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Action of Temperature-Sensitive Mutants of Myeloproliferative Sarcoma Virus Suggests That Fibroblast-Transforming and Hematopoietic Transforming Viral Properties Are Related

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The myeloproliferative sarcoma virus is molecularly related to the Moloney sarcoma virus (Pragnell et al., J. Virol. **38**:952–957, 1981), but causes both fibroblast transformation in vitro and leukemic changes—including spleen focus formation—in adult mice. The fibroblast transforming properties of myeloproliferative sarcoma virus were used to select viral temperature-sensitive mutants at 39.5°C, the nonpermissive temperature. These mutants are temperature sensitive in the maintenance of the transformed state. This was also shown by cytoskeletal changes of the infected cells at permissive and nonpermissive temperatures. Viruses released from cells maintained at both the permissive and nonpermissive temperature are temperature sensitive in fibroblast transformation functions. All temperature-sensitive mutants show only a low reversion rate to wild-type transforming function. The myeloproliferative sarcoma virus temperature-sensitive mutants are inefficient in causing leukemic transformation (spleen enlargement, focus formation) in mice at the normal temperature. A method to maintain a low body temperature (33 to 34°C) in mice is described. One temperature-sensitive mutant was checked at low body temperature and did not induce leukemia. These data thus indicate that the same or related viral functions are responsible for hematopoietic and fibroblast transformation.

One important aspect of oncogenesis induced by retroviruses is whether viral oncogenes act in a general manner, e.g., by an effect on cell proliferation (10), or whether transforming retroviruses may carry genes related to oncogenes that are expressed in cells on specific differentiation pathways (11). Duesberg (10) has divided acutely transforming retroviruses into the following two groups: viruses that interact and transform several cell types, one of these usually fibroblast cells; and those, similar to Friend spleen focus-forming virus, whose transforming activity appears limited to one differentiation pathway (24, 35, 36). Viral oncogenes of the latter class would seem related to genes whose activity is restricted to a single pathway, whereas those capable of wider transformation might represent genes perhaps controlling proliferation. The expression of such genes would influence many cell types (22). The myeloproliferative sarcoma virus (MPSV) (25), Abelson virus (28, 32), the Harvey, Kirsten, and BALB/c sarcoma viruses (1, 31), and also the avian erythroblastosis virus (29) all transform at least two different cell types, fibroblasts and hematopoietic cells. The action on two or more cell types could be due to a general effect of a single oncogene including cellular proliferation in those tissues or of one differentiation-specific gene acting on one type of cell. One gene could also act differentiation-specifically on a limited number of different cell types comparable to the presence of certain differentiation antigens in common to several cell types (38). Finally, two or more distinct oncogenes may be present in one viral genome, both of which may or may not act differentiation specific, as suggested for avian erythroblastosis virus (3, 18).

MPSV induces a large increase in the number of late erythroid precursor cells, hematopoietic stem cells, and granulocyte-macrophage precursor cells on infection of mice (17, 25). Furthermore, proliferation of erythroid early precursor cells (BFU-E), myeloid precursor cells (CFU-C), and stem cells (CFU-S) is stimulated in vitro (12, 21). The analysis of the MPSV genome has revealed the presence of sequences related to fibroblast-transforming Moloney murine sarcoma virus (Mol-MuSV) and further sequences absent in Mol-MuSV (27; A. Stacey, C. Arbuthnott, R. Kollek, L. Coggins, and W. Ostertag, submitted for publication), but related to the Moloney murine leukemia virus (Mol-MuLV). Mol-MuSV has not been shown to transform hematopoietic cells of adult mice (25). The additional MPSV sequences related to Mol-MuLV could thus be involved in hematopoietic transformation. It would be of great interest to determine whether MPSV—a virus related to, and possibly a variant of, the ancestor of Mol-MuSV (7)—has one or two oncogenes within its genome. One way of testing this is to isolate temperature-sensitive (*ts*) mutants of MPSV, which can be used to obtain information as to whether fibroblast transformation and leukemogenesis are caused by the same or different viral genes. In this paper we describe a novel method to isolate such *ts* mutants. Four independent mutants were tested and characterized. All four mutants are defective in fibroblast and in leukemic transformation.

MATERIALS AND METHODS

Cell culture and viruses. All cells were grown in modified Eagle medium supplemented with 10% fetal calf serum. Infectious virus was obtained from tissue culture supernatants and in some cases was concentrated 10- to 100-fold (25). The cell lines used in these studies are as follows: cell line 6-6#3 is an NRK nonproducer cell clone transformed by

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cloned MPSV (25). *ts* mutant and wild-type (WT) virus was rescued by Friend helper murine leukemia virus (F-MuLV) unless specified. Cell line 643/22N is an SC-1 cell line that releases F-MuLV, cloned twice (27). Cell line 1.1A is an NIH 3T3 cell clone infected with cloned Mol-MuLV. This Mol-MuLV was used for some experiments as indicated in the legends. For other experiments we used virus released by an NIH 3T3 cell line transfected by molecularly cloned Mol-MuLV (Mov-3 locus of mice) (15); 10 μg of molecularly cloned Mol-MuSV (m-1) DNA (37) and 10 μg of Mov-3 DNA were used to transfect NIH 3T3 cells. The viral supernatant of transformed cells releasing virus, as measured by determining the viral reverse transcriptase activity (25), was utilized for measuring ratios of fibroblast-transforming activity and spleen focus-forming activity (none detected) (Table 1). The same virus was used to infect NRK cells at endpoint dilution for focus formation in 24-well plates, and transformed nonproducer cell clones of m-1 virus were isolated (25). Nonproducer cell clones were infected with F-MuLV (of 643/22N), and virus of these cell clones was used again to infect NRK fibroblasts and DBA/2 mice to determine the ratios of fibroblast and spleen focus-forming activity (25). Mol-MuSV 124 refers to virus released by the murine TB cell line (G8-124). NIH 3T3, G8-124, and 1.1A cells were obtained through the courtesy of R. Weinberg. F4-6 cells are Friend virus-transformed cells (25). Cloning of NRK cells and transformants was as described previously (25).

Spleen and fibroblast focus formation. Spleen focus formation was assayed by injecting viral supernatants into the lateral tail vein of DBA/2 mice or as indicated in the legends to the tables and figures. MPSV spleen focus-forming units (FFU) were counted 16 days (25) postinjection. The fibroblast FFU assay was performed as described previously (4).

Mutagenesis of MPSV virus. Concentrated WT 6-6#3 virus (10^5 to 10^6 FFU/ml) was treated with different amounts of *N*-methyl-*N*-nitro-*N*-nitrosoguanidine (MNNG) for 20 min at 30°C in 0.2 M Na phosphate–0.002 M EDTA buffer (pH 6.0). This solution was then dialyzed against 50 volumes of Tris buffer (0.1 M NaCl, 0.01 M Tris-hydrochloride [pH 7.6], 0.001 M EDTA, 10% fetal calf serum) for 3 h at 4°C. The virus solution was filtered through 0.2-μm Acrodisc filters. Supernatants were then checked for fibroblast focus formation. The addition of 1.6 μg of MNNG per ml inactivated 40% of the FFU as compared with untreated virus control suspensions; 12.8 and 51.2 μg of MNNG per ml inactivated to 3×10^{-2} and 7×10^{-3} of the FFU of untreated virus, respectively. MNNG of 2 μg/ml (~50% inactivation) was then used for the actual experiment to minimize the number of mutational lesions. NRK cells at the log phase of growth (5×10^5 cells per ml) were then exposed to the mutagenized virus at 32°C (2×10^5 FFU/ml) for 2 h when the medium was changed. The different independently treated cultures were maintained for 2 weeks at 32°C, when all cells appeared transformed. Cells (3×10^5 cells per ml) were mixed with 1.6% methocel in medium with 15% fetal calf serum and 1.2% dimethyl sulfoxide and diluted with medium to give 1.3% methocel with 3×10^5 cells per ml. The methocel-cell suspension was plated onto petri dishes on top of 0.9% agar in medium. Plates were incubated at 39.5°C for 40 h. Bromodeoxyuridine (2×10^{-4} M, final concentration) was added, and the cultures were gently agitated to obtain a homogeneous solution of methocel-bromodeoxyuridine. Cells were incubated for 82 h at 39.5°C. Hoechst stain 33258 was then added at 1 μg/ml. Cultures were transferred to 4°C 3 h later. Methocel and cells were then suspended in 4 volumes of cold medium and spun at 300 × *g* for 10 min. The cell pellet was suspended in phosphate-buffered saline without phenol red. Cells were counted and then diluted to 10^5 cells per ml in phosphate-buffered saline and transferred to several T 75 flasks. Cells were irradiated with fluorescent (near-visible) light with an X-ray illuminator for 1 h at a distance of 10 cm. Cells were then pelleted and suspended in medium at a density of 2×10^5 cells per ml. Cells were diluted to 1×10^5 cells per ml with 0.6% agar-containing medium (at 45°C) to make 0.3% agar and plated on top of a 0.5% agar base (in medium) in petri dishes. Cells were incubated at 32°C for 10 to 21 days until colonies were visible. Colonies were isolated individually and maintained at 32°C.

TABLE 1. Spleen focus formation induced by pseudotypes of MPSV and Mol-MuSV[a]

Transforming		Helper virus	Ratio SFFU/FFFU		FFFU tested
Virus	Variant		Avg	Range	
Mol-MuSV	124	Mol-MuLV	0	0	2×10^6 (2)
	124	F-MuLV	0	0	5×10^7 (4)
	m-1	Mol-MuLV	0	0	5×10^7 (6)
	m-1	F-MuLV	2×10^{-5}	$0–7.2 \times 10^{-5}$	8×10^7 (8)
MPSV	6-6#3	Mol-MuLV	0.11	0.08–0.25	10^4 (3)
	6-6#3	F-MuLV	0.12	0.07–0.30	4×10^4 (10)

[a] Virus was concentrated and tested for reverse transcriptase activity (data not shown), for fibroblast FFU (FFFU) and spleen FFU (SFFU, 16 days after infection of DBA/2J mice). The SFFU/FFFU ratio is sometimes 10- to 100-fold lower if foci on spleens are counted 8 to 9 days after injection of virus into mice (14). The numbers within parentheses indicate the numbers of experiments.

Lowering of mouse body temperature. C57BL mice were maintained in a cold room at 10°C, not more than two animals per cage. Chlorpromazine, a tranquilizer drug, was added to the drinking water at a concentration that was adjusted individually (average 0.6 mg/ml), depending on the body temperature of each mouse. The body temperature of 20 C57BL mice thus treated was at an average 31 to 33°C. The variability in temperature is rather large, but should still permit the testing of *ts* mutants. C57BL Fv-2^r mice had a mortality of about 50% within the first 4 weeks of treatment. They are, however, unsuited since they are resistant to MPSV (23). We therefore used DBA/2J animals for the actual experiments with MPSV. Mortality was even higher with DBA/2J mice, but body temperature was again 30 to 34°C throughout the experiment.

Serological methods. Mouse monoclonal antibodies with binding affinity against vimentin were kindly provided as hybridoma culture supernatants by M. Franko (National Institutes of Health, Bethesda, Md.). Fluorescein-labeled reagents were purchased from the Institut Pasteur (Paris, France). Immunofluorescence preparations and photographic recordings were done by already published methods (30).

RESULTS

MPSV differs from Mol-MuSV by its high efficiency in inducing spleen focus formation. Kirsten and Harvey sarcoma viruses (31), but not Mol-MuSV, cause spleen enlargement in some inbred strains of adult mice and in all newborn mice. Moreover, virus-dependent induction of large erythroid colonies (BFU-E) in vitro has been reported for all three virus isolates, except for Mol-MuSV (12–14). We previously have shown that MPSV differs from Kirsten and Harvey sarcoma viruses by its higher efficiency of spleen focus formation in adult mice (25). Now we have carried out

a more systematic study to compare spleen focus-forming activity and fibroblast focus formation on MPSV with two different Mol-MuSV virus isolates, the Ball and the m-1 virus, with two different pseudotypes (F-MuLV and Mol-MuLV helper) (Table 1). Only MPSV was efficient in causing spleen foci in adult mice (ratio of spleen/fibroblast FFU, 0.07 to 0.3) regardless of pseudotype. No spleen foci could be found with the Mol-MuSV (Mol-MuLV) pseudotype virus, but surprisingly focus and spleen enlargement, albeit at a very low efficiency (ratio of spleen/fibroblast FFU, 0 to 7.2 $\times 10^{-5}$), occurred with the Mol-MuSV (F-MuLV) m-1 variant.

Isolation of MPSV *ts* mutants. Concentrated MPSV (F-MuLV) virus of the MPSV strain 6-6#3 (25) was mutagenized with a concentration of MNNG (2 μg/ml) that caused about 50% reduction of focus formation on NRK fibroblasts. Three different batches of cells exposed to mutagenized virus were grown at 32°C for 2 to 4 weeks. To select for cells harboring a *ts* MPSV genome, we used the procedure outlined by Wyke (39) for replication-competent avian sarcoma virus. Uninfected fibroblasts usually do not grow in suspension cultures in soft agar or methyl cellulose, whereas virus-transformed fibroblasts do. At the permissive temperature (32°C), virtually all cells can grow in suspension, whereas cells with virus that does not express the transforming function at the nonpermissive temperature (39.5°C) will not grow in methocel. Cells transformed at 39.5°C incorporate bromodeoxyuridine, whereas cells with a *ts* defective virus will not. The cells that incorporate bromodeoxyuridine into DNA are killed by irradiation with light; this effect is increased about 100-fold by the addition of the Hoechst dye 33258 (8). The surviving cells were cloned immediately after treatment in methocel at the permissive temperature, 32°C. Clones were established, and duplicate cultures of each were grown in liquid medium at 32 and 39.5°C. A total of 33 of 272 clones grown were attached and had fibroblast-like morphology at 39.5°C, but appeared transformed at 32°C.

MPSV, as used here, is a sarcoma virus complex consisting of the replication-deficient, transforming component and the replication-competent, but nontransforming, FMuLV helper virus. The expression of temperature sensitivity could possibly be due to a mutation in either (40). Moreover, helper virus infection of some nonproducer cells containing a *ts* virus was reported to overcome temperature sensitivity (33). We decided to test the release of helper virus from the remaining clones by measuring reverse transcriptase activity at both the permissive and nonpermissive temperatures (Table 2), to identify cell lines possibly containing *ts* F-MuLV. Only clones releasing comparable levels of virus at each temperature were studied further. Cell clones releasing very low titers of transforming virus and other clones releasing virus that transformed NRK cells equally well at 32 and 39.5°C were not further utilized. Cell clones 22, 29, 124, 143, 152, 159, 252, and 259 released virus able to transform cells much better at permissive than at nonpermissive temperatures. The viral supernatant of cell clones 124, 143, 159, and 259 was used to infect NRK cells at endpoint dilution to obtain nonproducer cell lines.

Isolation of nonproducer cell lines infected with *ts* MPSV mutants. *ts* MPSV-infected nonproducer NRK cell clones free of helper virus were isolated to confirm that temperature sensitivity was a property of the defective transforming MPSV subunit. The *ts* virus of cell lines 124, 143, 159, and 259 was serially diluted. Cells in wells with only one focus at the endpoint dilution were cloned in soft agar at 32°C, and colonies with the transformed phenotype were isolated and

TABLE 2. Virus release and fibroblast FFU titers[a]

Cell clone	FFFU/ml[b] 32°C	FFFU/ml[b] 39.5°C	FFFU ratio[c] 32°C/39.5°C	RT ratio[d] 32°C/39.5°C
F4-6[e]	0	0		2.1
6-6#3[e]	1.8×10^4	1.3×10^4	1.4	2.2
22[f]	2.5×10^2	0	High	18 a
26	1.9×10^4	0	High	10 a
29	5.2×10^2	0	High	5 a
124[f,g]	9.8×10^4	7.5×10^1	1,300	2 b
142[f]	2.0×10^4	5×10^1	400	NT[h] c
143[f,g]	2.8×10^3	8×10^1	35	1.9 c
152[f]	1.8×10^3	0	High	2.5 c
159[f,g]	4.8×10^4	0	High	1.2 c
252[f]	9×10^1	0	High	4.4 a
259[f,g]	1.6×10^4	3.2×10^1	500	2.4 a

[a] Titers at 32 and 39.5°C of some selected clones that appear transformed at 32°C and grow attached at 39.5°C.
[b] FFU, Fibroblast FFU on NRK cells.
[c] Ratio of fibroblast of cell supernatant of cells grown at 32 and 39.5°C, corrected for cell number.
[d] Ratio of reverse transcriptase activity (RT), as in footnote *c*.
[e] Wild-type MPSV of cell line 6-6#3 (infected with F-MuLV of cell clone 643/22N = 6-6#3) and Friend virus (of cell clone F4-6) were used as controls.
[f] These cell clones were further studied.
[g] Clones utilized for further subcloning. The letters in the last column identify cell lines that have been isolated from the same batch of mutagenized virus. Clones of the same batch have the same letters.
[h] NT, Not tested.

grown in tissue culture flasks. Only transformed cell clones that did not release virus were maintained. Samples of cells of these clones were subcultured at the permissive and nonpermissive temperatures. Nonproducer cell clones were obtained with *ts*159, *ts*143, and *ts*124 virus; none has been cloned as yet with *ts*259. All nonproducer cell clones, when grown at the nonpermissive temperature, were attached. At 39.5°C, 159 nonproducer cells were entirely attached, and 143 and 124 cells were predominantly attached. All of the cell lines had the transformed morphology at 32 to 33°C. The 159 cells were entirely in suspension, even more so than the nonproducer WT 6-6#3 cell clone; the 143 cells at low density were attached, but appeared obviously transformed. They were entirely in suspension at high density: the 124 cells all appeared morphologically transformed; about 30 to 70% of the cells, depending on growth conditions, were in suspension.

The 124 cells showed higher spontaneous focus formation at 39.5°C (clone 124/1, 7.6 foci per 10^4 cells; clone 124/2, 40 foci per 10^4 cells). The 159 cells were the most stable cells. They became epithelioid at the nonpermissive temperature and did not pile up at high density; their growth was then completely arrested, and only very few spontaneous foci appeared (<0.2 foci per 10^4 cells). *ts* mutant 159 was therefore utilized extensively for experiments on changes in cell morphology with temperature.

Shift-up and shift-down experiments were done with all cell clones. The cells regained the nontransformed (attached) phenotype on shift-up to 39.5°C within 24 to 48 h. Clone 159 cells attached within 12 h. Shift-down experiments led to the suspended (clone 159) or transformed (clones 124, 143, and 159) phenotype within 24 h.

Cytoskeletal organization of NRK cells infected with *ts* mutant MPSV. Extensive cytoskeletal rearrangements in transformed fibroblasts with wild-type and *ts* sarcoma virus

at the permissive temperature have been observed (6). Alterations in the cytoskeleton have repeatedly been discussed as a cause or as a consequence of retrovirus-mediated transformation of fibroblasts (2, 20, 34). We restrict ourselves to a short characterization of *ts* nonproducer and virus-infected nonproducer cells of cell clone 159/5/1 at the permissive and nonpermissive temperature (Fig. 1).

Cultures of cells grown at each temperature were split into equal parts; one was maintained at 32°C, and the other was maintained at 39.5°C. Cells were stained 72 h later. NRK control cells showed the typical cytoskeletal structure of fibroblasts at both temperatures. WT virus-transformed cell clone 6-6#3 was difficult to judge, since most cells were in suspension at either temperature. The attached 6-6#3 cells had transformed morphology at both temperatures. Staining for 10-nm filaments usually resulted in compact fluorescence with little structure. The 159/5/1 cells grown for 72 h at the permissive temperature (shift-down experiment) showed complete absence of recognizable supramolecular organization (actin cables, microtubules, and 10-nm filaments); they were indistinguishable from WT 6-6#3 cells (Fig. 1). The phenotype reverted to normal NRK phenotype at the nonpermissive temperature, as judged by stained microtubules, and to almost normal on staining of the 10-nm filaments.

The 159/5/1, 143, and 124 cells were infected with F-MuLV helper virus and maintained until most or all of the cells were infected with helper virus and then reexamined. No morphological changes were apparent for F-MuLV-infected as compared with uninfected cells.

Growth in semisolid media at the permissive and nonpermissive temperature. The original selection for our *ts* mutants favored cells unable to grow on semisolid methocel medium at the nonpermissive temperature (see above). We therefore expected to observe differences in cell cloning properties of *ts* nonproducer cells in semisolid medium at permissive and nonpermissive temperature. Cloning of nonproducer cells in 0.4% agar showed a 20-fold reduction in clonability for WT nonproducer cells at 32°C as compared with that at 36.5 to 38.5°C, but almost equal cloning efficiencies at both temperatures for the *ts* nonproducer clones. This result was unexpected, but confirmed when cloning in methyl cellulose medium on a solid agar support, in a manner similar to the initial selection conditions.

We then rechecked the clonability of normal NRK cells at

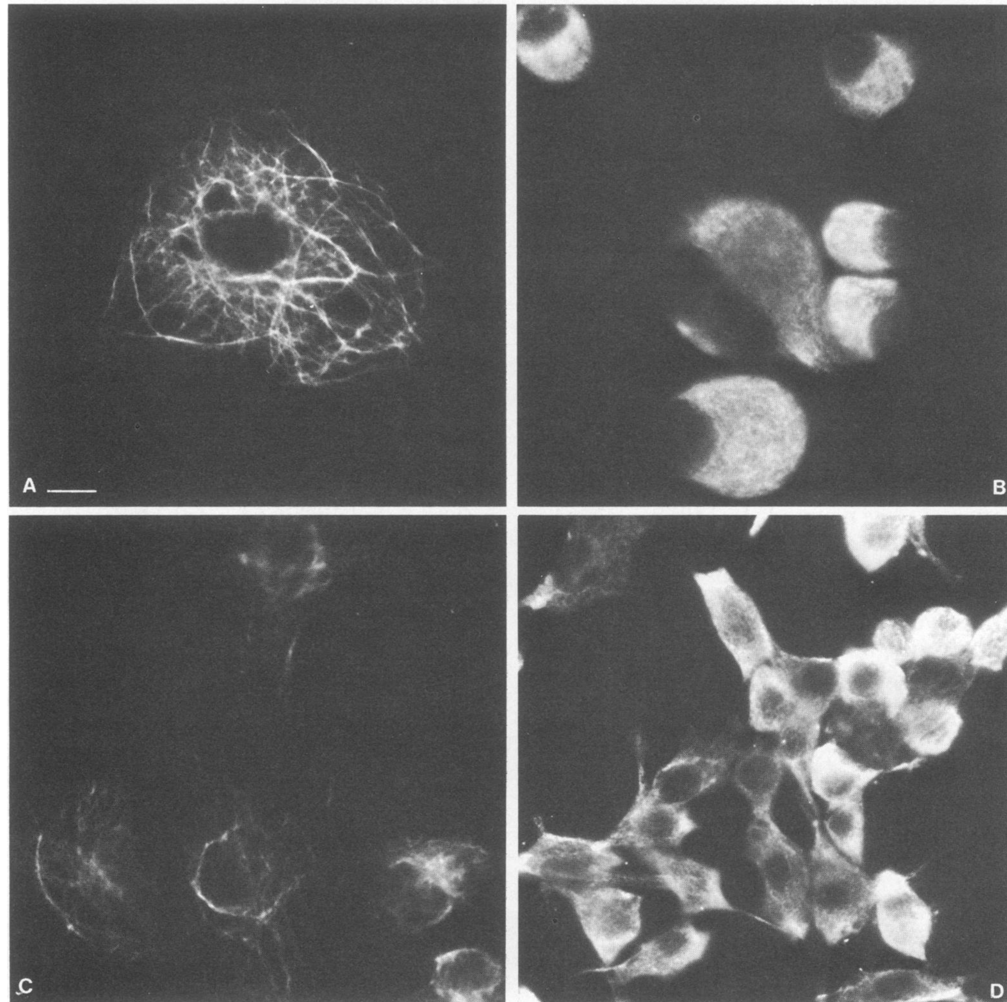

FIG. 1. Immunofluorescence pattern of 10-nm filaments. Cover slip cultures were fixed with 1% formaldehyde solution in phosphate-buffered saline and permeabilized by digitonin treatment (0.01% in phosphate-buffered saline). Labeling was done with a mouse monoclonal antibody with binding affinity for vimentin. (A) Normal NRK cells at 39.5°C. (B) WT 6-6#3 cells at 39.5°C; note the cap-like accumulation of vimentin-containing structures. (C) *ts*159/5/1 cells maintained at 39.5°C; a very fine netlike structure was seen. (D) *ts*159/5/1 cells at 32°C; no fine organization could be seen in most of the cells. Bar, 10 μm.

low passage number (passage 8) and at higher passage number (passage 25). Clonability in semisolid support (0.4% agar) of NRK cells was < 5% with early passage cells and 82% with late passage cells at 38.5°C. NRK cells (passage 18), when they were used for infection with *ts* MPSV to obtain *ts* nonproducer cells, thus may have already been in a state amenable to cloning in agar.

Retransformation of 159/5/1 nonproducer cells by WT MPSV at the nonpermissive temperature. Cells transformed by *ts*159 virus have lost their potential to express a transformed phenotype at the nonpermissive temperature. This is not an irreversible loss, as WT 6-6#3 virus can transform 159/5/1 cells at the nonpermissive temperature. The 159/5/1 cells were infected at 39.5°C with WT 6-6#3 virus. Transformed foci appeared in both 159/5/1 and the control NRK infected cultures. Transformed foci appeared as round refractile cells that adhered poorly to the plastic T flask and had a tendency to float off into the medium. Foci were counted 5 days postinfection to avoid the contribution of spontaneously developing foci to the count. The number of foci on infection with WT 6-6#3 virus in three independent experiments was 1.8- to 3-fold higher in 159/5/1 cells than in regular NRK cells. This indicates a greater sensitivity either to focus formation or to infection of 159/5/1 cells than of regular NRK cells. *ts* 159/5/1 virus, as expected, did not induce any foci in the 159/5/1 cell line. The 124 and 143 nonproducer cell clones could also be retransformed by WT 6-6#3 virus (data not shown).

MPSV *ts* mutants are deficient in the maintenance of the transformed state. Two sets of experiments were done to confirm that our MPSV *ts* mutants are deficient in maintaining the transformed state in NRK cells. The first set of experiments was designed to resolve the question of whether the virus released at 32°C was as temperature sensitive as the virus released at 39.5°C. For this purpose we collected virus of *ts* nonproducer cells infected by F-MuLV. One culture each of every *ts* cell clone raised at 32°C was split, and the two cultures were maintained for 2 months at 32 and 39.5°C. Viral supernatants were checked for their ability to form fibroblast foci at 32 and 39.5°C. The data of one set of experiments are summarized in Table 3. WT 6-6#3 virus showed a 1.2- to 3-fold ratio (1.4-fold in this particular experiment) of FFU, if obtained from cells at 32°C and compared with that of cells growing at 39.5°C. Virus of 124 cells grown at 39°C appeared to yield eightfold more FFU at 39.5°C (ratio 32°/39°C, 2,500) than that grown at 32°C (ratio, 290). The difference in ratios was only 1.8-fold in a second set of experiments. *ts*159 and *ts*143 virus showed no significant difference when obtained from cells grown at 32°C or, alternatively, at 39.5°C. In conclusion, no significant difference in the ratio of fibroblast FFU at 32 and 39.5°C was found when *ts* virus was isolated from cells grown at either permissive or nonpermissive temperatures. These data thus exclude the hypothesis that the temperature sensitivity of the *ts* virus is a function of the stability of the genome of the defective virus at 32 and 39.5°C.

We then performed several experiments to confirm that the virus produced by one of our *ts* cell clones (159/5/1 cells) was actually equally infective at 32 and 39.5°C. These experiments were also done to further demonstrate that *ts*159 virus was deficient in maintaining the transformed state at the nonpermissive temperature. Serial dilutions of MPSV *ts*159 virus (released by cells grown at 37°C) were used to infect multiple samples of NRK cells at 32°C (control) and at 39.5°C. Cultures of cells infected at 39.5°C were transferred to 32°C at different times after infection and maintained for a total of 2 weeks (at 32 and 39.5°C). *ts*159 virus, plated at 32°C on NRK cells and maintained at 32°C (controls), yielded a titer of 1.2×10^4 FFU/ml per original virus suspension. No foci were seen when cells were infected and maintained at 39.5°C. Cells maintained for 2 or 4 h and up to 3 days after infection at 39.5°C and then shifted to the permissive temperature showed a similar number of foci (1.1×10^4 FFU/ml after 2 and 4 h at 39.5°C; 7×10^3 FFU/ml after 3 days at 39.5°C), regardless of the time they were maintained at 39.5°C. The foci obtained in infected NRK cells, maintained at 39.5°C for 3 days and at 32°C for 11 days, were morphologically clearly distinguishable from those foci when cells after infection were kept at 39.5°C for 24 h or less. Foci of infected cells grown for 3 days at the nonpermissive temperature, even though as large as control foci, consisted not predominantly of round suspended cells, but of attached cells, only slightly rounded, growing in a criss-cross pattern. These cells reverted to the suspended phenotype after detachment by trypsin and reculturing. This set of experiments proves that *ts*159 virus is equally infective at both temperatures, that it is maintained in cells at 39.5°C, and that it will express its transforming protential only at 32°C.

Stability of MPSV *ts* mutants. One inherent disadvantage of working with *ts* mutants of Mol-MuSV is the high reversion rate of some of the best-characterized *ts* mutants (5). We therefore tried to test the stability of our MPSV *ts* mutants by two sets of experiments. First, we followed the temperature sensitivity of our *ts* mutant virus through repeated virus cloning (Tables 2 and 4). Second, foci obtained at the nonpermissive temperature were picked, and the virus obtained from these cells was checked for temperature sensitivity (Table 5).

The ratios of fibroblast FFU at 32° to 39.5°C were similar throughout three sets of independent experiments at different times. No evidence of increased focus formation at the nonpermissive temperature with viral transfer to new cells was seen (Tables 2 and 4). *ts*159/5/1 virus was also repeatedly used to reinfect NRK cells at a high multiplicity. Virus produced by these cell lines again yielded *ts*159 virus causing focus formation at 32°C, but not at 39.5°C. Molecularly cloned *ts*159/5/1 virus was equally stable (unpublished results).

We attempted in the second set of experiments to isolate *ts* virus revertants by cloning cells of foci obtained at the nonpermissive temperature (Table 5). Virus released by these transformed producer clones was tested for focus formation at 32 and 39.5°C. Several of these potential revertants of each *ts* mutant were tested (Table 5). Most of

TABLE 3. Fibroblast focus-forming properties of *ts* virus in cells grown at 32 and 39.5°C[a]

Virus of clone:	Growth temp (°C)	FFFU/ml		FFFU ratio 32°C/39.5°C
		32°C	39.5°C	
6-6#3	39.5	4.6×10^3	1.5×10^3	3.1
	32	2.4×10^5	1.9×10^5	1.3
124/1	39.5	2.5×10^3	1	2.5×10^3
	32	4×10^4	1.4×10^2	2.9×10^2
143/1/1	39.5	2×10^3	16	1.3×10^2
	32	3.9×10^4	1.9×10^2	205
159/5/1	39.5	3.5×10^2	0	∞
	32	8.2×10^5	0.8	$\sim 10^6$

[a] Symbols and abbreviations as for Table 2.

TABLE 4. Fibroblast and spleen FFU titers[a]

Virus	Fibroblast transformation			SFFU/ml in DBA/2 mice at 37.5°C	Ratio SFFU/ FFFU
	FFFU/ml		FFFU ratio 32°C/ 39.5°C		
	32°C	39.5°C			
WT 6-6#3	1.8×10^4	1.3×10^4	1.4	2.6×10^3	1.4×10^{-1}
*ts*124/1	5.6×10^7	5.9×10^4	940	3.4×10^2	6.1×10^{-6}
*ts*143/1/1	1.6×10^5	8×10^2	200	13	8.1×10^{-5}
*ts*159/5/1	9.8×10^6	6.5×10^3	1,500	0	0

[a] MPSV *ts* mutants were recloned. Symbols and abbreviations as for Table 2. SFFU were counted at mouse body temperature and FFFU were counted at 32°C by using samples of the same virus suspension.

the 143 and 124 revertant cell clones released virus with unchanged *ts* properties. Two 143 and one 124 revertant cell lines, however, released virus that had partially lost temperature sensitivity. Focus formation of virus of potential 159/5/1 revertants tested so far appears to be temperature sensitive. The revertant phenotype thus appears to be a property of the cellular response, not of secondary virus mutation ("reversion"). Eight potential revertant clones infected with *ts*159 virus released *ts* virus as the original (cellular variants), one clone *ts* virus with a higher background of focus formation at nonpermissive temperature (viral mutant) (Table 5). With these data the reversion rate was calculated for each of the *ts* mutants. Reversion rates were low for all *ts* mutants, $< 1.2 \times 10^{-5}$ for *ts*159 and highest for *ts*143 (2×10^{-3}).

Temperature sensitivity of MPSV *ts* mutants as a function of temperature. MPSV *ts* mutants were isolated mainly to test whether mutant virus deficient in the establishment of the transformed state in fibroblast cells was equally deficient in inducing leukemia in animals at 37.5°C body temperature and, if possible, at a lowered body temperature of 32 to 34°C (see below). It therefore would be advantageous to obtain data on the differential temperature sensitivity of the *ts* mutants between 32 and 39.5°C. NRK cells were infected at different temperatures with the same virus collection with three of our *ts* mutants, i.e., *ts*143, *ts*124, and *ts*159 virus, and compared with cells infected with WT 6-6#3 virus (Fig. 2). WT 6-6#3 virus has slightly higher focus-forming capacity at 35 and 37°C than at 32 or 39.5°C. *ts*159 virus shows a continuous increase of transforming potential with decreasing temperature (Fig. 2). *ts*143 and *ts*124 virus show similarly high transforming potential at 32 to 35°C. This potential decreases gradually above 35°C. All viral *ts* mutants have reduced transformation capacity at 37 to 38°C, the normal mouse body temperature.

MPSV *ts* mutant viruses are deficient in leukemic transformation in mice maintained at normal body temperature. *ts* mutant and control WT virus were injected into mice to determine spleen focus formation (and induction of splenomegaly) (Table 4). The same virus suspension was also tested for fibroblast focus formation at 32 and 39.5°C and with a different batch of the same virus at 37°C (Fig. 2). The ratio of spleen focus formation in DBA/2J mice (37.5°C) to that of fibroblast focus formation as determined at 32°C, the permissive temperature, was 1.4×10^{-1} for WT 6-6#3. It was at least 3 orders of magnitude lower for *ts*124 and *ts*143. *ts*159 virus never induced spleen foci (Table 4). Splenomegaly could be obtained 2 months postinjection with *ts*143 and *ts*124 virus, but not with *ts*159 virus. The three *ts* mutants of MPSV, *ts*124, *ts*143, and *ts*159, are thus deficient not only in fibroblast transformation but also in inducing splenomegaly and spleen focus formation at the nonpermissive temperature.

An assay for sarcoma formation at lowered (permissive) tail temperature was developed by Klarlund and Forchhammer (16). However, no method is at present available to test the effect of MPSV (or of any other virus) at the permissive body temperature of the animal. We devised a method to overcome this drawback (see above).

DBA/2 mice maintained at 30 to 34°C and at normal body temperature were used to inject *ts* and WT virus (Table 6). WT 6-6#3 virus induced spleen foci with equal efficiency in mice maintained at 30 to 34°C and at 37.5°C 14 days postinjection. *ts*259 virus did not induce splenomegaly or spleen focus formation at either 32 or 37.5°C (mouse body temperature). The high mortality of DBA/2 mice at lowered body temperature makes more extensive experiments with the other *ts* mutants difficult.

DISCUSSION

Several *ts* transformation-deficient mutants of the myeloproliferative sarcoma virus were isolated. These mutants

TABLE 5. Stability of MPSV *ts* mutants[a]

Virus of clones	Revertant cell clone(s)	Ratio of FFU at 32°C/39.5°C		Calculated reversion rate
		Average	Range (*n*)	
6-6#3		1.85	1.3–3.2 (7)	
259		610	500, 720 (2)	0
	R1	470	470 (1)	
	R2	850	850 (1)	
159/5/1		Very high	1.5×10^3–∞ (6)	$<1.2 \times 10^{-5}$
	8 clones (R)	Very high	2×10^4–∞ (8)	
	RA1	41	41 (1)	
143/1/1		148	35–205 (5)	2×10^{-3}
	5 clones (R)	180	120–274 (5)	
	RA1	2.1	1.5–3 (3)	
	RA2	2.3	2.3 (1)	
124/1		1,260	290–2,500 (4)	1.6×10^{-4}
	4 clones (R)	1,070	350–2,100 (4)	
	RA1	10.2	8.4, 12 (2)	

[a] MPSV *ts* mutant virus was plated on NRK cells at the nonpermissive temperature (39.5°C) by serial dilutions. Cells of individual foci close to the endpoint dilution were isolated and regrown (RA cell lines). Alternatively, cells of "revertant" foci at the nonpermissive temperature were cloned in soft agar (R cell clones). Supernatant virus was tested for focus formation at 32 and 39.5°C. The virus supernatants of 23 individual potential "revertant" clones were tested; only four cell lines released virus with an increased probability of focus formation at the nonpermissive temperature (*ts*159/5/1 RA1, *ts*143/1/1 RA1 and RA2, *ts*124/1 RA1). Two of these clones released virus that had not reverted entirely to WT ratios (*ts*159 RA1, *ts*124/1 RA1); only two revertants of *ts*143 had ratios of FFU similar to those of WT virus. The range of the ratio of focus formation of several experiments is indicated in one column, and the number of individual experiments used to obtain the range and the average ratio is indicated within parentheses. An estimate of the reversion rate to WT virus was obtained by calculating the proportion of foci with revertant virus at 39.5°C and relating this value to the foci present at the permissive temperature (32°C). The reversion rate for *ts*159 virus was difficult to calculate; no foci occurred at the nonpermissive temperature in four of seven experiments. We therefore calculated a maximum value for the reversion rate by assuming that one focus would have been found at the nonpermissive temperature at the next higher virus concentration.

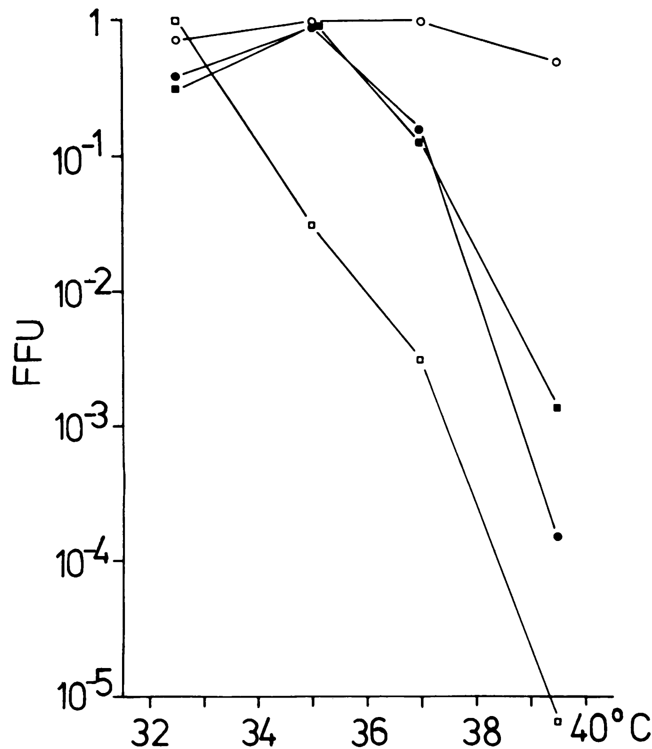

FIG. 2. Temperature sensitivity of virus-induced transformation when MPSV *ts* mutants were plated on NRK cells at 32, 35, 37, and 39.5°C. Fibroblast foci were counted 6, 9, 12, and 16 days after infection (see the text). The highest titer at any temperature was used as a reference (value of #1) and related to focus formation at other temperatures. Symbols: ○, WT 6-6#3 control; ●, *ts*124/1; ■, *ts*143/1/1; □, *ts*159/5/1.

showed a reduction (*ts*259, *ts*143, *ts*124) or an almost complete loss (*ts*159) of transformation potential at nonpermissive temperatures. The reversion rate to WT transformation levels appears to be very low (*ts*143 and *ts*124) or at a level undetectable by the usual methods (*ts*159). These *ts* mutants compare favorably with similar *ts* mutants of Mol-MuSV (5, 16) and will be useful for a study of the putative, but as yet unidentified, transformation protein of MPSV or Mol-MuSV (26).

All of our MPSV *ts* mutants are deficient in the maintenance of the transformed state, which is unaltered on infection with helper virus. This is in contrast to some of the other *ts* mutants of MuSV that have been described (33, 40).

Our selection procedure to isolate *ts* mutants involves the preferential killing of cells containing nonmutant virus at the nonpermissive temperature. These cells, which are transformed, grow in semisolid medium and incorporate bromodeoxyuridine into cellular DNA, in contrast to cells containing *ts* mutant virus, a procedure described for Rous sarcoma virus by Wyke (39). We modified the method by the addition of Hoechst dye 33258; this increased the lethality of light irradiation 100-fold. This procedure has previously been used to select for Friend cell mutants (8). We anticipated obtaining viral *ts* mutants with temperature sensitivity in colony formation of infected cells in semisolid medium. Unexpectedly, this was not observed and may have been a consequence of using NRK cells at a late passage number to obtain *ts* nonproducer cells. At this stage, control NRK cells are also capable of forming colonies in soft agar. Primary rat thyroid epithelial cells, however, if transformed with the MPSV *ts* mutant 159, behave as expected: they show clonal growth in agar at the permissive temperature, but not at the nonpermissive temperature (A. Fusco, personal communication).

MPSV is a unique Moloney sarcoma virus variant (Table 1) (17, 25), that not only induces efficient fibroblast transformation and causes sarcomas on intramuscular injection of adult mice (unpublished data), but also causes a multitude of leukemic changes in the adult sensitive mouse. These changes include an early 10- to 100-fold increase in the relative proportion of erythroid and granulocyte-macrophage precursor as well as of stem cells in the spleen, but not the bone marrow, of infected mice (17, 19, 25). These changes may be a consequence of a direct virus-induced change in proliferation of infected hematopoietic cells (23) or could, alternatively, be caused by infection of fibroblast-like cells ("hematopoietic microenvironment") secreting growth factors that are required for hematopoietic cell proliferation (21). The first alternative (direct action of MPSV on hematopoietic cells) may imply that two different transformation genes are in fact responsible for the dual action of MPSV on hematopoietic cells and on fibroblasts. The second alternative (transformation only of fibroblast-like cells and consequent release of hematopoietic growth factors) would require only one type of target cell and would imply the presence of a single transformation gene in MPSV.

All of the *ts* mutants of MPSV studied so far not only are deficient in causing fibroblast focus formation but also fail to induce spleen focus formation or leukemic changes. The deficiency of our MPSV *ts* mutants appears even more pronounced with respect to leukemic transformation; one mutant virus (*ts*259), which was injected and maintained at the inferred permissive temperature in the mouse—utilizing a novel procedure to lower mouse body temperature—was unable to induce leukemia. The association of leukemic transformation with fibroblast transformation with a mutant

TABLE 6. Fibroblast and spleen FFU titers at permissive and nonpermissive titers[a]

Virus	FFFU in NRK cells			SFFU in DBA/2J mice		SFFU/FFFU at 37.5°C and 32°C
	32°C	39.5°C	Ratio 32°C/39.5°C	30 to 34°C	37.5°C	
WT 6-6#3	1.8×10^4	1.3×10^4	1.4	3.1×10^3	2.6×10^3	1.2
*ts*259	1.6×10^4	3.2×10^1	500	0	0	0

[a] Wild-type 6-6#3 and *ts*259 virus are MPSV (F-MuLV) pseudotypes. Twenty DBA/2J mice were maintained at a temperature of 29.6 to 34°C as described. Two days after the lowering of body temperature (see the text), 12 DBA/2J mice were each injected with different dilutions of WT 6-6#3 virus; and 0.5 ml of virus suspension of the highest dosage availabe (1.6×10^4 fibroblast FFU). Two spleens were examined for spleen focus formation after 10 and 14 days, and 3 spleens were examined 28 days after injection; no foci were detected. Injection of WT and *ts*259 virus at normal mouse body temperature into three mice each for five fivefold serial dilutions of virus suspensions. Abbreviations as in Table 2.

virus does not permit us to separate the two transforming functions of MPSV. We have also sequenced the 3′ half of cloned WT MPSV and have found no indication of a second transforming gene in the MPSV genome (Stacey et al., submitted for publication).

Recombination of regions of the DNA of *ts* and WT virus genomes and with Mol-MuSV DNA will allow us to pinpoint the deficiency at a molecular level. The *ts* mutant MPSV isolates will also be an essential tool for further studies on the action of MPSV with Dexter-type stem cell cultures (9, 21) or direct assay systems of transformation (12). This also will help us to understand the causation of the myeloproliferative disease in humans, which is similar to the disease induced by MPSV in mice (19).

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