

# Friend murine leukemia virus-immortalized myeloid cells are converted into tumorigenic cell lines by Abelson leukemia virus

(oncogene/retrovirus/growth factor/leukemogenesis/tumor progression)

ALLEN OLIFF, OLGA AGRANOVSKY, MARTIN D. MCKINNEY, V. V. V. S. MURTY, AND ROBERT BAUCHWITZ

DeWitt Wallace Research Laboratory, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021

Communicated by Paul A. Marks, February 12, 1985

**ABSTRACT** Friend murine leukemia virus (Fr-MuLV) is a replication-competent murine retrovirus that induces acute nonlymphocytic leukemias in NFS/n mice. Fr-MuLV disease is divided into two stages based on the ability of the leukemia cells to grow in culture and transplant into syngeneic mice. Hematopoietic cells taken from the early stage of disease after Fr-MuLV infection grow as immortal myeloid cell lines in the presence of WEHI-3 cell-conditioned medium (CM) or interleukin 3. These growth factor-dependent cell lines do not grow in culture in the absence of CM and do not form tumors in syngeneic animals. If these Fr-MuLV-infected cells are superinfected with Abelson murine leukemia virus (Ab-MuLV), they lose their dependence on WEHI-3 CM and proliferate in culture in the absence of exogenous growth factors. Concomitant with the loss of growth factor dependence in culture, the Ab-MuLV-infected cell lines become tumorigenic in syngeneic mice. This secondary level of transformation is Ab-MuLV specific. Fr-MuLV-immortalized myeloid cell lines superinfected with Harvey murine sarcoma virus (Ha-MuSV) or amphotropic virus remain dependent on WEHI-3 CM for growth *in vitro* and are not tumorigenic *in vivo*. Neither Ab-MuLV- nor Ha-MuSV-infected normal mouse myeloid cell cultures produce growth factor-independent or tumorigenic cell lines. We conclude that at least two genetic events are needed to convert a murine myeloid precursor into a tumorigenic cell line. The first event occurs in Fr-MuLV-infected mice, generating cells that are growth factor dependent but immortal *in vitro*. The second event, which can be accomplished by Ab-MuLV infection, converts these immortal myeloid precursors into growth factor-independent and tumorigenic cells.

Friend murine leukemia virus (Fr-MuLV) is a replication-competent type C retrovirus that induces a rapidly fatal acute nonlymphocytic leukemia in NFS/n mice (1–3). Fr-MuLV disease is divided into two stages based on the growth properties of the virus-infected leukemia cells. Stage I cells proliferate in diseased animals but will not transplant into syngeneic mice. Stage I cells do not grow in culture under standard cell culture techniques. Stage II cells are morphologically indistinguishable from stage I cells. But stage II cells will transplant into syngeneic animals and form continuous cell lines in culture (4). The specific genetic events responsible for stage I and stage II transformation of hematopoietic precursors are unknown.

Hematopoietic cells obtained from mice with stage I leukemia will grow as immortal cell lines if cultured in the presence of WEHI-3 cell-conditioned medium (CM) or interleukin 3 (IL-3) (5). These cell lines are not identical to the Fr-MuLV-infected leukemia cells found in mice. Fr-MuLV disease *in vivo* manifests as an erythroleukemia. CM-depen-

dent cell lines grown *in vitro* consist of myeloblasts and other elements of the myeloid cell lineage. However, these CM-dependent myeloid cells exhibit growth properties similar to the stage I erythroleukemia cells of mice. CM-dependent cell lines do not form tumors in syngeneic mice, and they do not grow in culture in the absence of CM. After >18 months in culture, these cells remain absolutely dependent on CM for proliferation. In an attempt to alter the growth factor requirements of the CM-dependent cell lines, we superinfected these cells with two acutely transforming retroviruses—Abelson murine leukemia virus (Ab-MuLV) and Harvey murine sarcoma virus (Ha-MuSV).

## METHODS

**Animals.** NFS/n mice were originally obtained from the small animal facility of the National Institutes of Health (Bethesda, MD). Subsequently, mice were bred by brother/sister matings in our own laboratory.

**Cells.** NIH 3T3 cells were obtained from the American Type Culture Collection. WEHI-3 cells were a gift from Edward Scolnick of Merck Sharp & Dohme. Conditioned medium from WEHI-3 cells was obtained 5 days after passaging WEHI-3 cells in Dulbecco's modified Eagle's medium (DME medium) with 10% fetal calf serum. WEHI-3 cells were removed by centrifugation (1000 × *g* for 10 min), and the clarified supernatant was frozen at –20°C until needed. All CM-dependent cell lines were grown in DME medium with 10% fetal calf serum and 10% WEHI-3 CM. Bone marrow cultures were established in 60-mm Petri dishes by flushing the contents of two femurs into 5 ml of CM-dependent cell line medium. These cultures were demi-depopulated every 3–4 days and fed with an equal volume of fresh media.

**Viruses.** Fr-MuLV is a replication-competent, NB-tropic, ecotropic, type C retrovirus obtained by transfection of molecularly cloned Fr-MuLV DNA into NIH 3T3 cells (2). Amphotropic virus (designated "Ampho") is a replication-competent, N-tropic, amphotropic, type C retrovirus obtained by the transfection of molecularly cloned Ampho DNA into NIH 3T3 cells (6). Ha-MuSV/Ampho and Ha-MuSV/Fr-MuLV pseudotype viruses were prepared from an Ha-MuSV-nonproducer NIH 3T3 cell (a gift from Douglas Lowy of the National Cancer Institute) by superinfection with Ampho or Fr-MuLV virus, respectively. Virus stocks of Ha-MuSV/Ampho contain 10<sup>4</sup> focus-forming units (ffu) of transforming virus per ml of culture fluid as assayed on NIH 3T3 cells. Ha-MuSV/Fr-MuLV stocks contain 10<sup>5</sup> ffu/ml. Ab-MuLV/Ampho and Ab-MuLV/Fr-MuLV pseudotype viruses were prepared from Ann-1 (7) Ab-MuLV-nonproducer

cells (a gift from Albert DeLeo of Sloan-Kettering Institute) by superinfection with Ampho or Fr-MuLV virus, respectively. Virus stocks of Ab-MuLV/Ampho and Ab-MuLV/Fr-MuLV contain  $10^4$  and  $10^5$  ffu/ml of culture fluid, respectively.

**Animal Studies.** Transplant recipients were injected intravenously with  $10^6$  viable cells; 2–5 weeks later, the mice were sacrificed and their spleens were removed. Foci of tumor cells were dissected out of the diseased spleens for further analysis.

Preparation of cellular DNAs and RNAs and blot hybridizations were performed as described (8). Molecular clones of *v-abl*-specific sequences (pABI-9) and IL-3 cDNA sequences (pIL-3) used for probe synthesis were provided by Stephen Goff of Columbia University and Hiam Aviv of Biotechnology General, respectively. Reverse transcriptase assays were performed as described by Scolnick *et al.* (9).

## RESULTS

Two CM-dependent cell lines were chosen for analysis. Single-cell derivatives of these cell lines were isolated by soft-agar cloning (IO<sub>3</sub> and E1c) and were tested for the ability to propagate in the absence of WEHI-3 CM. IO<sub>3</sub> and E1c cells were removed from culture in mid-logarithmic-phase growth, washed, and resuspended in fresh medium without CM. After 3 days in suspension culture, no viable cells were visible. After 3 weeks in soft-agar cultures, no colonies were present. Parallel cultures of IO<sub>3</sub> and E1c cells suspended in medium with CM yielded  $1-2 \times 10^6$  viable cells per ml of suspension culture and 100–300 colonies per  $10^6$  cells plated in soft agar with CM.

IO<sub>3</sub> and E1c are derived from Fr-MuLV-infected mice and produce Fr-MuLV. Therefore, IO<sub>3</sub> and E1c cannot be superinfected either by Fr-MuLV or by other ecotropic murine leukemia viruses (10). To overcome this problem, we prepared pseudotypes of Ab-MuLV and Ha-MuSV, using an amphotropic murine retrovirus—Ampho (11). IO<sub>3</sub> and E1c cells were removed from culture, washed, and resuspended in fresh medium with CM and  $10^4$  ffu of Ab-MuLV/Ampho or Ha-MuSV/Ampho or  $10^5$  infectious units of Ampho virus. After 1 week in culture with CM, the Ab-MuLV/Ampho- and Ha-MuSV/Ampho-infected cells were assayed for growth in the absence of CM. Suspension cultures of IO<sub>3</sub> and E1c cells superinfected with Ab-MuLV/Ampho continued to grow to high cell density ( $1-2 \times 10^6$  cells per ml) in the absence of CM. These cells also produced dense colonies of IO<sub>3</sub> and E1c cells in soft agar without CM. However, the IO<sub>3</sub> and E1c cells superinfected with Ha-MuSV/Ampho or Ampho alone did not grow in the absence of CM in suspension cultures or in soft agar (Table 1).

To prove that the Ab-MuLV/Ampho and Ha-MuSV/Ampho viruses had infected the IO<sub>3</sub> and E1c cells, the culture

media from these cells were assayed for infectious focus-forming virus on NIH 3T3 cells. Both the Ab-MuLV/Ampho- and Ha-MuSV/Ampho-superinfected IO<sub>3</sub> and E1c cells produced focus-forming virus (Table 1). Focus-forming viruses were not detectable in culture media from untreated or Ampho-superinfected IO<sub>3</sub> and E1c cells. To further analyze the Ab-MuLV in the CM-independent IO<sub>3</sub> and E1c cells, clonal isolates of these cell lines were prepared in soft-agar cultures. Two such isolates (clones 1 and 4, designated cl-1 and cl-4) were picked and expanded in suspension culture. High molecular weight DNA and poly(A)<sup>+</sup> RNA was isolated from cl-1 and cl-4 cells and probed for the presence of Ab-MuLV sequences. Southern blot analysis revealed multiple Ab-MuLV bands in cl-1 and cl-4 that were not present in the parental IO<sub>3</sub> cells (Fig. 1). Similarly, RNA blot analysis revealed high levels of Ab-MuLV-specific transcripts in cl-1 and cl-4 cells (Fig. 2). Parallel filters hybridized with a murine leukemia virus long terminal repeat and *gag* probe recognized the same RNA species as did the *abl* probe (data not shown). These studies indicate that Ab-MuLV is present and is actively transcribed in cl-1 and cl-4.

Since IO<sub>3</sub> and E1c cells are dependent on IL-3 for growth in culture, we asked if the growth factor-independent derivatives of IO<sub>3</sub> and E1c make their own IL-3. Southern blot analysis of the IL-3 gene locus in cl-1, cl-4, and IO<sub>3</sub> showed the same pattern of bands comprising the IL-3 coding sequences in all three cell lines (Fig. 3). RNA dot-blot analysis of poly(A)<sup>+</sup> RNA from cl-1, cl-4, and IO<sub>3</sub> revealed the same abundance of IL-3-specific sequences in the growth factor-dependent and -independent cell lines (Fig. 4). These experiments indicate that the IL-3 gene is not rearranged or expressed at elevated levels in Ab-MuLV/Ampho-infected factor-independent cells. To further test the possibility that these cell lines produce a self-stimulating growth factor, CM was prepared from cl-1 and cl-4 cells and tested for the ability to stimulate proliferation of two IL-3-dependent cell lines—IO<sub>3</sub> and FD (12). Cell proliferation was assayed by viable cell counts and [<sup>3</sup>H]thymidine incorporation into cellular DNA. Neither cl-1 nor cl-4 CM stimulated cell proliferation in either IO<sub>3</sub> or FD cells (data not shown).

Table 1. Growth parameters of IO<sub>3</sub> and E1c cell lines

Cell line	Superinfecting virus	Continuous growth <i>in vitro</i>			Tumorigenic <i>in vivo</i>	ffu/ml*
		With CM	Without CM			
IO <sub>3</sub>	—	+	—	—	<1	
E1c	—	+	—	—	<1	
IO <sub>3</sub>	Ab-MuLV/Ampho	+	+	+	100	
E1c	Ab-MuLV/Ampho	+	+	+	100	
IO <sub>3</sub>	Ha-MuSV/Ampho	+	—	—	1000	
E1c	Ha-MuSV/Ampho	+	—	—	1000	
IO <sub>3</sub>	Ampho	+	—	—	<1	
E1c	Ampho	+	—	—	<1	

\*ffu titers were determined on NIH 3T3 cells with 0.5 ml of freshly harvested culture fluid.

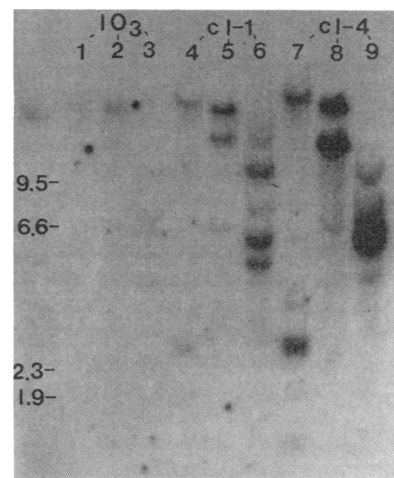


FIG. 1. Southern blot analysis of IO<sub>3</sub>, cl-1, and cl-4 DNAs probed with *abl*-specific sequences. Genomic DNA (10  $\mu$ g) from each cell line was digested with *Eco*RI (lanes 1, 4, and 7), *Xba*I (lanes 2, 5, and 8), and *Ava*I (lanes 3, 6, and 9) and were electrophoresed through a 0.5% agarose gel at 35 V for 18 hr. The digested DNAs were blot-transferred to nitrocellulose paper and hybridized to nick-translated radiolabeled molecularly cloned *abl* DNA. Filter washes were performed at high stringency: 0.1% NaDodSO<sub>4</sub>/0.015 M NaCl/0.0015 M sodium citrate, pH 7, at 55°C. Note the Ab-MuLV bands present in cl-1 and cl-4 but absent from the uninfected IO<sub>3</sub>. Size markers indicate the location of *Hind*III-digested wild-type phage  $\lambda$  DNA fragments (in kilobases) run in an adjacent lane.

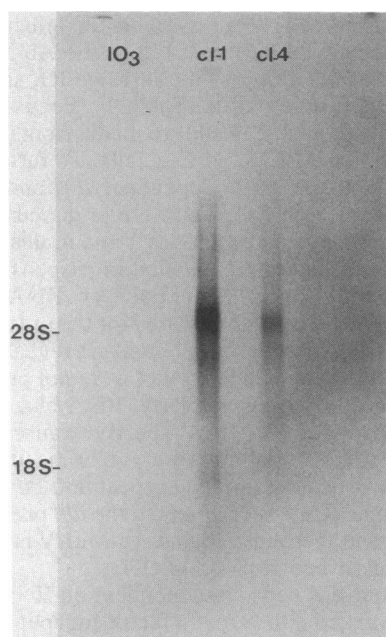


FIG. 2. RNA gel analysis of IO<sub>3</sub>, cl-1, and cl-4 RNA probed with *abl*-specific sequences. Poly(A)<sup>+</sup> RNA (15 μg) was denatured in 2.2 M formaldehyde/50% formamide and electrophoresed through a 0.8% agarose/formaldehyde gel at 50 V for 18 hr. The RNAs were blot-transferred to nitrocellulose paper and hybridized and washed as in Fig. 1. Molecular size markers indicate the migration distances of the 28S and 18S rRNAs present as contaminants in each preparation of poly(A)-selected RNA.

To test the tumorigenic potential of the growth factor-independent cell lines,  $1 \times 10^6$  cl-1 or cl-4 cells were injected intravenously into syngeneic mice. Control mice were injected with  $1 \times 10^6$  IO<sub>3</sub>, E1c, Ha-MuSV/Ampho-infected IO<sub>3</sub>, or Ampho-infected IO<sub>3</sub> cells. The mice injected with cl-1 and cl-4 developed multiple macroscopic foci of tumor cells in their spleens after 2–3 weeks. These tumor foci progressively enlarged, resulting in diffuse splenomegaly after 3–5 weeks. Microscopic examination of these spleens revealed

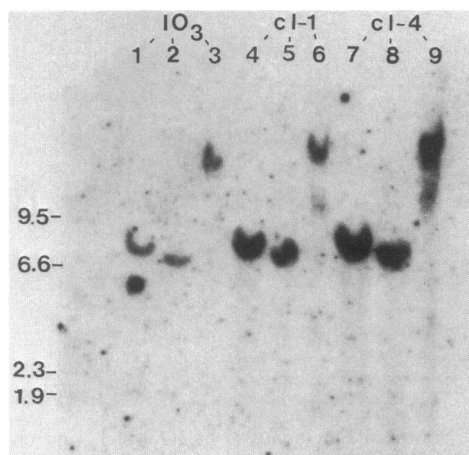


FIG. 3. Southern blot analysis of IO<sub>3</sub>, cl-1, and cl-4 DNAs probed with IL-3 cDNA. Genomic DNA (10 μg) from each cell line was digested with *Eco*RI (lanes 1, 4, and 7), *Xba*I (lanes 2, 5, and 8), and *Ava*I (lanes 3, 6, and 9). Electrophoresis and blot-transfer were performed as in Fig. 1. Hybridization was performed by using a radiolabeled nick-translated molecularly cloned IL-3 cDNA. Filter washings were carried out as described in Fig. 1. Size markers indicate the location of *Hind*III-digested wild-type phage λ DNA fragments (in kilobases) run in an adjacent lane.

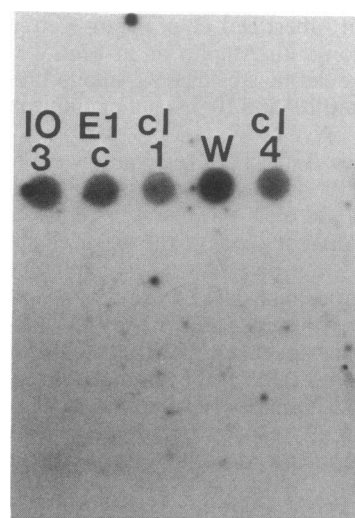


FIG. 4. RNA dot-blot analysis of IO<sub>3</sub>, E1c, cl-1, cl-4, and WEHI-3 cells probed with IL-3 cDNA. Poly(A)<sup>+</sup> RNA (15 μg) was denatured as described in Fig. 2 and dotted onto nitrocellulose paper by using a Bethesda Research Labs dot-blot apparatus. Filter hybridization and washings were performed as described in Fig. 1.

>90% hematopoietic blasts replacing the normal spleen cells. To prove that the tumor cells were of donor-cell origin and not transformed host cells, the sex of the tumor cells was determined by cytogenetics. cl-1 and cl-4 were derived from male mice. The syngeneic animals used in this study were all females. XY karyotypes were present in 17 of 25 metaphase cells found in the diseased spleens. Thus, the majority of the dividing cells in these spleens must be derived from the cl-1 or cl-4 cells injected into the female mice. None of the mice injected with IO<sub>3</sub>, E1c, Ha-MuSV/Ampho-infected IO<sub>3</sub>, or Ampho-infected IO<sub>3</sub> developed tumor foci or splenomegaly.

IO<sub>3</sub> and E1c are growth factor-dependent, immortal, myeloid cell lines obtained from leukemic mice. Ab-MuLV infection converted these cell lines into growth factor-independent tumorigenic cells. We next asked if Ab-MuLV infection of normal myeloid cells grown under the same conditions as IO<sub>3</sub> and E1c cells would also produce growth factor-independent tumorigenic cells. Suspension cultures of normal mouse bone marrow were established in the presence of WEHI-3 CM. The suspended cells were passaged twice weekly into new culture dishes. After 1–2 weeks, this process yielded a self-renewing population of myeloid cells in the absence of attached cells or feeder layers. These cultures consisted of 5–10% myeloblasts and 90–95% mature myeloid elements (metamyelocytes, stabs, and mature granulocytes). Myeloid cultures prepared in this manner exhibited the same factor dependence for WEHI-3-CM as did IO<sub>3</sub> and E1c cells. However, the myeloid cultures obtained from normal mouse bone marrow are not immortal and invariably die out within 3–6 weeks. To evaluate the effect of Ab-MuLV infection on these normal myeloid cells, four independently established bone marrow cultures were infected with either Fr-MuLV or Ampho pseudotypes of Ab-MuLV. Parallel cultures also were infected with Ha-MuSV/Fr-MuLV, Ha-MuSV/Ampho, Fr-MuLV, or Ampho. Successful infection was monitored by reverse transcriptase levels and the recovery of focus-forming virus activity from the Ab-MuLV- and Ha-MuSV-infected cultures. All six viruses successfully infected the normal myeloid cultures (Table 2). However, no CM-independent cell lines were established from these cultures, and no tumor cell foci developed in the spleens of mice injected with these cells.

IO<sub>3</sub> and E1c are myeloid cell lines. The myeloid nature of these cells was established by histochemical staining and

Table 2. Growth parameters of normal myeloid cell cultures

Infecting virus	Growth <i>in vitro</i>		Tumorigenic <i>in vivo</i>	Reverse transcriptase, cpm	ffu/ml*
	With CM	Without CM			
Ampho	+	-	-	45,000	<1
Fr-MuLV	+	-	-	62,000	<1
Ab-MuLV/Ampho	+	-	-	36,000	100
Ab-MuLV/Fr-MuLV	+	-	-	68,000	100
Ha-MuSV/Ampho	+	-	-	27,000	100
Ha-MuSV/Fr-MuLV	+	-	-	45,000	1000
None	+	-	-	900	<1

\*ffu titers were determined on NIH 3T3 cells with 0.5 ml of freshly harvested culture fluid.

morphologic examination (5). Exposure of IO<sub>3</sub> and E1c to inducers of differentiation (e.g., 1.3% dimethyl sulfoxide or 2 mM hexamethylene bisacetamide) shifted these cell populations from 95–99% myeloblasts and 1–5% mature myeloid elements to 50–60% myeloblasts and 40–50% mature cells. Ab-MuLV-infected IO<sub>3</sub> and E1c cells retained this potential for differentiation albeit to a lesser extent than did their parental cell lines. Dimethyl sulfoxide treatment of cl-1 and cl-4 shifted these cells from 99% myeloblasts and 1% mature myeloid elements to 75–85% myeloblasts and 15–25% mature cells.

## DISCUSSION

Friend virus-induced leukemias are divided into distinct stages based on the growth properties of the leukemia cells (13, 14). In the early stage (stage I disease) of leukemia, the blast cells are unable to grow outside their normal hematopoietic environment (i.e., bone marrow or spleen). In the late stage of leukemia (stage II disease), leukemia cells can grow at any site in the mouse and will form continuous cell lines *in vitro* (15). The genetic basis for this progression from restricted to unrestricted cell growth is unclear. The present study suggests that the activation of particular oncogenes (e.g., *abl*) can alter the growth conditions for Fr-MuLV-immortalized leukemia cells. It is possible that the tumor cell progression seen in Fr-MuLV-induced leukemias (stage I to stage II) is due to the sequential activation of several oncogenes. As more oncogenes are activated, tumor cell growth becomes less restricted and the cancer becomes more aggressive. Although the implications of our observations for human cancer are not clear, it is known that many human cancer cells transcribe more than one oncogene (16–18). The malignant phenotype may result from the combined effects of these genes.

The case for a sequential effect of transforming genes in the pathogenesis of fully malignant murine leukemia is further substantiated by the observation that Ab-MuLV does not convert myeloid cells obtained from normal mouse bone marrow into CM-independent tumorigenic cell lines. At least one other genetic change, in addition to expression of the *abl* gene, is needed to transform myeloid precursors into tumorigenic cells. Fr-MuLV-infected myeloid cell lines IO<sub>3</sub> and E1c possess this additional genetic change since Ab-MuLV infection transforms these cells into growth factor-independent tumorigenic cell lines. It is unclear what this additional genetic change is. However, Fr-MuLV infection *per se* is not sufficient to alter the growth properties of normal myeloid cell cultures. None of the normal myeloid cell cultures grown in CM and infected with Fr-MuLV developed into immortal cell lines (Table 2). Fr-MuLV is more likely needed to activate an endogenous cellular oncogene via insertional mutagenesis (19, 20). This type of Fr-MuLV-induced mutation is likely to be a rare genetic event and may not occur during the limited life span of normal myeloid cell cultures (3–6 weeks). However, *in vivo* many more myeloid precursors

are exposed to Fr-MuLV infection, thus increasing the likelihood that Fr-MuLV will integrate near a critical gene in at least one myeloid precursor.

Several investigators have shown that at least two oncogenes are needed to transform primary mouse embryo fibroblasts into tumorigenic cells (21–23). One gene provides so called “immortalizing” functions. The second gene is then capable of inducing malignant cell transformation, but only in the presence of the immortalizing gene. One or more of these “immortalization” oncogenes may be activated in IO<sub>3</sub> and E1c cells.

Ab-MuLV and Ha-MuSV transform both fibroblasts and hematopoietic cells *in vitro* and *in vivo* (24–31). Little is known about the mechanism of cell transformation used by these viruses. We have found that Ab-MuLV but not Ha-MuSV converts IL-3-dependent myeloid cell lines into growth factor-independent cells. Concomitant with the loss of growth factor dependence, the Ab-MuLV-infected cells become tumorigenic in syngeneic animals. This study suggests that the cellular basis of Ab-MuLV-induced myeloid cell transformation is elimination of the requirement for growth factors for cell division. Ha-MuSV-induced hematopoietic cell transformation must be accomplished by a different mechanism since Ha-MuSV-infected cell lines remain dependent on WEHI-3 CM for cell proliferation. The molecular basis of Ab-MuLV-induced IL-3 independence is unclear. However, analysis of cl-1 and cl-4 cells fails to support the “autocrine” model of tumorigenesis (32). CM obtained from these cells does not support the growth of hematopoietic factor-dependent cell lines. No evidence of altered IL-3 transcription or rearranged IL-3 DNA sequences is found in cl-1 or cl-4. Instead of triggering the production of IL-3, Ab-MuLV appears to make IL-3 superfluous for cell proliferation.

We thank Drs. Paul Marks and Richard Rifkind for their support and advice in performing these studies. This work was supported in part by the David Schwartz Foundation, the Robert and Helen Kleberg Foundation, and grants from the American Cancer Society (MV-170A) and the National Cancer Institute (CA-16599 and CA-08748).

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