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A Cell Mutant That Exhibits Temperature-Dependent Sensitivity to Transformation by Various Oncogenes

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We previously isolated a Fisher rat fibroblast mutant, B812, that has the unique property of temperature-dependent transformation by various oncogenic retroviruses. At the permissive temperature (35°C), this mutant was sensitive to oncogenic transformation and formed foci on a dish at the same frequency as did the parental fibroblast cell line. When Kirsten murine sarcoma virus (Ki-MSV) was applied to the cells, the frequency of focus formation decreased more than 25-fold at the nonpermissive temperature (39°C), whereas the cells expressed nearly the same level of the *ras* transcript as well as the *ras* protein. The temperature-restricted focus formation was fully reversible and was completely suppressed upon fusion with the wild-type parent cell. In addition to *ras*, the *mos*, *fos*, *src*, and *erbB*-2 oncogenes transformed this mutant with the same temperature dependence as described above; polyomavirus middle T antigen, adenovirus type 12, and human papillomavirus 16-E67 also transformed, but without temperature dependence. These results suggest that *ras*, *fos*, *mos*, *src*, and *erbB*-2 use a common cellular pathway for transforming cells.

Characterization of more than 40 distinct oncogenes thus far reported has greatly contributed to an understanding of the mechanism of oncogenic transformation (for reviews, see references 2, 19, and 34). Despite the evidence for the tumorigenic potential of these oncogenes, the molecular mechanism of oncogene-induced transformation remains unclear.

One approach to investigating the molecular mechanism is to study cell mutants that have an inactivated cellular factor. Some cellular factors may play such crucial roles that cells cannot be transformed without their participation even when oncogenes are fully expressed and functional. Two kinds of cellular mutants have recently been isolated from mammalian cell lines transformed by viral oncogenes: flat revertants (3, 7, 20, 22, 24, 25, 28, 38) and conditional mutants (27, 32, 35). In these mutants, viral oncogenes seem to function normally yet fail to induce transformation, suggesting that their transformation-resistant phenotype is due to a mutation(s) in a cellular gene.

We previously isolated temperature-dependent cell mutants that showed a reduced frequency of focus formation at a nonpermissive temperature (39°C) when they were infected with oncogenic retroviruses (15). One of these mutants, B812, was reported to have a temperature-sensitive cellular factor regulating viral transcription in PyMLV (a chimeric retrovirus containing polyomavirus middle T antigen) infection (14). Here we report that temperature-sensitive regulation of viral transcription seems to function only at the early stage of infection and that the temperature-dependent transformation of B812 was a result of a mutation in a cellular factor(s) that could be a component of a common cellular pathway for transformation by several oncogenes.

MATERIALS AND METHODS

Cells. No. 7 (15) is a clone of the rat fibroblast cell line F2408 (12). B812 is a cellular mutant of no. 7 isolated previously (15). K1 is a transformant clone obtained by infecting no. 7 with Kirsten (Ki-) murine sarcoma virus (MSV) (Eco). All cell lines were maintained in Dulbecco modified Eagle minimal essential medium supplemented with 5% fetal calf serum.

Viruses. Ki-MSV, Moloney (Mo)-MSV, Harvey (Ha)-MSV, PyMLV (9), murine Rous sarcoma virus (MRSV) (1), and Z16E67 virus (37) were prepared by rescuing the viruses from nonproducing cell lines by superinfection with the indicated helper viruses. The nonproducing cell lines of PyMLV and MRSV, as well as adenovirus type 12 (Ad12), were obtained from W. Eckhart (Salk Institute), T. Honjo (Kyoto University Faculty of Medicine), and K. Shiraki (Institute of Medical Science, University of Tokyo), respectively.

Infectious virus assay. A total of 5×10^5 cells of the K1 and KTS clones were seeded into 60-mm-diameter dishes and incubated for 3 days at 35 and 39°C. Cultured cells (5×10^5) were reseeded into 60-mm-diameter dishes and incubated overnight at the temperature used for the first incubation. After a change of medium, the cultures were incubated for an additional 12 h. The cultured medium harvested from each dish was centrifuged to remove cells, and then the supernatant of serial dilutions was used for the focus assay with no. 7 at 37°C. The number of focus-forming units per milliliter was determined by the number of transformed foci observed 8 days after infection.

DNA and DNA transfection. Plasmid pPMTneo was constructed by inserting the polyomavirus middle-T-antigen (Py-MT)-coding sequence into pSV2neo (31). Plasmid pFBJ-2neo was constructed by excising a *Bam*HI-*Hin*dIII fragment containing the FBJ MSV provirus sequence from pFBJ-2 (29) and by inserting it into pSV2neo. pFBJ-2 was kindly provided by K. Nose (Institute of Medical Science, University of Tokyo). pSVerbB-2VE (activated form of *erbB*-2) was constructed and kindly provided by T. Yamamoto and K. Toyoshima (Institute of Medical Science, University of Tokyo). pcDsrc was constructed by inserting a 2.9-kilobase (kb) *Eco*RI fragment of pSRA-2 (6) into the pcD vector (26). Transfection of cells was carried out as follows. No. 7 or KTS3 (4×10^5 cells) was seeded into 60-mm-diameter plastic dishes. The next day, the cells were transfected with plasmid DNA by coprecipitation with calcium phosphate (4). For transfection with pSV2neo, pPMTneo,

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and pFBJ-2neo, 1 μg of each plasmid DNA was used with 19 μg of calf thymus carrier DNA. For pSVerbB-2VE and pcDsrc transfection, 10 μg of each plasmid DNA was cotransfected with 1 μg of pSV2neo and 9 μg of carrier DNA. The transfected cells were selected in G418 medium (0.5 mg/ml). After 3 weeks of selection at 35°C, 10^4 cells from surviving colonies were inoculated into a 0.33% agar medium and incubated at 35 or 39°C for 3 weeks. The number of colonies larger than 0.125 mm in diameter was then scored by examination under an inverted microscope.

Infection and temperature shift experiments. Infection was done as described previously (15). Briefly, 2×10^5 cells were seeded into a 60-mm-diameter plastic dish and preincubated at 35 or 39°C. The next day, cells were infected with 0.2 ml of virus preparation for 1 h at 37°C. After incubation for various periods postinfection, the incubation temperature was raised or lowered to 39 or 35°C, and incubation was continued for various periods of time. Transformed foci were determined by examination under an inverted microscope.

Preparation of RNA and Northern (RNA) blotting. Total cytoplasmic RNA was prepared as described by Chirgwin et al. (5). Poly(A)$^+$ RNA was selected by passage through an oligo(dT)-cellulose column (10). A 3-μg sample of poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel. The gel was blotted onto a nylon filter in 20× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) without pretreatment and hybridized with the Ki-*ras*-specific fragment HiHi-380 (11) and human β-actin DNA (23) ^{32}P labeled with the multiprime labeling system (Amersham Corp.) in 4× SSC containing 5× Denhardt solution (0.1% bovine serum albumin, 0.1% Ficoll 400, 0.1% polyvinylpyrrolidone), 0.2% sodium dodecyl sulfate (SDS), 50 μg of denatured herring sperm DNA per ml, and 50% formamide at 42°C for 18 h. The filters were washed four times each with 2× SSC–0.1% SDS and 0.1× SSC–0.1% SDS and then exposed to a X-ray film for 1 day.

Immunoprecipitation assay. No. 7 and KTS3 (2×10^5 cells of each) were seeded into 60-mm-diameter plastic dishes and preincubated for 3 days at 35 or 39°C. Cells were labeled and immunoprecipitated as described previously (14). Briefly, the cultures were starved in methionine-free medium for 2 h and labeled with 125 μCi of [^{35}S]methionine per dish in methionine-free medium for 3 h at the respective temperatures. The cells were lysed with RIPA buffer, and then cleared lysates were incubated at 4°C with rat anti-T-antigen serum or monoclonal antibody Y13-259 (Oncogene Sciences) (13). The immunocomplexes were absorbed to protein A-Sepharose, heated at 100°C for 2 min, and resolved by electrophoresis in 12.5% SDS-polyacrylamide gels. The resulting gel was exposed to X-ray film for 3 weeks at −80°C.

RESULTS

Reversible temperature-dependent focus formation by B812 cells. B812 was isolated from a rat fibroblast cell line (no. 7) after being mutagenized by UV irradiation (15). It showed temperature-dependent focus formation when infected with Mo-MSV, PyMLV, Ki-MSV, and Ha-MSV (15; data not shown). At the nonpermissive temperature, the frequency of focus formation decreased more than 14-fold in those virus infections. Somatic cell hybridization with the parental cell line no. 7 indicated that the temperature-dependent focus formation of B812 is recessive (14). Cell morphology and the rates of cellular growth, DNA replication, and protein synthesis of B812 were comparable to those of no. 7 at both permissive and nonpermissive temperatures (14), suggesting that the temperature-dependent focus formation of B812 was not due to a nonspecific decrease in cell viability at the high temperature.

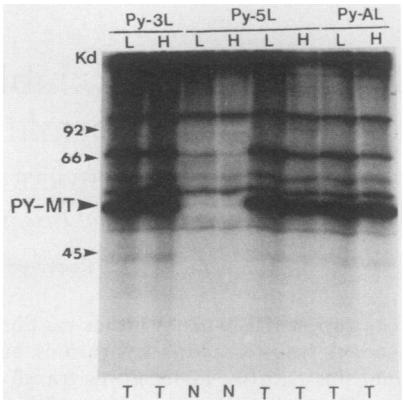

FIG. 1. Expression of Py-MT in foci induced by PyMLV. Py-3, Py-5, and Py-AL were cells from foci isolated from temperature shift experimental groups of 3L, 5L, and AL of B812 (see legend to Fig. 2), respectively, 7 days after infection. After expansion at 37°C, cells were divided into two groups and incubated at 35°C (L) and 39°C (H) for 3 days. They were then labeled with [^{35}S]methionine and immunoprecipitated with either normal rat serum (N) or rat anti-T serum (T) as described in Materials and Methods. The immunoprecipitated proteins were electrophoresed on a 12.5% SDS-polyacrylamide gel, and the gel was exposed to X-ray film for 3 weeks at −80°C.

We previously reported a possible involvement of a temperature-sensitive transcriptional factor in the temperature-dependent transformation of B812 by various oncogenic retroviruses (14). Further studies, however, showed the temperature-sensitive regulation was detected only at the early stage of infection by PyMLV. When B812 foci induced by PyMLV were isolated and examined for Py-MT expression at both permissive and nonpermissive temperatures, an identical level of Py-MT was detected in all experimental groups (Fig. 1); once formed, PyMLV-induced B812 foci did not exhibit temperature dependence.

However, we found that although the same promoter was used to drive v-Ki-*ras* expression, focus formation induced by Ki-MSV was fully reversible (Fig. 2). When the incubation temperature was either raised or lowered various days after infection with Ki-MSV, the number of foci increased or decreased, depending on the incubation period at each temperature. Reversible focus formation was also observed in infection by Ha-MSV, Mo-MSV, and MRSV (data not shown). On the other hand, temperature-dependent focus formation was not detected in any experimental group of no. 7.

Characterization of Ki-*ras* transformant clones of B812. To characterize the temperature-dependent phenotype of B812, we isolated transformed foci of B812 after Ki-MSV infection. After 20 days of incubation postinfection, a total of 135 transformed foci were picked. After sequential cloning (three times), cells from six (designated KTS1 to KTS6) foci were selected as representative clones and characterized (Table 1).

At 35°C, the KTS cells were fully transformed with an apparent lack of contact inhibition (Fig. 3) and formed colonies in a soft agar at frequencies of 99 to 62% of K1 levels (Table 1). At 39°C, they were flat and fully contact inhibited (Fig. 3) and grew to almost the same saturation densities as did untransformed no. 7 and B812 (Table 1).

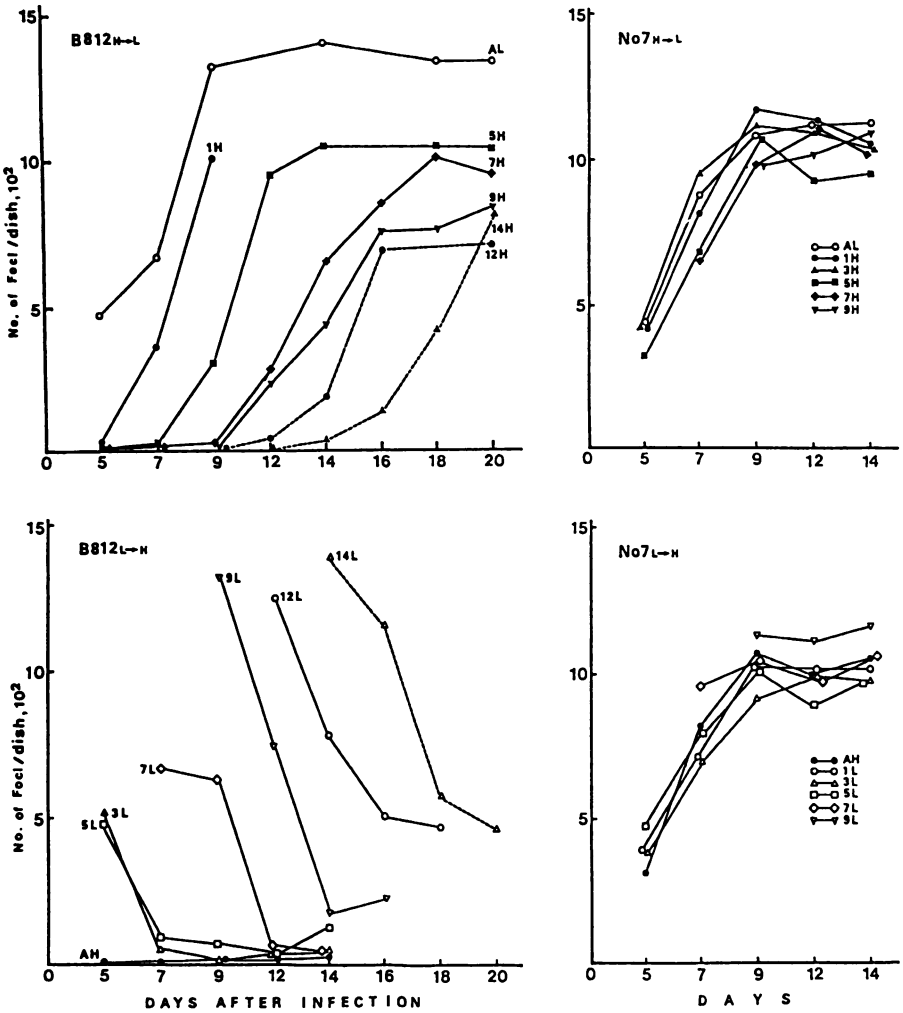

FIG. 2. Temperature shift experiments with Ki-MSV. After overnight preincubation at 35°C (L) or 39°C (H), no. 7 and B812 cells were infected with Ki-MSV and incubated at the temperatures used for preincubation. Various days after infection, the incubation temperature was either raised or lowered. The numbers before L and H indicate days of incubation at the respective temperatures. The number of transformed foci of each culture done in duplicate was counted and averaged on the day indicated.

TABLE 1. Characteristics of KTS cells

Cell line	Saturation density[a] at 39°C (10^2 cells/mm^2)	Soft agar growth[b] (no. of colonies/dish)			v-Ki-*ras* mRNA	p21$^{v\text{-Ki-}ras}$
		35°C	39°C	39°C/35°C		
No. 7	3.63	4	0	0	−	−
B812	3.39	5	0	0	−	−
K1		1,885	1,675	0.890	+	+
KTS1	2.68	1,160	20	0.018	+	+
KTS2	2.62	1,766	24	0.014	+	+
KTS3	2.93	1,756	184	0.105	+	+
KTS4	4.65	1,865	122	0.065	+	+
KTS5	1.22	1,650	65	0.039	+	+
KTS6	2.70	1,848	166	0.090	+	+

[a] Cells (5×10^5) were inoculated into a 60-mm-diameter plastic dish, incubated at 39°C for 5 days, trypsinized, and counted. All experiments were repeated two to four times, and average values were calculated.

[b] Cells (10^4) were inoculated into soft agar and incubated at 35 or 39°C. Three weeks later, colonies larger than 0.125 mm in diameter were counted.

Furthermore, their frequencies of colony formation in a soft agar diminished to 1 to 11% of K1 levels (Table 1).

Transcription levels of the v-Ki-*ras* oncogene in the six KTS clones were examined by Northern blotting (Fig. 4). A Ki-*ras* transcript of 6.6 kb was detected in all clones but KTS2, which contained a 3.5-kb transcript. Judging from the unchanged β-actin mRNA level used as a internal standard, v-Ki-*ras* mRNA levels at 39°C were slightly decreased compared with those at 35°C in the KTS clones. A comparable reduction of v-Ki-*ras*-specific mRNA levels was also observed in K1 at 39°C.

The expression of p21$^{v\text{-Ki-}ras}$, a product of the v-Ki-*ras* oncogene, in the KTS clones was examined by immunoprecipitation and Western blotting (immunoblotting) (Fig. 5 and data not shown). The levels of p21$^{v\text{-Ki-}ras}$ in KTS cells were slightly reduced at the nonpermissive temperature. A similar reduction of the level of p21$^{v\text{-Ki-}ras}$ was also observed in K1.

The temperature-dependent phenotype of KTS cells was not a result of temperature-sensitive viral replication, including transcription of the viral oncogene. Rescued viruses from KTS cells showed infectious virus production comparable to that of K1 cells (Table 2). Titers (focus-forming units per

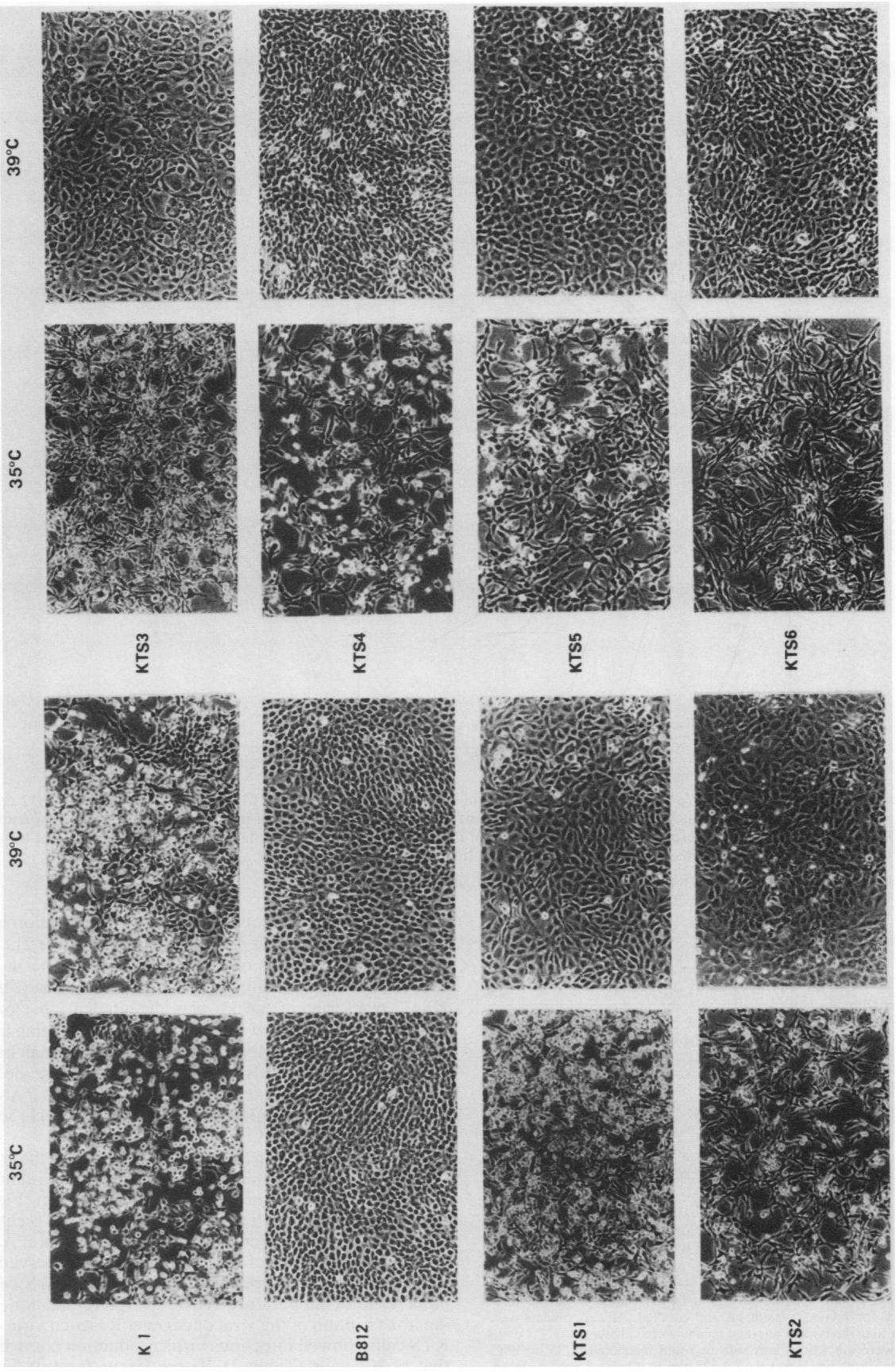

FIG. 3. Morphology of KTS clones. A total of 5×10^5 cells of each clone were inoculated into a 60-mm-diameter plastic dish and incubated at 35 or 39°C for 3 days.

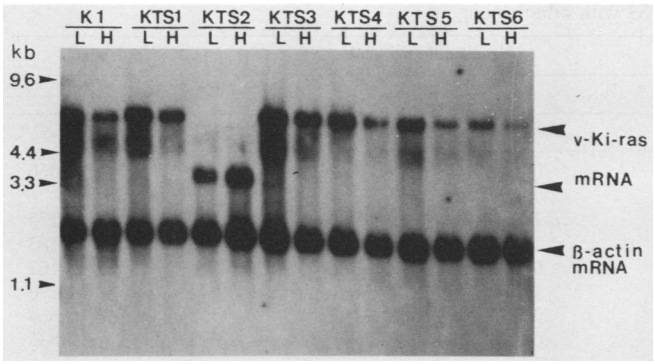

FIG. 4. Northern blots. Poly(A)$^+$ RNA was isolated from cells cultured at either 35°C (L) or 39°C (H) for 3 days. Approximately 3 μg of poly(A)$^+$ RNA was electrophoresed on a 1% agarose gel, transferred to a nylon filter, and then hybridized with the ^{32}P-labeled v-Ki-*ras*-specific fragment HiHi-380 and β-actin DNA. v-Ki-*ras* mRNA species are approximately 6.6 and 3.5 kb; β-actin mRNA is approximately 2.0 kb.

milliliter) of viruses rescued from K1 and KTS cells were both lower at 39°C than at 35°C.

Transforming viruses rescued from KTS clones at the permissive temperature were examined for focus-forming ability at both permissive and nonpermissive temperatures. None of them was temperature sensitive (data not shown), confirming that the temperature-dependent phenotype of B812 was not of viral origin.

Ki-*ras* transformants of B812 were insensitive to transformation by Ha-*ras*, *mos*, *src*, *fos*, and *erbB*-2 at the nonpermissive temperature but sensitive to transformation by Py-MT, Ad12, and HPV16-E67. To examine the sensitivity of B812 to other oncogenes, KTS3 was either superinfected with oncogene-carrying retroviruses or transfected with oncogene-carrying plasmids (Table 3). The frequency of colony formation of KTS3 cells infected with viruses carrying Ki-*ras*, Ha-*ras*, or *mos* decreased 10-fold at the nonpermissive

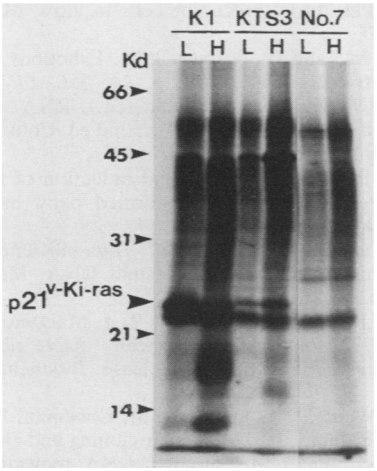

FIG. 5. Expression of p21$^{v\text{-}Ki\text{-}ras}$ in K1 and KTS cells. No. 7, K1, and KTS3 (5×10^5 cells of each) were inoculated into 60-mm-diameter dishes and incubated at 35°C (L) or 39°C (H) for 3 days. The cells were labeled with [^{35}S]methionine at each temperature and immunoprecipitated with monoclonal antibody Y13-259 as described in Materials and Methods. The immunoprecipitated proteins were electrophoresed on a 12.5% SDS-polyacrylamide gel, and the gel was exposed to X-ray film for 3 weeks at −80°C.

TABLE 2. Infectious virus assay[a]

Cell line	Focus-forming units/ml		
	35°C	39°C	39°C/35°C
K1	2.68×10^4	4.58×10^3	0.169
KTS1	1.07×10^4	2.69×10^3	0.250
KTS2	4.38×10^3	6.20×10^2	0.142
KTS3	3.15×10^5	2.93×10^4	0.093
KTS4	9.50×10^4	7.35×10^3	0.077
KTS5	5.70×10^4	6.02×10^3	0.105
KTS6	1.50×10^2	2.50×10	0.167

[a] A total of 5×10^5 cells per 60-mm-diameter dish were incubated at the indicated temperature for 3 days. Then 5×10^5 cultured cells were inoculated into 60-mm-diameter dishes and incubated at the same temperature. The medium was changed the next day, and cells were harvested 12 h later for use in the focus formation assay.

temperature. When infected with viruses carrying polyomavirus, Ad12, and human papillomavirus type 16 (HPV16) transforming genes, however, KTS3 cells formed colonies at 39°C with the same frequency as at 35°C. Essentially the same results were obtained when the experiments were carried out with transfection using plasmid DNAs containing these oncogenes and when B812 instead of KTS3 was used for infection or transfection with various oncogenes (data not shown).

DISCUSSION

Characteristics of Ki-*ras* transformants of B812. This study reports the characterization of a mutant cell line, B812, that displays fully reversible temperature-dependent focus formation upon infection with various retroviruses (Fig. 2). The temperature-dependent phenotype does not seem to be caused by a reduction in the level of oncogene expression and viral replication at a nonpermissive temperature. As demonstrated, the levels of *ras* oncogene expression and infectious virus production in the Ki-MSV transformants isolated from B812 (KTS cells) were reduced at a nonpermissive temperature. A similar degree of reduction was observed in a Ki-MSV transformant isolated from no. 7 (K1) (Fig. 4 and 5; Table 2), suggesting that the lower *ras* oncogene expression and infectious virus production reflected more the heat-labile nature of the oncogene message and viral infectivity than any reduction in *ras* oncogene expression or viral replication at a nonpermissive temperature. It is unlikely that loss of the transformed phenotype of KTS cells at the nonpermissive temperature resulted from a lowered level of v-Ki-*ras* oncogene expression below the threshold of transformation because superinfection of KTS3 cells by Ki-MSV did not result in a loss of temperature dependence (Table 3). Therefore, the mutation responsible for the temperature-dependent phenotype of B812 must be in a cellular gene crucial for the oncogene-induced transformation. Since it is suppressed upon fusion with normal parental cells, this mutant phenotype is perhaps the result of a temperature-dependent loss of a cellular gene function(s).

We previously detected a temperature-sensitive transcriptional factor in B812 that seemed to be involved in the regulation of PyMLV transcription only at the early stage of infection. After viruses somehow overcome such a cellular regulation (possibly by an increase in amounts of the mutated cellular factor, resulting in the accumulation of leaked normal function, or by the induction of a different cellular factor involved in transcription) and infection is completed, the factor no longer influences viral transcription. KTS3 lost

TABLE 3. Interaction of KTS3 with other oncogenes

Virus or plasmid	Oncogene	No. of colonies/dish					
		No. 7			KTS3		
		35°C	39°C	39°C/35°C	35°C	39°C	39°C/35°C
None		1	0	0	630	38	0.060
Virus[a]							
Ki-MSV (Ampho)	v-Ki-*ras*	440	574	1.305	513	102	0.175
Ha-MSV (Xeno)	v-Ha-*ras*	605	763	1.261	348	36	0.103
Mo-MSV (Hix)	v-*mos*	463	303	0.654	570	58	0.102
PyMLV (Xeno)	Py-MT	1,498	1,814	1.211	488	574	1.176
Ad12	E1A.E1B	1,040	746	0.717	1,450	963	0.664
Z16E67 (Ampho)	HPV16-E67	1,243	1,085	0.873	1,560	1,303	0.835
Plasmid[b]							
pFBJ-2neo	v-*fos*	1,569	1,595	1.017	815	61	0.075
pSVerbB-2VE	*erbB*-2	1,608	1,094	0.681	1,385	130	0.094
pcDsrc	v-*src*	2,688	1,475	0.549	1,155	61	0.053
pPMTneo	Py-MT	2,975	3,013	1.013	1,683	1,205	0.716

[a] No. 7 and KTS3 (2×10^5 cells of each) were inoculated into 60-mm-diameter plastic dishes and preincubated at 35 or 39°C for 24 h. The cultured cells were then infected with viruses at 37°C for 1 h. After an additional 2 days of incubation, 10^5 cells of each culture were inoculated into soft agar. Three weeks later, colonies larger than 0.125 mm in diameter were counted.

[b] Plasmids were transfected into 5×10^5 cells of no. 7 and KTS3 in 60-mm-diameter plastic dishes by the calcium phosphate technique at 35°C. The transfected cells were inoculated into two 100-mm-diameter dishes and selected in G418 medium for 3 weeks at 35°C. Then 10^4 surviving cells were inoculated into a soft agar and incubated at 35 or 39°C. Three weeks later, colonies were counted as described above.

its temperature dependence after PyMLV infection and pPMTneo transfection, indicating that it is not temperature sensitive to transformation by Py-MT (Table 3). The mechanism of temperature sensitivity of B812 in PyMLV infection is therefore different from the mechanism of the temperature-dependent phenotype in other retrovirus infections, indicating that B812 may carry double mutations.

Involvement of the mutated factor(s) in transformation by various oncogenes. The defect observed in B812, perhaps resulting from a mutation of a cellular factor(s), seems to lie in a common cellular pathway for transformation inducible by several oncogenes, including Ki-*ras*, Ha-*ras*, *mos*, *src*, *fos*, and *erbB*-2 (Table 3). The cellular components of these oncogenes appear to constitute a signal transduction pathway for the regulation of cell proliferation and differentiation and to acquire oncogenic activities by a deregulation of their normal functions. *erbB*-2 is a membrane-associated epidermal growth factor receptor-like protein with tyrosine kinase activity (33). *src* is a tyrosine kinase associated with the internal plasma membrane (2, 18). *ras* is a membrane-associated GDP-binding protein involved in cellular signal transduction (2, 36). *mos* is localized in the cytoplasm and has serine-threonine kinase activity (16, 17). *fos* is localized in the nucleus and binds to transcriptional factors (8, 21).

Zarbl et al. (38) recently reported the diverse sensitivity of the v-*fos* revertants to retransformation by various oncogenes. The revertants were retransformed by *trk* and Py-MT but not by *fos*, Ha-*ras*, *abl*, and *mos*. It is worth noting that all of the tested oncogenes that failed to transform KTS3 at the nonpermissive temperature were unable to transform the v-*fos* revertants. In addition, Py-MT could transform both the v-*fos* revertants and KTS3. This finding suggests that the mutated cellular factors in the v-*fos* revertant and KTS3 may be the same or lie in the same transformation pathway. Smith et al. (30) reported that a monoclonal antibody against $p21^{ras}$ reversibly suppressed transformation induced by the *fes*, *fms*, *ras*, *src*, and bovine papillomavirus-E6 oncogenes but not by the *mos* and *raf* oncogenes and simian virus 40 T antigen, indicating that *ras* constitutes a signal transduction pathway which involves the *fes*, *fms*, *ras*, *src*, and bovine papillomavirus-E6 oncogenes. Together, their results and ours suggest that the *erbB*-2, *abl*, *ras*, *src*, *mos*, *fes*, *fms*, *fos*, and bovine papillomavirus-E6 oncogenes are likely to transform cells through a common cellular pathway that normally functions as a regulatory signal transduction pathway of cell proliferation and differentiation.

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