Virus purified for radioimmunoassay was concentrated by centrifugation at 18,000 rpm for 90 min in the type 19 rotor at 4°C and suspended in TEN buffer (20 mM Tris-HCl at pH 7.6, 1 mM ethylenediaminetetraacetate, 100 mM NaCl). The particles were then purified by equilibrium centrifugation followed by velocity sedimentation in TEN buffered sucrose.
Purity of these virus preparations was assessed by polyacrylamide gel electrophoresis (15).

Preparation of Cells for Radioimmunoassay. Cells grown in 75-cm² T-flasks to subconfluence were washed twice with warm Ca²⁺, Mg²⁺-free phosphate buffered saline. The flasks were then incubated with 10 ml of TEN buffer at 37°C for 5 min. Flasks were agitation vigorously and the cells were harvested by low-speed centrifugation. Cell pellets were suspended in 10 ml of TEN buffer, enumerated, and concentrated by centrifugation. Vital staining with trypan blue indicated that these cells remain intact and viable throughout this procedure. Cell packs were suspended in a minimal amount of TEN (0.5 to 1 × 10⁶ cells per ml) and suspensions were kept on ice or frozen at −20°C prior to solubilization.

Radioimmunoassay. Assay of viral protein was carried out by radioimmunoassay as described by Hunter (16). The reaction mixtures contained the following: 0.005 ml of normal rabbit serum, 0.01 ml of 125I-labeled virus protein (1 or 2 ng of protein containing 10¹⁰ to 10¹² cpm/ng), either 0.15 ml of cell extract or 0.01 ml of purified virus or viral protein as competing proteins, and 0.01 ml of rabbit anti-Theilen FeLV serum as the final addition at a concentration which precipitated approximately 50% of the 125I-labeled antigen. The total volume of the assay was adjusted to 0.2 ml with TEN buffer containing 0.2% Triton X-100 and 2 mg/ml of crystalline bovine serum albumin. Dilutions were made in TEN buffer which contained 0.2% Triton X-100 and either 20 mg/ml of carrier bovine serum albumin for antisera, purified virus or viral proteins, or 2 mg/ml for cell extracts. The reaction mixture was incubated at 37°C for 15–18 hr, after which 0.04 ml of goat anti-rabbit IgG serum was added. Incubation was continued at 37°C for an additional 2 hr followed by overnight incubation at 2–4°C. Cold TEN buffer (0.5 ml) was added and the precipitate was collected by centrifugation at 4°C. The pellet was washed twice with 0.5 ml of TEN buffer and the 125I-labeled antigen present in the precipitate was measured in a gamma counter.

Growth on Normal Monolayers. This property of MuSV transformed cells was examined essentially as described by Aaronson and Todaro (17). Appropriately diluted cells (1, 100, 500) were simultaneously plated on two sets of 60-mm plastic petri dishes, one set containing a confluent monolayer of BALB/c 3T3 cells. Growth medium was changed on the fourth and eighth day. Plates were fixed with absolute methanol, stained with hematoxylin, and colonies were scored macroscopically on day 10. Results were expressed as the ratio of the colony-forming efficiency (CFE) on monolayers to the CFE on plastic dishes. The plating efficiency of NRK cells in these experiments was approximately 80%.

Saturation Densities. Saturation densities were the equilibrium densities maintained in 75-cm² plastic T flasks or 60-mm petri dishes, 3 days after uninfected NRK cells reached confluence. Fresh medium was added every other day, and the pH of all cultures was monitored and when needed adjusted to pH 7.4 daily.

Sugar Uptake. Uptake of 4H-labeled 2-deoxyglucose was measured under conditions to be described elsewhere (Bilillo et al., unpublished observations). Briefly 5 × 10⁴ cells were plated in the wells of a Linbro disposetray (Linbro Chemical, New Haven, Conn.); after 48 hr, the cells were washed with glucose-free Earle's balanced salt solution (EBSS) and incubated in glucose-free medium. Cells were pulse labeled with 0.2 μCi/ml of ³H-labeled 2-deoxyglucose for 30 min, washed three times with chilled EBSS and lysed in 0.15% sodium dodecyl sulfate. Samples were counted in a liquid scintillation spectrometer while an additional aliquot was assayed for total protein by the Lowry method.

RESULTS

Selection of clones of KiMuSV transformed nonproducer NRK cells

The isolation of transformed foci was carried out with NRK cells because foci develop from clonal expansion of single transformed cells rather than from recruitment of the neighboring cells by infection with progeny virus (18). NRK cells were infected with a limiting dilution of a KiMuSV/KiMuLV stock as described in Materials and Methods. Transformed cells were obtained by selecting random foci from plates containing fewer than 10 foci. The initial test for virus synthesis (productive infection) or the lack of virus synthesis (nonproductive infection) in confluent cultures was carried out by examination of tissue culture fluids for infectious virus. Nine foci isolated from four plates all yielded nonproducer cells and these were chosen for further characterization.

To confirm the nonproducer phenotype, all of the clones were analyzed for the release of C-type particles by testing the culture fluids for infectious virus, RNA directed DNA polymerase activity, and by radioimmunoassay for viral p30 protein. Nonproducer clones showed no evidence of virus release by these criteria under conditions where productively infected cells yielded 10⁶ to 10⁷ focus-forming units of virus and incorporated >200 cpm/ng ml of culture medium. All of the clones were passed 1 month or longer prior to testing. Over the course of these investigations, none of the nonproducer clones has ever released infectious virus as monitored by infectivity and/or infectious center assay.

Synthesis of viral specific proteins

Synthesis in the transformed cell of the two principal structural proteins of C-type particles, the major internal protein (p30) and the major viral envelope glycoprotein (gp69/71), was measured by radioimmunoassay as described in Materials and Methods. This assay was based upon a heterologous antigen-antibody system, that is anti-feline leukemia virus serum and the murine virus antigen, so that antibodies reacting specifically with common interspecies antigenic determinants could be utilized. The heterologous assay system measured common antigenic determinants of all murine viruses tested and provides a quantitative measurement of the p30 or gp69/71 of Kirsten virus despite the use of Rauscher virus proteins as labeled antigens (15, 16).

The p30 protein was not detected in extracts of the nonproducer cells (Fig. 1a). Internal controls show that antibody binding of 125I-labeled Rauscher MuLV p30 was blocked by unlabeled purified p30, purified KiMuSV/KiMuLV, and extracts of producer cells, and that 1% of the p30 present in producer cells could have been detected by this assay. Extracts of one of the uninfected, control NRK-9 cells contained p30 protein at a concentration which was approximately 9% that of the producer cell extract. This p30 protein may be attributable to the expression of the endogenous rat C-type genome.

In contrast to the absence of Kirsten p30, approximately half of the transformed cell clones contained gp69/71 at concentrations approximately equal to that of the productively
Fig. 1. Radioimmunoassay of mammalian C-type virus proteins in cell extracts. (a). The p30 competition assay was performed as described in Materials and Methods with 1 ng of 125I-labeled R-MuLV p30 and rabbit anti-FeLV serum (1:5000). (b) Competition assay for gp69/71 protein was performed with 2 ng of 125I-labeled R-MuLV gp69/71 and rabbit anti-FeLV (1:1500). The competing purified proteins, Triton X-1000, disrupted KiMuSV/KiMuLV, and cell extracts of NRK and (KiMuSV/KiMuLV) NRK were added as indicated. Ki-NRK transformed cell (Fig. 1b). This viral envelope protein appears to be a component of the transformed cell, since gp69/71 was not removed from the expressor cells by two 5-min incubations with trypsin-ethylenediaminetetraacetate (0.05% and 0.02%, respectively) followed by low-speed centrifugation. Experiments were also carried out to determine if gp69/71 could have been removed from some of the cells by the procedure employed to collect cells for radioimmunoassay. Carrier crystalline bovine serum albumin (50 μg/ml) was added to each wash, ammonium sulfate was added to 75% saturation, and the precipitated proteins concentrated by centrifugation. The precipitates were dissolved in 0.5 ml of TEN buffer and assayed for gp69/71. None of the washes from non-expressors contained gp69/71.

Further controls have also shown that the gp69/71 of these Kirsten-transformed NRK cells is murine and not rat. Radioimmunoassay analysis of the homology of murine and rat gp69/71 interspecies determinants showed that virus derived from NRK cells did not compete significantly with the murine gp69/71 for binding of anti-FeLV antibodies, indicating that few if any of the interspecies determinants common to feline and murine gp69/71 are shared by the rat virus protein (Fig. 2).

We conclude that approximately 50% of the KiMuSV non-productively transformed cells express the viral envelope glycoproteins in the absence of the major internal structural protein and of virus particles.

**Growth characteristics of nonproducer clones**

Morphologically, the producer, and the two nonproducer cell lines S+p30−gp69/71+ and S+p30−gp69/71− were indistinguishable; all contain refractile, round and spindle shaped cells. Growth characteristics of producer and nonproducer clones were examined by a variety of assays (Table 1). The saturation densities of the clones fell within a narrow range that was approximately 3.5-fold higher than that of the uninfected NRK cells. These values are minimal, however, as the transformed cells float off into the medium as the cultures become dense. Similarly, the rate of labeled 2-deoxyglucose uptake by nonproducer clones did not differ significantly from transformed producer cells. Clones plated upon confluent BALB/c 3T3 monolayers showed slightly broader distribution of plating efficiencies, which did not correlate with the expression of the glycoprotein. Uninfected NRK cells did not form colonies upon BALB/c 3T3 monolayers.

**Table 1. Properties of NRK cells and KiMuSV focus-derived lines**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Ratio CFE†</th>
<th>2-deoxy-uptake (cpm/ mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Saturation</td>
<td>density (10⁶ cells/cm²) to CFE on empty dishes</td>
</tr>
<tr>
<td>Normal</td>
<td>3.26</td>
<td>0.59</td>
</tr>
<tr>
<td>NRK CI-3</td>
<td>96,687</td>
<td>ND</td>
</tr>
<tr>
<td>NRK-0</td>
<td>80,227</td>
<td>ND</td>
</tr>
<tr>
<td>S+p30−gp69/71−</td>
<td>74,155</td>
<td>ND</td>
</tr>
<tr>
<td>Ki-NRK 1</td>
<td>68,176</td>
<td>ND</td>
</tr>
<tr>
<td>Ki-NRK 2</td>
<td>68,176</td>
<td>ND</td>
</tr>
<tr>
<td>Ki-NRK 3</td>
<td>68,176</td>
<td>ND</td>
</tr>
<tr>
<td>Ki-NRK 4</td>
<td>68,176</td>
<td>ND</td>
</tr>
<tr>
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<td>68,176</td>
<td>ND</td>
</tr>
<tr>
<td>1255-B-7</td>
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<td>ND</td>
</tr>
<tr>
<td>S+p30−gp69/71+</td>
<td>104,433</td>
<td>ND</td>
</tr>
<tr>
<td>Ki-NRK 6</td>
<td>104,433</td>
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</tr>
<tr>
<td>Ki-NRK 7</td>
<td>104,433</td>
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</tr>
<tr>
<td>Ki-NRK 8</td>
<td>104,433</td>
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</tr>
<tr>
<td>Ki-NRK 9</td>
<td>104,433</td>
<td>ND</td>
</tr>
<tr>
<td>KiMuSV/KiMuLV</td>
<td>95,333</td>
<td>ND</td>
</tr>
<tr>
<td>producer</td>
<td>95,333</td>
<td>ND</td>
</tr>
</tbody>
</table>

* Experimental details were as described in Materials and Methods.
† CFE, colony forming efficiency.
ND, not determined.
The presence of the KiMuSV genome in representative gp69/71 expressor and nonexpressor clones was detected by direct co-cultivation with Woolly leukemia virus (WLV) NRK cells. All produced low levels of focus-forming virus after 5–7 days. Preliminary experiments appear to demonstrate that both gp69/71 positive and negative clones are inducible with IdUrd. Experiments are in progress to determine the phenotype of cells infected with virus induced by IdUrd or rescued from individual nonproducer clones.

Representatives from both classes of nonproducer KiMuSV clones have been tested for their tumor inducing ability in vivo by inoculating freshly trypsinized cell suspensions into adult Osborne-Mendel rats. Both classes of nonproducer cells initiated progressively growing tumors at the inoculation site within 14 days.

Stability of gp69/71 expression in nonproducer clones

The stability of the expressor and nonexpressor phenotypes was tested by recloning one representative clone of each type. Twenty-four randomly chosen subclones of S +p30-gp69/71 -cell type were propagated. None were found to contain p30 or gp69/71 as tested by radioimmunoassay. Similar assays were performed with ten randomly selected S +p30-gp69/71 + subclones. All remained positive for gp69/71 at concentrations equal to that of the producer cell line (Fig. 3a). Three of the gp69/71 positive subclones previously negative for p30 now expressed this protein at concentrations of approximately 1–5% or less than that of the producer cell line, which may be attributable to the induction or synthesis of endogenous rat virus p30 (Fig. 3b). Supernatants from these subclones have been tested for infectious virus, and no focus forming activity was found. All of the original clones, now approximately 25 passages from isolation, have remained free of p30.

**DISCUSSION**

Analysis of a number of individual Kirsten transformants revealed a high proportion of clones which do not produce virus particles or the viral p30 protein, yet they contain concentrations of the viral envelope gp69/71 protein equal to that of productively transformed cells. Several alternative models can be suggested to explain this finding: (i) There is a class of KiMuSV which contains the genetic information for the glycoprotein and transformation and in which their expression is closely linked, whereas a second class of KiMuSV does not contain the information for gp69/71. (ii) A single class of virus genome exists which contains genes for both functions, and the difference between the two phenotypes in expression of gp69/71 is controlled by some other viral or host function. (iii) The genes are on separate nucleic acid segments that may or may not be coordinately integrated or expressed in the infected cell. It is also possible, in spite of the fact that foci were isolated at limiting dilution, that cells expressing gp69/71 were simultaneously infected with both a sarcoma virus and a (p30-gp69/71 +) defective leukemia virus. Whichever of these or other phenomena explain the finding, it is noteworthy that expression of the p30 protein is distinct from that of gp69/71, indicating that these proteins are unlinked or noncoordinate expressed. The interpretation of these findings has implications as well for the models of viral genome structure proposed by Vogt (19) and Duesberg et al. (20).
In the context of the model recently proposed by Scolnick et al. (21), where the Kirsten sarcoma information was derived from an in vivo recombinational event between a Kirsten murine leukemia virus genome and rat cell information, the S'p30-gp69/71-, S'p30-gp69/71+, and several other of the reported phenotypes (i.e., S'+L-, S'+H-) appear to represent the loss of leukemia virus information. Theoretically, any number of other phenotypes might be expected to occur as a result of the loss of information or a block at different stages of viral gene expression, transcription, translation, or assembly. For example, in addition to the S'+L- and the different nonproducer phenotypes, Ball et al. (1) recently described a MuSV which can persist in the cell, but will produce MuSV and transform only upon superinfection with MuLV. There also can be differences in phenotype related to variable expression of transformation gene information; both flat and intermediate revertants of MuSV transformed cells (22-24) and intermediate SV40 transformants (25) have been described. In the present experiments the lack of cell clones intermediate in the expression of transformation and/or gp69/71 may be attributed to the selective nature of the focus assay.

Rescue of infectious MuSV appears to require helper virus replication (24, 26) and the progeny sarcoma virus can acquire both the host range and antigenic specificity of the helper (5, 6, 27). Early experiments with the Bryan high titer strain of RSV (28-30) led to the suggestion that a defective virus is unable to synthesize envelope glycoproteins necessary for infectivity, and that the role of the helper virus is to specify the envelope by phenotypic mixing. Isolation of S'p30-gp69/71+ cells demonstrates that in some cases a sarcoma virus may direct the synthesis of the envelope glycoproteins in the absence of p30 or virus particles, and that in these cells the mechanism of the helper virus rescue may involve other leukemia virus functions.

There is considerable evidence that the gp69/71 glycoproteins are viral envelope surface antigens (31, 32). Since the assembly of C-type RNA viruses occurs at the periphery of the cell, gp69/71 expressed at the cell surface might well be concerned with the expression of the transformed phenotype. However, in these studies the expression of the viral envelope glycoprotein does not appear to modify the phenotype of the transformed cell as measured by in vitro transformation assays. Nevertheless, expression of this phenotype in vivo could be expected to have an effect upon the nature of the host immune response to the transformed cell. For example, transformed cells producing gp69/71 in the absence of virus synthesis may possibly explain the observations of MuSV induced tumors which did not produce virus but which were rejected by syngeneic animals previously immunized with MuSV (33-35).

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