Expression of Kirsten Murine Sarcoma Virus in Transformed Nonproducer and Revertant NIH/3T3 Cells: Evidence for Cell-Mediated Resistance to a Viral Oncogene in Phenotypic Reversion

JOHN D. NORTON, 1, 4 FRANK COOK, 1 PETER C. ROBERTS, 2 JON P. CLEWLEY, 3 AND ROGER J. AVERY 2

Department of Biological Sciences, University of Warwick, Coventry CV4 7AL, 1 and Virus Reference Laboratory, Central Public Health Laboratory, Colindale, London NW9 5HT, 2 England, and Veterinary Research Laboratory, Montana State University, Bozeman, Montana 59717 2

Received 28 July 1983/ Accepted 20 January 1984

Expression of the provirus in a clonally related series of Kirsten murine sarcoma virus-transformed NIH/3T3 nonproducer cell lines was examined at both the transcriptional and translational levels. All cells expressed high levels of genome-sized viral RNA with little variation between cell lines despite differences in provirus integration site and copy number. Expression of K-ras RNA was estimated to be at least 10- to 20-fold higher than that of the mouse cellular homolog of the viral transforming gene. Levels of the virus-coded transforming protein, p21, were similarly elevated, with little variation between nonproducer cells. In two revertant cell lines containing a normal provirus and a rescuable transforming gene, no impairment in expression at either the transcriptional or translational level was found. After superinfection with Kirsten murine sarcoma virus, one revertant became more tumorigenic, whereas the other remained non-tumorigenic. These results show that cell transformation by Kirsten murine sarcoma virus is invariably associated with elevated expression of the virus-coded oncogene and that one of the revertants is resistant to the action of the viral transforming gene.

Strongly oncogenic retroviruses transform cells in culture, rapidly induce neoplasms in animals at high frequency, and are usually replication defective (42). These properties correlate with the acquisition by the genomes of these viruses of normal host cell sequences, generally at the expense of viral genes required for replication (1, 2, 9). The cell-derived sequences (collectively termed protooncogenes) (9, 37) encode a set of transforming proteins. Under the transcriptional control of retroviral regulatory sequences, these proteins are expressed at high levels in virus-transformed cells (3, 8, 10, 30). Consequently, a model of oncogenesis has been proposed whereby overexpression of a cryptic cellular protooncogene is responsible for initiation and maintenance of the transformed state of the cell (27, 29). This model has been further strengthened by the finding that protooncogenes are themselves oncogenic in an in vitro transfection assay when linked to retroviral regulatory sequences, which enhance expression (4, 12).

The genome of the strongly oncogenic Kirsten murine sarcoma virus (KiMSV) arose from the weakly oncogenic Kirsten murine leukemia virus (KiMLV) by transduction of two types of rat cellular sequences (6, 20). The major component of the rat-derived sequences is a rodent retrovirus-like genetic element of no known function, whereas the remaining sequences represent the transforming gene of the virus (6, 16). This latter gene (K-ras) is a member of the ras family of oncogenes (16), and in common with other ras genes, encodes a 21,000-molecular weight (MW) protein associated with nucleotide-binding activity (18, 23, 33, 34).

Recently, it was shown that qualitative differences in ras protooncogenes can also account for oncogenicity (28, 31, 36). However, the mechanisms by which these sequences exert their effect remains unknown.

The isolation and characterization of a series of KiMSV-transformed and revertant nonproducer fibroblasts derived from twice-cloned NIH/3T3 cells have been previously described (24). Because these cell lines are all clonally related, they allow the study of viral gene expression in relation to the phenotype of the cell against a constant background of cell genetic information. We have found most transformed nonproducers to contain a single, variably located provirus and the revertants (which contain a rescuable, biologically active sarcoma virus) possess a provirus indistinguishable from that in the parental transformed line (25a). In this report, we describe the analysis of viral gene expression at both the transcriptional and translational levels in transformed and revertant cells. Our results show that transformation is invariably associated with elevated viral gene expression, implicating this as an essential feature of KiMSV transformation. In two revertants, unaltered viral gene expression was found, suggesting that loss of the transformed phenotype is attributable to changes in cellular factors required for transformation. This was confirmed for one revertant cell line, which was shown to be impervious to retransformation by the homologous virus.

MATERIALS AND METHODS

Cells and viruses. NIH/3T3 cells and KiMSV (KiMLV) were generously provided by J. Levy (University of California, San Francisco) and S. Aaronson (National Institutes of Health, Bethesda, Md.), respectively. The establishment and properties of transformed and revertant KiMSV-infected cell lines have been described previously (24). All cells were maintained in Dulbecco modified medium containing 10% newborn calf serum (Flow Laboratories Ltd., Irvine, Scotland).

Control NIH/3T3 cells and revertants were infected with KiMSV (KiMLV) at a multiplicity of 10 focus-forming units per cell with a virus stock propagated on NRK cells. This stock contained an approximately 100-fold excess of KiMSV to KiMLV as determined by biological assay. Cells were treated with DEAE-dextran (25 μg/ml at 37°C) for 30 min.
was washed with medium, and then infected with 2.0 ml of stock virus. Mock-infected cell lines were treated identically, except that 2.0 ml of medium was substituted for virus. Infected and mock-infected cell lines were passaged several times and then grown for DNA extraction and tumorigenicity assay.

**Preparation of RNA.** Cytoplasmic fractions were prepared from cells by homogenization in 10 mM Tris, pH 7.4–10 mM NaCl–1.5 mM MgCl₂ containing 0.5% Nonidet P-40 and removal of nuclei by low-speed centrifugation (500 × g for 5 min). Nucleic acid was purified from the cytoplasmic fraction by treatment with proteinase K (200 μg/ml) for 15 min at 20°C and extraction with phenol-chloroform. Total cytoplasmic RNA was recovered from the aqueous phase by ethanol precipitation.

**Quantitation of KiMSV-specific RNA.** Duplicate 5-μg samples of cytoplasmic RNA in 0.5 ml of 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate; pH 7.0) were immobilized by filtration through 13-mm nitrocellulose disk filters (Sartorius) as described by Thomas (38). A 4.6-kilobase-pair cloned probe (K-smα) specific for the rat-derived sequences of KiMSV (25) was 32P labeled by nick translation (32) to a specific activity of at least 10⁸ cpm/μg. The filters were prehybridized in sealed vials at 43°C overnight in hybridization buffer that contained 50% (vol/vol) formamide, 3× SSC, 50 mM HEPES (N-2-hydroxyethylpipеразин-N'-2-етансульфоная кислота), 0.5 mg of yeast RNA per ml and 10 μg of sheared salmon sperm DNA per ml and was supplemented with Denhardt reagents (13); the prehybridization solution was overlaid with paraffin oil. Hybridization with the nick-translation probe (10⁶ cpm per filter) was performed in 0.5 ml of hybridization buffer containing 10% (wt/vol) dextran sulfate for 70 h at 43°C. After hybridization, the filters were washed twice for 15 min at room temperature in each of the following: 3× SSC–0.1% sodium dodecyl sulfate (SDS); 1× SSC–0.1% SDS; and 0.3× SSC–0.1% SDS. Finally, filters were washed twice for 15 min at 65°C in 0.1× SSC–0.1% SDS. After the filters were washed, hybrids were detected by liquid scintillation counting. Serial dilutions of a KiMSV (KiMLV) virion RNA preparation (containing 40% KiMSV-specific sequences) were mixed with 5 μg of NIH/3T3 cytoplasmic RNA and assayed as described above. The calibration curves obtained (see Fig. 1) were used to determine the absolute quantities of KiMSV-specific sequences in the hybridization assay.

**Restriction enzyme digestion and agarose gel electrophoresis of DNA.** High-molecular-weight DNA was prepared from fibroblasts by lysis with SDS, phenol-chloroform extraction, and RNase treatment. DNA (10 μg) was digested with an excess of restriction enzyme HindIII (New England Biolabs) under conditions recommended by the suppliers. After the addition of SDS to a concentration of 0.5%, digests were electrophoresed overnight on a 1.0% (wt/vol) horizontal agarose slab gel in 40 mM Tris–20 mM sodium acetate–2.0 mM EDTA (adjusted to pH 7.7 with acetic acid). The gel was stained with ethidium bromide for visualization of size markers, and the DNA was blotted onto a nitrocellulose filter. Filters were annealed with 32P-labeled K-smα probe for detection of KiMSV-specific sequences exactly as described above.

**Immunoprecipitation of p21.** Cells (ca. 10⁶) in a 5-cm dish were radiolabeled with 500 μCi of [35S]methionine (Radiochemical Centre, Amersham, England; specific activity, 800 μCi/mmol) for 2 h at 37°C in 0.8 ml of Earle saline containing 20 mM HEPES and 1% Glasgow modified Eagle medium. Cells were then lysed as described previously (34), and p21 was immunoprecipitated with saturating amounts of p21 antiserum kindly donated by E. M. Scollnick (Merck Sharp & Dohme Research Laboratories, West Point, Pa.) for 12 h at 4°C. Antigen-antibody complexes were precipitated (19) and recovered by resuspending, heating the pellet at 95°C for 3 min in a buffer containing 10% (wt/vol) SDS, 25% (vol/vol) 2-mercaptoethanol, 0.25 M Tris, pH 6.8, 20% glycerol, and 0.002% bromophenol blue. Immunoprecipitated polypeptides were analyzed on 10 to 30% (wt/vol) polyacrylamide gradient gels with a Tris-glycine buffer system (22). After electrophoresis, gels were dried under suction onto a filter paper (without fixing) before exposure to X-ray film.

**Determination of tumorigenicity.** The tumorigenicity of revertant, KiMSV-infected revertant, and control cell lines was tested by subcutaneous injection of 10⁶ cells into 2-day-old NIH mice (OLAC 1976 Ltd., Shaw’s Farm, Blackthorn, Bicester, Oxfordshire, England). The mice were scored after 12 days for appearance of tumors and were considered positive when the tumor diameter was ca. 1 cm. At this point, positive groups of mice were killed to avoid further suffering. Otherwise, mice were maintained for an additional 7 weeks, unless tumors appeared in a significant proportion of mice within a group, in which case they were killed.

**RESULTS**

**KiMSV intracellular RNA in nonproducer cells.** We have previously examined the integrated proviruses in ca. 19 KiMSV-transformed nonproducer lines (25a). Most lines contained a single provirus located at a different site in the cellular DNA in each case. It was, therefore, of interest to determine the variation in levels of intracellular viral RNA in different transformed nonproducer cell lines. Two revertant cell lines (R2-1 and R5-5-1) derived from the transformed cell line CCl were previously found to be phenotypically normal and nontumorigenic (24). However, these lines contain a normal provirus and a resuable transforming gene (24, 25a). Therefore, we also sought to determine whether there was any reduction in virus-specific RNA in the revertants, which might account for the loss of the transformed phenotype.

Cytoplasmic RNA from a number of nonproducer cell lines and from the two revertants was assayed by quantitative hybridization with a cloned probe specific for the rat-derived sequences of KiMSV (25). By comparison with the hybridization obtained with purified virion RNA (Fig. 1), the absolute quantity of KiMSV-specific RNA could be estimated. Figure 2 shows the results of this analysis. All the nonproducer cell lines except CB3 and DH9 contain a single normal provirus (25a). CB3 contains a deletion of sequences nonessential for transformation (8a), and DH9 contains multiple proviruses (two to three per cell). It can be seen from Fig. 2 that the intracellular levels of KiMSV RNA in different transformed nonproducer cells fell within a narrow range (between 0.003 and 0.01%) of cytoplasmic cell RNA. Similar results were obtained with total cellular RNA (data not shown). Also, there appears to be no dependence on provirus copy number, since in DH9 the level of KiMSV RNA was no higher than in those cell lines containing only a single provirus. A low level of hybridization was detected with control uninfected NIH/3T3 cell RNA (0.0005%). This is largely attributable to transcription from the mouse K-ras gene(s), which is expressed as 2- and 5-kilobase mRNA species in normal mouse cells (15). Taking into account the fact that the coding sequence for the K-ras p21 is ca. 0.6 kilobase (40), we calculated that expression of K-ras-containing transcripts from the KiMSV provirus occurs at about
a 10- to 20-fold higher level than from the mouse K-ras counterpart.

The two revertant cell lines, R2-1 and R5-5-1, both showed a lower level of intracellular KiMSV RNA than did the parental transformed line, CC1 (Fig. 2). However, even in R2-1, which showed the greatest reduction, the level of expression was still within the range displayed by other transformed cell lines. Therefore, decreased expression of provirus does not appear to be a likely mechanism to explain the phenotypic reversion.

By using the northern blotting procedure (38), we were able to detect genome-sized RNA in all of the lines examined (data not shown). However, for technical reasons, we would not unequivocally rule out the existence of subgenomic RNAs.

Levels of p21 transforming protein in nonproducer cells. Although KiMSV transcripts of the correct size were found in the revertant cells at levels comparable to those in other transformed nonproducer lines, it is possible that this mRNA is inefficiently translated. This would result in reduced intracellular levels of the 21,000-MW transforming protein and the consequent loss of the transformed phenotype. Such a mechanism, perhaps involving a minor sequence change reflected at the level of mRNA, would nevertheless be compatible with the ability to rescue transforming viruses from these cells. When [35S]methionine-labeled CC1 cells were lysed and immunoprecipitated with saturating amounts of p21 antiserum, the 21,000-MW protein was clearly detectable after polyacrylamide gel electrophoresis (Fig. 3, lane d). This protein was not seen in the same cell lysate treated with control sera (lane c). Similarly, the revertant cell line R2-1 yielded a comparable level of the 21,000-MW transforming protein (lane f). R5-5-1 (lane h) appeared to contain only about half the level of the protein found in the parental transformed line. However, we do not believe this reduction to be significant, since the level of p21 was within the range of levels of the protein (ca. a three- to fourfold variation) displayed by a number of other transformed cell lines examined (data not shown). In this connection, we have noted no correlation between the intracellular levels of viral RNA and transforming protein synthesized within individual cell lines. However, since the variations between different...
cell lines extended over only a limited range, this result was not unexpected. The results of our analysis of p21 levels argues against reduced amounts of this protein being responsible for loss of the transformed phenotype in the revertants.

A 21,000-MW protein encoded by the mouse homolog of the K-ras oncogene (15) was not detected in control uninfected NIH/3T3 cells (Fig. 3, lane b). We can, therefore, only state as a minimal estimate that intracellular levels of the 21,000-MW transforming protein are 10- to 30-fold higher in KiMSV-transformed nonproducer cells than in normal NIH/3T3 cells (based on quantitation of p21 bands; data not shown).

Susceptibility of revertants to retransformation by KiMSV.

The preceding biochemical characterization detected no apparent change in viral gene expression in the revertant cells. Therefore, the reversion event may have involved an alteration in cellular factors required for transformation, which resulted in the cells becoming resistant to transformation by the p21 protein. To investigate this possibility, we attempted to retransform revertant cells by reinfection with KiMSV. Shortly after high-multiplicity infection of control NIH/3T3 cells and the two revertant lines, newly acquired KiMSV proviruses were detected in high-molecular-weight DNA, as shown in Fig. 4. The enzyme, HindIII, used in this analysis cleaves proviral DNA at two sites, generating an internal 1.6-kilobase-pair fragment (26) together with two junction fragments of variable size (depending on the particular integration site in cell DNA). Figure 4, lane b, shows a digest of DNA from the parental transformed line CCI, known to contain a single provirus. In the three superinfected cell lines (lanes c through e), the intensity of the internal 1.6-kilobase-pair HindIII fragment indicates the presence of ca. 10 to 20 additional proviruses, whereas the heterogeneous smear shows the majority of newly acquired proviruses to be integrated nonspecifically in cell DNA. The various discrete bands can be accounted for as being the products of digestion of residual unintegrated linear and circular viral DNAs present in the preparation. These results clearly show that the revertants are not resistant to infection with the homologous virus. In addition, the superinfected cell lines were found to be positive for reverse transcriptase activity (data not shown), further illustrating normal expression of viral genes.

Table 1 summarizes the results of a tumorigenicity trial with the superinfected revertants and a number of control cell lines. As expected, after 12 days, no tumors had appeared in mice inoculated with either mock-infected NIH/3T3 cells or either of the two revertant cell lines, whereas the parental transformed CCI cells and the KiMSV-infected NIH/3T3 cells induced tumors. However, after the trial was extended for a longer period of time, tumors began to appear in a significant portion of the control mock-infected R2-1 cells. This was an unexpected result, since a complete lack of tumorigenicity was previously reported for this cell line (24). The discrepancy is due to the fact that the earlier trial was terminated after only 2 weeks. The superinfected R2-1 cell line induced tumors in the majority of mice at day 12 (Table 1), showing that its tumorigenicity had been increased as a result of acquiring additional proviruses. Superinfection of R5-5-1, in contrast, resulted in no increase in tumorigenicity. The mice inoculated with superinfected R5-5-1 cells were maintained for a period of 9 weeks, and no tumors were scored. Thus, the R5-5-1 cell line appears to be resistant to the action of the transforming protein, as determined by the criterion of tumorigenicity (14).

**DISCUSSION**

KiMSV has been previously reported to transcribe a single genome-sized RNA species in transformed mink cells (7). In transformed NIH/3T3 cells, we have found a genome-sized transcript, but we cannot definitively rule out the existence of a subgenomic mRNA species which may have escaped detection. This possibility must remain open, since a precedent for host cell-determined differences in provirus transcription has been reported for Rous sarcoma virus, which produces three viral RNA species in permissive avian cells but only two RNA species in nonpermissive vole cells (21). All transformed nonproducer cells were found to express high levels of both viral RNA and p21 transforming protein (at least 10- to 30-fold higher than endogenous mouse ras expression) with little variation between cell lines. This lack of variation was somewhat unexpected and contrasts markedly with results of studies on the expression of other retrovirus proviruses (5, 17, 41). We can envisage two

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Tumor incidence on day 12*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mock-infected NIH/3T3</td>
<td>0/10</td>
</tr>
<tr>
<td>KiMSV-infected NIH/3T3</td>
<td>0/10</td>
</tr>
<tr>
<td>CCI</td>
<td>0/10</td>
</tr>
<tr>
<td>Mock-infected R2-1</td>
<td>0/6 (4/6 on day 24)</td>
</tr>
<tr>
<td>KiMSV-infected R2-1</td>
<td>10/13</td>
</tr>
<tr>
<td>Mock-infected R5-5-1</td>
<td>0/8</td>
</tr>
<tr>
<td>KiMSV-infected R5-5-1</td>
<td>0/6</td>
</tr>
</tbody>
</table>

* Data indicate number of mice showing tumors out of number of mice tested.
explanations to account for this observation. The first invokes a lack of dependence of provirus transcription on the cell DNA integration site. In this connection, the nucleotide sequences of presumed proviral regulatory elements within the long terminal repeat of KiMSV DNA show some differences compared with those of other murine retroviruses (J. D. Norton, unpublished observations), although we cannot as yet assess the possible functional significance of these differences. A second, more likely explanation is that the various nonproducer cells were originally selected as being transformed and are remarkably invariant in this property (24). Therefore, the relative uniformity of elevated expression within these cells may simply reflect a requirement for viral transformation. This observation is of some importance in understanding the mechanism of p21-mediated cell transformation. Recently, the cellular and viral homologs of the ras gene family have been shown to possess differences which could alter the enzymatic activities of p21 in cells (28, 31, 36). Specifically, normal cellular homologs have a glycine residue at amino acid position 12 from the N terminus of the protein, whereas oncogenic cellular variants and the viral ras p21 proteins have a different amino acid substituted at the same position (31, 36, 40). According to recently proposed three-dimensional models of the p21 polypeptide chain, such a replacement of glycine for any other amino acid (possessing a side chain) would lead to an altered mode of nucleotide binding and disruption of the normal functioning or control of p21 (43). This, in turn, would account for oncogenic activation in the absence of elevated expression. Such a mechanism implies that elevated expression of retroviral ras genes is not a requirement for cell transformation. However, it seems equally possible from the results we present that the presence of a serine residue in place of glycine in the p21 of KiMSV (40) may be insufficient in itself to account for the highly oncogenic properties of the virus and that elevated expression is also an important factor.

In the revertant cell lines, we have previously found no loss or alteration of proviral sequences (25a), in contrast to KiMSV revertants that have been described by others (39). In the present study, expression at the level of both RNA and the p21 transforming protein was found to be essentially the same as that in the parental transformed line. Upon superinfection with the homologous virus, one of the revertants, R2-1, regained tumorigenicity comparable to that of the parental transformed line. The original R2-1 cell line was, however, found to be weakly tumorigenic itself, with tumors appearing after an extended latency period. We do not understand the basis for this apparent partial revertant phenotype displayed by R2-1. However, a possibly similar class of revertants of avian sarcoma virus-transformed cells has been described in which, like our KiMSV revertants, the cells contained a resuable sarcoma virus (11). Thus, a working hypothesis to explain the properties of R2-1 would be that an alteration in the transforming gene has occurred such that the efficiency of its action on cellular targets is reduced.

The other revertant line, R5-5-1, retained a normal phenotype with regard to tumorigenicity upon reinfection with KiMSV. Since there was no block to either infection by or expression of the virus, we can conclude that the reversion is due to a change in cellular factors required for transformation. A possibly similar type of phenotypic reversion of KiMSV-transformed cells has been described previously (35). Assuming that a single cellular lesion is involved in the reversion of R5-5-1, it follows that common mechanism(s) are involved whether transformation is due to a structural alteration of p21 or elevated expression of this protein. These mechanisms are blocked in the R5-5-1 cell line. Whatever the precise mechanism involved in KiMSV transformation, cellular mutants such as this should prove invaluable for delineating cellular targets of the ras oncogene product in the mouse system.

ACKNOWLEDGMENTS

This work was supported by the Cancer Research Campaign of Great Britain.

We thank E. M. Scolnick for the gift of antiserum to p21.

LITERATURE CITED


