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Characterization of a preleukemic state induced by Moloney murine leukemia virus: Evidence for two infection events during leukemogenesis

(hemopoietic progenitors/preleukemia)

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ABSTRACT **A preleukemic state in mice inoculated with Moloney murine leukemia virus (Mo-MuLV) was characterized. Six to 10 weeks after neonatal inoculation, animals developed mild splenomegaly and generalized hematopoietic hyperplasia. The hyperplasia was evident from myeloid and erythroid progenitor assays. A nonleukemogenic variant, Mo+PyF101 Mo-MuLV, did not induce the hyperplasia; this suggests that the hyperplasia is a necessary event in Mo-MuLV leukemogenesis. Another variant, MF-MuLV, which contains the long terminal repeat of Friend MuLV and causes erythroid leukemia instead of T-cell lymphoma, also induced the preleukemic hyperplasia. A model for Mo-MuLV leukemogenesis is presented in which two infection events are necessary: the first leads to generalized hematopoietic hyperplasia, and the second results in site-specific insertion and long terminal repeat activation of cellular protooncogenes.**

Retroviruses that lack oncogenes induce hematopoietic neoplasms with long latency (1). This long latency may reflect multiple steps in the leukemogenic process. In the case of Moloney murine leukemia virus (Mo-MuLV), which induces T-cell lymphoma, one of these steps is site-specific proviral integration adjacent to cellular protooncogenes (2–5). This results in transcriptional activation of the protooncogenes by promoters or enhancers in the viral long terminal repeat (LTR; refs. 2, 3). In at least some instances, this may be a relatively late event in leukemogenesis (6, 7). Leukemogenesis may also involve recombination with endogenous retroviral sequences to generate dual-tropic (MCF) MuLVs (7–9). The thymotropic enhancers in the Mo-MuLV LTR are also important determinants in the specific type of leukemia (T-lymphoma) induced (10, 11). Other steps may also be involved in the leukemogenic process.

We have generated Mo-MuLV derivatives that have insertions or substitutions of enhancer sequences from the PyF101 variant of polyomavirus within the Mo-MuLV LTR, Mo+PyF101 and ΔMo+PyF101, respectively (12, 13). Neither of these viruses are leukemogenic, although they establish infection in inoculated animals normally. These viruses are minimal variants, since the alterations do not involve protein coding sequences. It is likely that the nonleukemogenicity results from an alteration in the tissue tropism of these viruses such that they are restricted from expression in some critical cell type whose infection is necessary for Mo-MuLV leukemogenesis. Thus, these viruses may allow identification of critical leukemogenic events and target cells. In the experiments presented here, these and other variants of Mo-MuLV were used to identify a preleukemic stage in Mo-MuLV leukemogenesis.

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MATERIALS AND METHODS

Viruses and Inoculation of Mice. Generation of wild-type and Mo+PyF101 Mo-MuLV has been described (12). MF-MuLV and FM-MuLV viral stocks (11, 14) were generously provided by Nancy Hopkins. All stocks were culture supernatants from productively infected NIH 3T3 cells. Infectivity titrations were performed by the UV/XC plaque assay (15). For all experiments, neonatal NIH Swiss or inbred NFS mice were inoculated subcutaneously with 2.5×10^4 XC plaque-forming units (pfu) of virus.

Hematopoietic Colony Assays. Assays for myeloid colony-forming cells (myeloid CFC) and erythroid colony-forming units (CFU-E) were performed essentially according to Metcalf (16). Myeloid CFC were measured by agar colony assays in medium containing WEHI-3B cell supernatant as a source of growth factors. Colonies (>50 cells) were scored microscopically after 7 days of incubation. CFU-E were measured in methylcellulose colony assays in medium containing concentrated supernatant from pokeweed mitogen-stimulated spleen cells and erythropoietin as growth and differentiation factors. CFU-E (densely packed colonies containing a minimum of eight small cells, at least 50% benzidine positive) were scored after 2–3 days.

Infectious Center Assays. Infectious center assays for splenocytes and thymocytes have been described previously (17). Bone marrow cells were flushed from mouse femurs, passed three or four times through a 23-gauge needle to obtain single cell suspensions, and assayed as for the splenocytes and thymocytes.

RESULTS

The organizations of the MuLVs used in these experiments are shown in Fig. 1. In the first experiments, wild-type and Mo+PyF101 Mo-MuLVs were compared. Recently, we showed that Mo+PyF101 Mo-MuLV establishes high levels of infection in thymocytes at early (preleukemic for wild-type Mo-MuLV) times even though it does not induce leukemia or cause preleukemic changes in the thymus (17). These data are not consistent with a model in which thymic infection and LTR activation of protooncogenes is by itself sufficient for Mo-MuLV leukemogenesis. Another event(s) in addition to thymocyte infection must also be important, and Mo+PyF101 Mo-MuLV is apparently defective in that step. Recently, Storch *et al.* (18) described an early preleukemic state in the spleens of Mo-MuLV-inoculated mice, typified by mild splenomegaly and increased immature lymphoid and nonlymphoid cells. We therefore tested mice inoculated with

Abbreviations: MuLV, murine leukemia virus; Mo-MuLV and F-MuLV, Moloney and Friend MuLV, respectively; FM-MuLV and MF-MuLV, derivatives of F- and Mo-MuLV, respectively; LTR, long terminal repeat; pfu, plaque-forming unit(s); CFC, colony-forming cells; CFU-E, erythroid colony-forming unit(s).

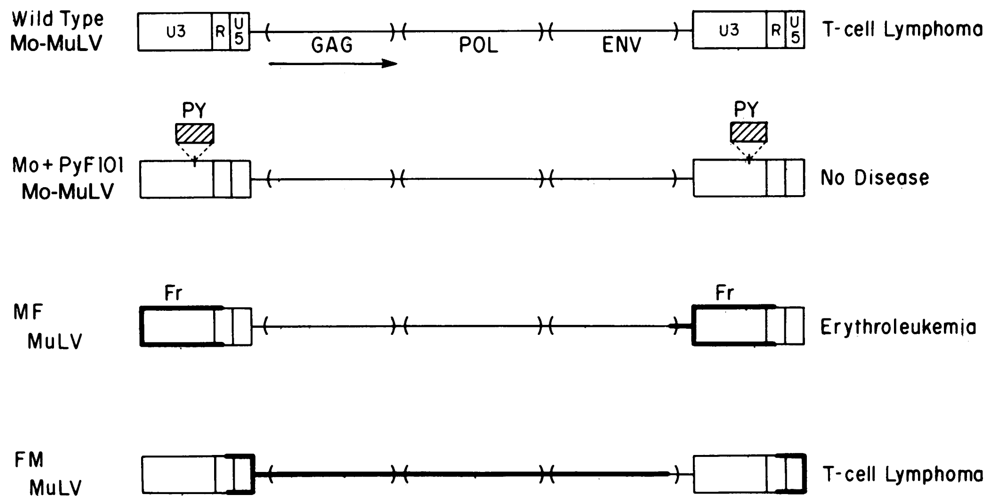

FIG. 1. Mo-MuLV and derivatives that have altered pathogenic potential. The genomes of the viruses are shown in DNA form. The LTR sequences are indicated by open boxes. Wild-type Mo-MuLV is a standard replication-competent retrovirus that induces T-cell lymphoma with a latency of 3–4 months under the conditions used here. Mo+PyF101 Mo-MuLV contains enhancer sequences from the F101 mutant of polyoma virus inserted at −150 base pairs in the U3 region of the Mo-MuLV LTR. MF-MuLV is identical to wild-type Mo-MuLV except for the substitution of sequences from Friend MuLV (shown in bold lines) containing the U3 and R sequences from the LTR and also a small portion of the envelope p15E region. FM-MuLV is the reciprocal recombinant. We have confirmed that MF-MuLV and FM-MuLV induce erythroleukemia and T-lymphoid leukemia in neonatally inoculated NIH Swiss mice, as reported previously (14).

wild-type and Mo+PyF101 Mo-MuLV for preleukemic changes in the spleen.

Mice inoculated with wild-type Mo-MuLV showed splenic enlargement (a factor of 2 or 3) 4–9 weeks after inoculation, well before development of leukemia. Flow cytometry indicated that the enlargement was accompanied by a shift to greater numbers of cells that were not mature T or B lymphocytes (negative for surface immunoglobulin or for Thy 1.2 antigen—"null cells;" a 6- to 8-fold increase compared with a control animal, data not shown), in agreement with Storch *et al.* (18). To test whether this reflected increased proliferation of multiple hematopoietic lineages, myeloid and erythroid stem cells were quantified by colony assays (Tables 1 and 2). Mice inoculated with wild-type Mo-MuLV showed a 4- to 10-fold increase in total myeloid colonies (myeloid CFC), and a 10- to 20-fold increase in total erythroid colonies

Table 1. Hematopoietic progenitors in preleukemic mice: Myeloid

Virus inoculated	Time to sacrifice, wk	Spleen: XC pfu/10^5 cells*	Spleen: CFC/10^5 cells†	Spleen: Total cells ($\times 10^{-8}$)‡	Spleen: Total CFC ($\times 10^{-3}$)†	Bone marrow: CFC/10^5 cells†§
			Experiment 1			
None	6		0.6	2.7	1.6	13
wt Mo-MuLV	6	ND	4.1 (6.8×)	2.3	9.4 (5.9×)	36 (2.8×)
			Experiment 2			
None	7		2.1	2.1	4.4	67
wt Mo-MuLV	7	ND	5.4 (2.6×)	2.8	15 (3.4×)	54 (0.8×)
			Experiment 3			
None	≈7.5		0.4	1.2	0.5	41
wt Mo-MuLV	≈7.5	ND	2.0 (5.0×)	3.0	6.0 (13×)	141 (3.4×)
			Experiment 4			
None	≈8.5		1.6	2.6	4.2	
wt Mo-MuLV	≈8.5	7.5×10^3	6.8 (4.3×)	2.6	18 (4.2×)	
wt Mo-MuLV	≈8.5	ND	8.3 (5.2×)	4.3	36 (8.5×)	
Mo+PyF101 Mo-MuLV	≈8.5	$<10^0$	1.2 (0.8×)	2.0	2.4 (0.6×)	
Mo+PyF101 Mo-MuLV	≈8.5	4.5×10^2	2.3 (1.4×)	2.3	5.3 (1.3×)	
			Experiment 5			
None	9		4.0	2.8	11.6	
wt Mo-MuLV	9	1.8×10^4	11.5 (2.9×)	3.3	38 (3.4×)	
Mo+PyF101 Mo-MuLV	9	3.2×10^3	2.5 (0.6×)	2.7	6.8 (0.6×)	
Mo+PyF101 Mo-MuLV	9	$<10^0$	3.5 (0.9×)	3.0	10.5 (0.9×)	

Neonatal NIH Swiss mice were inoculated subcutaneously with 2.5×10^4 XC pfu of the indicated (wt, wild type) viruses. Animals were sacrificed at the times indicated (weeks after inoculation). Single-cell suspensions were prepared from total spleen by grinding the organ through a fine wire mesh. ND, not determined.

*Numbers of cells per 10^5 splenocytes that could act as infectious centers (by the XC plaque assay) when plated on NIH 3T3 cells.

†Concentrations of CFC for virus-inoculated animals relative to a control uninoculated animal in the same experiment are indicated in parentheses.

‡Total numbers of cells recovered from the spleen were determined by counting a diluted sample with a hemocytometer.

§It was not possible to determine total numbers of bone marrow cells, because recovery from the femurs was incomplete. Thus, only concentrations of CFC are shown.

Table 2. Hematopoietic progenitors in preleukemic mice: Erythroid

Virus inoculated	Time to sacrifice, wk	Spleen		
		CFU-E/ 10^5 cells*†	Total cells ($\times 10^{-8}$)	Total CFU-E ($\times 10^{-3}$)†
None	≈10.5	0.8	2.6	2.1
wt Mo-MULV	≈10.5	5.0 (6.3×)	4.7	23.5 (11×)
wt Mo-MULV	≈10.5	10.6 (13×)	3.6	38.2 (18×)
Mo+PyF101 Mo-MuLV	≈10.5	0.8 (1×)	1.8	1.4 (0.7×)
Mo+PyF101 Mo-MuLV	≈10.5	0.4 (0.5×)	2.2	0.9 (0.4×)

For legend, see Table 1.

*The criterion for scoring CFU-E was relatively stringent; 20–50 times more colonies showed at least one or two benzidine-positive cells and were potentially CFU-E as well. Thus, absolute numbers appear lower than those reported by others.

†Concentrations of CFU-E for virus-inoculated animals relative to a control uninoculated animal in the same experiment are indicated in parentheses.

(CFU-E). Thus, Mo-MuLV induced hyperplasia of multiple hematopoietic lineages, including those that do not ultimately develop neoplasms. (Mo-MuLV induces exclusively T-lymphoma when inoculated under the conditions used here.) Importantly, Mo+PyF101 Mo-MuLV did not induce splenic enlargement or increased numbers of myeloid or erythroid colonies (Tables 1 and 2). This strongly supports the hypothesis that hematopoietic hyperplasia is a necessary early event in Mo-MuLV leukemogenesis. Furthermore, the presumptive cell for which Mo+PyF101 Mo-MuLV is restricted appears to be involved in establishment of the hyperplasia.

Bone marrow from some but not all Mo-MuLV-inoculated animals also showed increases in myeloid CFC. As for the spleen, none of three Mo+PyF101 Mo-MuLV-inoculated mice analyzed showed any increase in bone marrow myeloid CFC (see Table 4).

One explanation for the hyperplasia could be an immunological response to Mo-MuLV infection (19). It should be emphasized that wild-type and Mo+PyF101 Mo-MuLV virus particles are identical, so the two viruses present the same antigenic epitopes. Thus, an immunological basis for Mo-MuLV hyperplasia predicts very low levels of target tissue infection for Mo+PyF101 Mo-MuLV-inoculated animals. This is examined in Tables 1 and 2 and in more detail in Table 3. Many Mo+PyF101 Mo-MuLV-inoculated mice showed high levels of infection, often within 5-fold of the level for a wild-type Mo-MuLV-inoculated animal. Furthermore, when individual Mo+PyF101-inoculated animals were compared, those with high splenocyte infection showed no increases in myeloid colonies, just as for those with low or undetectable splenocyte infection (Tables 1 and 2). Thus, the Mo-MuLV-induced hyperplasia does not appear to simply reflect an immune response. However, it is formally possible that a minority population of infected cells are responsible for antigen presentation to the immune system and that Mo+PyF101 Mo-MuLV is defective for expression in them.

The results with Mo+PyF101 Mo-MuLV suggested a model for Mo-MuLV leukemogenesis involving two independent infections (Fig. 2). The first infection, in the bone marrow or spleen, leads to generalized hematopoietic hyperplasia evident in the spleen (Tables 1 and 2). Thymic leukemia results from migration of hyperplastic lymphoid precursors to the thymus and infection (or reinfection) by Mo-MuLV or an MCF derivative. Then, during the second infection, proviral integration adjacent to one or more cellular protooncogenes occurs, resulting in transcriptional activation by the Mo-MuLV LTR and development of the final tumor. According to this model, thymic leukemia results from the specific ability of the Mo-MuLV LTR and its enhancers to function (and activate protooncogenes) in mature thymocytes during the second infection. By extension, the disease specificity of other MuLVs may also reflect second-infection events. In this model, MCF derivatives provide for efficient infection of Mo-MuLV-induced hyperplastic cells if they are already infected, since MCF viruses infect cells by using distinct receptors. Mo+PyF101 Mo-MuLV is apparently defective in the first infection event leading to hematopoietic hyperplasia. In addition, since Mo+PyF101 Mo-MuLV can establish high level infection of thymocytes *in vivo* and productively infect thymocytes *in vitro* (17), it does not appear defective for second-stage infections.

In light of this model, it was interesting to examine preleukemic states induced by MuLVs with altered disease specificity. In particular, Chatis *et al.* (14) have substituted the LTR of Friend MuLV (F-MuLV) into Mo-MuLV to generate the derivative MF-MuLV (Fig. 1). MF-MuLV causes erythroleukemia due to the specificity of the F-MuLV

Table 3. Levels of infectious virus in hemopoietic organs of wild-type Mo-MuLV- and Mo+PyF101 Mo-MuLV-inoculated mice

Age, wk	Thymus		Spleen		Bone marrow	
	Wild type	Mo+PyF101	Wild type	Mo+PyF101	Wild type	Mo+PyF101
2	6×10^2	5×10^1	3×10^3	3×10^1	7×10^2	3.5×10^2
	1×10^3	$<10^{-1}$			3×10^3	5×10^1
5	3.8×10^4	2×10^3	6×10^3	$<10^0$	1.3×10^4	$<10^0$
		4×10^4		4.9×10^3		2.8×10^3
≈8.5			7.5×10^3	$<10^0$		
				4.5×10^2		
9	1.6×10^5	1.1×10^4	1.8×10^4	3.2×10^3		
		$<10^1$		$<10^1$		

Mice were inoculated with wild-type or Mo+PyF101 Mo-MuLV at birth, and infectious centers from thymus, bone marrow, and spleen cells were measured at the times indicated. Values represent infectious centers (XC pfu) per 10^5 cells. Values from the various organs of an animal inoculated with either wild-type or Mo+PyF101 Mo-MuLV are shown on the same horizontal line. All animals inoculated with Mo+PyF101 Mo-MuLV were successfully infected, since assay of blood sera detected circulating levels of infectious virus.

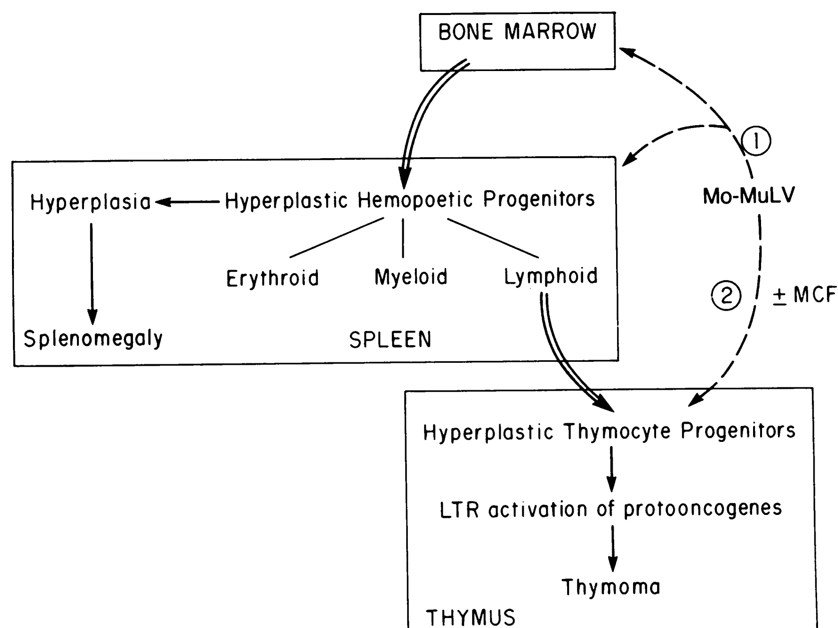

FIG. 2. Two-step infection model for Mo-MuLV-induced leukemogenesis. Dashed lines indicate the putative targets for Mo-MuLV infection. Double lines indicate the migration of hyperplastic hematopoietic progenitors from one organ to another. Solid lines indicate the progression of events within hematopoietic organs.

enhancer sequences, but the virus particles are virtually identical to those of Mo-MuLV. In our conditions, MF-MuLV also induced preleukemic splenic enlargement and increase in null cells. The results of myeloid and erythroid colony assays for preleukemic MF-MuLV-inoculated mice (as well as data from wild-type and Mo+PyF101 Mo-MuLV-inoculated animals) are summarized in Table 4. MF-MuLV induced increases in both myeloid and erythroid stem cells in preleukemic spleen, equivalent to that observed for wild-type Mo-MuLV. Importantly, preleukemic spleens from MF-MuLV-inoculated mice did not show a significantly larger increase in CFU-E relative to myeloid CFC, even though the virus ultimately caused erythroleukemia. Thus, the F-MuLV enhancer sequences do not appear to change the spectrum of hyperplastic progenitor cells in preleukemic animals. This is consistent with the LTR enhancers exerting their tissue-specific influence on disease specificity at a second infection event.

Since all of the experiments presented thus far involved variants of Mo-MuLV, it was interesting to test whether other strains of MuLV induce the same kind of preleukemic state. For these experiments, another MuLV derived by Chatis and co-workers (11, 14) was useful: FM-MuLV, Friend MuLV containing a Mo-MuLV LTR (Fig. 1). This virus induces T-lymphoid leukemia, even though all viral proteins are encoded by F-MuLV. Sitbon et al. (20) recently showed that the *env* gene of F-MuLV itself induces early erythroid hyperplasia, so CFU-E assays were not performed. However, as shown in Table 4, FM-MuLV also induces increased myeloid CFC. Thus, the generalized hematopoietic hyperplasia is apparently induced by MuLVs with viral proteins different from M-MuLV.

DISCUSSION

In these experiments, a preleukemic state induced by Mo-MuLV infection consisting of generalized hematopoietic hyperplasia was documented. Other investigators have described this hyperplasia (18). However, the failure of nonleukemogenic Mo+PyF101 Mo-MuLV to induce it provides strong evidence that it is a necessary step in the leukemogenic process. These results, coupled with the fact that Mo+PyF101 Mo-MuLV efficiently establishes infection in mature thymocytes, led to a model for Mo-MuLV leukemogenesis involving two independent infections. The model provides a role for MCF derivatives and predicts that tissue specificity of disease results from the second infection event. The fact that MF-MuLV induced the same hyperplasia as wild-type Mo-MuLV is consistent with disease specificity being determined by the second infection. It seems possible that leukemogenesis by other nonacute retroviruses may involve similar mechanisms.

Table 4. Increase in preleukemic hematopoietic progenitors induced by MuLVs with different disease specificity

	Myeloid CFC			Erythroid CFU-E	
	Spleen		Bone marrow	Spleen	
Virus inoculated	Conc.	Total	Conc.	Conc.	Total
None	1× (9)	1× (8)	1× (5)	1× (2)	1× (2)
Wild type Mo-MuLV	4.2× (11)	6.1× (10)	1.7× (6)	10.3× (3)	14.8× (3)
Mo+PyF101 Mo-MuLV	1.1× (7)	0.9× (7)	0.8× (3)	0.8× (2)	0.6× (2)
MF-MuLV	3.9× (4)	5.9× (4)	1.6× (1)	15.0× (2)	20.6× (2)
FM-MuLV	10× (2)	19.5× (2)	ND	ND	ND

Values are shown relative to levels of CFC or CFU-E in uninoculated animals in the same assay. The values are averages from different animals, all between 6 and 10 weeks after inoculation. Numbers of animals tested for each entry are shown in parentheses. ND, not determined.

These results are consistent with results of Asjo *et al.* (21), who tested for preleukemic cells in Mo-MuLV-inoculated mice by transplantation into irradiated recipients. Preleukemic cells were first detected in the spleen or bone marrow and only at later times in the thymus. This is supportive of the model described here, if the preleukemic cells are related to hyperplastic lymphoid stem cells. The identification of potential preleukemic cells in the bone marrow has also been described for the AKR, F-MuLV, and radiation-induced leukemia virus systems (22–24). Infection *in vitro* of long-term marrow cultures by Mo-MuLV has also been reported to lead to increased myeloid progenitors (25).

It is unclear whether the critical infection events leading to Mo-MuLV-induced hyperplasia occur in the spleen or bone marrow. In fact, the spleen consistently showed greater increases in concentrations of myeloid and erythroid stem cells than did bone marrow. This suggests that the important infection event occurs in the spleen. Alternatively, the bone marrow could be the main target, but hyperplastic stem cells might migrate to the spleen and undergo proliferation and differentiation. It is also possible that hyperplastic lymphoid stem cells could migrate directly from the bone marrow to the thymus.

The mechanism by which Mo-MuLV induces hematopoietic hyperplasia has yet to be determined. If the preleukemic cells identified by Asjo *et al.* (21) are related to the increased hematopoietic stem cells described here, this suggests that the preleukemic stem cells are abnormal in some way. Normal spleen or bone marrow (which contain normal hematopoietic progenitors) did not give leukemias in the transplantation experiments (21).

Preleukemic hyperplasia could result from direct infection by Mo-MuLV of hematopoietic stem cells or alternatively by infection of cells that produce hematopoietic growth factors. Greenberger *et al.* (25) have reported that Mo-MuLV infection of long-term marrow cultures leads to increased production of the myeloid growth factor colony-stimulating factor. On the other hand, preliminary flow cytometry experiments indicate that a large fraction of cells detected in the myeloid CFC assays are infected, which is consistent with direct infection of stem cells. In any event, both possibilities appear to require growth stimulation by Mo-MuLV, which does not contain an oncogene. Identification of the cell types that are restricted for Mo+PyF101 Mo-MuLV infection will allow more detailed analysis of Mo-MuLV-induced leukemogenesis.

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