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Myeloproliferative Virus, a Cloned Murine Sarcoma Virus with Spleen Focus-Forming Properties in Adult Mice

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Myeloproliferative virus, derived from Moloney sarcoma virus, causes erythroleukemia and myeloid leukemia in adult mice. This virus is also capable of fibroblast transformation in vitro. The virus consists of two separable biological entities which have been cloned. The helper virus component caused no visible changes in adult mice, whereas the defective virus induced both spleen focus formation and a large increase in erythroid precursor cells but retained the sarcoma virus property of transforming fibroblasts in vitro. Thus, myeloproliferative virus is the first murine sarcoma virus which induces erythroleukemia in adult animals.

The availability of RNA tumor viruses which have specific effects on the differentiation of normal cells has stimulated considerably research in this area. Although in avian systems a number of defective leukemia virus strains have been shown to transform both fibroblasts and hematopoietic cells (13, 14, 24), only Abelson murine leukemia virus transforms both fibroblasts and lymphatic B cells (23, 26). Kirsten and Harvey murine sarcoma viruses (MSV-Kirsten and MSV-Harvey, respectively) induce erythroid proliferation of spleen cells only in newborn mice (2, 25; this paper), and these erythroid cells cannot be transplanted and do not grow as permanent cell lines (unpublished observations). Rauscher and Friend viruses are of particular interest since they induce spleen foci and erythropoiesis in adult *Fv-2^s/2^s* mice (9, 22, 27). A few of the erythroid tumor cells produced in the course of the erythroleukemia elicited by these viruses are transplantable and can then be used to establish permanent cell lines (5, 6, 10, 11, 18). The Friend and Rauscher virus complexes do not transform fibroblasts. We describe here a cloned variant, myeloproliferative virus derived from Moloney sarcoma virus [MPV(MSV)] (4; M. C. LeBousse-Kerdiles, F. Smadja-Joffe, B. Klein, B. Caillou, and C. Jasmin, Eur. J. Cancer, in press), which is shown to initiate spleen foci in adult *Fv-2^s/2^s* mice but not in C57BL (*Fv-2^r/2^r*) mice. MPV(MSV) induced a lowered requirement for erythropoietin in early (erythroid burst-forming units [BFU-E]) and an increase in later (erythroid colony-forming units [CFU-E]) erythroid precursor cells in adult mice. These cells still require a low dose of erythropoietin for colony formation in vitro (this paper; B Fagg, W. Ostertag, K. Vehmeyer, W. Paetz, C. Jasmin, F. Smadja-Joffe, M. C. Le Bousse, and B. Klein, manuscript in preparation). In addition, MPV(MSV) transforms fibroblasts in vitro, a property which can be used to generate mutants which are temperature sensitive for the fibroblast transformation or erythropoiesis-stimulating properties or both. Fibroblast and erythroid transformations (spleen focus formation) are both properties of the same defective viral subunit of the MPV(MSV) complex.

MATERIALS AND METHODS

Cell culture and viruses. All cell lines were grown in modified Eagle medium supplemented with 10% fetal calf serum (18). Infectious virus was obtained from spleens and tissue culture supernatants as described previously (19). Unless otherwise stated, the virus was used unconcentrated. Concentrated virus was used in some of the experiments in Table 1 and 2. Virus from tissue culture supernatants was concentrated 10- to 100-fold with an Amicon DC2 hollow-fiber apparatus and in a second step with Amicon type XM 300 membranes. Almost no reverse transcriptase activity is lost by using this procedure. The concentrates are usually 30 to 50% lower in relative biological activity than virus used before concentrating the supernatants. This was measured by infectivity of the virus in fibroblasts or by focus formation in fibroblasts or in mouse spleens.

The cell lines used in these studies and referred to in the figures and text are as follows. Cell line 643/22 is an SC1 cell line which releases Friend helper virus, LLV-F, cloned twice. 643/22N and 643/22F are cell

clones of 643/22 (20). Cell line 1.1A is an NIH/3T3 cell clone infected with cloned Moloney murine leukemia virus (LLV-Mol). MSV-Ball refers to virus released by the murine TB cell line (G8-124) of CFW/D mice coinfected with Moloney sarcoma virus (MSV-Mol) and LLV-Mol. MSV-Mol is in excess to LLV-Mol. Both cell lines (MSV-Ball and 1.1A) were obtained through the courtesy of R. Weinberg (Massachusetts Institute of Technology, Cambridge). MSV-Kirsten is a rat NRK cell line obtained from John Bilello (University of Hamburg, Hamburg, West Germany) and releases both Kirsten murine leukemia virus and Kirsten sarcoma virus (MSV-K). MSV-K+F is derived from an NRK nonproducer MSV^+LLV^- cell line transformed by MSV-K. MSV-K in this cell line is rescued by cloned LLV-F. MSV-Harvey-infected nonproducer MSV^+LLV^- cell line HT3 was obtained through the courtesy of J. Harvey. HT3 cells were superinfected with cloned LLV-F as described above. F4-6 cells are Friend spleen focus-forming virus (SFFV)-transformed erythroid cells of DBA/2J mice releasing high levels of virus particles (21). F4-6 cells can be treated with dimethyl sulfoxide (Me_2SO) to induce a 120-fold increase in the SFFV titer as described previously (19).

Reverse transcriptase assays. Reverse transcriptase activity was calculated as described previously (21) and corrected for variations in the assay by using a standard viral supernatant of Friend F4-6 cells (21) which was kept frozen at −80°C in aliquots.

Spleen focus formation. Spleen focus formation was assayed by injecting Gelman Acrodisc-filtered viral supernatants into the tail vein of mice as indicated in Tables 1 to 3. Serial 1:5 dilutions were made, and six mice were injected with 0.5-ml viral suspensions for each concentration of the virus. With SFFV, spleen foci were detected 10 days after injection, whereas MPV(MSV)-induced foci were detectable and counted after 16 days.

Focus-forming assay. The fibroblast focus-forming unit (FFU) assay was performed as described previously with NRK fibroblasts (3).

CFU-E and BFU-E assays. The procedure followed for CFU-E and BFU-E assays was essentially that described by Iscove and Sieber (12).

Spectrin assays. The assay for spectrin, using spectrin antisera, has been described previously (7).

RESULTS

Properties of uncloned MPV(MSV). MPV(MSV) was originally isolated by Chirigos et al. (4) from the plasma of adult BALB/c mice after multiple in vivo cellular and acellular passages of a tumor induced in adult mice by an MSV-Mol. MPV(MSV) induces a neoplasia in DBA/2 or BALB/c mice mainly characterized by a modification of hematopoietic organs (spleen, liver, thymus, peripheral blood, and bone marrow). Granulocytic and erythroid lines seem to be most affected by this pathological process (LeBousse-Kerdiles et al., in press). The hematological perturbations are always associated with an invasion of hematopoietic and non-hematopoietic organs by "tumor-like" nodules which destroy the normal architecture of the affected organ. This invasive phase usually occurs when the spleen weight increases from 0.5 to 1.2 g. The disease is also characterized by a progressive myelofibrosis of spleen and bone marrow. No significant increase in spleen weight is detectable after MPV(MSV) injection into C57BL mice. Spleens of DBA/2J mice which were about 1 g in weight were minced, extracted with buffer, and filtered. The filtered supernatant was used for the cloning experiments. Cloned MPV(MSV) (see below) with a titer of 10^3 spleen focus-forming units (SFFU) usually causes a doubling of spleen size by 16 days after injection; the spleen size increases from then until it is about 2 to 3 g in weight, usually 4 to 5 weeks after injection of the virus. The mice die 4 to 6 weeks postinjection. We call this phase the terminal phase of the disease.

Cloning of MPV(MSV). When inoculated onto mouse embryonic fibroblasts, MPV(MSV) induced foci of transformed cells similar to those seen with MSV-Mol. MPV(MSV) was cloned in rat NRK cells. Rat NRK cells (passage no. 14 to 20) were grown under log-phase growth conditions. A total of 2.5×10^3 NRK cells were seeded in each well of Costar 24-well plates. Medium was replaced 1 day after seeding with fresh medium containing 1% Me_2SO, only 5% fetal calf serum, and 6 μg of Polybrene per ml. Virus was added 1 day later, the same dilution of virus-containing medium to 12 different wells. Ten fivefold-dilution steps of the virus suspension were thus assayed. Cells infected with virus were kept for another 9 days (one or two medium changes) in 1% Me_2SO containing low-serum medium. Foci were then counted in all wells. The supernatant of cells of all wells after growth in T flasks was also assayed for reverse transcriptase activity. The titer of the original MPV(MSV) was 4×10^4 fibroblast FFU per ml of spleen cell supernatant (Table 1). The titer estimate of fibroblast-transforming particles was strictly proportional to the dose (Fig. 1). At endpoint dilution, 3 of 12 wells each contained 1 focus of transformed fibroblasts (Fig. 2). These cells were kept in 1% Me_2SO–5% serum-containing medium, with three medium changes per week for 4 weeks, and then transferred. Only transformed fibroblastic cells survived in two of the three wells which originally contained 1 focus of transformed cells. These cells and all of the cell clones derived from these were virus negative by reverse transcriptase assays (Table 1). The plating of helper virus as assayed by reverse transcriptase activity also followed a single-hit kinetics (Fig. 1). We can calculate the relative biological titer of both viruses if we assume that the helper virus and the sarcoma

TABLE 1. *First cloning of MPV(MSV) on NRK fibroblasts: fibroblast and spleen focus-forming titers*[a]

Cell line	Cell type	Reverse transcriptase		MSV		SFFV				
		Virus released	pmol of TTP per 10 μl (1, 2)	Fibroblast-transforming property	FFU/ml on NRK cells	Spleen focus-forming property	SFFU/ml in DBA/2J mice	FFU/pmol of TTP	SFFU/pmol of TTP	SFFU/FFU
Spleen	Infected with MPV(MSV)	Yes	ND	Yes	3.7×10^4	Yes	1.5×10^4	ND	ND	0.4
NRK 4-3	NRK fibroblast	Yes	5–22	0	0	0	0	0	0	—
NRK 4-3 (2)		Yes	1,800 (2)	0	0	0	0	0	0	—
MPV 6-1	NRK cells each with 1 focus	0	0	0	0	0	0	0	0	—
MPV 6-6		0	0	0	0	0	0	0	0	—
MPV 6-1#5	Cloned sarcoma cells of above	0	0	0	0	0	0	0	0	—
MPV 6-1#11		0	0	0	0	0	0	0	0	—
MPV 6-6#3		0	0	0	0	0	0	0	0	—
MPV 6-6#7		0	0	0	0	0	0	0	0	—
MPV 6-1#5+F	Cloned cells like above but	Yes	38	Yes	4.8×10^3	Yes	1.5×10^3	1.26	0.40	0.32
MPV 6-1#11+F	infected with LLV-F	Yes	22	Yes	3.0×10^3	Yes	1×10^3	1.37	0.45	0.33
MPV 6-6#3+F		Yes	9	Yes	1.3×10^3	Yes	4×10^2	1.44	0.44	0.30
MPV 6-6#3+F (2)		Yes	400 (2)	Yes	ND	Yes	1×10^4	ND	0.25	ND
MPV 6-6#7+F (2)		Yes	27 (2)	Yes	ND	Yes	8×10^2	ND	0.30	ND
MPV 6-1#11+M (2)	Sarcoma cells infected with	Yes	12 (2)	Yes	ND	Yes	3×10^2	ND	0.25	ND
MPV 6-6#3+M (2)	LLV-Mol	Yes	14 (2)	Yes	ND	Yes	2.5×10^2	ND	0.18	ND
MPV 6-6#7+M (2)		Yes	3 (2)	Yes	ND	Yes	5×10^1	ND	0.17	ND
643/22F (3)	Fibroblasts infected with LLV	Yes	430 (2)	0	0	0	0	0	0	—
1.1A (LLV-Mol) (4)		Yes	20	0	0	0	0	0	0	—
MSV-Ball (4)	Sarcoma cells	Yes	510 (2)	Yes	ND	0	0	ND	0	0
MSV-Kirsten (5)		Yes	2,400 (2)	Yes	ND	0	0	ND	0	0
MSV-K+F (5, 6)		Yes	3,000 (2)	Yes	ND	0	0	ND	0	0
MSV-Harvey (7)		Yes	1,700 (2)	Yes	ND	0	0	ND	0	0
F4-6 (8)	Friend cells	Yes	16	0	0	Yes	1.2×10^3	0	0.75	—
F4-6+Me_2SO (9)		Yes	22	0	0	Yes	1.5×10^5	0	68.00	—

[a] Columns 1 and 2 describe the cell type (organ) in which the virus was propagated. The MPV(MSV)-infected spleen was of DBA/2J origin. The MPV(MSV)-infected cell lines and their origin are described in the legend to Fig. 2. Columns 3 and 4 relate to the measurements of reverse transcriptase activity in the supernatants (21). Columns 5 and 6 refer to fibroblast-transforming properties of the virus released by the cell lines. Columns 7 and 8 refer to spleen focus-forming properties of the same virus. The numbers within parentheses refer to the following: (1) reverse transcriptase activity calculated as described previously (21) and corrected for variations in the assay by using a standard viral supernatant of Friend F4-6 cells (21) which was kept frozen at −80°C in aliquots; (2) virus was further concentrated 10- to 100-fold with an Amicon DC2 hollow-fiber apparatus and in a second step with Amicon type XM 300 membranes; (3) LLV-F 643/22N virus was usually used for rescue, except for the rescue of virus of p5-8#1, when we used 643/22F virus; (4) cloned LLV-Mol; (5) Kirsten murine leukemia virus and MSV-K; (6) MSV-K rescued from a nonproducer cell line by LLV-F; (7) MSV-Harvey rescued from a nonproducer cell line by LLV-F; (8) F4-6 virus released by F4-6 Friend cells (21); (9) F4-6 virus released during Me_2SO treatment of F4-6 cells; (10) 4-3 indicates virus of cell line NRK 4-3 (Table 1 and Fig. 2). This is the original helper virus of the MPV(MSV) complex. The origin and description of the cell lines and viruses are given in detail in the text. ND, Not determined. —, These supernatants do not contain virus particles with focus-forming activity on fibroblasts.

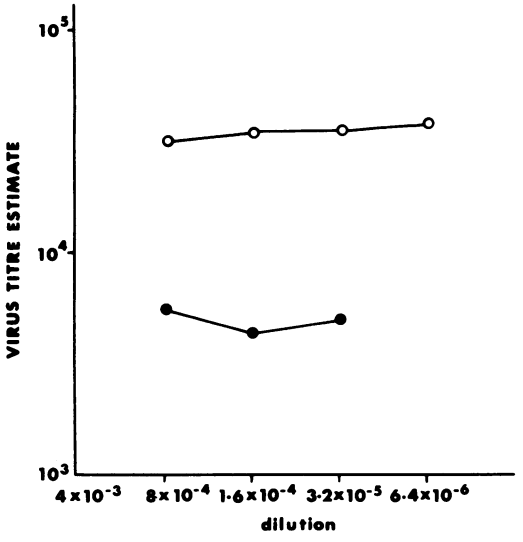

FIG. 1. *Dose response of the sarcoma and helper component of MPV(MSV). MPV(MSV) was plated on wells with NRK cells at different dilutions. Symbols: ○, titer estimate of MSV; ●, titer estimate of the helper virus. MSV-induced foci were counted, and the titer was caluclated at the dilutions as indicated. The helper virus was determined by measuring reverse transcriptase activity of individual wells of a 24-well plate of NRK cells. The number of wells with cells with no virus release (zero fraction) was used to calculate the titer by the Poisson distribution.*

virus were equally efficient in infection of rat NRK cells: the titer for the helper virus was one-eighth that of the sarcoma component (Fig. 1), that is, 5×10^3/ml. This titer may be a 100-fold underestimate of the titer if measured in murine fibroblasts (15).

Fibroblast focus formation in murine SC1 cells was not much higher than that in rat NRK cells. We measured the focus formation of the MPV(MSV) clonal isolate of cell line 6-6≠3 infected with LLV-F to estimate the relative titers of the fibroblast-transforming viral component in rat and murine fibroblasts. With one particular cell supernatant, we obtained 1.03×10^4 FFU/ml in NRK cells and 2.9×10^4 FFU/ml in murine SC1 cells, using a similar cell number of rat and mouse cells per well during infection.

The sarcoma virus component is responsible for the spleen focus-forming activity of MPV(MSV). MPV(MSV) injected into the lateral tail vein of adult DBA/2J or C3H/HeJ mice induced visible spleen foci within 12 to 16 days. These foci were distinct and grossly similar in appearance to those caused by Friend SFFV (Tables 1 and 3). NRK cells transformed by MPV(MSV) at endpoint dilution were cloned in agar. Four cloned cell lines of two different foci at endpoint dilution were used for the experiments described (Fig. 2 and Table 1). All of these cell clones were reverse transcriptase negative and were also shown to be virus negative by electron microscope observation. All four cell clones could be superinfected by either cloned LLV-F or cloned LLV-Mol (8) (Table 1). All three transformed cell clones released fibroblast-transforming virus on superinfection (Table 1). The rescued transforming virus of the four cloned sarcoma cell lines, when either cloned LLV-F or LLV-Mol (1.1A) was used for rescue, was able to induce spleen foci after intravenous injection of the virus into adult $Fv\text{-}2^s/2^s$ DBA/2J mice (Table 1). The spleen foci, 0.2 to 2 mm in diameter at 16 days after injection into adult mice, were indistinguishable from those obtained by injecting a supernatant of uncloned MPV(MSV).

One cell line, NRK 4-3 (Table 1 and Fig. 2), was isolated at a virus dilution of 1.6×10^{-4}. This well did not show fibroblast foci. All 11 sister wells contained more than 1 focus. NRK 4-3 cells released high titers of virus (see below). The virus did not have spleen focus-forming properties. SFFV of MPV(MSV), if it had been at a much higher excess to the sarcoma component and coded by a separate genomic subunit, should have been present at this virus dilution and should be associated with the helper virus of NRK 4-3. In contrast, all viruses rescued from sarcoma cell lines which were each obtained from 1 focus at a 25-fold-lower MPV(MSV) dilution (6.4×10^{-6}), however, did show spleen focus-forming activity (Table 1; see above).

The transforming virus of two of the sarcoma clones rescued by LLV-F was used again for MPV(MSV) cloning. At endpoint dilution, 6 of 24 wells [using the same dilution of MPV(MSV)] contained 1 sarcoma focus and another well contained 2 sarcoma foci if the rescued virus complex of nonproducer cell clone MPV 6-1≠5 was used. All of the supernatants of the cells in the wells at endpoint dilution of MSV were negative in the reverse transcriptase assay. The cells of these foci were again cloned in agar. All of these clones were reverse transcriptase negative as expected (Table 2). MPV(MSV) of cell clone 6-1≠5 is thus in excess compared with LLV-F. Cells of clone MPV 6-1≠11 were also superinfected with LLV-F, and the sarcoma component was cloned at endpoint dilution (Fig. 2). MPV(MSV)-transformed fibroblasts of wells each with 1 focus were again cloned in agar. Some of these cell clones released virus; i.e., they were infected by LLV-F. LLV-F was thus in excess of MSV in the MPV 6-1≠11 cells which were superinfected with LLV-F. The supernatant of four of these cell clones was checked for

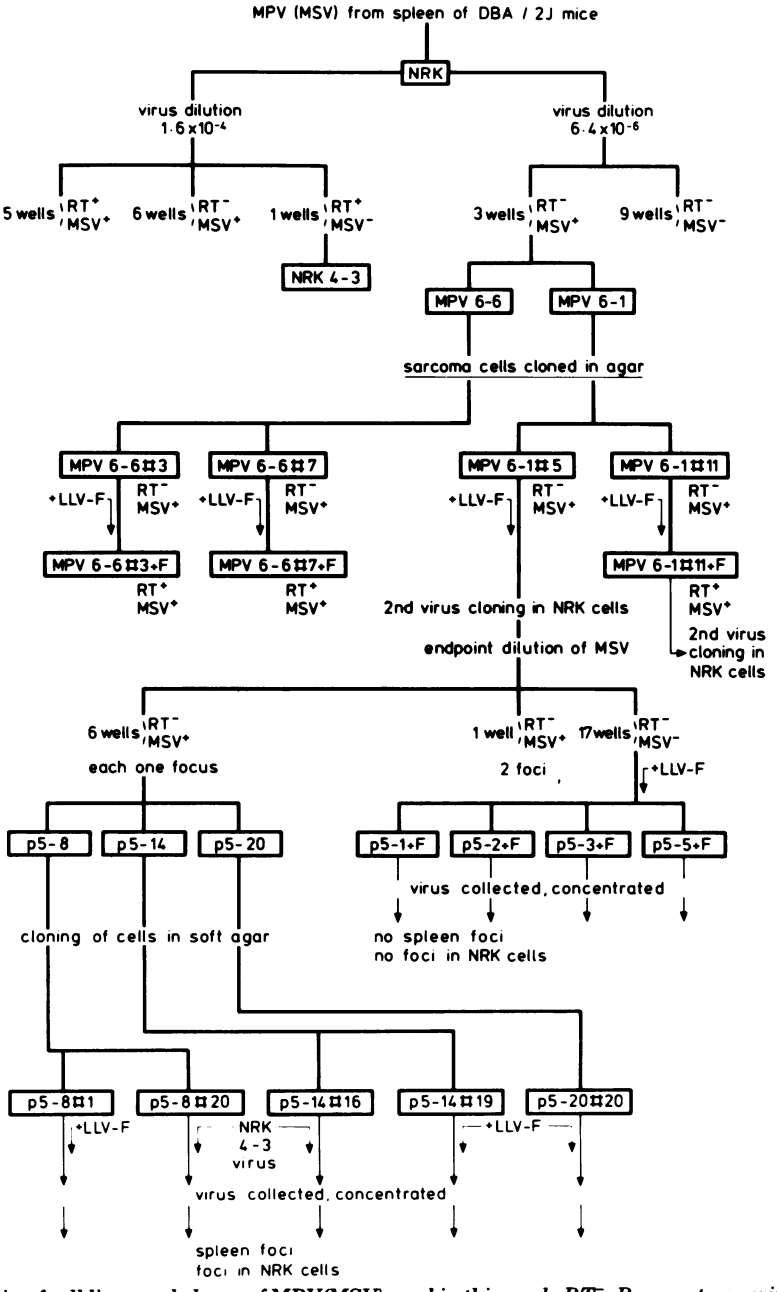

FIG. 2. *Origin of cell lines and clones of MPV(MSV) used in this work. RT⁻, Reverse transcriptase-negative ("virus-negative") cell lines; RT⁺, virus-producing cell lines; MSV⁻, cells not transformed by MPV(MSV); MSV⁺, cells transformed by MPV(MSV); LLV-F, cloned Friend helper virus as described previously (20); NRK virus, virus of cell line NRK 4-3 = cloned helper virus of the original MPV(MSV).*

focus formation in fibroblasts and in mouse spleen cells. All of the cloned sarcoma cells released virus with focus-forming properties in fibroblasts and in DBA/2J spleens (data not shown). Rescue experiments with the nonproducing cloned sarcoma cells obtained after infection with the cloned virus of MPV 6-1≠5 origin (cell clones p5–8, p5–14, and p5–10) (Fig. 2) showed that the rescued virus and not LLV-F was the SFFV (Table 2): all of the supernatants were able to induce spleen focus formation and transformed NRK fibroblasts.

TABLE 2. *Second cloning of MPV(MSV): fibroblast and spleen focus-forming titers*[a]

Cell line	Type of cell line	Reverse transcriptase		MSV		SFFV		SFFU/ pmol of TTP
		Virus released	pmol of TTP per 10 μl (1, 2)	Fibroblast-transforming property	FFU/ml on NRK cells	Spleen focus-forming property	SFFU/ml in DBA/2J mice	
MPV 6-1#5+F	Cloned sarcoma cells infected with LLV-F of cell line 643/22F	Yes	38	Yes	4.8×10^3	Yes	1.5×10^3	0.40
p5-1	NRK cells at endpoint dilution of MSV in wells with no MSV foci	No	0	No	0	No	0	0
p5-2		No	0	No	0	No	0	0
p5-3		No	0	No	0	No	0	0
p5-5		No	0	No	0	No	0	0
p5-1+F (2)	Same cells as above but infected with LLV-F	Yes	6,000 (2)	No	0	No	0	0
p5-2+F (2)		Yes	1,200 (2)	No	0	No	0	0
p5-3+F (2)		Yes	2,500 (2)	No	0	No	0	0
p5-5+F (2)		Yes	1,600 (2)	No	0	No	0	0
p5-8	NRK cells at endpoint dilution of MSV with 1 focus per well	No	0	No	0	No	0	0
p5-14		No	0	No	0	No	0	0
p5-20		No	0	No	0	No	0	0
p5-8#1	Cloned sarcoma cells of p5-8	No	0	No	0	No	0	0
p5-8#20		No	0	No	0	No	0	0
p5-8#1+F (2)	As above but helper virus infected	Yes	1,600 (2)	Yes	ND	Yes	3.9×10^4	0.25
p5-8#20+4-3 (2, 10)		Yes	15 (2, 4)	Yes	ND	Yes	7×10^1	0.42
p5-14#19	Cloned sarcoma cells of p5-14	No	0	No	0	No	No	0
p5-14#16		No	0	No	0	No	No	0
p5-14#19+F (2)	As above but helper virus infected	Yes	700 (2)	Yes	ND	Yes	1.7×10^3	0.03
p5-14#16+4-3 (10)		Yes	150 (2)	Yes	ND	Yes	3×10^1	0.002
p5-20#20	Cloned sarcoma cells of p5-20	No	0	No	0	No	No	0
p5-20#20+F (2)	As above but infected with LLV-F	Yes	150 (2)	Yes	ND	Yes	5×10^3	0.33

[a] The MPV(MSV)-infected cell lines and clones and their origin are described in the legend to Fig. 2 and footnote *a* of Table 1. The legends are the same as those used for Table 1.

We also decided to test cells of wells which did not contain fibroblast foci of transformed cells to exclude the already unlikely possibility that we had copurified a second defective viral putative SFFV subunit of the MPV(MSV) complex which may have been in excess to the MSV subunit. The viral supernatant was further concentrated to detect minute amounts of contaminating virus. None of these supernatants transformed fibroblasts and spleen cells of adult mice. The sarcoma-transforming and spleen focus-forming properties should therefore reside in the same viral RNA subunit.

The helper virus of the MPV(MSV) complex is not responsible for spleen focus formation. We wished to exclude the possibility that both helper and transforming viruses of the MPV(MSV) complex are spleen focus forming. Therefore, we looked at the property of the original helper virus of the first cloning experiment at endpoint dilution of the reverse transcriptase activity. We obtained a cell line, NRK 4-3, from one of the reverse transcriptase-positive wells after serial passage which did not contain any transformed fibroblasts (Fig. 2). The reverse transcriptase-positive supernatant of this cell line (Table 1) did not induce any spleen foci when injected into the lateral tail vein of DBA/2J mice. The virus was still biologically active since rescue of the transforming virus in nonproducer sarcoma cells with twice-cloned MPV(MSV) was possible (Table 2).

We thus conclude that the MPV(MSV) complex consists of at least two entities: (i) a helper virus, which does not transform mouse embryonic fibroblasts in vitro and does not induce spleen foci in adult mice, but can rescue MPV(MSV) of nonproducer $MPV^{+}LLV^{-}$ cells; (ii) a defective fibroblast-transforming virus, which is also SFFV [MPV(MSV)].

Host range restriction of SFFV in *Fv-2^r/2^r* mice. The *Fv-2^r* locus controls the sensitivity of mice to Friend or Rauscher SFFV. SFFV does not induce any spleen foci in C57BL *Fv-2^r/2^r* mice (17, 27). The *Fv-2^r* locus is recessive to the *Fv-2^s* locus. The action of the *Fv-2^r* locus is believed to be related to a restriction of virus replication in homozygous hematopoietic cells (17, 28) of *Fv-2^r/2^r* mice. We examined the spleen focus formation in *Fv-2^s/2^s* and *Fv-2^r/2^r* mice. Both Friend SFFV and MPV(MSV) formed spleen foci in *Fv-2^s* animals (Table 3). No foci were obtained in *Fv-2^r* homozygous C57BL mice. The spleen focus-forming activity of both virus isolates with SFFV activity thus seems to be restricted by the *Fv-2^r* locus unless a second gene locus of C57BL mice is involved in the restriction of MPV(MSV). We are currently testing the focus formation of MPV-(MSV) in *Fv-2^s* mice with C57BL background and in *Fv-2^r* mice with DDD background (1, 17). The *Fv-2^r* genotype does not restrict fibroblast transformation by the myeloproliferative virus (unpublished data).

Effect of MPV(MSV) on erythroid cell proliferation. The polycythemia-inducing Friend virus strain (FV-P) of Mirand alters the response of virus-infected erythroid cells to the hormone erythropoietin (16). Bone marrow or spleen cells of mice which are infected by FV-P mature autonomously without a requirement for erythropoietin (Table 4). This autonomy seems to result from the infection of erythroid precursor cells with SFFV and not the helper virus, since Friend SFFV cloned in fibroblasts and rescued by a variety of type C helper virus isolates has identical properties in this respect (B. Fagg and W. Ostertag, submitted for publication).

Spleen and bone marrow cells of mice infected with uncloned and cloned MPV(MSV) were cloned in methylcellulose as described by Iscove and Sieber (12) with and without erythropoietin. None of the cultures showed a large increase in CFU-E or BFU-E in the absence of erythropoietin (Table 4). However, when a low dose of erythropoietin (0.15 U/ml) was added to MPV-infected spleen cells, we obtained a more than 100-fold increase in BFU-E and an up to 10-fold increase in CFU-E cells compared with cells of control spleens. This increase was even higher when a correction was made for the 10- to 20-fold size increase of the spleen in MPV(MSV)-infected mice. The erythroid bursts (BFU-E) caused by MPV(MSV) infection in mouse spleens did not differ in appearance from normal 10-day BFU-E except that they were usually even larger. There was only an increase in BFU-E cells but no increase in CFU-E cells when we assayed for colony formation in vitro with bone marrow cells of MPV(MSV)-infected mice (Table 4). This correlates with the hematological changes which are observed during MPV(MSV) injection of mice (LeBousse-Kerdiles et al., in press; see below). The number of BFU-E cells which were detected in MPV(MSV)-infected mice with a low dose of erythropoietin was approximately the same as that found in normal mice with a high dose of erythropoietin (2 U/ml) (Table 4). However, the number of BFU-E cells per infected mouse was at least 10- to 20-fold larger per spleen when a correction was made for the increase in spleen size. A detailed analysis of the type of erythroid cell which is stimulated by MPV(MSV) and its response to erythropoietin will be published elsewhere. Staining with spectrin antiserum of both spleen and bone marrow cells of mice infected with uncloned and cloned MPV(MSV) of cell line MPV 6-6≠3+F (Fig. 2) showed a marked increase in the proportion of spectrin-positive cells, whereas the number of benzidine-staining cells was decreased. Spleen and bone marrow cells of mice in the terminal phase of the disease (4 weeks after injection of the virus) were used for the spectrin assay. A total of 56% of the MPV 6-6≠3-infected spleen cells and 60% of the bone marrow cells contained spectrin. Only 6% of the spleen cells and 20% of the bone marrow cells of control DBA/2J mice were spectrin positive. The presence of spectrin is an indicator of the presence of both early and late erythroid cells and not of any other differentiated cell (7). Most of the bone marrow and spleen cells of mice infected with MPV(MSV) are thus erythroid.

DISCUSSION

Our data on the MPV(MSV) virus complex show the following: (i) MPV(MSV) consists of at least two viral species, a replication-competent helper virus and a defective transforming virus; (ii) the helper component does not cause spleen focus formation in adult mice; (iii) the cloned sarcoma virus induces spleen foci in adult mice; (iv) the spleen focus formation of MPV(MSV) seems to be restricted by the *Fv-2^r* locus like the SFFV of the Friend virus complex; (v) MPV(MSV) induces a greater than 100-fold increase in CFU-E cells in the spleen. The num-

TABLE 3. *Spleen focus formation of MPV(MSV) in FV-2^r/2^r and FV-2^s/2^s mice*[a]

Origin of virus	Type of virus	SFFU/ml		
		DBA/2J	C3H/HeJ	C57BL
DBA/2J spleen	Original MPV(MSV)	1.5×10^4	1.0×10^3	0
MPV 6-1#11 + LLV-F	Cloned MPV(MSV)	1.2×10^3	ND	0
MPV 6-6#3 + LLV-F	Cloned MPV(MSV)	4×10^3	ND	0
F4-6	Friend SFFV	3×10^5	ND	0

[a] Spleen focus formation was determined as described in Table 1, footnote *a*. Spleen foci were counted 16 days [MPV(MSV)] or 10 days (SFFV) after injection of the viral suspension into lateral tail veins of DBA/2J, C3H/HeJ, or C57BL mice. The mice were 3 to 8 months of age during injection and were all obtained from the Jackson Laboratory, Bar Harbor, Maine. The F4-6 virus was obtained from an Me_2SO-treated differentiating F4-6 culture (19). C3H/HeJ mice were included in this study since Chirigos et al. (4), in the original publication on MPV(MSV), indicated that C3H/HeJ mice were resistant to MPV(MSV). We could not confirm this report but found that DBA/2J mice were more sensitive to MPV(MSV). Similar titer differences between C3H/HeJ and DBA/2J mice are obtained with Friend SFFV of cell line F4N (18); DBA/2J mice are *Fv-2^s/2^s* and *Fv-1^n/1^n*; C57BL mice are *Fv-2^r/2^r* and *Fv-1^b/1^b*. The *Fv-1* genotype had no influence on the number of foci which were obtained; the helper virus that we used was NB tropic.

TABLE 4. *CFU-E and BFU-E cells in spleens and bone marrow of mice infected with MPV(MSV) and Friend SFFV*[a]

Tissue	Virus used for infection	Avg spleen wt (g)	CFU-E				BFU-E			
			0		+Epo		0		+Epo	
			Colonies counted	CFU-E/10^5 cells	Colonies counted	CFU-E/10^5 cells	Colonies counted	BFU-E/10^5 cells	Colonies counted	BFU-E/10^5 cells
Bone marrow	None	0.12	8	0.8	3300	177	0	0	0	0
	Original virus = MPV(MSV)	1.53	46	0.3	224	32	0	0	46	1.9
	MPV 6-1#5+F	1.15	28	2.0	410	38	0	0	96	6.7
	MPV 6-1#11+F	1.50	28	1.1	1860	56	0	0	12	1.1
	MPV 6-6#3+F	2.06	446	3.6	4900	335	0	0	54	4.5
	Friend SFFV	2.45	11,100	2,454	8100	2,246	0	0	0	0
Spleen	None	0.12	0	0	750	43	0	0	0	0
	Original virus = MPV(MSV)	1.53	104	12.7	9880	1,840	0	0	138	11.5
	MPV 6-1#5+F	1.15	44	3.4	1030	169	0	0	58	3.3
	MPV 6-1#11+F	1.50	236	11.0	9400	507	0	0	131	9.5
	MPV 6-6#3+F	2.06	504	45.9	1400	724	0	0	122	7.5

[a] The results in this table represent the summary of more extensive data which will be published elsewhere (Fagg et al., in preparation). Mice were infected with the virus of the cell lines indicated in column 2. Mice with a spleen weight of 1.1 to 2.5 g were used. This size of spleen was usually obtained at 1 to 3 weeks after injection of FV-P (SFFV) of cell line F4-6 and at 1 to 6 weeks after injection of MPV(MSV). The titer of MPV(MSV) was usually increased before injection by a hollow-fiber Amicon concentration as described in the text. The MPV(MSV)-transformed cell lines which were used for obtaining the virus are listed in Table 1 and Fig. 2. Bone marrow or spleen cells of the infected mice were plated by the methods described by Iscove and Sieber (12) for quantitating normal erythropoietic cells with methylcellulose. The colonies were counted after 2 days (CFU-E) or 10 days (BFU-E). Erythropoietin, which we prepared from the urine of patients with Fanconi anemia (Fagg et al., in preparation) was added to the bone marrow or spleen cells during plating (+Epo) in concentrations which promote CFU-E but not BFU-E formation with bone marrow cells of normal DBA/2J mice. A 10-fold-higher concentration of erythropoietin was required for bone marrow or spleen cells of normal mice to obtain any BFU-E. At this concentration, a maximum of 2 to 6 10-day BFU-E per 10^5 bone marrow cells was obtained if cells of normal mice were used.

ber of BFU-E cells is also increased. The BFU-E cells require a low concentration of erythropoietin for in vitro growth. However, Friend SFFV (FV-P) induces proliferation of erythropoietin-independent (autonomous) erythropoiesis. Thus, the action of MPV(MSV) in inducing erythroid proliferation in the spleens of infected mice is unique to MPV(MSV).

The cloning procedure that we used employs rat NRK cells. These are known to replicate murine viruses rather inefficiently. Spleen focus formation and fibroblast-transforming properties, if dependent on two separate genomic entities, may also have been obtained if the putative separate SFFV entity had been in large excess to the MSV entity or if it had infected rat NRK cells preferentially as compared with the MSV particles. This possibility is extremely improbable because of the following reasons. (i) No SFFV properties were found with cell line NRK 4-3 which, during the first virus cloning, replicated helper virus but did not contain MSV properties at a 25-fold-higher concentration of plating virus than that used for endpoint dilution of the MSV particles which transform NRK cells. (ii) All independently cloned MSV particles of the MPV(MSV) complex also had the properties of spleen focus formation in adult mice. We listed nine of those clones in Fig. 2 and Tables 1 and 2. We also cloned the sarcoma virus of MPV 6-6#3 (Fig. 2) twice and recloned this virus a third time. All independent clones which have been checked transformed fibroblasts and formed foci in spleens of adult mice (data not shown). (iii) We would have expected to also obtain some SFFV activity by rescue of cells derived from sister wells to wells with transformed fibroblasts if the putative separate SFFV entity had been in excess to MSV. We therefore infected cell lines obtained from sister wells which contained no foci (cell lines p5-2, p5-3, and p5-5) with LLV-F. Efficient replication was monitored by measuring the reverse transcriptase activity of these supernatants. These were further concentrated 50- to 200-fold to obtain maximum detectability of SFFV acitivity in mice. None was detected in any of the four supernatants. (iv) Focus formation in rat fibroblast NRK and murine SC1 cells is similarly efficient. Statistical reasons thus make it extremely unlikely that spleen focus formation and fibroblast transformation are caused by different viral genomes of the MPV(MSV) complex.

The only way to explain our data (MSV and SFFV properties) on a two-virus genome basis would thus be to assume that the putative separate genomes of MPV(MSV) and of a new type of SFFV would, in fact, always be linked as a functional unit similar to the situation found with balanced lethal genes in *Drosophila melanogaster,* for example. This model can possibly be excluded by generating temperature-sensitive mutants for the fibroblast-transforming property of the virus. It can also be tested by an analysis of the genomic subunits.

The isolation and characterization of MPV(MSV) has shown that an MSV-Mol has been modified, presumably by a recombination event or mutation within the genome. This represents the first case of a modified sarcoma virus causing erythroleukemia in adult mice. Nucleic hybridization data have indicated that although all or part of the sarcoma genome is retained, other new virus sequences have been added to the MPV(MSV) genome. These do not appear to be in the Friend SFFV genome (Pragnell et al., manuscript in preparation).

A histological and hematological study of the disease induced by the cloned virus is necessary to determine whether this isolate can induce the same complex disease as can the uncloned virus. The property of MPV(MSV) to transform fibroblast cells will permit the selection of temperature-sensitive mutants of MPV(MSV) for the fibroblast-transforming gene as described for diverse avian sarcoma viruses (29). This may give some insight into the mechanism of action of the virus on spleen focus formation and the stimulation (transformation) of erythroid precursor cells.

MPV(MSV)-transformed spleen cells form transplantable tumors (unpublished data). Hematopoietic stem cells from MPV(MSV)-infected mice are serially transplantable in irradiated mice. These observations indicate that it may be possible to isolate hematopoietic cell lines transformed with temperature-sensitive mutants of MPV(MSV). These cell lines could be used to study differentiation of granulocytic and erythroid murine cells in vitro at permissive and nonpermissive temperature.

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