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p21 of Kirsten Murine Sarcoma Virus Is Thermolabile in a Viral Mutant Temperature Sensitive for the Maintenance of Transformation

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We have recently described an intracellular protein, p21, in nonproducer cells transformed by either the Kirsten (Ki-MSV) or Harvey (Ha-MSV) strain of murine sarcoma virus (Shih et al., Virology, in press). The p21 is phosphorylated and has been shown to be coded for by either Ki-MSV or Ha-MSV. In this report, we compare the thermal stability of the newly synthesized [^{35}S]methionine-labeled p21 in cells transformed by the wild-type Ki-MSV or by a mutant of Ki-MSV(*ts* 371) which is temperature sensitive in a viral function required for the maintenance of several properties of the transformed phenotype. The immunoprecipitability of the p21 coded for by the *ts* 371 Ki-MSV was markedly more thermolabile than the p21 of the wild-type Ki-MSV when the cell extracts are heated in vitro. The present finding suggests that the p21 is required for the maintenance of transformation induced by Ki-MSV.

Both Kirsten murine sarcoma virus (Ki-MSV) and Harvey murine sarcoma virus (Ha-MSV) are fibroblast-transforming and sarcoma-inducing viruses isolated by passages of murine type C helper viruses through rats. Molecular analyses of these viral genomes have established that both are recombinant viruses with the rat genetic sequences as large inserts between sequences derived from the type C helper viruses at both ends of the genomic viral RNA (13, 14, 17; Y. H. Chien, M. Lai, T. Y. Shih, I. M. Verma, E. M. Scolnick, and N. Davidson, submitted for publication). Recently, we have identified a phosphoprotein of 21,000 (21K) daltons (p21) apparently encoded by the rat insert sequences in nonproducer cells transformed by either Ha-MSV or Ki-MSV (16). Antisera from rats bearing tumors induced by syngeneic transplantation of Ha-MSV-transformed nonproducer cells specifically precipitate the virus-encoded p21 from nonproducer cells transformed by either Ha-MSV or Ki-MSV and from polypeptides synthesized by in vitro translation of either virion RNA.

An important question is whether the p21 of Ki-MSV and Ha-MSV is the transforming *src* protein of these sarcoma viruses. In this paper, we report the characterization of a Ki-MSV mutant, *ts* 371, temperature-sensitive for the maintenance of several properties of the transformed cell phenotype, and demonstrate that the p21 encoded by the *ts* 371 Ki-MSV is significantly more thermolabile than the wild-type (wt) Ki-MSV p21.

MATERIALS AND METHODS

Cells and viruses. Two contact-inhibited mouse cells were used, NIH 3T3 cells and C127 cells, a contact-inhibited cell line derived from an RIII mouse (9). Normal rat kidney cells (NRK) have been described (6). All cells were grown in Dulbecco-Vogt-modified Eagle medium with 10% calf serum (Flow Laboratories).

A clone of ecotropic Moloney murine leukemia virus (Mo-MuLV) was the gift of Nancy Hopkins (Massachusetts Institute of Technology, Cambridge, Mass.) and was routinely propagated in NIH 3T3 cells. A Ki-MSV-transformed nonproducer NIH 3T3 cell has been described (2, 14). XC plaque assays were performed on NIH 3T3 cells and focus assays were performed on C127 cells as previously described (8, 12).

Isolation of temperature-sensitive mutants. A culture of approximately 10^6 NIH 3T3 nonproducer cells transformed by Ki-MSV was superinfected in a 75-cm^2 flask with approximately 100 XC PFU of the ecotropic Mo-MuLV. The helper-independent Mo-MuLV was allowed to replicate for 5 days until the cells reached confluence. Approximately 5% of the cells were then subcultured to a new 75-cm^2 flask, and a virus stock was prepared when these cells again reached confluence. To prepare Ki-MSV/Mo-MuLV mutagenized by 5-bromodeoxyuridine, a 24-h collection of virus was prepared from the above culture, and approximately 5×10^6 NIH 3T3 cells were infected at 34°C with approximately 5×10^6 focus-forming units (FFU) and 5×10^6 XC PFU of the Ki-MSV/Mo-MuLV virus complex. Infection was carried out during the first 24 h in the absence and in the presence of 50 μg of 5-bromodeoxyuridine per ml. After 24 h, both cultures were fed with fresh media. Two days later the cultures were refed, and a virus collection was made

18 h later. Using C127 cells as indicator cells, the titer of the virus stock prepared in the absence of 5-bromodeoxyuridine was approximately 2×10^5 FFU/ml and 2×10^5 XC PFU/ml, and in the presence of 5-bromodeoxyuridine, it was 5×10^2 FFU/ml and 10^2 XC PFU/ml. The 5-bromodeoxyuridine-treated virus stock was used to infect NRK cells at a low multiplicity of infection, and approximately 1,000 nonproducer foci were isolated at 34°C from this virus stock. Each nonproducer NRK cell was reinfected with wt Mo-MuLV, and the virus progeny released were titrated on new NRK cells for FFU at 34°C and 39 to 39.5°C. Initial titrations were performed by infecting two parallel plates of NRK cells with a single dilution of virus at 34°C; 24 h later, one culture was transferred to 39 to 39.5°C. Foci were scored at day 7 after infection at 39°C and at day 9 at 34°C. Cells grown at 34°C were fed twice a week and those grown at 39 to 39.5°C were fed three times per week. Methods for growth of cells in 0.3% (wt/vol) soft agar in Falcon microtest plates have been described (1).

Preparation of antisera. Antisera containing antibodies to the p21 were prepared from rats bearing tumors induced by syngeneic transplantation of NRK cells transformed by Ki-MSV in newborn Osborne-Mendel rats following procedures previously described for Ha-MSV antisera (16). A total of 2×10^7 cells were inoculated into 10-day-old weanling rats, and sera were obtainted by cardiac puncture 3 to 6 weeks after tumors had appeared.

Cell labeling and preparation of cell extracts. Procedures for cell labeling and preparation of cell extracts were fully described previously (16). Cell stocks infected with wt Ki-MSV or *ts* 371 Ki-MSV were maintained at 34°C. A total of 2×10^6 cells were seeded in each 100-mm plastic petri dish, or 5×10^5 cells were seeded in each 60-mm dish. Forty-eight hours later cells were labeled with [^{35}S]methionine (400 to 800 μCi/ml) in methionine-free Dulbecco-Vogt-modified Eagle minimal essential medium supplemented with 1 or 2% dialyzed fetal calf serum for 4 h at 34 or 39°C as indicated. To label phosphoprotein, cells were incubated with [^{32}P]orthophosphate (0.5 mCi/ml) in phosphate-free medium supplemented with 2% dialyzed fetal calf serum for 4 h at 34 or 39°C.

To prepare cell extracts for heat inactivation experiments, cells grown at 34°C in 100-mm dishes were lysed by three passages through a 22-gauge needle in 2.5 ml of buffer containing 1% Triton X-100, 0.1 M NaCl, 0.02 M Tris-hydrochloride (pH 7.2), 0.005 M $MgCl_2$, and a protein inhibitor, Trasylol (200 U/ml; Calbiochem, San Diego, Calif.). The cell lysates were centrifuged at 2,000 × *g* for 20 min to remove nuclei and cell debris. To reduce nonspecific immunoprecipitation, the cell extracts were precleaned by incubation with 0.6 ml of formaldehyde-fixed *Staphylococcus aureus* suspension (10%, vol/vol) per 2.5 ml of extract for 1 h. It was then cleared by centrifugation at 150,000 × *g* for 30 min. All these operations were carried out at 4°C. Samples of 8×10^7 incorporated cpm (ca. 60 μl) were stored in liquid nitrogen. After thawing the lysates on ice before use, heat inactivation was performed for 5 min in a water incubator preset at indicated temperatures. To prepare cell extracts for other immunoprecipitation, cells were lysed directly in immunoprecipitation buffer containing 1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 0.01 M phosphate buffer (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and 200 U of Trasylol per ml. Cell lysates were clarified as above. Under present labeling conditions, the C127 cells infected with wt Mo-MuLV, wt Ki-MSV/wt Mo-MuLV, or *ts* 371 Ki-MSV/wt Mo-MuLV incorporated approximately the same 1.5×10^8 cpm of [^{35}S]methionine in extracts from 10^6 cells at 34°C, or 10^8 cpm from 10^6 cells at 39°C.

Immunoprecipitation and polyacrylamide gel electrophoresis. Cell extracts containing 6×10^6 to 60×10^6 cpm of incorporated [^{35}S]methionine in 0.1 to 0.5 ml of immunoprecipitation buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 0.01 M NaH_2PO_4-Na_2HPO_4 [pH 7.5], 0.1 M NaCl, 1 mM EDTA, and 200 U of Trasylol per ml) were incubated with 7.5 to 15 μl of antisera at 4°C for 15 h. The antigen-antibody complexes were precipitated by addition of 50 μl of a 10% (vol/vol) suspension of formaldehyde-fixed *S. aureus* (Cowan strain I) containing protein A as described by Kessler (10). After 2 h at 4°C, precipitates were washed three times with the same buffer and extracted in gel electrophoresis buffer containing sodium dodecyl sulfate and 2-mercaptoethanol at 95°C for 3 min. The precipitated proteins were analyzed by discontinuous polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. The detailed procedures for immunoprecipitation and gel electrophoresis were described in our previous publication (16).

RESULTS

Isolation of a conditional mutant of Ki-MSV. To obtain a mutant of Ki-MSV temperature sensitive for the maintenance of transformation, a virus stock of Ki-MSV/Mo-MuLV mutagenized with 5-bromodeoxyuridine was prepared as described in Materials and Methods. Individual nonproducer foci were isolated as described and rescued with unmutagenized Mo-MuLV. Each virus stock was then titrated at 34°C and 39 to 39.5°C. The permissive temperature was chosen since the mouse cells replicated relatively well (within a twofold doubling rate) at this temperature compared to 37°C. The nonpermissive temperature of 39.5°C was the highest temperature at which mouse cells could be propagated without significant toxicity. Of approximately 1,000 foci examined, only one clone, 371, was temperature sensitive. The titration of *ts* 371 Ki-MSV and wt Ki-MSV is shown in Table 1, and pictures of C127 cells producing wt Mo-MuLV, wt Ki-MSV/wt Mo-MuLV, or *ts* 371 Ki-MSV/wt Mo-MuLV are shown in Fig. 1.

The titer of the *ts* 371 Ki-MSV/wt Mo-MuLV released from NRK cells was 5×10^4 to 1×10^5 FFU/ml at 34°C on C127 or NRK indicator cells. When titrations were performed at 39.5°C, an approximately 500-fold-lower titer was observed on each indicator cell. wt Ki-MSV/wt

TABLE 1. *Focus-forming titers of wild-type and ts 371 mutant of Ki-MSV*[a]

Virus used	Temp (°C)	Titer (FFU/ml)		
		Monolayer assays		Agar assays (NRK)
		C127	NRK	
wt Ki-MSV/wt Mo-MuLV	39	2×10^5	1×10^5	2×10^5
	34	2×10^5	1×10^5	2×10^5
ts 371 Ki-MSV/wt Mo-MuLV	39	1×10^2	2×10^2	1×10^1-2×10^1
	34	5×10^4	1×10^5	1×10^5

[a] Virus titrations were performed using an ecotropic wt Mo-MuLV pseudotype of wt Ki-MSV or *ts* 371 Ki-MSV propagated at 34°C on NRK cells. Growth in soft agar was performed as described (1). Each virus assay was performed in duplicate determinations which were within 15% of each other.

Mo-MuLV yielded titers of approximately 2×10^5 FFU/ml on either indicator cell at either temperature. Titrations of wt Ki-MSV and *ts* 371 Ki-MSV were also performed using NRK cells suspended in soft agar at 34 versus 39°C. NRK cells were infected at 34°C with the wt Ki-MSV/wt Mo-MuLV or *ts* 371 Ki-MSV/wt Mo-MuLV; 18 h later, cells were suspended in soft agar and half the plates were transferred to 39.5°C. Under these conditions, using NRK cells and wt Ki-MSV/wt Mo-MuLV, we have found that focus-forming virus titers are comparable in soft-agar assays and in monolayer assays. The wt Ki-MSV/wt Mo-MuLV yielded titers of 2×10^5 FFU/ml at both temperatures (Table 1). The *ts* 371 Ki-MSV/wt Mo-MuLV yielded 10^5 FFU/ml at 34°C and only 10 to 20 small foci at 39.5°C. The results indicate that clone 371 Ki-MSV was temperature sensitive for transformation. We could not obtain an efficient agar assay with C127 cells.

C127 cells infected at 34°C and producing wt Mo-MuLV, wt Ki-MSV/wt Mo-MuLV, or *ts* 371 Ki-MSV/wt Mo-MuLV were grown at 34°C and shifted, once transformed, to 39.5°C. The cells are shown in Fig. 1. Cells infected with wt Ki-MSV/wt Mo-MuLV are round, refractile, and piled one upon another at both temperatures (Fig. 1D and E). Cells infected with *ts* 371 Ki-MSV/wt Mo-MuLV are almost as round and are piled upon each other at 34°C (Fig. 1A), as are the C127 cells infected with wt Ki-MSV/wt Mo-MuLV. When these cells are shifted to 39.5°C, however, the cells infected with the mutant (Fig. 1B) are flat and form a monolayer much like the C127 cell infected only with wt Mo-MuLV (Fig. 1F). When the flat, mutant-infected C127 cells are further subcultured at 34°C, they once again resume the rounded, heaped-up morphology (Fig. 1C). Maximum cell densities given in Table 2 indicate almost a 10-fold difference between normal C127 cells and C127 cells infected with wt Ki-MSV/wt Mo-MuLV at either 34 or 39°C. The cells infected with *ts* 371 Ki-MSV/wt Mo-MuLV have an equally high maximum cell density at 34°C but resemble C127 cells at 39.5°C.

To perform soft-agar assays, NRK cells nonproductively transformed at 34°C with either wt Ki-MSV or *ts* 371 Ki-MSV were tested for growth in soft agar at 34 and 39.5°C. Ki-NRK_{wt} grew at almost 100% efficiency in soft agar at 34 or 39.5°C (Table 2). Ki-$NRK_{ts\,371}$ formed colonies well in agar at 34°C but virtually none at 39.5°C. Even colonies that were visible at 39.5°C in soft agar were small, containing only 4 to 8 cells per colony compared to wild-type colonies, which contained 30 to 60 cells per colony. The results indicate that clone 371 Ki-MSV is a mutant temperature sensitive for the maintenance of properties of the transformed phenotype.

Thermolability of p21 of the *ts* 371 Ki-MSV mutant. Since *ts* 371 Ki-MSV is temperature sensitive for the maintenance of the transformed cell phenotype, we have been interested in determining whether the p21 specified by the temperature-sensitive mutant is thermolabile. C127 cells infected with *ts* 371 Ki-MSV/wt Mo-MuLV or wt Ki-MSV/wt Mo-MuLV were grown at the permissive temperature of 34°C. As a control, C127 cells infected with wt Mo-MuLV alone were also used. Cellular proteins were labeled with [^{35}S]methionine for 4 h at 34°C, and cell lysates were prepared at 4°C. Samples of the cell lysates were heated at temperatures ranging from 36 to 48°C for the same period of 5 min. A sample left at 0°C on ice without any heating was used as a control. Each extract was then immunoprecipitated at 4°C with Ki-MSV antiserum. Figure 2 depicts the virus-specific p21 precipitated after these treatments. p21 of the wt Ki-MSV was precipitated equally well over the whole range of temperatures as can be seen in Fig. 2A: lanes 5 and 6, 0°C; lane 7, 36°C, lane 8, 39°C; lane 9, 42°C; lanes 10 and 11, 45°C; and lane 12, 48°C. No protein of the size of 21K is visible for the same extract reacted with the normal rat serum at either extreme temperature (lane 4, 0°C; lane 13, 48°C). It is noticeable in this figure that heating of cell extracts at increasing temperatures causes increased aggregation and denaturation of many cellular proteins, which considerably increases the background level in the electrophoretogram. This, however, does not seriously interfere with the resolution of low-molecular-weight proteins, such as p21, in which we are interested (see inserts, Fig. 2, for autoradiogram of longer exposure). The antisera prepared from rats bearing tumors induced by nonproducer cells transformed by Ki-MSV or

Ha-MSV do not react with any viral protein of the Mo-MuLV helper type C virus, since no Mo-MuLV virion protein labeled with [^{35}S]methionine was precipitated by the antisera and no additional protein from Mo-MuLV-infected cells, as compared with uninfected control cells, was precipitated by the antisera (16; unpublished data). This is consistent with the known

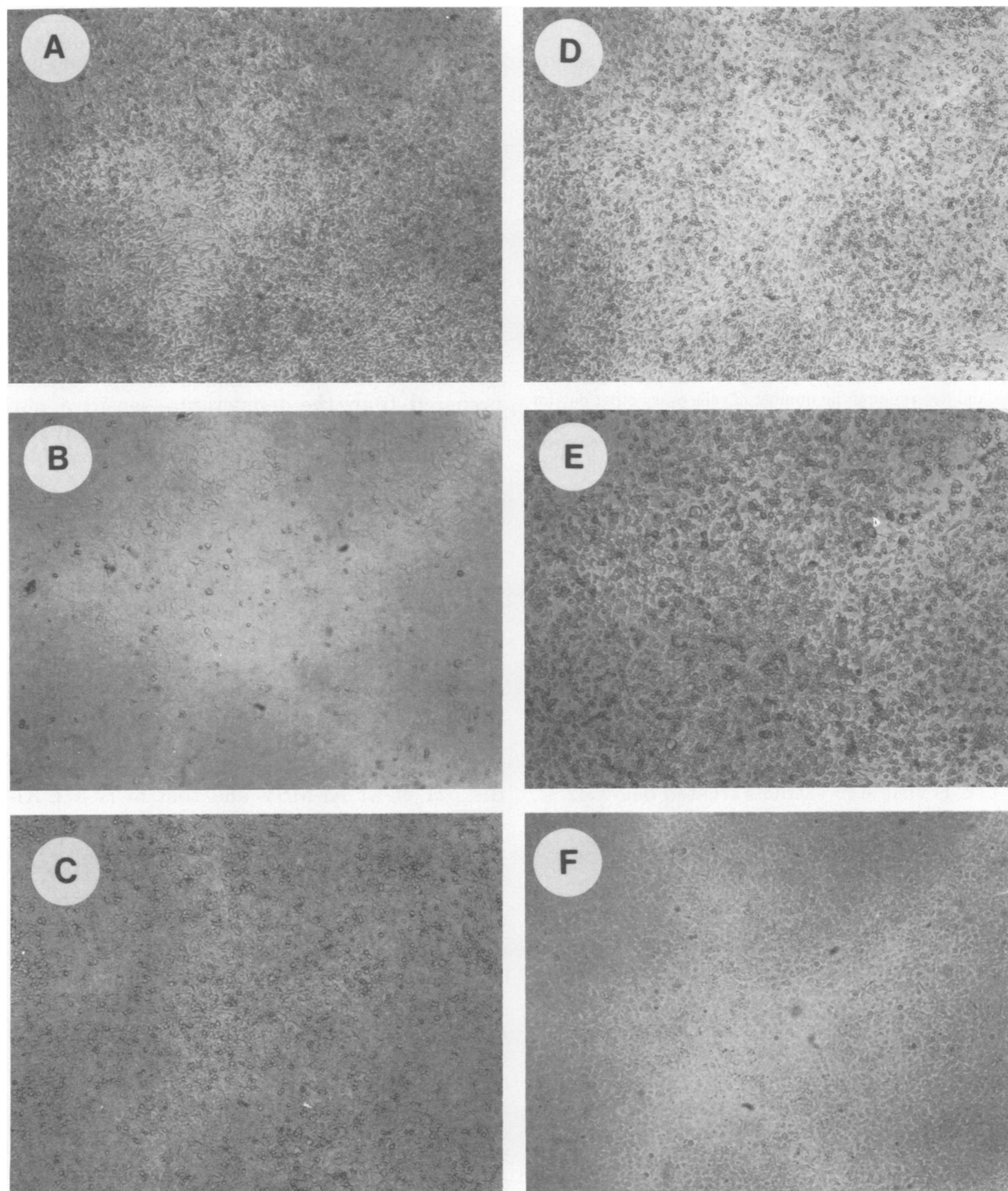

FIG. 1. *Morphology of C127 cells infected with wt Ki-MSV or ts 371 Ki-MSV. C127 cells infected with wt Mo-MuLV, wt Ki-MSV/wt Mo-MuLV, or ts 371 Ki-MSV/wt Mo-MuLV were grown for approximately 7 days at 34°C. At that time, 3 × 10^5 cells of each culture were seeded on 60-mm petri dishes and grown for 48 h at either 34 or 39.5°C, at which time photographs were taken at approximately ×60 magnification. The ts 371 Ki-MSV-containing cells at 39.5°C were subcultured again at 3 × 10^5 cells per 60-mm dish at 34°C and photographed again 60 h later. (A) ts 371 Ki-MSV/wt Mo-MuLV, 34°C; (B) ts 371 Ki-MSV/wt Mo-MuLV, 34 → 39°C; (C) ts 371 Ki-MSV/wt Mo-MuLV, 34 → 39 → 34°C; (D) wt Ki-MSV/wt Mo-MuLV, 34°C; (E) wt Ki-MSV/wt Mo-MuLV, 34 → 39°C; (F) wt Mo-MuLV alone, 34°C.*

TABLE 2. *Growth efficiency of cells transformed by wild-type and ts 371 mutant of Ki-MSV*[a]

Cells used	Temp (°C)	Efficiency of growth in agar	Maximum cell density (cells/plate)
Ki-NRK_{wt}	39	0.92	ND
	34	0.98	ND
Ki-$NRK_{ts\,371}$	39	<0.001	ND
	34	0.90	ND
C127(wt Mo-MuLV)	39	ND	1.0×10^6
	34	ND	1.1×10^6
C127(wt Ki-MSV/wt Mo-MuLV)	39	ND	1.0×10^7
	34	ND	1.1×10^7
C127(*ts* 371/wt Mo-MuLV)	39	ND	1.5×10^6
	34	ND	1.0×10^7

[a] Efficiency of growth is the number of colonies that grow divided by the number of colonies seeded in agar. Plates were scored on day 7 at 39°C and on day 12 at 34°C. Maximum cell density was determined by seeding 10^5 cells per 60-mm petri dish and determining the number of cells every other day for 12 days at 34°C and for 10 days at 39°C. Cells were fed every 3 days during that period on days 3, 6, and 9. Each cell assay was performed in duplicate determinations which were within 15% of each other. ND, Not determined.

structure of Ki-MSV and Ha-MSV genomes which do not retain any helper coding sequences of the *gag*, *pol*, and *env* genes (17; Chien et al., submitted for publication). One of the results obtained from C127 cells infected with wt Mo-MuLV alone is presented in Fig. 2A. Lane 1 is the extract at 0°C without heating, lane 2 is that heated at 42°C, and lane 3 is that heated at 48°C reacted with the Ki-MSV antiserum. No protein of 21K is visible in extracts treated below 42°C, although at the highest temperature of 48°C an aggregation product with mobility slightly slower than that of p21 is visible.

When cell extracts prepared from C127 cells infected with *ts* 371 Ki-MSV/wt Mo-MuLV were heated in parallel to extracts of wt Ki-MSV/wt Mo-MuLV, a marked decrease in immunoprecipitation of the p21 band of the *ts* 371 Ki-MSV was seen (Fig. 2B). This result has been demonstrated in three separately prepared extracts. Without heating at 0°C, the mutant p21 is precipitable with Ki-MSV antiserum (lanes 5 and 6). After heating even at 36°C for 5 min, the p21 band of *ts* 371 Ki-MSV is markedly reduced in intensity (lane 7), and at higher temperatures (lane 8, 39°C; lane 9, 42°C; lanes 10 and 11, 45°C; lane 12, 48°C) the intensity is decreased to a background level. Lanes 4 and 13 are the extracts at both extreme temperatures of 0°C and 48°C reacted with a normal rat serum. Lanes 1, 2, and 3 are the extracts from C127 cells infected with Mo-MuLV alone reacted with the Ki-MSV antiserum (lane 1, 0°C; lane 2, 42°C; lane 3, 48°C). It is apparent from the autoradiograph with longer exposure, presented in the insert of Fig. 2B, that there is a residual 21K band even after the mutant extract is heated at 48°C. The residual 21K band in extracts heated at higher than 39°C may be due to residual Ki-MSV p21, to some aggregation products, or to a similarly sized protein always noted in most mammalian cells labeled with [^{35}S]methionine and immunoprecipitated with Ki-MSV or Ha-MSV antisera (16). Therefore, disappearance of the p21 band may be more pronounced than the data presented here show. The results of an experiment with lower background can be seen in Fig. 4C. In other experiments not shown, heat inactivation experiments performed with freshly prepared extracts without freezing in liquid nitrogen and thawing yielded similar results. The fact that thermolability of the *ts* 371 Ki-MSV p21 is not due to the altered milieu in the extracts prepared from the temperature-sensitive mutant-infected cells but rather to the intrinsic property of the temperature-sensitive mutant p21 is shown by a mixing experiment. The [^{35}S]methionine-labeled wt Ki-MSV/wt Mo-MuLV extract was mixed with an equal amount of unlabeled extracts prepared either from the *ts* 371 Ki-MSV/wt Mo-MuLV-infected cells or as a control from cells infected with wt Mo-MuLV alone. The mixtures were then heated at 39 and 45°C. No loss of immunoprecipitability of the [^{35}S]methionine-labeled wt Ki-MSV p21 was noted in these mixtures as compared with the control at 0°C. In conclusion, these studies indicate a marked difference in the lability of the p21 of wt Ki-MSV and that of *ts* 371 Ki-MSV.

Other altered properties of p21 of the *ts* 371 Ki-MSV mutant. p21 of *ts* 371 Ki-MSV appears to be more thermolabile than wt Ki-MSV p21 upon in vitro heat inactivation. We next examined the characteristics of the p21 in cells infected with wt Ki-MSV or *ts* 371 Ki-MSV. C127 cells infected with either wt Ki-MSV/wt Mo-MuLV or *ts* 371 Ki-MSV/wt Mo-MuLV propagated at 34°C were seeded in new plates, and half of those plates were shifted up to 39°C. After 2 days of growth at 39 or 34°C, cells were labeled with [^{35}S]methionine at the respective temperatures for 4 h. Cell lysates were then prepared at 4°C. Lysates from the same number of cells growing at 39 and 34°C were immunoprecipitated with the same amount of antisera. The results of this experiment are shown in Fig. 3A. As expected, a normal rat serum precipitated no band of 21K from either 39°C or 34°C extracts of cells infected with wt Ki-MSV/wt Mo-MuLV. Ki-MSV antiserum precipitated a typical p21 band from cells growing at both 39 and

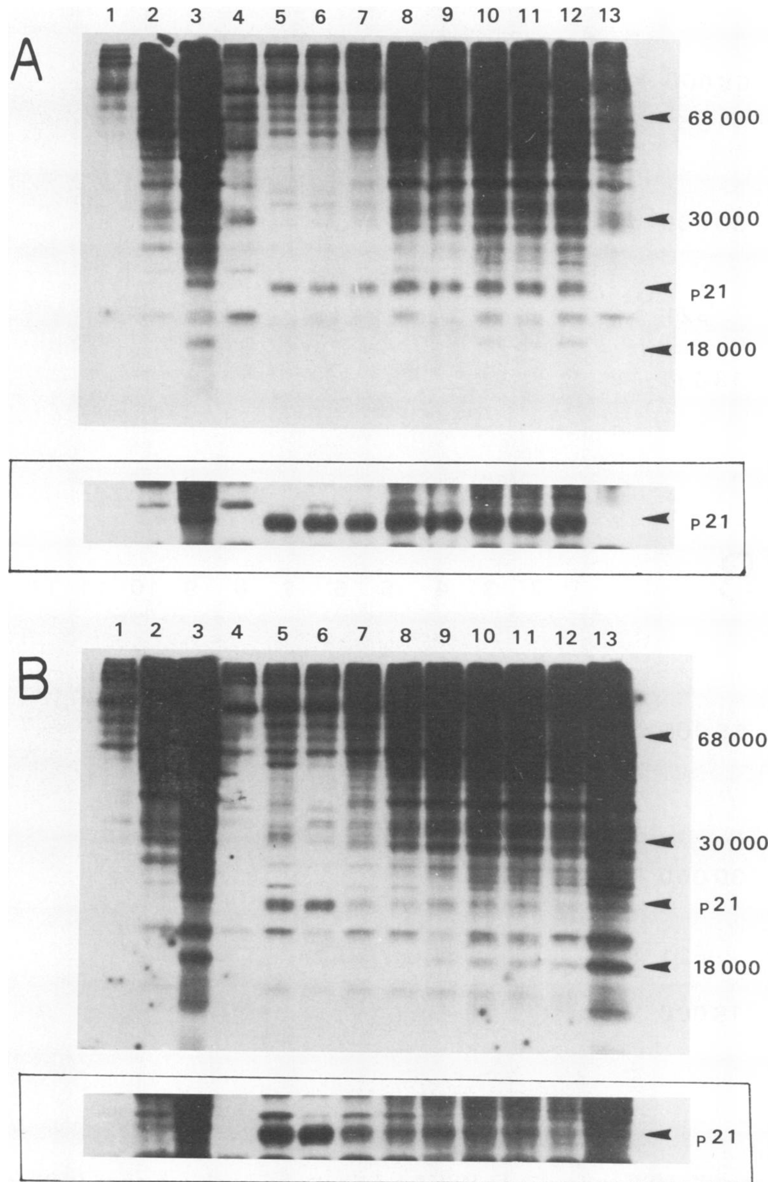

FIG. 2. *In vitro heat inactivation of Ki-MSV p21. (A) Cell lysates of C127 cells infected with wt Ki-MSV/wt Mo-MuLV or with wt Mo-MuLV alone. Cells were labeled at 34°C and lysates were prepared at 4°C as described in the text. Aliquots of the extracts containing 8×10^7 incorporated cpm were heated at various temperatures (indicated below) for exactly 5 min. Portions containing 6×10^6 cpm (10^5 cells) were incubated with 7.5 µl of Ki-MSV antiserum or normal rat serum as the control. The precipitated proteins were subjected to electrophoresis in 13% polyacrylamide gel with ^{125}I-labeled bovine serum albumin (68K), F-MuLV p30 (30K), and bovine milk β-lactoglobulin (18K) as molecular weight markers. Fluorography was for 3 days (insert, 7 days). Cells infected with wt Mo-MuLV and with the Ki-MSV antiserum: lane 1, 0°C; lane 2, 42°C; lane 3, 48°C. Cells infected with wt Ki-MSV/wt Mo-MuLV and with the Ki-MSV antiserum: lanes 5 and 6, 0°C; lane 7, 36°C; lane 8, 39°C; lane 9, 42°C; lanes 10 and 11, 45°C; lane 12, 48°C. The same lysate and with a normal rat serum: lane 4, 0°C; lane 13, 48°C. (B) Lysates of C127 cells infected with ts 371 Ki-MSV/wt Mo-MuLV and C127 cells infected with wt Mo-MuLV alone. After heating, portions of 12×10^6 cpm (2×10^5 cells) were incubated with 7.5 µl of antiserum. Procedures were the same as in (A). Cells infected with wt Mo-MuLV and with the Ki-MSV antiserum: lane 1, 0°C; lane 2, 42°C; lane 3, 48°C. Cells infected with ts 371 Ki-MSV/wt Mo-MuLV and with the Ki-MSV antiserum: lanes 5 and 6, 0°C; lane 7, 36°C; lane 8, 39°C; lane 9, 42°C; lanes 10 and 11, 45°C; lane 12, 48°C. The same lysate and with a normal rat serum: lane 4, 0°C; lane 13, 48°C.*

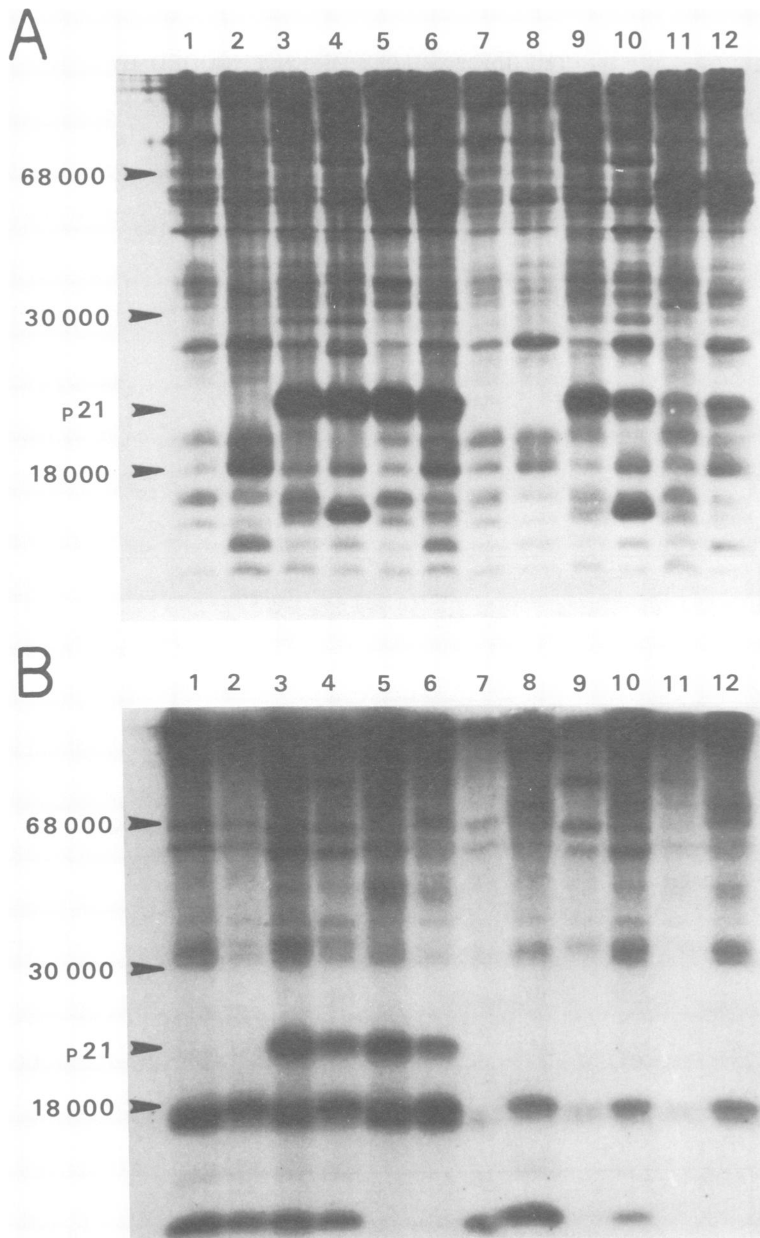

FIG. 3. *p21 of wt Ki-MSV and ts 371 Ki-MSV labeled with [^{35}S]methionine and [^{32}P]orthophosphate. (A) [^{35}S]methionine labeling: C127 cells infected with wt Ki-MSV/wt Mo-MuLV or ts 371 Ki-MSV/wt Mo-MuLV were labeled at 39 or 34°C for 4 h, and extracts were prepared at 4°C. Cell lysates from 3 × 10^5 cells were reacted with 10 μl of antisera. The precipitated proteins were subjected to electrophoresis in 13% gel with markers indicated in Fig. 2. Fluorography was for 2 days. Extracts from cells infected with wt Ki-MSV/wt Mo-MuLV and reacted: with normal rat serum, 39°C (lane 1), 34°C (lane 2); with Ki-MSV antiserum, 39°C (lane 3), 34°C (lane 4); with Ha-MSV antiserum, 39°C (lane 5), 34°C (lane 6). Extracts from cells infected with ts 371 Ki-MSV/wt Mo-MuLV and reacted: with normal rat serum, 39°C, (lane 7), 34°C (lane 8); with Ki-MSV antiserum, 39°C, (lane 9), 34°C (lane 10); with Ha-MSV antiserum, 39°C (lane 11), 34°C (lane 12). (B) [^{32}P]orthophosphate labeling: sister plates of C127 cells infected with wt Ki-MSV/wt Mo-MuLV or ts 371 Ki-MSV/wt Mo-MuLV were labeled with [^{32}P]orthophosphate at 39 and 34°C. All conditions were similar to those for (A). Autoradiography was for 1 day with Dupont intensifying screen. Extracts from cells infected with wt Ki-MSV/wt Mo-MuLV and reacted: with normal rat serum, 39°C (lane 1), 34°C (lane 2); with Ki-MSV antiserum, 39°C (lane 3), 34°C (lane 4); with Ha-MSV antiserum, 39°C (lane 5), 34°C (lane 6). Extracts from cells infected with ts 371 Ki-MSV/wt Mo-MuLV and reacted: with normal rat serum, 39°C (lane 7), 34°C (lane 8); with Ki-MSV antiserum, 39°C (lane 9), 34°C (lane 10); with Ha-MSV antiserum, 39°C (lane 11), 34°C (lane 12).*

34°C (lanes 3 and 4). An Ha-MSV antiserum (16) also brought down the p21 equally well from cells infected with the wt Ki-MSV at both 39 and 34°C. From lysates prepared from C127 cells infected with *ts* 371 Ki-MSV/wt Mo-MuLV and growing at 39 or 34°C, the normal rat serum did not precipitate any protein of 21K (lane 7, 39°C; lane 8, 34°C). The Ki-MSV antiserum, however, precipitated the p21 protein at both 39 and 34°C and failed to discriminate this protein in cells growing at nonpermissive and permissive temperatures, although the same antiserum did discriminate the thermolability of the p21 in in vitro inactivation studies. Importantly, when the same *ts* 371 Ki-MSV extract was reacted with a heterologous Ha-MSV antiserum, a loss of p21 immunoprecipitability from the *ts* 371 Ki-MSV lysate of 39°C compared to that of 34°C can be detected (lanes 11, 39°C; lane 12, 34°C). The results indicate that in vivo thermolability of the p21 of the *ts* 371 Ki-MSV can be demonstrated by using this Ha-MSV antiserum, which may possess fewer antigen recognition sites in the heterologous reaction, but not with Ki-MSV antisera. It is of interest that different anti-$p60^{src}$ sera of Rous sarcoma virus also discriminate differently the $p60^{src}$ of wt and temperature-sensitive Rous sarcoma virus in this type of in vivo thermolability study (3, 11).

Since p21 of both Ki-MSV and Ha-MSV are phosphorylated and phosphorylation may be involved in modulation of its function, it is of interest to examine whether the extent of Ki-MSV p21 phosphorylation is altered in the temperature-sensitive mutant. A set of sister plates as described in Fig. 3A was labeled with [^{32}P]orthophosphate for 4 h at both 39 and 34°C; the results are shown in Fig. 3B. As expected, the p21 of the wt Ki-MSV was labeled at both 39 and 34°C and was immunoprecipitated with both Ki-MSV antiserum (lane 3, 39°C; lane 4, 34°C) and Ha-MSV antiserum (lane 5, 39°C; lane 6, 34°C). A normal rat serum did not precipitate any protein band at 21K (lane 1, 39°C; lane 2, 34°C). In contrast, from C127 cells infected with the mutant, no p21 band is noticeable from reaction with normal rat serum (lane 7, 39°C; lane 8, 34°C), Ki-MSV antiserum (lane 9, 39°C; lane 10, 34°C), or Ha-MSV antiserum (lane 11, 39°C; lane 12, 34°C). In experiments of a sister set of plates labeled simultaneously with [^{35}S]methionine, the p21 of the *ts* 371 Ki-MSV mutant was clearly precipitated (Fig. 3A). It can be concluded that even at the permissive temperature the p21 of the mutant is deficient in phosphorylation or, alternatively, the mutant p21 dephosphorylates readily or the phosphorylated form of p21 of the mutant is not precipitable by our antitumor sera. Although the experiments of Fig. 3 were performed on producer cells at 34°C, essentially similar results were obtained on producer cells grown at 31°C, or with NRK nonproducer cells transformed by the wt Ki-MSV versus the *ts* 371 Ki-MSV. The physiological significance of the inability to precipitate a p21 from the *ts* 371 Ki-MSV-transformed cells at 34°C is not clear. Nevertheless, the results again indicate that the p21 of the *ts* 371 Ki-MSV is altered as compared with that of the wt Ki-MSV.

p21 of a wild-type revertant of the *ts* 371 Ki-MSV. To further examine the role of p21 in Ki-MSV-induced transformation, we isolated a revertant of *ts* 371 Ki-MSV which had lost the temperature-sensitive phenotype. C127 cells infected with *ts* 371 Ki-MSV/wt Mo-MuLV were grown at 39.5°C for 14 days. Foci which appeared on the flat monolayer at this time were isolated and recultured at 34°C. The virus progeny of each focus were tested for their transforming titers at 34 and 39.5°C on new indicator cells. In addition, the individual foci from the monolayer were also subcultured at 39.5°C. The virus released from one focus, called revertant 1, had lost the temperature-sensitive phenotype. C127 cells infected with this revertant at either 34 or 39.5°C were rounded and morphologically transformed. The titer of FFU per milliliter obtained from a culture of this revertant was only five- to seven fold lower at 39.5°C than at 34°C in monolayer assays on C127 cells, as opposed to the 500-fold difference observed in the *ts* 371 Ki-MSV. Thus, we concluded that this Ki-MSV clone had significantly reverted toward properties of the wt Ki-MSV.

We were interested in determining whether the thermostability of the revertant p21 had restored to that of the wt Ki-MSV p21. [^{35}S]-methionine-labeled extracts were prepared in parallel from C127 cells grown at 34°C which were infected with the wt Mo-MuLV alone or with wt Ki-MSV/wt Mo-MuLV, *ts* 371 Ki-MSV/wt Mo-MuLV, or revertant Ki-MSV/wt Mo-MuLV. In vitro heat inactivation was performed as described above, and the immunoprecipitability of the p21 by a Ki-MSV antiserum is shown in Fig. 4. Again, the p21 band of the wt Ki-MSV was precipitated equally well over the whole range of temperatures from 0 to 45°C (Fig. 4A). The p21 band of the *ts* 371 Ki-MSV once again lost its precipitability after heating above 36°C (Fig. 4C). The p21 of the revertant Ki-MSV is present after heat inactivation, and only a slight loss of precipitability was noted after heating the extract at 45°C (Fig. 4B). The results indicate that the p21 of the revertant

from *ts* 371 Ki-MSV regained its thermostability in immunoprecipitation. In other studies not shown, the phosphorylated form of the revertant p21 could be readily detected in the transformed cells, as was the case for the wt Ki-MSV.

DISCUSSION

For all the replication-defective transforming retroviruses, no clear deletion mutant which has lost the transforming function has been isolated.

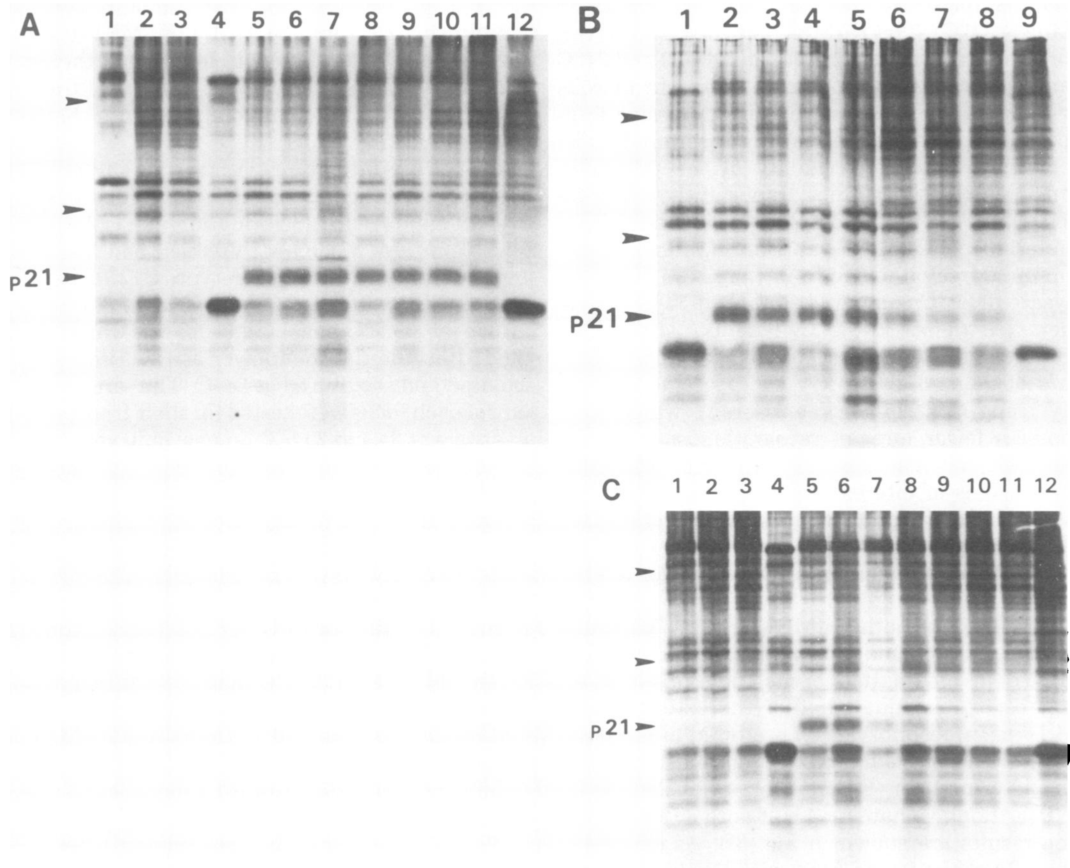

FIG. 4. *In vitro heat inactivation of the p21 of a wild-type revertant of the ts 371 Ki-MSV mutant. Cells were labeled with [^{35}S]methionine at 34°C, and cell lysates were prepared at 4°C as described in the text. Aliquots of extracts were heated at indicated temperatures for 5 min and immunoprecipitated with a Ki-MSV antiserum or a normal rat serum. The precipitated proteins were subjected to electrophoresis in 13% gel. The location of the molecular weight markers are indicated by arrows in this figure: 68K bovine serum albumin (upper arrow) and 30K F-MuLV p30 (lower arrow). Fluorography was for 3 days. (A) Lysates of C127 cells infected with wt Ki-MSV/wt Mo-MuLV or with wt Mo-MuLV alone. Lysates containing 6 × 10^6 cpm (10^5 cells) were precipitated with 7.5 μl of sera. Cells infected with wt Mo-MuLV alone and precipitated with a Ki-MSV antiserum: lane 1, 0°C; lane 2, 42°C; lane 3, 45°C. Cells infected with wt Ki-MSV/wt Mo-MuLV and precipitated with a Ki-MSV antiserum: lanes 5 and 6, 0°C; lane 7, 36°C; lane 8, 39°C; lane 9, 42°C; lanes 10 and 11, 45°C. The same lysate precipitated with a normal rat serum: lane 4, 0°C; lane 12, 45°C. (B) Lysates of C127 cells infected with revertant ts 371 Ki-MSV/wt Mo-MuLV. Lysates containing 6 × 10^6 cpm (10^5 cells) were precipitated with 7.5 μl of sera. Cells infected with revertant ts 371 Ki-MSV/wt Mo-MuLV and precipitated with a Ki-MSV antiserum: lanes 2 and 3, 0°C; lane 4, 36°C, lane 5, 39°C; lane 6, 42°C; lanes 7 and 8, 45°C. The same lysate precipitated with a normal rat serum: lane 1, 0°C; lane 9, 45°C. (C) Lysates of C127 cells infected with ts 371 Ki-MSV/wt Mo-MuLV or with wt Mo-MuLV alone. Lysates containing 1.2 × 10^7 cpm (2 × 10^5 cells) were precipitated with 7.5 μl of sera. Cells infected with wt Mo-MuLV alone and precipitated with a Ki-MSV antiserum: lane 1, 0°C; lane 2, 42°C; lane 3, 45°C. Cells infected with ts 371 Ki-MSV/wt Mo-MuLV and precipitated with a Ki-MSV antiserum: lanes 5 and 6, 0°C; lane 7, 36°C; lane 8, 39°C; lane 9, 42°C; lanes 10 and 11, 45°C. The same lysate with a normal rat serum: lane, 4, 0°C; lane 12, 45°C.*

Only a very few conditional mutants of these replication-defective viruses, temperature sensitive for the maintenance of the transformed cell phenotype, have been reported. These include some heat-sensitive mutants of Ki-MSV isolated in this laboratory (4, 15), a cold-sensitive mutant of Moloney sarcoma virus isolated by Somers and Kit (18), and a heat-sensitive mutant of avian erythroblastosis virus isolated by Graf et al. (7). The recent identification of a nonvirion 21K phosphoprotein (p21) in nonproducer cells encoded by Ki-MSV and Ha-MSV (16) prompted us to characterize the p21 of a mutant of Ki-MSV, which is clearly a viral mutant in a function required for the maintenance of fibroblast transformation. This particular mutant, *ts* 371 Ki-MSV, is readily transmissible as opposed to earlier mutants of Ki-MSV and thus can be studied in the background of many cell types and demonstrates a rather stringent restriction of properties of transformation at 39 to 39.5°C. Thus, it was chosen to determine if the p21 could be directly related to the fibroblast-transforming function of Ki-MSV.

Three experimental results demonstrate that the p21 of *ts* 371 Ki-MSV is structurally altered as compared with the p21 of wt Ki-MSV: (i) in vitro, the p21 of *ts* 371 Ki-MSV shows a marked thermal inactivation of its ability to be immunoprecipitated by Ki-MSV antisera as compared with the p21 of wt Ki-MSV; (ii) in vivo at 39°C, the p21 of *ts* 371 Ki-MSV is less precipitable by a heterologous Ha-MSV antiserum as compared with the p21 of wt Ki-MSV; and (iii) in vivo at both 34 and 39°C, the phosphorylated form of the p21 of *ts* 371 Ki-MSV is not detected by our antisera, although comparable amounts of [^{35}S]methionine-labeled p21 of both viruses are detected. The results suggest that the p21 of *ts* 371 Ki-MSV is altered as compared with the wt Ki-MSV p21. The simplest conclusion is that the p21 of Ki-MSV is required for the maintenance of fibroblast transformation induced by this virus.

There are, however, two important qualifications to consider in drawing this conclusion. The inadequacies of genetic analysis of the replication-defective Ki-MSV preclude us from eliminating the possibility that *ts* 371 Ki-MSV carries two mutations, a mutation in the gene coding for the p21 and another mutation in the "true" *src* gene of the virus. The structural changes in the p21 would be adventitious based on this model and unrelated to the transforming function of Ki-MSV. We cannot exclude this model since fine analysis of this type of replication-defective virus has not been possible up to now. However, the results obtained on the p21 of a revertant of *ts* 371 Ki-MSV make this possibility less likely. The revertant has largely lost the temperature-sensitive phenotype, and simultaneously the p21 has regained the increased thermostability in immunoprecipitation. These results support the hypothesis that alterations in the p21 of *ts* 371 Ki-MSV are directly related to its conditional transformation properties rather than the alternative that the biochemical changes are simply adventitious. The second qualification is that even if the p21 is required for the maintenance of Ki-MSV-induced transformation, the results do not preclude a second gene in Ki-MSV which is required concomitantly. The size of the genome of either Ki-MSV (7.5 kilobases) or Ha-MSV (6 kilobases) is considerably greater than the genetic information required to code for a p21 protein. Thus, although there are no positive data to make us think there is a second protein coded for by Ki-MSV, we must consider the possibility that this is a virus which requires more than one gene to maintain cell transformation.

Finally, it is of interest to note that even at the permissive temperature, a phosphorylated p21 is not detected in cells transformed by the *ts* 371 Ki-MSV, although p21 protein is present. It is possible that our antisera cannot precipitate the phosphorylated p21 in such cells due to structural alterations which have changed its antigenicity. It is also possible that it is the p21 rather than the phosphorylated p21 which is the active form of this protein, although studies on the $p60^{src}$ of avian sarcoma virus indicate that the phosphorylated form, $pp60^{src}$, is associated with a kinase activity (5, 11). We cannot distinguish between these alternatives at present. However, it is worth emphasizing that thermolability of the p21 of *ts* 371 Ki-MSV is observed with proteins labeled with [^{35}S]methionine, and most likely it represents the property of the nonphosphorylated form of the p21 protein.

In conclusion, the thermolability of Ki-MSV p21 in a mutant temperature sensitive for transformation suggests that the p21 is required for the transforming function of Ki-MSV. Further studies on this mutant by complementation with molecularly cloned and amplified viral DNA fragments will enable us to gather insight on the precise location of the *src* gene and to determine whether p21 is indeed the *src* gene product. Purification and microinjection of the p21 of both wt and temperature-sensitive Ki-MSV into fibroblasts may further illuminate the function of this protein.

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