Abelson Murine Leukemia Virus Mutants Deficient in Kinase Activity and Lymphoid Cell Transformation

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Abelson murine leukemia virus transforms both lymphoid cells and fibroblasts *in vitro* and induces a unique type of thymus-dependent lymphoma *in vivo*. Four fibroblast-transforming strains of Abelson murine leukemia virus were identified, based on the sizes of the Abelson murine leukemia virus-specific phosphoproteins produced by these isolates. Two of these strains, the standard P120- and the P160-producing viruses, transformed lymphoid cells efficiently *in vitro* and induced Abelson disease *in vivo*. Two other strains, which synthesized small Abelson murine leukemia virus-specific proteins with molecular weights of 90,000 (P90) and 100,000 (P100), transformed lymphoid cells very poorly both *in vitro* and *in vivo*. The reduced oncogenic potentials of these isolates were correlated with a high level of synthesis of fairly unstable P90 and P100. In addition, neither P90 nor P100 functioned efficiently in protein kinase assays. The correlation of abnormal metabolism and deficient protein kinase activity with the reduced oncogenic potentials of these virus strains supported a direct role for these proteins and the kinase activity in transformation. Furthermore, these results suggested that the requirements for lymphoid cell transformation and fibroblast transformation are different.

Abelson murine leukemia virus (A-MuLV) is a replication-defective virus that transforms lymphoid cells and some fibroblasts *in vitro* and induces a unique thymus-independent lymphoma *in vivo* (13). This virus arose in a steroid-treated mouse injected with Moloney murine leukemia virus (M-MuLV), probably by recombination of the M-MuLV with cellular DNA (D. Baltimore, A. Shields, G. Otto, S. Goff, P. Besmer, O. Witte, and N. Rosenberg, Cold Spring Harbor Symp. Quant. Biol., *in press*). The genome of the standard, clonally derived A-MuLV strain, which was originally isolated in NIH/3T3-transformed nonproducer cell line ANN-1 (18), is 5.5 kilobases long and has regions of precise homology with the M-MuLV genome which are 1,320 bases long at the 5' end and 720 bases long at the 3' end. The central portion of the genome is related to unique sequences in normal mouse DNA (19; Baltimore et al., *in press*). This A-MuLV strain encodes a single protein of 120,000 molecular weight (P120), which shares homology with the M-MuLV gene products p15, p12, and part of p30, in addition to containing about 90,000 daltons of A-MuLV-specific determinants (9, 23, 24). The ANN-1-

derived A-MuLV strain has been designated A-MuLV-P120 (15; O. N. Witte, S. Goff, N. Rosenberg, and D. Baltimore, Proc. Natl. Acad. Sci. U.S.A., *in press*).

A second strain of A-MuLV, which was originally detected in cell lines transformed by tumor extracts independent of the extracts used to produce the ANN-1 cell line, encodes an A-MuLV-specific protein of 160,000 molecular weight (P160) that is analogous to P120. In addition, two other strains of A-MuLV, which were derived from A-MuLV-P120 and encode smaller A-MuLV-specific proteins of 100,000 (P100) and 90,000 (P90) molecular weight, have also been isolated (15).

P120 isolated by immunoprecipitation or chromatography contains an *in vitro* phosphorylation activity that transfers the γ phosphate of ATP to tyrosine residues on the P120 molecule (22). This activity is similar to the protein kinase activity associated with Rous sarcoma virus-encoded pp60^{src} and polyoma virus middle T antigen (3, 4, 6). The functional similarity of P120 to pp60^{src} and the inability to detect other A-MuLV-encoded proteins suggest that P120 and the other related A-MuLV-specific proteins are the transforming proteins of these viruses.

All A-MuLV strains transformed lymphoid and fibroblast cells *in vitro* (15). However, about 50% of the A-MuLV-specific sequences present

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in P160 are missing in P90. Thus, if this protein is the transforming protein, A-MuLV-P160 appears to retain, in a stable fashion, a large amount of information that is not required by the virus. To examine this apparent paradox and the role of these A-MuLV-specific proteins in transformation more fully, we studied the oncogenic potentials of the different A-MuLV strains by using quantitative *in vitro* assays for lymphoid and fibroblast transformations and *in vivo* assays for Abelson disease induction. These experiments showed that although all of the A-MuLV strains are highly efficient in fibroblast transformation, both A-MuLV-P100 and A-MuLV-P90 are deficient in the ability to transform lymphoid cells *in vitro* and in the ability to induce Abelson disease *in vivo*. In addition, the metabolism and functional properties of the smaller A-MuLV-specific proteins are altered. The amounts of P90 and P100 synthesized by lymphoid cells transformed by these strains are not only abnormally high, but the molecules are also less stable than P120. Both P100 and P90 are also deficient in protein kinase activity. Thus, the loss of a portion of the A-MuLV-specific determinants in these proteins correlates with a reduction in their transformation potentials and suggests that the additional determinants expressed in A-MuLV-P160 and A-MuLV-P120 play an important role in transformation. The correlation among altered metabolism, deficient kinase activity, and reduced transformation potential supports a direct role for these proteins and the protein kinase activity in transformation. In addition, the ability of A-MuLV-P90 and A-MuLV-P100 to transform fibroblasts efficiently suggests that there are functional differences for transformations of fibroblasts and lymphoid cells.

MATERIALS AND METHODS

Cell lines and viruses. A-MuLV-P160 was prepared either from RAW309 Cr cells (a gift from Peter Ralph, Memorial Sloan-Kettering Institute, Rye, N.Y.) or from an NIH/3T3 nonproducer cell line transformed by A-MuLV-P160. This cell line was derived by infecting NIH/3T3 cells with A-MuLV released from RAW309 Cr at a multiplicity of infection of 0.1 focus-forming unit (FFU) per cell. After adsorption of virus, the infected cells were trypsinized and plated into microtiter wells at a density of 0.3 cell per well. Transformed nonproducer clones were identified by morphology and the absence of infectious virus in the culture fluid. The absence of helper virus-encoded proteins was confirmed by sodium dodecyl sulfate (SDS)-polyacrylamide gel analyses of [³⁵S]methionine-labeled immunoprecipitates. A-MuLV-P160 was rescued from this cell line with M-MuLV clone 2 (12). A-MuLV-P100 and A-MuLV-P90 were rescued from AbNRK clones carrying these strains by using the

amphotropic virus 292 (8), and NIH/3T3 nonproducer cell lines transformed by these viruses were prepared and rescued as described above.

M-MuLV clone 2 was prepared from a chronically infected culture of NIH/3T3 cells. All viruses were prepared from 24-h supernatants of exponentially growing cultures and filtered through 0.45- μ m filters before use.

The titer of M-MuLV in all preparations was determined using the S⁺L⁻ assay (2) and is expressed as the number of plaque-forming units per milliliter; the titer of A-MuLV was determined by the NIH/3T3 transformation assay (18) and is expressed as the number of FFU per milliliter.

Transformation of bone marrow cells. Bone marrow cells from 6- to 8-week-old BALB/c mice were infected with A-MuLV at a density of 10⁶ nucleated cells per ml of RPMI 1640 medium supplemented with 20% heat-inactivated (56°C, 30 min) fetal calf-serum (Sterile Systems, Ogden, Utah) and 50 μ M mercaptoethanol (20p medium), in the presence of 4 μ g/ml of polybrene per ml, as described previously (11, 12). After a 1.5-h adsorption period, the cells were either plated into liquid medium at a density of 10⁶ cells per ml in 35-mm dishes or diluted and mixed with agar-containing medium at a final density of 10⁵ cells per ml. Cells were plated onto 35-mm dishes or into 16-mm wells. The agar (Difco Laboratories, Detroit, Mich.) was at a final concentration of 0.27% in 20p medium.

The cultures were fed 5 to 6 days after plating. Macroscopic foci of transformed cells in agar cultures were scored 10 days after plating. In some cases, the cultures were fed after counting on day 10 and rescored 4 to 5 days later. Liquid cultures were observed for 3 weeks, with feeding at days 5, 10, and 15. Usually, large numbers of rapidly growing lymphoid cells were present 8 to 10 days after infection.

Tumor induction. BALB/cJ and NIH/Swiss mice were bred in our colony at the Tufts Cancer Research Center. C57L mice were purchased from Jackson Laboratory, Bar Harbor, Maine. Newborn animals (less than 48 h old) were each injected intraperitoneally with 0.1 ml of filtered virus stock and observed at least twice weekly. Animals with symptoms of disease, such as lymphadenopathy, splenomegaly, cachexia, or paralysis, were sacrificed by cervical dislocation, and autopsies were performed. In some cases, tumor tissue was removed sterilely and dissociated in 20p medium. After the concentration of viable cells was determined by using trypan blue, the cells were plated at densities of 1 $\times$ 10⁶ to 2 $\times$ 10⁶ cells per ml in 20p medium. After 7 to 10 days of culture, the cells could be subcultured at regular intervals. A portion of each tumor cell suspension was also washed with phosphate-buffered saline and prepared for [³⁵S]methionine labeling.

Labeling and kinase studies. Viral protein synthesis was monitored by using [³⁵S]methionine labeling, immunoprecipitation, and SDS-polyacrylamide gel electrophoresis, as previously described (15, 21). For tumor cell suspensions, the labeling mixture was adjusted to 2 mM EDTA and 5 mM phenylmethylsulfonyl fluoride just before cell lysis. For kinase reactions, cell extracts were reacted with anti-M-MuLV virion serum, and the immune precipitates were col-

lected by using *Staphylococcus aureus*. The pellets were washed in 50 mM Tris (pH 8.0) and suspended in 25 μ l of a solution containing 20 mM Tris (pH 8.0), 10 mM MnCl₂, and 1 μ Ci of [γ -³²P]ATP at 30°C for 5 min. The samples were washed in 50 mM Tris (pH 8.0) and processed for SDS gel electrophoresis in the standard fashion.

RESULTS

Transformation of bone marrow cells with different A-MuLV strains. The A-MuLV strains encoding P160, P120, P100, and P90 all transforming fibroblasts, and high-titer transforming virus stocks are obtained from these cells. Virus stocks with titers of 1×10^5 to 5×10^5 FFU/ml and 1×10^5 to 10×10^5 PFU/ml were routinely harvested from all of the producer cultures (data not shown). All four of the strains also transformed bone marrow cells in vitro (15). However, our initial experiments showed that there were important differences in the efficiencies of transformation among the different A-MuLV strains. When the liquid transformation assay (11, 14) was used, none of the cultures infected with A-MuLV-P100 or A-MuLV-P90 contained significant numbers of transformed lymphoid cells by day 10 (Table 1). As expected, A-MuLV-P120- and A-MuLV-P160-infected cultures, although infected with 5- to 10-fold less A-MuLV, were all positive by day 10 and contained $>10^6$ transformed lymphoid cells per ml. Some of the A-MuLV-P90-infected cultures did contain transformed cells after a prolonged (17 day) incubation. As reported previously, no rapidly dividing lymphoid cells were noted in mock-infected cultures or in cultures infected with helper virus alone (11, 14).

When the transformation potentials of the four A-MuLV strains were examined by

using the agar transformation assay (11) and BALB/c bone marrow cells, both A-MuLV-P90 and A-MuLV-P100 were much less efficient than the P120 and P160 strains (Table 1). Equal virus doses were used in these experiments, and the numbers of foci observed with A-MuLV-P90 and A-MuLV-P100 were 7- to 20-fold fewer than the number of foci observed with A-MuLV-P160 and 4- to 10-fold fewer than the number observed with A-MuLV-P120. No foci were observed in mock-infected or M-MuLV-infected cultures. Because the foci in A-MuLV-P90- and A-MuLV-P100-infected cultures were generally smaller than those in cultures infected with A-MuLV-P120 or A-MuLV-P160, all of the assay plates were refed at day 10 and rescored after an additional 7-day incubation to insure that any slowly growing foci in these cultures, which were too small to be observed after 10 days of incubation, were not missed. Although the sizes of the foci increased during this time, we observed no change in the total number of foci in any of the cultures.

Individual foci can be removed from agar and adapted to grow in liquid medium as continuous cells lines (11, 12). When a series of foci transformed by A-MuLV-P90 and A-MuLV-P160 were picked and adapted to grow in vitro, no differences in the plating efficiencies of the foci transformed by the two A-MuLV strains were observed and, as reported previously (15), all isolates synthesized the A-MuLV-specific protein of appropriate size (data not shown).

Tumor induction by the A-MuLV strains. The various A-MuLV strains were tested for the ability to induce Abelson disease in newborn mice. Several virus stocks were used, and in some cases both NIH/Swiss and BALB/c mice were examined. A-MuLV-P120 and A-MuLV-

TABLE 1. Transformation of lymphoid cells by A-MuLV strains^a

A-MuLV strain	Liquid transformation ^b				Agar transformation ^c		
	Expt 1		Expt 2		Expt 1	Expt 2	Expt 3
	Day 10	Day 17	Day 10	Day 17			
P160	5/5	5/5			36	19	22
P120	6/6	6/6	10/10	10/10	11	7	9
P100	0/4	0/4			2	1.5	
P90	0/4	3/4	0/10	4/10	3	3	0.9

^a Transformation was monitored by using liquid cultures and A-MuLV-P160 prepared on RAW309 Cr cells, A-MuLV-P120 prepared on ANN-1 cells rescued with 292 virus, and A-MuLV-P90 and A-MuLV-P100 prepared on transformed NIH/3T3 cells with 292 virus as a helper. The P160 and P120 strains had titers of 10^4 FFU/ml and 5×10^4 PFU/ml. The P100 and P90 strains had titers of 10^5 FFU/ml and 3×10^5 PFU/ml. For the agar assays, all virus stocks were prepared with M-MuLV clone 2 as a helper virus and had titers of 3×10^5 to 5×10^5 FFU/ml and 1×10^6 to 2×10^6 PFU/ml.

^b Liquid transformation was scored on days 10 and 17 postinfection as the number of transformed cultures/number of infected cultures. Cultures were infected with 1 ml of undiluted virus.

^c Agar transformation was scored as the number of lymphoid foci per 10^5 fibroblast FFU.

P160 were highly efficient in inducing Abelson disease (Table 2). The diseases in these cases were similar and were characterized by lymphadenopathy of peripheral lymph nodes and cranial tumors. The thymus was spared in all instances. This picture is typical of Abelson lymphoma when newborn animals are inoculated with virus, and the latent periods observed are consistent with those observed by us and others when these two mouse and virus strains have been used (1, 7, 10, 12, 18).

Both A-MuLV-P100 and A-MuLV-P90 were deficient in the ability to induce Abelson disease in newborn mice, even though very high titers of virus were used in our experiments (Table 2). A-MuLV-P100 was examined in NIH/Swiss mice, and none of the 13 animals tested developed Abelson disease. None of the six NIH/Swiss mice inoculated with A-MuLV-P90 developed Abelson disease either. A small number of BALB/c mice did develop Abelson disease when they were inoculated with A-MuLV-P90 after a fairly long latent period. In these cases the disease was similar to the typical Abelson disease pattern observed in adult BALB/c mice, a disease characterized by paralysis of the hind limbs, tumors of the lower vertebral column, and sparing of the thymus (10; Clark and Rosenberg, unpublished data).

A significant proportion of the animals inoculated with A-MuLV-P90 and A-MuLV-P100 that failed to develop Abelson disease did develop thymic tumors characteristic of M-MuLV-induced lymphomas when they were held for sufficient periods of time. The gross pathology of the disease in these animals, which was characterized by large thymic tumors, hepatosplenomegaly, and often large mesenteric tumors, was readily distinguishable from that of Abelson disease. The latent period for thymoma induction in these animals was somewhat longer than the latent period in animals injected with M-MuLV alone. The possible significance of this longer latent period is under study.

Synthesis of A-MuLV-specific proteins by tumor cells. Synthesis of A-MuLV and M-MuLV-encoded proteins by some of the tumors induced in mice was examined by an SDS-polyacrylamide gel analysis of [³⁵S]methionine-labeled immunoprecipitates. We examined Abelson tumors from nine mice injected with A-MuLV-P160 and six mice injected with A-MuLV-P120, as well as all three of the Abelson tumors observed in A-MuLV-P90-inoculated mice. In most cases, the tumors were labeled immediately after autopsy. However, no differences were observed when cultured tumor cells were tested. The labeling mixtures were ad-

TABLE 2. Disease induction by the A-MuLV strains^a

Virus strain	Titer inoculated ^b		Mouse strain	Disease			
	FFU	PFU		Abelson		Thymoma	
				MLP ^c	Incidence	MLP	Incidence
A-MuLV-P160	1 × 10 ²	7 × 10 ³	BALB/c	37	6/7 ^d		
A-MuLV-P120	2 × 10 ⁴	2 × 10 ⁵	BALB/c	21	7/7		
	7 × 10 ²	1 × 10 ⁴	NIH/Swiss	34	7/7		
A-MuLV-P100	2 × 10 ⁴	3 × 10 ⁴	NIH/Swiss		0/6 ^e		
	7 × 10 ³	5 × 10 ³	NIH/Swiss		0/7 ^f	142	5/7
A-MuLV-P90	4 × 10 ⁴	7 × 10 ⁴	NIH/Swiss		0/6	149	4/6
	8 × 10 ³	3 × 10 ⁴	BALB/c	54	1/6	101	5/6
	1 × 10 ⁴	7 × 10 ⁴	BALB/c	63	2/6	112 ^g	1/6
M-MuLV		3 × 10 ⁴	NIH/Swiss			92	6/6
		6 × 10 ³	NIH/Swiss			96	9/9
		3 × 10 ³	NIH/Swiss			125	10/10
		6 × 10 ²	NIH/Swiss			153	10/10

^a Newborn mice were inoculated with filtered virus stocks, and autopsies were performed when signs of disease were noted.

^b The A-MuLV component of the virus stock was assayed on NIH/3T3 cells and is given as FFU; the helper component of the virus stock was assayed on FG-10 cells and is given as PFU.

^c MLP, Mean latent period.

^d Number of animals with tumors/number of animals injected.

^e Animals held 75 days.

^f Animals held 260 days.

^g Experiment in progress. Animals were observed after 120 days.

justed to 2 mM EDTA and 5 mM phenylmethylsulfonyl fluoride just before cell lysis because mixing experiments showed that the A-MuLV-specific phosphoproteins were particularly sensitive to degradation in the presence of either normal or tumor-bearing bone marrow (data not shown). The addition of phenylmethylsulfonyl fluoride and EDTA to the cell extracts allowed detection of A-MuLV-specific proteins in all of the tumor cells examined. Once the tumor cells were adapted to *in vitro* growth, the addition of phenylmethylsulfonyl fluoride and EDTA to the lysis mixture was not required.

Analyses of the viral proteins in representative tumors are shown in Fig. 1. Extracts of tumors from mice injected with A-MuLV-P160, which were precipitated with anti-M-MuLV serum (Fig. 1, lanes 4 to 8), synthesized P160, and extracts of tumors from mice injected with A-MuLV-P120 (Fig. 1, lanes 9 to 12) synthesized P120. The shifts in the mobilities of *pr65^{gag}*, *pr80^{env}*, and *p30* in lanes 11 and 12 reflect the presence of the wild mouse virus 292, the helper virus present in the P120 stock used to induce these tumors. These altered mobilities are characteristic of 292 virus in the absence of A-MuLV (data not shown). An analysis of the viral pro-

teins in a representative A-MuLV-P90-induced tumor is shown in Fig. 1, lanes 13 through 15. Both anti-M-MuLV virion serum (lane 13) and anti-p15 serum (lane 14) precipitated P90, whereas anti-gp70 serum (lane 15) did not, which is consistent with the previously described characteristics of P90 (15).

Several of the thymomas arising in mice injected with A-MuLV-P100 and A-MuLV-P90 were also examined for viral proteins. None of the four tumors examined synthesized A-MuLV-specific protein, although all of them synthesized characteristic M-MuLV-encoded proteins. In addition, these cell lines failed to synthesize detectable amounts of virus that induced foci on NIH/3T3 cells (data not shown). The tumors did release large amounts of virus that produced plaques in the *S*⁺*L*⁻ assay. These results are in direct contrast to those obtained with the Abelson tumors, all of which released virus that did transform NIH/3T3 cells (data not shown).

P90 production by A-MuLV-transformed cells. The biological data show that A-MuLV-P90 and A-MuLV-P100 interact with lymphoid cells in an abnormal fashion. To determine whether these biological effects might correlate with changes in production and stability of the

FIG. 1. Analyses of viral proteins in tumors induced by the different A-MuLV strains. Tumors from animals with typical Abelson disease were labeled with [³⁵S]methionine (50 μ Ci/5 $\times$ 10⁶ cells) for 1 h at 37°C and extracted as previously described (20), except that the solutions were adjusted to 5 mM phenylmethylsulfonyl fluoride and 2 mM EDTA just before lysis; 2.5 $\times$ 10⁶ cells were immunoprecipitated with 5 μ l of goat anti-M-MuLV virion serum (lanes 1 to 13), 5 μ l of rabbit anti-p15 serum (lane 14), or 5 μ l of rabbit anti-gp70 serum (lane 15). Precipitates were collected by using *S. aureus*, denatured, and analyzed on 10% SDS-polyacrylamide gels; development was by fluorography (14, 20). The standards were A-MuLV-transformed lymphoid cell lines derived by *in vitro* infection with A-MuLV-P120 (lane 1), A-MuLV-P160 (lane 2), and A-MuLV-P90 (lane 3). The tumors were induced with A-MuLV-P160 (lanes 4 through 8), A-MuLV-P120 (lanes 9 through 12), A-MuLV-P90 (lanes 13 through 15).

A-MuLV-specific phosphoproteins, we compared the metabolism of P90 with that of P120 in a series of pulse-chase experiments. An example of such an experiment is shown in Fig. 2. The amount of P90 precipitated from a C57L-derived lymphoid cell clone labeled with a 20-min [35 S]methionine pulse (Fig. 2b, lane A) was two- to threefold higher than the amount of P120 (Fig. 2a, lane A) precipitated from a lymphoid cell clone derived from the same pool of C57L bone marrow cells. Both cell lines incorporated equivalent amounts of [35 S]methionine into trichloroacetic acid-precipitable protein (data not shown). In addition, the apparent stability of P90 was also reduced; it had a half-life of about 2 to 3 h (Fig. 2b, lanes B to D), compared with 5 to 7 h for P120 (Fig. 2a, lanes B to D). These data were confirmed in multiple experiments with several P120- and P90-synthesizing lymphoid clones (data not shown). No discrete cleavage products from either P90 or P120 were observed, even when an anti-Abelson tumor serum that recognized the A-MuLV-specific portion of these phosphoproteins was used (data not shown). P160 resembled P120 in its stability (data not shown).

Kinase activities of the A-MuLV-specific proteins. A property associated with P120 and P160 proteins is the ability of these molecules or molecules closely associated with them to transfer the γ phosphate of ATP to tyrosine residues on the P120 and P160 molecules (22; O. N. Witte, L. Sun, N. Rosenberg, and D. Baltimore, Cold

Spring Harbor Symp. Quant. Biol., in press). This activity can be measured by using an *in vitro* assay in which [3 H]leucine-labeled cells are extracted and immunoprecipitated with anti-M-MuLV virion serum. For these experiments, one-half of the sample is added to an *in vitro* reaction mixture containing [γ - 32 P]ATP, and after the reaction both portions of the sample are analyzed in SDS-polyacrylamide gels; the 32 P-labeled material is visualized by autoradiography, and the [3 H]leucine-labeled material is developed by fluorography.

To study the kinase activity associated with P90, [3 H]leucine-labeled extracts of NIH/3T3 fibroblast clones transformed by A-MuLV-P90, A-MuLV-P120, and A-MuLV-P160 were immunoprecipitated with anti-M-MuLV virion serum. These extracts were adjusted to contain about the same number of [3 H]leucine counts (Fig. 3, lanes A to C), one-half of the material was used in an *in vitro* kinase reaction, and then both sets of samples were analyzed on SDS-polyacrylamide gels. The amount of 32 P incorporation in the P90 sample was much lower (Fig. 3, lane D) than the amounts of incorporation in the P120

FIG. 2. Pulse-chase analyses of P120 and P90. Lymphoid cells transformed by A-MuLV-P120 (a) and A-MuLV-P90 (b) were labeled for 20 min with [35 S]methionine ($50 \mu\text{Ci}/5 \times 10^6$ cells) (lanes A) and then chased after the addition of 15 mg of cold methionine per liter for 1, 3, and 5 h (lanes B, C, and D, respectively); 2.5×10^6 cells were precipitated with goat anti-M-MuLV virion serum for each time point. The samples were analyzed as described in the legend to Fig. 1.

FIG. 3. Kinase activities of the A-MuLV-specific proteins. Cell lines were labeled with [3 H]leucine ($100 \mu\text{Ci}/5 \times 10^6$ cells), extracted, and immunoprecipitated with goat anti-M-MuLV virion serum. The samples were adjusted to contain about the same number of [3 H]leucine counts, and one-half of each sample was incubated with [γ - 32 P]ATP, as described in the text. All samples were analyzed on 10% SDS-polyacrylamide gels; the [3 H]leucine-labeled samples were developed by fluorography, and the 32 P-labeled samples were developed by autoradiography. [3 H]leucine-labeled extracts from cells expressing P90, P120, and P160 (lanes A to C) were used for the kinase reactions in lanes D to F. [3 H]leucine-labeled extracts from cells expressing P120 and P100 (lanes G and H) were used for the kinase reactions in lanes I and J.

and P160 samples (Fig. 3, lanes E and F, respectively). A similar experiment compared the kinase activities in equivalent amounts of [^3H]-leucine-labeled P120 and P100 (Fig. 3, lanes G and H, respectively). As with P90, the kinase activity associated with P100 (Fig. 3, lane J) was less than the activity associated with P120 (lane I). The lower-molecular-weight bands in Fig. 3, lanes I and J, represent ^{32}P -labeled fragments of P120 and P100 (Witte, unpublished data).

The transkinase assay, an assay that monitors the ability of A-MuLV-specific proteins to act as donors and acceptors of ^{32}P (Witte, Sun, Rosenberg, and Baltimore, in press), was also used to monitor the functional capabilities of P90 and P100. In this assay, an immunoprecipitate of one protein is heated and then added to an immunoprecipitate containing an A-MuLV-specific protein of a different size. The ability of the unheated protein to catalyze transfer of ^{32}P to the heated protein (an enzymatically inactive protein) is monitored. When this assay was used, both P100 and P90 were deficient in catalyzing the labeling of heated P160 and P120 as they were in the standard autokinase assay. However, both P100 and P90 were efficient acceptors of ^{32}P when heated immunoprecipitates of these proteins were mixed with active P120 or P160 (Witte, Sun, Rosenberg, and Baltimore, in press).

DISCUSSION

A-MuLV is unique among murine retroviruses in its ability to transform both fibroblasts and lymphoid cells stably in vitro. The only A-MuLV-encoded gene product that has been identified rigorously is a large phosphoprotein that mediates the transfer of the γ phosphate of ATP to tyrosine residues on this protein in vitro (22). This enzymatic activity is similar to an activity associated with Rous sarcoma virus-encoded pp60^{src} protein and polyoma middle T antigen (3, 4, 6, 16, 20).

The role of the A-MuLV-encoded phosphoproteins in transformation is strongly supported by the results presented here. The two virus strains that synthesize small proteins (A-MuLV-P100 and A-MuLV-P90) are deficient in the ability to induce lymphoid transformation in vitro. The number of foci observed in the agar assay is reduced, and the appearance of large numbers of transformed cells in infected liquid cultures is delayed. This delay probably reflects the time required for the few transformants in the cultures to grow to the number required for recognition of transformation. Once lymphoid cells are transformed by these viruses, they are similar to cells transformed by A-MuLV-P120

and A-MuLV-P160 with respect to expression of lymphoid differentiation markers (15) and with respect to growth in vitro (unpublished data). Thus, the block in efficient transformation by these virus stocks appears to lie in their ability to carry out the early steps in the transformation process.

The deficiency in the oncogenic potentials of A-MuLV-P90 and A-MuLV-P100 can also be detected in vivo. We observed no tumors induced by either of these viruses in NIH/Swiss mice, and a reduced number of tumors was observed in BALB/c mice inoculated with A-MuLV-P90. The difference in susceptibility between these two mouse strains may be related to the Av-1 and Av-2 genotypes of BALB/c mice (10). BALB/c mice and closely related strains of mice are susceptible to Abelson disease induction when they are inoculated as adults, whereas most other mouse strains, including NIH/Swiss, are resistant to A-MuLV when they are injected as adults (10). The pathology of Abelson disease noted in A-MuLV-P90-injected BALB/c mice is consistent with a late disease onset. Since NIH/Swiss mice resist Abelson disease when they are inoculated as adults, they would be expected to develop this type of tumor at a very low frequency. Thus, the P100 strain would be expected to induce Abelson disease at a low frequency in BALB/c mice. The observation that A-MuLV-P100 does induce some transformation of NIH/Swiss bone marrow cells in vitro (unpublished data) is also consistent with this hypothesis.

The deficiency in transformation potential is a stable property of these virus stocks. Virus rescued from lymphoid cells transformed by A-MuLV-P90 or from an A-MuLV-P90-induced tumor is also deficient in the bone marrow transformation assay (unpublished data), which excludes the possibility that lymphoid transformation selects for more oncogenic variants within the A-MuLV-P90 population.

The in vitro agar transformation experiments suggested that A-MuLV-P160 may be somewhat more oncogenic than A-MuLV-P120. The number of BALB/c-derived bone marrow foci was consistently higher in A-MuLV-P160-infected cultures. This result was not expected because A-MuLV-P120 has been used as the prototype oncogenic A-MuLV by several laboratories for some time. However, subtle differences in the abilities of A-MuLV-P160 and A-MuLV-P120 to transform BALB/c bone marrow cells are magnified when a more restrictive transformation system employing DBA/2 bone marrow cells is used. Bone marrow cells from this strain are quite resistant to in vitro transformation (11). When equivalent titers of A-MuLV-P160 and A-MuLV-P120 were compared in this system, 70%

of the cultures infected with the P160 strain were transformed, whereas transformation was observed in less than 5% of the A-MuLV-P120-infected cultures (L. Maker and N. Rosenberg, unpublished data). Thus, the additional 40,000 daltons in P160 appears to play an important role in transformation.

The ability of A-MuLV-P90 and A-MuLV-P100 to transform NIH/3T3 cells as efficiently as A-MuLV-P120 and A-MuLV-P160 suggests that the mechanisms for these two types of transformation are different. A conditional mutant of avian erythroblastosis virus that is deficient in erythroid but not fibroblast cell transformation has been reported (5). The helper virus present in A-MuLV stocks also affects lymphoid cell transformation but not fibroblast transformation (12, 17). Whether these differences in transformation are qualitative or quantitative remains to be determined. These comparisons all rely on measurements of infectivity as monitored by transformation. The ratio of A-MuLV RNA to M-MuLV RNA in A-MuLV-P120 stocks is similar to the ratio of infectious fibroblast-transforming virus to plaque-forming virus (A. Shields, N. Rosenberg, and D. Baltimore, unpublished data), but whether all A-MuLV strains are equally efficient in fibroblast transformation is unclear. Although we have no evidence to support the role of a second A-MuLV-encoded gene product in lymphoid or fibroblast transformation, this possibility cannot be excluded rigorously.

The metabolism of P90 and P100 is different than that of P120 and P160. The smaller proteins are less stable and are synthesized in larger amounts than P120 or P160. The large amounts of the smaller proteins synthesized in A-MuLV-transformed lymphoid cells may indicate that only clones arising from a cell-virus interaction that allows abnormally high expression of P90 or P100 go on to become transformed. Fibroblast cell lines, such as NIH/3T3, might not be as sensitive to levels of these phosphoproteins because these cells are already permanently growing. The A-MuLV-P90 clone used in the experiments described above contains a single copy of A-MuLV (S. Goff and D. Baltimore, personal communication), indicating that elevated P90 production does not result from expression of multiple A-MuLV genomes in these cells.

The small A-MuLV proteins also function poorly in protein kinase assays. This deficiency is manifested by a markedly reduced ability of the proteins to catalyze the transfer of [γ - 32 P]-ATP. The ability of the proteins to accept phosphate is not reduced. Thus, the ability to catalyze phosphate transfer is probably much more important in transformation than the ability to

accept phosphate on a tyrosine residue in the A-MuLV-specific protein. Furthermore, the separation of these two properties indicates that different portions of the molecule may be involved in these two functions.

The correlation of abnormal metabolism and reduced protein kinase function with reduced oncogenic potential supports a direct role for these proteins and their protein kinase functions in transformation. Further support for the interrelationship of these properties comes from the isolation of a transformation-defective A-MuLV that does not transform fibroblasts or lymphoid cells and lacks detectable protein kinase activity (Witte, Goff, Rosenberg, and Baltimore, in press).

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