# THE EFFECT OF HELPER VIRUS ON ABELSON
# VIRUS-INDUCED TRANSFORMATION OF LYMPHOID CELLS*

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In the transformation of fibroblasts by defective sarcoma viruses, the role of the helper virus has usually been interpreted as the provision of virion proteins. These may include the internal structural components, the external glycoproteins, and/or the reverse transcriptase (1-4). The ease with which nonproducer, transformed cells can be isolated (5, 6) has suggested that the helper genome plays no role in transformation by defective sarcoma viruses and in certain cases direct examination of transformed cells has confirmed the absence of certain helper nucleic acid sequences (7, 8). Thus, we view fibroblast transformation solely as a consequence of the action of a gene or genes of the defective virus, a view which is supported by the apparent independence of the avian *src* gene from the rest of the nondefective Rous sarcoma virus genome (9, 10). With this background we have been analyzing the helper specificity for transformation of lymphocytes by the Abelson murine leukemia virus (A-MuLV).[1]

A-MuLV, originally isolated from a tumor arising in a prednisolone-treated BALB/c mouse inoculated with Moloney murine leukemia virus (M-MuLV), induces a thymus-independent lymphoma 3-5 wk postinoculation (11). A-MuLV resembles rapidly transforming murine sarcoma viruses in its mode of replication on fibroblast cell lines. The virus stocks from A-MuLV-induced tumors contain a defective virus which transforms some fibroblasts and an oncogenic thymic lymphoma helper virus, M-MuLV. The transforming component of A-MuLV can be obtained free of replicating helper virus by isolating nonproducer fibroblast cell lines (12). Several of these cell lines lack significant sequences of the helper virus genome (7, 8).

A-MuLV is unique in that it also transforms lymphoid cells in vitro (13). A quantitative semisolid culture system which monitors the frequency of transformation has been developed (14). To analyze the role of helper virus in A-MuLV-induced transformation, we tested the oncogenic activity and the transforming

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[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine luekemia virus; M-MuLV, Moloney murine leukemia virus; DME-CS, Dulbecco's modified Eagle's medium supplemented with 10% calf serum; DME-IFS, Dulbecco's modified Eagle's medium supplemented with 10% heat inactivated fetal calf serum; E-MuLV, BALB/c endogenous virus WN1802N-C135; F-MuLV, Friend MuLV; G-MuLV, Gross-MuLV; moi, multiplicity of infection; FFU, focus-forming units; PFU, plaque-forming units; PBS, phosphate-buffered saline; RPMI medium, RPMI-1640 supplemented with 20% heat inactivated fetal calf serum and 50 $\mu$M 2-mercaptoethanol.

TABLE I
*Origin of Helper Viruses*

| Virus stock | Abbreviation used |
|---|---|
| Moloney murine leukemia virus, clone 1 (reference 18) | M-MuLV-1 |
| Moloney murine leukemia virus, clone 2 | M-MuLV-2 |
| Friend murine leukemia virus (N-tropic), clone 13 | F-MuLV-N |
| Friend murine leukemia virus (B-tropic), clone 10 | F-MuLV-B |
| BALB/c endogenous virus WN1802N-C135 (references 19 and 20) | E-MuLV |
| Gross murine leukemia virus | G-MuLV |

ability of A-MuLV stocks prepared with several different helper viruses. For fibroblasts, all of the murine retroviruses tested were equivalently effective helpers and A-MuLV stocks prepared with helpers that are highly oncogenic were found to be highly oncogenic in vivo and to transform lymphoid cells efficiently. We were surprised, however, to find that A-MuLV prepared with helpers that are weakly oncogenic alone is very inefficient as a transforming agent either in vivo and in vitro. This result suggests that the helper virus may play a more active role in lymphoid cell transformation than in fibroblast transformation.

## Materials and Methods

*Cell Cultures.* Most fibroblast cell cultures were maintained in Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, N. Y.) supplemented with 10% calf serum (Microbiological Associates, Walkersville, Md.) (DME-CS). JLS-V9 and CCL64 mink lung cells were maintained in DME supplemented with 10% heat inactivated (at 56°C for 30 min) fetal calf serum (Microbiological Associates) (DME-IFS). 5H cells, an A-MuLV-transformed nonproducer fibroblast clone, were derived by infecting JLS-V9 cells at a multiplicity of infection (moi) of 3 focus-forming units (FFU) and 2 plaque-forming units (PFU) with A-MuLV(M-MuLV-1) (see Table I for a description of the helper viruses used). After a 1.5-h adsorption period, the cells were washed two times with phosphate-buffered saline (PBS) (200 mg/liter KCl, 200 mg/liter $KH_2PO_4$, 100 mg/liter $MgCl_2 \cdot 6H_2O$, 8,000 mg/liter NaCl, 1,150 mg/liter $Na_2HPO_4 \cdot 2H_2O$), trypsinized for 7 min at 37°C, and plated in microtiter wells at a concentration of 0.33 cells/well. Transformed clones were isolated 14 days later and screened for virus production by using infectivity and reverse transcriptase assays. 5H cells are morphologically transformed but supernates from these cells contain no detectable infectious virus or reverse transcriptase. The cells contain the A-MuLV genomes as demonstrated by rescue experiments with M-MuLV-2.

*Lymphoid Cell Lines.* A-MuLV-transformed lymphoid cell lines were derived by removing single foci from agarose transformation plates with a Pasteur pipette. The cells were transferred to 16-mm plastic wells (Corning Glass Works, Corning, N. Y.), suspended in 1 ml of RPMI-1640 (Grand Island Biological Co.), supplemented with 20% heat inactivated fetal calf serum and 50 $\mu$M 2-mercaptoethanol (Eastman Kodak Co., Rochester, N. Y.) (RPMI medium). Cultures were observed daily for growth and maintained at densities of $5 \times 10^5$–$1 \times 10^6$ cells/ml until the cultures grew vigorously, doubling every 12 h. The length of this adaptation period usually varied from 3 to 4 days to 1–2 wk. Once established, cells were passaged at a 1:100 split ratio when they reached a density of 2.5–3.5 $\times 10^6$ cells/ml, about every 3rd day.

BR48 is an A-MuLV-transformed lymphoid nonproducer cell line derived from A-MuLV(M-MuLV-1)-infected C57BR/cdJ (Fv-1$^{n/n}$) bone marrow cells. 2M is another A-MuLV-transformed nonproducer cell line derived from A-MuLV(M-MuLV-2) infected BALB/c (Fv-1$^{b/b}$) bone marrow cells. Both of these cell lines contain the A-MuLV genome as demonstrated by recovery of oncogenic, transforming virus after M-MuLV-2 superinfection.

L691 cells, a gift of Dr. Paul Arnstein, California State Department of Health, were derived from a radiation-induced thymoma in a C57L mouse (15). These cells express markers commonly associated with T-cell tumors such as Thy-1, TL, and Lyt-4 antigens, and the enzyme terminal

deoxynucleotidyl transferase (16, 17; A. Silverstone et al., unpublished results). No infectious ecotropic virus has been isolated from the cells, but xenotropic virus can be recovered by cocultivation of L691 cells with CCL64 mink lung cells or other non-murine cell lines (15; N. Rosenberg and D. Baltimore, unpublished results).

*Virus Stocks.* M-MuLV-1, M-MuLV-2, Friend-MuLV(F-MuLV)-N, Gross-MuLV(G-MuLV), G-MuLV-1, and E-MuLV were all grown as NIH/3T3 carrier cell cultures (Table I). F-MuLV-B was maintained as a BALB/3T3 carrier culture. M-MuLV-1 was originally isolated from JLS-V11 cells by end point dilution as previously described (18). M-MuLV-2 was isolated from M-MuLV-1 by cloning NIH/3T3 cells after adsorption of virus at low multiplicity. E-MuLV was isolated by end point dilution of WN1802N (19) as previously described (20). G-MuLV, originally obtained from Dr. Nancy Hopkins, M.I.T., was cloned by infecting NIH/3T3 cells at a moi of 0.3 PFU. After a 1.5-h adsorption period, the infected cells were trypsinized and plated in microtiter plates at a concentration of 0.3 cells/well. 10 days later, all wells containing cells were assayed for virus production by the XC plaque assay (21). 2 of 51 clones released infectious virus, and one of these, G-MuLV-1, was selected for use in experiments. Cloned F-MuLV stocks originally obtained from Dr. Robert Eckner, Boston University School of Medicine, Boston, Mass. were a gift of Dr. Alfred Bothwell, M.I.T. F-MuLV-N and F-MuLV-B were isolated by low moi and cloning of SC-1 cells. C57Bl/KaB (22), a clone of fibrotropic C57Bl/6 virus, was a gift of Doctors Henry Kaplan and Alain Declève, Stanford University, Stanford, Calif.

A-MuLV stocks were prepared by superinfecting mass cultures of nonproducer fibroblast cells with helper virus. After a 2-h adsorption period, the cells were maintained at high densities with frequent medium changes for 1.5-2 wk to allow virus spread. Both helper virus and A-MuLV stocks were prepared from 24-h harvests of exponentially growing cultures. The virus supernates were centrifuged for 15 min at low speed, filtered through a 0.45-$\mu$ filter (Millipore Corp., Bedford, Mass.), and frozen at $-70°C$ for subsequent use.

For quantitative rescue experiments with lymphoid nonproducer cells, $2 \times 10^6$ cells were resuspended directly in 1 ml of the helper virus preparations supplemented with 8 $\mu$g/ml polybrene (Aldrich Chemical Co., Milwaukee, Wis.). After a 1.5-h adsorption period, the cells were collected by low speed centrifugation, washed once with 10 ml of PBS, and treated with 0.25% trypsin, 0.2% EDTA for 7-10 min at 37°C. Trypsin-EDTA was removed by low speed centrifugation and the cells were plated in 4 ml of RPMI medium. Subsequently, culture fluids were collected for virus assay at 24-h intervals. The cells were collected and replated at the initial cell density ($5 \times 10^5$ cells/ml) in fresh medium for the next collection interval.

*Virus Assays.* Helper viruses were assayed on NIH/3T3 and BALB/3T3 cells by using the standard XC assay or by mouse S+L- assay with FG-10 cells (23). FG-10 assays were carried out by plating $1 \times 10^5$ cells in 60-mm plastic dishes. 24 h later, the cells were infected with 0.5 ml of virus. After a 2-h adsorption period in the presence of 8 $\mu$g/ml polybrene, the cells were fed 4 ml of DME-CS and incubated. The S+L- plaques were visualized by hematoxylin staining 5 days postinfection. The titers obtained with the XC and FG-10 assays were equivalent for all the virus stocks (data not shown).

Fibroblast transformation assays were used to standardize the FFU in A-MuLV stocks. Either $1 \times 10^5$ NIH/3T3 or BALB/3T3 cells, plated in 60-mm dishes 24 h previously, were infected with 0.5-ml portions of virus. After a 2-h adsorption period in the presence of 8 $\mu$g/ml polybrene, the plates were fed fresh DME-CS, and incubated. Medium was changed 5 and 10 days postinfection and foci were scored in live cultures by using low power magnification ($\times$ 100) at 15 days postinfection (12). The amount of helper virus present in A-MuLV stocks was determined by using SC or S+L- assays.

All helper virus and A-MuLV preparations were screened for the ability to replicate on xenogeneic cells. For this assay, CCL64 mink lung cells, plated 24 h previously at $1 \times 10^5$ cells/60 mm dish, were infected with 0.5 ml of undiluted virus stock containing 8 $\mu$g/ml polybrene. After a 2-h adsorption period, the cells were fed fresh DME-IFS and incubated. Cells were passaged twice weekly and screened for the presence of virus by using a standard reverse transcriptase assay 6 wk postinfection.

*Bone Marrow Transformation.* Transformation of mouse bone marrow cells was carried out as previously described (14). Briefly, $2 \times 10^6$ nucleated cells were plated in 35-mm dishes in 1 ml of RPMI medium. 1 ml of virus suspension was added to the dishes and the plates were incubated. $\frac{1}{2}$ ml of fresh RPMI medium was added to the cultures 6 and 12 days postinfection. Transformation was recognized by rapid proliferation of immature lymphoid cells in the culture fluid.

*Mice.* The mice used for most experiments were supplied by our breeding colony at M.I.T. NIH/Swiss mice were originally obtained from the Division of Research Services, National Institutes of Health, Bethesda, Md. BALB/cAN mice were obtained from Dr. Herman Eisen, M.I.T. CD-1/Swiss mice were purchased from Charles River Breeding Laboratories, Wilmington, Mass. For certain experiments, C57BR/cdJ mice were purchased from The Jackson Laboratory, Bar Harbor, Maine.

For induction of leukemia all animals were inoculated intraperitoneally with 0.1 ml of 0.45 $\mu$m of filtered virus suspension at less than 48 h of age. Animals were observed weekly for cachexia, anemia, lymphadenopathy, or splenomegaly. Mice inoculated with M-MuLV clones and F-MuLV clones remaining disease free were sacrificed 6 mo postinoculation. Animals inoculated with E-MuLV and G-MuLV were held for 15 mo. Mice inoculated with all A-MuLV stocks were observed for 3 mo. Gross pathologic findings were noted for all animals either when illness was noted or at the termination of the experiments. Spleen, thymus, lymph node, liver, and any other abnormal tissues from representative animals were also fixed and processed for histologic examination according to routine procedures.

For studies on recovery of A-MuLV from mice, several NIH/Swiss litters were randomly mixed and then redivided for virus inoculation. Each mouse received equivalent doses of either A-MuLV(M-MuLV-1) or A-MuLV(G-MuLV). Animals were sacrificed by cervical fracture 4, 10, 15, and 21 days postinfection (and, in the case of A-MuLV(M-MuLV-1), when disease was evident). The animals were examined for evidence of disease and spleen, thymus, femur, and tibia were removed and frozen immediately on dry ice. Virus extracts, prepared in RPMI containing no serum, were made by using a dounce homogenizer or a mortar and pestle. The extracts were centrifuged twice at 2,500 rpm for 30 min and filtered through a 0.45-$\mu$m Millipore filter before infectivity studies. Protein concentration in the extracts was determined by the standard Lowry et al. procedure (24).

*Reverse Transcriptase Assays.* 24-h supernates from exponentially growing cell cultures were clarified by low speed centrifugation. The supernates were then centrifuged at 100,000 $g$ for 45 min at 4°C. Pellets were resuspended in 0.5 ml of 0.01 M Tris-HCl (pH 7.5) and sonicated for 40 s in a Raytheon sonic oscillator, Raytheon Co., Portsmouth, R.I. A 50-$\mu$l portion of the virus suspension was assayed for exogenous reverse transcriptase activity in 0.1 ml reaction mixture containing 0.05 M Tris-HCl buffer (pH 8.3), 0.06 M NaCl, 6 mM MnCl$_2$, 0.02 M dithiothreitol, 1 $\mu$g poly(rA) (Miles Laboratories, Inc., Kankakee, Ill.), 0.5 $\mu$g oligo(dT) (Collaborative Research, Inc., Waltham, Mass.), 0.05% Nonidet P-40 (Gallard Schlesinger Chemical Mfg. Corp., Carle Place, N.Y.), and 10$^{-5}$ M [$^3$H]dTTP (52 Ci/mmol) (New England Nuclear, Boston, Mass.).

## Results

The tropism of the helper viruses described in Table I was determined by using the XC plaque assay method (21) on NIH/3T3 and BALB/3T3 cells (Table II). Both cloned stocks of M-MuLV produced equivalent numbers of large, clear plaques on the two cell lines and were therefore NB-tropic (25). The two F-MuLVs were of opposite tropism, one preferring NIH/3T3 cells (F-MuLV-N) and the other BALB/3T3 cells (F-MuLV-B). The G-MuLV stocks and E-MuLV clone were N-tropic, producing small, clear XC plaques preferentially on NIH/3T3 cells. All of the viruses replicated to high titer in fibroblast cells of the appropriate Fv-1 genotype. None of the virus stocks had an amphotropic host range as determined by their inability to establish an infection in mink cells.

The oncogenicity of helper virus preparations was determined by inoculating newborn mice of proper Fv-1 genotype with 0.45 $\mu$m filtered virus stocks (Table III).[2] Mice were sacrificed when signs of disease were evident and gross and histologic examinations were conducted. Both M-MuLV-1 and M-MuLV-2 induced disease in 96–100% of both Swiss and BALB/c mice after a 2.5–3.5-mo latent period. Hepatosplenomegaly and large mesenteric lymph node tumors

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[2] Jolicoeur et al. *J. Natl. Cancer Inst.* In press.

TABLE II
*Properties of Helper Viruses*

| Virus stock | Titer | | Growth on mink lung cells |
| --- | --- | --- | --- |
| | NIH | BALB | |
| M-MuLV-1 | $2 \times 10^6$ | $1 \times 10^6$ | — |
| M-MuLV-2 | $2 \times 10^6$ | $1 \times 10^6$ | — |
| F-MuLV-N | $4 \times 10^5$ | $1 \times 10^2$ | — |
| F-MuLV-B | $<10$ | $1 \times 10^6$ | — |
| E-MuLV | $1 \times 10^4$ | $1 \times 10^2$ | — |
| G-MuLV | $3 \times 10^5$ | $1 \times 10^2$ | — |
| G-MuLV-1 | $2 \times 10^5$ | $8 \times 10^2$ | |

0.5-ml portions of filtered virus dilutions were exposed to NIH/3T3 or BALB/ 3T3 cells plated 24 h earlier at densities of $5 \times 10^4$ cells or $7.5 \times 10^4$ cells/60 mm dish. After a 2-h virus adsorption period, cells were fed fresh DME-CS and incubated. 3 days later the plates were irradiated with ultraviolet light and $1 \times 10^6$ XC cells were added. Plaques were visualized by hematoxylin staining.
Growth of virus stocks on mink lung cells was monitored by reverse transcriptase assay 6 wk postinfection.

were prominent features of the disease and thymomas were noted in about 50% of the animals. Histologic examination of tumor tissue showed that the tumors were composed of lymphoblasts. M-MuLV-2 titrations in NIH/Swiss mice indicated that doses less than $6 \times 10^3$ were effective in disease induction (data not shown).

F-MuLV-B and F-MuLV-N were also oncogenic. Both viruses induced diseases confined to the spleen and liver. Mesenteric and peripheral lymph nodes and the thymus of inoculated mice were normal. Histologic examination of the diseased tissues showed the F-MuLV-B-induced tumors to be composed of lymphoblasts, morphologically indistinguishable from the cells in M-MuLV-induced tumors. As with M-MuLV, F-MuLV-B induced a disease after a 2.5–3.5-mo latent period. F-MuLV-N induced disease after a short, 5–6 wk, latent period. The types of cells in F-MuLV-N-induced tumors have not been fully characterized. The virus stocks did induce disease in adult mice but did not induce spleen foci characteristic of Friend virus complex with the standard 9 day focus assay (26) (data not shown).

Neither E-MuLV nor G-MuLV induced tumors in NIH/Swiss mice. Inoculated animals were observed for 15 mo and no gross or histologic evidence of tumors was noted.

*Characterization of Abelson Stocks.* Two A-MuLV-transformed nonproducer fibroblast cell lines were used to prepare A-MuLV stocks with the various helpers. ANN-1 cells (12), derived from NIH/3T3 cells, were used to prepare virus stocks with N- and NB-tropic helpers and 5H cells, derived from BALB/c JLS-V9 cells (27), were used to prepare virus stocks with B- and NB-tropic helpers. The focus-forming component of all virus stocks was monitored by transformation of NIH/3T3 or BALB/3T3 cells and the titers are represented as FFU. Helper virus titers were determined by using XC or S+L- plaque assays and are represented as PFU. All A-MuLV stocks transformed fibroblasts of

TABLE III
Oncogenicity of Helper Viruses

| Virus stock | Titer inoculated (PFU) | Mouse strain | Disease frequency | Latent period | |
|---|---|---|---|---|---|
| | | | | Mean | Range |
| | | | | days | |
| M-MuLV-1 | $1 \times 10^4$ | N | 15/15 | 102 | 78–118 |
| | $1 \times 10^4$ | B | 30/31 | 83 | 77–91 |
| M-MuLV-2 | $7 \times 10^3$ | N | 6/6 | 92 | 72–109 |
| | $3 \times 10^5$ | B | 6/6 | 109 | 94–128 |
| F-MuLV-N | $1 \times 10^4$ | N | 5/6 | 37 | 34–39 |
| E-MuLV | $3 \times 10^4$ | N | 0/11 | – | – |
| G-MuLV | $3 \times 10^4$ | N | 0/11 | – | – |

Neonatal mice were inoculated intraperitoneally with filtered virus stocks and observed for signs of disease. Animals inoculated with E-MuLV and G-MuLV were observed for 15 mo. The mouse strains used were either CD-1 or NIH/Swiss (N) and BALB/c (B). CD-1 and NIH/Swiss mice are equally susceptible to these lymphoma viruses.

appropriate Fv-1 genotype efficiently and reflected the tropism of the helper virus (Table IV). The proportion of helper virus in virus stocks varied with the preparation but most of the virus stocks had PFU/FFU ratios of 1–5. Virus stocks prepared with M-MuLV-1 and ANN-1 cells and one ANN-1-grown F-MuLV-N stock contained virus that could replicate in mink lung cells. Occasionally, preparations made with 5H cells contained virus that could replicate on mink cells after long-term infection (Table IV). Xenotropic virus probably arises as a low level contaminant from the rescued A-MuLV nonproducer cell lines. Most transformed lymphoid cell lines isolated with these virus stocks do not release virus which grows on mink lung cells (data not shown).

To determine the relationship between FFU and Abelson disease induction, an in vivo A-MuLV titration was performed initially by using A-MuLV(M-MuLV-2) and NIH/Swiss mice. Mice were sacrificed when cachexia, lymphadenopathy, paralysis of hind limbs, or meningeal tumors evident grossly as a bulging of cranial bones were noted. Animals with lymphosarcomas involving bone marrow and peripheral lymph nodes with slight splenomegaly and normal thymus and mesenteric lymph nodes were scored as positive for Abelson disease. An inoculum of $10^3$ FFU induced disease in more than 50% of the test animals while only 1 of 11 mice inoculated with $5 \times 10^2$ FFU developed Abelson disease (Table V).

A-MuLV prepared with the various helper viruses was then inoculated into newborn mice at doses expected to give a high disease frequency based on the A-MuLV(M-MuLV-2) titration. A-MuLV stocks prepared with both clones of M-MuLV induced disease efficiently in NIH/Swiss and BALB/c mice after a 3–5-wk latent period (Table VI). Typical Abelson lymphosarcomas, characterized by large tumors of peripheral lymph nodes, spine, and skull with slight splenomegaly and normal thymus, were found in these animals. Histologically, the tumors were composed of immature lymphoid cells. Disease after A-MuLV(F-MuLV-B) inoculation was similar to the syndrome observed when A-MuLV(M-MuLV) clones were used. The A-MuLV(F-MuLV-B) stocks were more efficient in BALB/c than NIH/Swiss mice.

TABLE IV
Properties of A-MuLV Stocks

| Virus stock | PFU Titer | | FFU Titer | | Growth on mink lung cells |
|---|---|---|---|---|---|
| | NIH | BALB | NIH | BALB | |
| ANN-1(M-MuLV-1) | $1 \times 10^6$ | $7 \times 10^5$ | $1 \times 10^6$ | $7 \times 10^5$ | + |
| ANN-1(M-MuLV-2) | $1 \times 10^6$ | ND* | $5 \times 10^5$ | $5 \times 10^5$ | ND |
| ANN-1(F-MuLV-N) | $2 \times 10^5$ | $4 \times 10^1$ | $7 \times 10^5$ | <10 | +/− |
| ANN-1(E-MuLV) | $2 \times 10^4$ | $3 \times 10^2$ | $4 \times 10^4$ | $1 \times 10^2$ | − |
| ANN-1(G-MuLV) | $8 \times 10^4$ | $3 \times 10^2$ | $1 \times 10^5$ | $8 \times 10^1$ | − |
| 5H(M-MuLV-2) | $3 \times 10^5$ | $2 \times 10^5$ | $9 \times 10^4$ | $4 \times 10^4$ | + |
| 5H(F-MuLV-B) | <10 | $1 \times 10^5$ | <10 | $2 \times 10^5$ | +/− |

0.5-ml portions of filtered virus dilutions were exposed to NIH/3T3 or BALB/3T3 cells for assay by the standard XC plaque test or the A-MuLV fibroblast focus assay. For the XC test, XC cells were added to monolayers irradiated with ultraviolet light 3 days postinfection and plaques were scored 3 days later. A-MuLV transformed foci were scored 15 days postinfection. Growth of virus on mink lung cells was monitored by reverse transcriptase assay 6 wk postinfection. The A-MuLV stocks are described by the nonproducer cell from which they were derived and, in parenthesis, the helper virus used for rescue.
* ND, not done.

NIH/Swiss mice inoculated with A-MuLV(F-MuLV-N) also developed typical Abelson disease (Table VI). However, in all of the mice inoculated with this virus stock, hepatosplenomegaly was a prominent feature of the disease. This type of pathology is very uncommon in Abelson disease and probably reflects the action of the F-MuLV-N helper virus in the preparation. The overlapping latent period for F-MuLV-N disease and Abelson disease makes it difficult to assess which cells have responded to which virus. Skull, spine, and peripheral lymph node tumors, characteristic of Abelson disease, and never noted in F-MuLV-N injected mice, were present in about 40% of the animals. The high titer of helper virus in the particular A-MuLV(F-MuLV-N) preparation used in these animal experiments may account for the high proportion of animals with hepatosplenomegaly.

Mice inoculated with A-MuLV(G-MuLV) did not develop any detectable disease during a 3-4-mo observation period. To determine if A-MuLV(G-MuLV) replicates in inoculated animals, lymphoid tissues from NIH/Swiss mice injected with this virus stock were extracted and examined for virus by using the S+L-assay and the NIH/3T3 focus assay (Fig. 1). Tissues from mice inoculated with an equivalent amount of A-MuLV(M-MuLV-1) served as controls. The virus dose used, $2 \times 10^4$ FFU, induced disease in 100% of the A-MuLV(M-MuLV-1) inoculated mice. A-MuLV(M-MuLV-1) replicated well in the mice and as early as 4 days postinjection both components of A-MuLV could be detected in bones of the animals. Helper virus was also recovered from spleen and thymus. As the incubation period increased, the titer of A-MuLV increased. The higher titer of A-MuLV in thymus tissue observed in the tumored mice probably arises from large parathymic lymph node tumors in these animals. No virus was recovered from bone, spleen, or thymus of A-MuLV(G-MuLV) injected mice. Litter mates of the A-MuLV(G-MuLV) mice sacrificed in these experiments are healthy 5 mo postinoculation.

TABLE V
Oncogenicity of A-MuLV(M-MuLV-2)

| Titer inoculated | | Disease fre-quency | Latent period | |
|---|---|---|---|---|
| PFU | FFU | | Mean | Range |
| | | | days | |
| $1.5 \times 10^5$ | $5 \times 10^4$ | 8/8 | 24 | 21–28 |
| $1.5 \times 10^4$ | $5 \times 10^3$ | 6/7 | 33 | 29–40 |
| $3 \times 10^3$ | $1 \times 10^3$ | 3/5 | 75 | 71–78 |
| $1.5 \times 10^3$ | $5 \times 10^2$ | 1/11 | – | 78 |

Neonatal mice were inoculated intraperitoneally with 0.1 ml of filtered virus stock. Animals were sacrificed when disease was evident. Animals were observed for at least 5 mo.

TABLE VI
Oncogenicity of A-MuLV Stocks

| Virus stocks | Titer inoculated | | Mouse strain | Disease frequency | Latent period | |
|---|---|---|---|---|---|---|
| | PFU | FFU | | | Mean | Range |
| | | | | | days | |
| ANN-1(M-MuLV-1) | $1 \times 10^5$ | $2 \times 10^4$ | N | 4/4 | 30 | 28–30 |
| | ND* | $2 \times 10^4$ | B | 7/7 | 19 | 18–20 |
| ANN-1(M-MuLV-2) | $1 \times 10^4$ | $5 \times 10^3$ | N | 8/8 | 37 | 29–43 |
| | | | B | ND | – | – |
| ANN-1(F-MuLV-N) | $2 \times 10^4$ | $4 \times 10^3$ | N | 11/11 | 41 | 38–55 |
| | | | B | ND | – | – |
| 5H(M-MuLV-2) | $3 \times 10^4$ | $6 \times 10^3$ | N | 5/9 | 54 | 28–87 |
| | | | B | 5/5 | 28 | 23–30 |
| 5H(F-MuLV-B) | $1 \times 10^4$ | $3 \times 10^4$ | N | 1/6 | 30 | 30 |
| | | | B | 5/6 | 31 | 24–47 |
| ANN-1(G-MuLV) | $8 \times 10^3$ | $1 \times 10^4$ | N | 0/14 | – | – |
| | | | B | ND | – | – |

Neonatal mice were inoculated intraperitoneally with 0.1 ml of filtered virus stock. Animals were sacrificed when disease was evident. Animals were observed for at least 3 mo. Mouse strains used were NIH/Swiss (N) and BALB/c (B). The A-MuLV stocks are described by the nonproducer cell from which they were derived and, in parentheses, the helper virus used for rescue.
* ND, not done.

*Transformation of Bone Marrow Cells.* To determine the effect of helper viruses on the ability of A-MuLV stocks to transform hematopoietic cells in vitro, bone marrow cells from 4- to 6-wk old mice were infected and plated in a quantitative agarose transformation assay. Macroscopic foci of lymphoid cells were counted 12 days postinfection (Table VII). The transformation frequency obtained with different virus preparations was standardized by the fibroblast FFU in the virus stocks. A-MuLV in combination with most of the helper viruses efficiently transformed lymphoid cells. Colonies arising in these cultures could routinely be established as continuous cell lines. Plating efficiency in most cases was 80–90% (data not shown).

Cultures infected with A-MuLV(E-MuLV) and A-MuLV(G-MuLV) had very few macroscopic foci (Table VII). In more than 200 A-MuLV(E-MuLV) infected
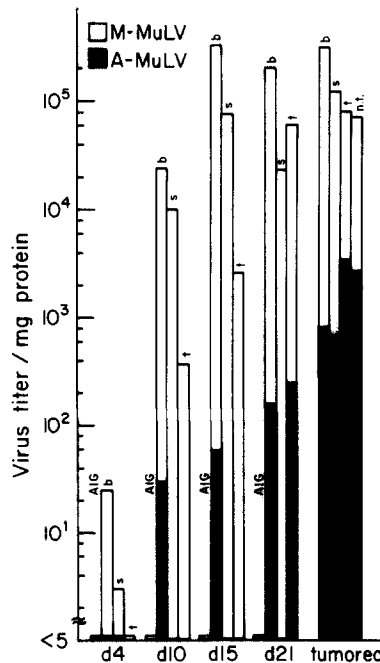
FIG. 1.   Recovery of A-MuLV from mice. Extracts of bone (b), spleen (s), and thymus (t) from mice injected with A-MuLV(M-MuLV-1) or A-MuLV(G-MuLV) were assayed for both components of A-MuLV in vitro. Lymph node tumors (n.t.) from terminal animals injected with A-MuLV(M-MuLV-1) were also examined. ■ (A-MuLV) indicates FFU and □ (M-MuLV) indicates PFU recovered in the various tissues from A-MuLV(M-MuLV-1)-injected mice. For mice injected with A-MuLV(G-MuLV), results are shown in one column (A/G) although all three tissues were assayed separately.

cultures, three foci were observed. None of these grew independently in liquid culture. One macroscopic focus has been noted in a culture infected with A-MuLV(G-MuLV). The cells in this focus did grow independently and were established as a cell line. The cells appeared to be similar to other A-MuLV transformed lymphoid cells with respect to growth and morphologic properties. The cells did not produce infectious virus, but did contain the A-MuLV genome which could be rescued by superinfection with M-MuLV (data not shown).

To further investigate the transformation potential of A-MuLV stocks, both A-MuLV(E-MuLV) and A-MuLV(G-MuLV) were used to infect mass cultures of bone marrow cells. In this transformation test, virus is mixed with bone marrow cells in liquid culture and the cells are incubated in the presence of input virus and any virus arising by subsequent rounds of replication. Under conditions where an equivalent dose of A-MuLV(M-MuLV-1) transformed 95–100% of the cultures, no transformation was observed in cultures infected with either A-MuLV(E-MuLV) or A-MuLV(G-MuLV).

The ratio of helper virus to fibroblast focus-forming virus in different A-MuLV stocks varies. However, the amount of helper virus in A-MuLV stocks does not appear to influence the transformation potential of the virus (Table VI). Addition of a competent helper virus such as M-MuLV-1 to A-MuLV(G-

TABLE VII

*A-MuLV-Induced Transformation of Bone Marrow Cells*

| Virus stock | $\dfrac{PFU}{FFU}$ | Lymphoid foci/$10^5$ fibroblast FFU | | Lymphoid foci NIH/Swiss marrow/Lymphoid foci BALB/c marrow | Oncogenic in vivo |
|---|---|---|---|---|---|
| | | NIH/Swiss | BALB/c | | |
| ANN-1(M-MuLV-1) | 6 | 92 | 48 | 1.7 | Yes |
| ANN-1(M-MuLV-2) | 5 | 28 | 26 | 1.0 | Yes |
| ANN-1(F-MuLV-N) | 3 | 20 | 0.25 | 200 | Yes |
| 5H(M-MuLV-2) | 3 | 148 | 88 | 1.6 | Yes |
| 5H(F-MuLV-B) | 0.5 | 8 | 103 | 0.006 | Yes |
| ANN-1(G-MuLV) | 0.7 | <0.01 | ND* | | No |
| ANN-1(E-MuLV) | 1.5 | <0.02 | ND | | ND |

NIH/Swiss or BALB/c mouse bone marrow was infected with A-MuLV in vitro and plated in the standard lymphoid cell transformation assay. Foci were scored 12 days postinfection. The A-MuLV stocks are described by the nonproducer cell from which they were derived and, in parenthesis, the helper virus used for rescue.

* ND, not done.

MuLV) virus at the time of infection did not overcome its inability in transformation (data not shown).

As has been observed for fibroblast cells, N-tropic A-MuLV stocks preferentially transform lymphoid cells from Fv-$1^{n/n}$ mice while B-tropic A-MuLV stocks are much more efficient on bone marrow cells from Fv-$1^{b/b}$ mice (Table VII). NB-tropic A-MuLV stocks transform bone marrow cells from both types of mice at high efficiency.

Results obtained in a different experimental system (28) have suggested that Fv-1-mediated restriction does not operate in lymphoid cells. The low transformation frequencies obtained with N- and B-tropic A-MuLV stocks on bone marrow cells of inappropriate Fv-1 genotype suggest that lymphoid cell transformation is subject to the effect of the Fv-1 gene.

*Rescue of A-MuLV from Nonproducer Cells.* Lymphoid nonproducer cell lines were isolated by removing single foci from agarose culture plates and adapting the cells to growth in liquid medium. Nonproducer cell lines were identified by the lack of reverse transcriptase-containing virus particles in the culture fluid. Two nonproducer cell cultures, BR48 and 2M, were readily superinfected with M-MuLV-2 (Table VIII). After addition of the helper virus, infection and release of both helper and focus-forming virus occurred within 12–15 h. Superinfected cultures could then be maintained as stable virus-producing cell lines.

Initial attempts to make virus-producing cultures of BR48 cells by using G-MuLV as a helper were unsuccessful (Table VIII). A standard fibroblast nonproducer rescue protocol was employed, growing the superinfected cells for 1.5–2 wk before assaying virus. More careful study revealed that G-MuLV did not form a stable association with lymphoid nonproducer cells. After superinfection with G-MuLV, virus production of both helper and focus-forming virus increased for the first 48-h and then declined (Fig. 2). In cultures superinfected with M-MuLV-2, virus production continued in a stable fashion. After virus

TABLE VIII
*Superinfection of A-MuLV Transformed Nonproducer Cells*

| Nonproducer | Helper virus | Virus Replication | |
|---|---|---|---|
| | | PFU | FFU |
| BR48(Fv-1$^{n/n}$) | M-MuLV-2 | $1 \times 10^5$ | $8 \times 10^4$ |
| | F-MuLV-N | $1 \times 10^3$ | $2 \times 10^2$ |
| | G-MuLV-1 | 9 | 2 |
| | E-MuLV | 0 | 0 |
| 2M(Fv-1$^{b/b}$) | M-MuLV-2 | $2 \times 10^5$ | $7 \times 10^4$ |
| | F-MuLV-B | $1.5 \times 10^6$ | $5 \times 10^5$ |
| | C57Bl/KaB | 0 | ND* |

Lymphoid nonproducer cell lines were infected with helper viruses at an moi of 0.5. After a 2-h adsorption period, cells were maintained as exponentially growing cultures for 10–14 days at which time fresh culture fluids were examined for the presence of PFU and FFU. Viruses produced by 2M cells were assayed on BALB/3T3 cells. Viruses produced by BR48 cells were assayed on NIH/3T3 cells.
* ND, not done.

production had ceased in BR48(G-MuLV) cultures, the population could be reinfected with G-MuLV or M-MuLV-2 suggesting that the culture has lost some if not all virus expression from the initial superinfection.

BR48 cells, an Fv-1$^{n/n}$ cell line, could be stably superinfected by F-MuLV-N but not by E-MuLV (N-tropic). 2M, an Fv-1$^{b/b}$ cell line, could be easily superinfected by both M-MuLV-2 and F-MuLV-B but not by the B-tropic C57Bl/KaB virus (Table VIII). G-MuLV, however, did infect a T-cell lymphoma cell line, L691-6, and replicated nearly as efficiently as M-MuLV-2 in these cells (Fig. 3).

## Discussion

These studies have shown that A-MuLV oncogenesis and lymphoid cell transformation is dependent on which helper virus rescued and accompanies the defective A-MuLV. Three MuLVs that readily caused lymphomas on their own acted as efficient helpers to produce Abelson disease in vivo and lymphoid cell transformation in vitro. Two MuLVs that induced no detectable disease by themselves also failed to act as efficient helpers for lymphoid cell transformation in vitro and the one that was tested, A-MuLV(G-MuLV), was also inactive in vivo. Neither component of the A-MuLV(G-MuLV) stocks established an infection in the inoculated mice. For fibroblast transformation, however, all five helpers were equally active when normalized to their PFU content. There is, therefore, an important difference between the requirements for A-MuLV transformation of lymphoid cells and of fibroblasts. Scher (29) has observed a similar distinction in studies with Kirsten sarcoma virus and A-MuLV.

Transformation of fibroblasts by A-MuLV is similar to transformation of fibroblasts by murine sarcoma viruses in that any MuLV that can replicate on its own will act as an effective helper (1, 2, 5, 6, 30). What then is the significance of the helper specificity for lymphoid cell transformation and oncogenesis by A-MuLV?
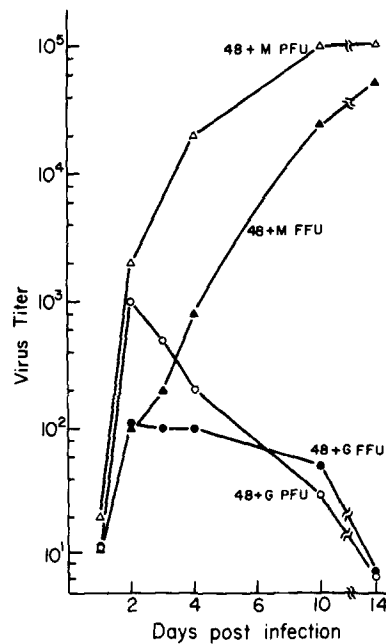
FIG. 2. Replication of virus in A-MuLV-transformed lymphoid nonproducer cells. BR48 cells were infected with M-MuLV and G-MuLV at an moi of 0.1. Virus in the culture fluids was monitored at intervals postinfection by using standard assays.

Two hypotheses are possible. One is that the helper is needed for effective penetration of the A-MuLV genome and that lymphoid target cells have receptors that distinguish the competent helpers from the incompetent ones. The other hypothesis is that the helper plays an intracellular role in initiating or maintaining the transformation.

The penetration hypothesis is supported by the inability of an added competent helper virus to elicit lymphoid transformation by an incompetent A-MuLV stock. Other explanations of this result, however, are possible and further experiments in this area are needed.

As a test of the penetration hypothesis, we examined the ability of A-MuLV transformed nonproducer lymphoid cells to be infected by G-MuLV and M-MuLV. Both viruses established infections in the A-MuLV-transformed cells therefore, it can be argued, that at least after transformation the cells have receptors for both viruses. We cannot test the cells before infection because we cannot make a pure culture of target cells. A further argument against the penetration hypothesis is the ability of G-MuLV and M-MuLV pseudotypes with vesicular stomatitis virus to infect the A-MuLV-transformed nonproducer cells with equal efficiency (P. Besmer et al., unpublished results).

The possibility that the "helper" may play a critical, on-going role in the transformation process receives some support from our studies of particle production by the A-MuLV-transformed lymphoid cell lines. Some of these lines are totally nonproductive but a majority do produce a noninfectious particle (A. Shields et al., unpublished results). This particle has a major internal protein closely related to that of M-MuLV suggesting that the M-MuLV genome may be
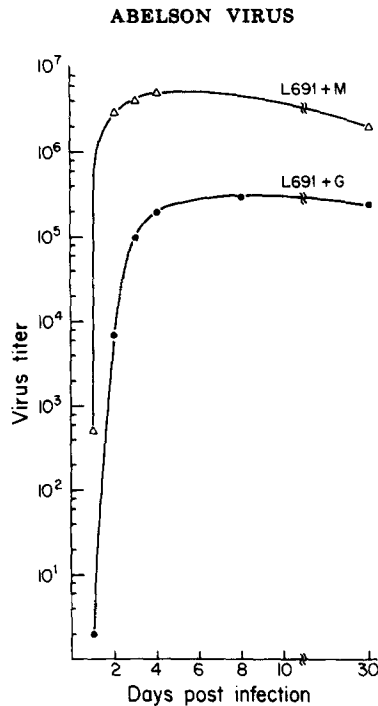
FIG. 3. Replication of helper virus in L691-6 thymoma cells. L691-6 cells were infected with M-MuLV-1 and G-MuLV at an moi of 0.1. Virus in culture fluids was monitored at intervals postinfection by using standard assays.

present in most of the lymphoid transformants. Nucleic acid hybridization experiments will be able to assess whether all transformants might contain some or all of the M-MuLV genome. If the genome is present, it could contribute to maintaining the transformed state. This possibility is not without precedent: certain murine sarcoma viruses only transform in the presence of a helper virus (6, 23).

The transitory production of virus after G-MuLV infection of the A-MuLV-transformed nonproducer cells (Fig. 2) is not understood. The loss of infectivity is so rapid that overgrowth of initially uninfected cells is an unlikely explanation. It seems that G-MuLV, although it can initiate infection, cannot stably maintain the infected state and whether the G-MuLV provirus can even integrate is not known. The inability of G-MuLV to stably infect lymphoid nonproducer cells does not reflect an inability of this virus to infect all lymphoid cells, however, because this virus replicates nearly as well as M-MuLV-2 in L691 lymphoma cells. This inability of G-MuLV to maintain a productive infection of the A-MuLV-transformed cells may be an explanation of its inability to act as an effective helper for A-MuLV transformation.

The inability of E-MuLV, another inefficient helper, to stably infect lymphoid nonproducer cells supports a correlation between a virus' inability to help A-MuLV transform lymphoid cells and its inability to stably associate with lymphoid nonproducer cells. Although the helper efficiency of C57Bl/KaB virus has not been determined, the virus is fibrotropic and nononcogenic in C57Bl/Ka mice (22). Thus, the inability of this virus to stably infect 2M cells probably also fits this pattern.

The correlation between the oncogenicity of a replication-competent MuLV and its ability to act as an efficient helper is striking. This could suggest that the helper virus is providing a critical leukemogenic function in the transformation process. Thus, the data might be reinterpreted by saying that A-MuLV is acting as a helper for the replication competent MuLV in producing the Abelson disease. The Abelson virus would then function to alter the cellular specificity of the leukemogenic virus, e.g. M-MuLV, and allow it to transform more immature lymphoid cells than those usually affected by that virus (17).

These speculations may seem premature but they serve to focus attention on the difference between lymphoid cell transformation and fibroblast cell transformation. Fibroblast transformation may be a relatively simple process whereby a single gene product establishes control of cellular growth but at present leukemia virus transformation has properties that suggest more complex interactions.

## Summary

Abelson murine leukemia virus (A-MuLV)-transformed fibroblast nonproducer cells were used to prepare A-MuLV stocks containing a number of different helper viruses. The oncogenicity of the A-MuLV stocks was tested by animal inoculation and their ability to transform normal mouse bone marrow cells was measured in vitro. All of the A-MuLV stocks transformed fibroblast cells efficiently. However, only A-MuLV stocks prepared with helper viruses that are highly oncogenic were efficient in vivo and in vitro in hematopoietic cell transformation. In addition, inefficient helpers did not establish a stable infection in lymphoid nonproducer cells. Thus, helper virus has a more central role in lymphoid cell transformation than in fibroblast cell transformation.

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