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Abelson Murine Leukemia Virus Variants with Increased Oncogenic Potential

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A number of strains of Abelson murine leukemia virus (A-MuLV) with various abilities to transform cells have been identified. Among these is the A-MuLV-P90 strain, a mutant derived from A-MuLV-P120 that encodes an A-MuLV protein missing sequences that are normally present at the extreme carboxy terminus of P120 (N. Rosenberg and O. N. Witte, J. Virol. 33:340–348, 1980). This virus transforms NIH 3T3 cells efficiently but does not transform a high frequency of lymphoid cells in vitro or in vivo. In this communication, we show that of the relatively few tumors induced by A-MuLV-P90 nearly all contained new variant viruses that stably expressed either larger or smaller A-MuLV proteins. Strains that expressed larger A-MuLV proteins behaved like A-MuLV-P120 in transformation assays, whereas those expressing smaller A-MuLV proteins induced a high frequency of tumors after a short latent period in vivo but failed to transform large numbers of lymphoid cells in vitro. Thus, these latter viruses separated the requirements for in vitro transformation of lymphoid cells from those for tumor induction. All of the variants differed from A-MuLV-P90 in the carboxy-terminal region of the A-MuLV protein, suggesting that sequences in this region play a key role in the ability of the virus to interact with hematopoietic cells in vivo and in vitro.

Abelson murine leukemia virus (A-MuLV) is a replication-defective retrovirus that arose by recombination of Moloney murine leukemia virus (M-MuLV) and the cellular c-*abl* proto-oncogene (6). As a consequence of the recombination event, A-MuLV acquired a unique set of biological properties, including the ability to transform both NIH 3T3 cells and pre-B lymphocytes in vitro and to induce a rapidly appearing lymphosarcoma in vivo (19).

A-MuLV encodes a single fusion protein composed of M-MuLV-derived *gag* gene determinants and v-*abl*-encoded determinants (18, 36). The molecule is a tyrosine protein kinase that phosphorylates both itself and other tyrosine-containing proteins in vivo (4, 26, 32, 34). Two strains of A-MuLV, A-MuLV-P160 and A-MuLV-P120, that encode A-MuLV proteins of 160,000 and 120,000 M_w, respectively, have been identified (24). Although A-MuLV-P120 has a 789-base in-frame deletion in v-*abl* (17, 30), both of these strains are considered wild type because of their high oncogenic potential (23).

The A-MuLV protein has been divided into four regions: I, the *gag*-derived portion; II, the kinase region; III, the A-MuLV-P160-specific region; and IV, the carboxy-terminal portion of the molecule (38). Analysis of a series of spontaneous and genetically engineered mutants has indicated that sequences in regions I, II, and IV play an important role in transformation of at least some cell types. For example, region II, which shares homology with other tyrosine kinases (1, 17), is required for all of the transforming functions of A-MuLV (13, 33), and sequences near the amino terminus of region I are required for lymphoid cell transformation in vivo and in vitro (14, 15). An intact region IV is associated with a lethal function of the virus (7, 29, 38) and with high-efficiency transformation of lymphoid cells in vivo and in vitro (14, 15, 23, 28).

The mechanism by which region IV mediates lethality and lymphoid cell transformation has yet to be fully elucidated. Examination of A-MuLV-P90, a region IV mutant derived from A-MuLV-P120 (24), shows that this virus transforms 5- to 10-fold fewer bone marrow cells than does A-MuLV-P120 and that it induces lymphomas at a low frequency after an extended latent period. P90 is less stable than P120 in at least some lymphoid cells and has a reduced activity in autophosphorylation assays (23). In addition, this molecule lacks one serine phosphopeptide present in both P160 and P120 (12). Taken together, these data suggest that impaired enzymatic activity or stability (or both) of the transforming protein may account for the biology of this virus strain.

As part of our effort to understand the role of region IV in lymphoid cell transformation in vivo and in vitro, we sought to derive viruses from A-MuLV-P90 that had regained high oncogenic potential. Because a strong selection for oncogenic viruses occurs in the course of tumor induction, we examined the viruses present in tumors arising in a series of A-MuLV-P90-injected mice. Analysis of the A-MuLV proteins present in these tumors revealed that the majority of them synthesized A-MuLV proteins that were either smaller or larger than P90 and that these changes were a stable property of the virus strain associated with the tumor. All of the new virus strains were similar to the wild-type parent of A-MuLV-P90, A-MuLV-P120, in their ability to induce tumors at high frequency after a short latent period. In addition, all of these strains were stable in vivo. However, only strains which encoded A-MuLV proteins larger than P90 transformed lymphoid cells in vitro at high efficiency, separating the properties required to induce tumors in vivo from those required to transform lymphoid cells in vitro. All of the changes in the A-MuLV proteins encoded by the variants involve region IV, indicating that changes in this region have a significant impact on the biology of the virus.

MATERIALS AND METHODS

Virus preparation. A-MuLV-P120 (25), A-MuLV-P160 (24), and A-MuLV-P90 (24) were prepared from clonally derived NIH 3T3 transformed virus nonproducer cell lines

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that were superinfected with M-MuLV-C12 (5). All virus stocks were prepared from 24-h culture fluid and filtered through 0.45-μm-pore-size filters (Nalgene Labware Div., Nalge/Sybron Corp.) before use. The titer of A-MuLV was determined by using the NIH 3T3 transformation assay (25), and the titer of M-MuLV-C12 was determined by using the FG-10 assay (2).

To prepare transformed NIH 3T3 cell cultures with tumor cell culture fluid, NIH 3T3 cells were plated at 10^5 cells per 60-mm-diameter petri dish and infected with 0.5 ml of 0.45-μm-pore-size-filtered culture fluid supplemented to contain 8 μg of Polybrene per ml. After a 1.5-h adsorption period, the cells were fed with fresh Dulbecco modified Eagle medium supplemented to contain 10% heat-inactivated (56°C, 30 min) fetal calf serum (DME-10) and were monitored for the appearance of morphologic transformation. Areas of transformed cells were usually visible at 48 to 96 h postinfection.

Virus nonproducer cell clones were prepared by infecting NIH 3T3 as described above, except that a multiplicity of infection of 0.3 focus-forming units (FFU) per cell was used. After the virus adsorption period, the cells were trypsinized and seeded in agar medium at a density such that fewer than 20 colonies appeared in each 60-mm-diameter culture dish. The medium consisted of DME-10 in a final concentration of 0.3% agar. The cells were plated over a base layer of DME-10 in 0.6% agar. Well-isolated colonies were picked 10 to 15 days after plating and were examined for the presence of virus. Virus nonproducer cultures were identified as morphologically transformed cells that did not produce infectious virus. The presence of A-MuLV protein and the absence of M-MuLV-encoded proteins in each of the cell clones were confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of [^{35}S]methionine-labeled cell extracts immunoprecipitated with antibodies directed against M-MuLV-encoded *gag* gene products.

Oncogenicity testing. The ability of the various virus stocks to transform lymphoid cells in vitro was examined by using both the agar transformation assay and liquid cultures of bone marrow cells as previously described (20, 22). In the agar assay, the number of colonies obtained with each of the virus stocks was linear with respect to virus dilution.

To examine the oncogenicity of the viruses in vivo, BALB/c mice from our colony at Tufts were injected via an intraperitoneal route with 0.1 ml of 0.45-μm-pore-size-filtered virus stock containing approximately 10^5 FFU/ml and 10^6 PFU of M-MuLV per ml. Animals were monitored daily for tumor development and sacrificed when signs of disease such as lymphadenopathy, cranial tumor, or hind-limb paralysis were evident. Animals that did not develop disease were sacrificed and autopsied 90 days after virus injection. Diagnosis of disease was based on gross pathological examination and the presence of A-MuLV protein in the tumor cells.

Tumor cell culture. Bone marrow from the femurs and tibias of diseased mice was harvested by flushing the cells from the bones with a needle and syringe. Single-cell suspensions were cultured in 35-mm-diameter dishes at a density of 10^6 cells per ml in RPMI 1640 supplemented to contain 20% heat-inactivated fetal calf serum and 50 μM 2-mercaptoethanol (RPMI medium). The size of the A-MuLV protein expressed by the cells was analyzed usually within several days. Cell lines were established from most of the tumor cultures by using the techniques described for establishing cell lines from in vitro-transformed bone marrow cells (20). The size of the A-MuLV protein expressed by the tumor cell lines remained stable on reanalysis.

Labeling and immunoprecipitation. The techniques used for metabolic labeling with [^{35}S]methionine and in vitro autophosphorylation with [^{32}P]ATP have been described previously (23, 32). For size determination, A-MuLV proteins were precipitated by using either goat anti-M-MuLV virion serum (23) or a monoclonal antibody directed against the M-MuLV-encoded p15 protein (L. Schiff-Maker and N. Rosenberg, submitted for publication), and the immune precipitates were analyzed on 10% SDS-polyacrylamide gels. The panel of v-*abl*-specific antibodies used to map regions of the A-MuLV protein were prepared in rabbits immunized with fragments of the A-MuLV protein fused to *trpE* or peptides homologous to portions of the A-MuLV protein and have been previously described (11).

For partial proteolytic peptide mapping, a modification of the procedure of Cleveland et al. (3) was used (34, 37). Briefly, immune complexes were recovered from *Staphylococcus aureus* by heating in 1% SDS–1% 2-mercaptoethanol–50 mM Tris (pH 6.8). A portion of each sample was mixed with either 10 μg of *S. aureus* V8 protease per ml or an equal amount of 50 mM Tris (pH 6.8) and was reacted for 30 min at 37°C. The reaction was stopped by adding an equal volume of 2% SDS–2% 2-mercaptoethanol and heating the mixture at 70°C for 10 min. The samples were analyzed on 10% SDS-polyacrylamide gels. Alternatively, the preparations were suspended in immunoprecipitation buffer, and fragments were immunoprecipitated with antibodies directed against M-MuLV- and v-*abl*-derived portions of the molecule.

RESULTS

Variants are generated by A-MuLV-P90 in vivo. Because we had previously demonstrated that A-MuLV-P90 is a weakly oncogenic virus strain (23), we undertook a systematic study to examine the virus recovered from tumors arising in mice injected with A-MuLV-P90 to determine whether animal passage would select variants with enhanced oncogenic potential. Animals injected with the two wild-type strains, A-MuLV-P160 and A-MuLV-P120, served as controls. All animals were injected at less than 48 h of age and monitored daily for signs of disease such as enlarged lymph nodes, splenomegaly, paraplegia, or cachexia. As expected, animals injected with each of the viruses developed tumors; however, all of the animals injected with A-MuLV-P160 and A-MuLV-P120 developed disease after a 3- to 4-week latent period, whereas only 62% (15 of 24) of the animals injected with A-MuLV-P90 developed disease during the 90-day observation period. At autopsy, the gross pathology was similar in all of the diseased animals and typical for A-MuLV disease (19). Enlarged peripheral lymph nodes and mild splenomegaly were evident, and the thymus appeared normal. Animals developing tumors relatively soon after injection often had evidence of cranial tumors, whereas those developing disease later often had spinal tumors with accompanying paralysis of the hind limbs.

Bone marrow was removed from each of the diseased animals and cultured as a representative sample of the tumor tissue. This tissue is the primary site of A-MuLV lymphoma and is predominantly composed of tumor cells in the late stages of the disease (19). After several days, a sample of each of the cultures was labeled with [^{35}S]methionine, extracted, and immunoprecipitated with a monoclonal antibody directed against the p15 *gag* protein (Schiff-Maker and

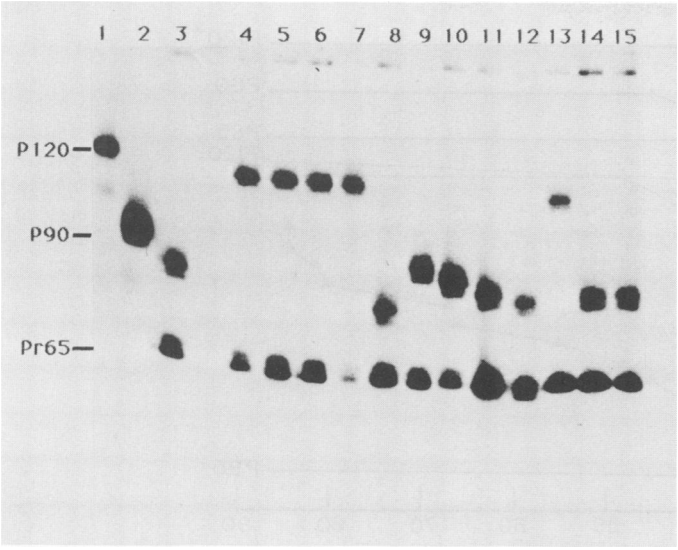

FIG. 1. A-MuLV proteins synthesized by tumors arising in A-MuLV-P120- and A-MuLV-P90-injected mice. Bone marrow cells derived from animals infected with A-MuLV were labeled with [^{35}S]methionine for 1 h. Extracts were clarified and immunoprecipitated with an anti-p15 monoclonal antibody (21; Schiff-Maker and Rosenberg, submitted). The immune complexes were collected by using IgSorb, washed, and analyzed on a 10% SDS-polyacrylamide gel. Extracts from the A-MuLV-P120-transformed lymphoid cell line 2M3 (21) (lane 1) and the A-MuLV-P90-transformed cell line 298-8 (Rosenberg, unpublished data) (lane 2) served as standards. Extracts from tumor cells recovered from A-MuLV-P90-infected mice are shown in lanes 3 and 8 to 15. Extracts from tumor cells recovered from A-MuLV-P120-infected mice are shown in lanes 4 to 7.

Rosenberg, submitted). This antibody precipitates the A-MuLV protein via the *gag*-derived sequences in the amino-terminal portion of the molecule (36). The immunoprecipitates were analyzed by SDS-PAGE. Each of the tumors induced with A-MuLV-P120 (Fig. 1, lanes 4 to 7) synthesized both the M-MuLV-encoded Pr65 *gag* precursor and a protein that comigrated with authentic P120 synthesized by a standard transformed virus nonproducer cell line (lane 1). However, none of the tumor cells shown in this representative analysis from the A-MuLV-P90-injected mice synthesized a protein that comigrated with the P90 protein synthesized by a standard A-MuLV-P90-transformed virus nonproducer cell clone (lane 2). Each of these cell extracts contained a molecule immunoprecipitated by the antibody that was either smaller (lanes 3, 8 to 12, 14, and 15) or larger (lane 13) than P90. As expected, all of these tumor cells also synthesized the M-MuLV-encoded Pr65 *gag* precursor. Similar results were observed when tumor tissue was labeled immediately after removal from the animal (data not shown).

Additional immunoprecipitation analyses demonstrated that the new proteins observed in the tumor cell cultures derived from A-MuLV-P90-injected mice could be precipitated by antisera directed against the *gag* gene proteins p15, p12, and p30 and by anti-Ab-T serum, an antiserum containing antibodies against v-*abl* determinants (35) but not by antisera directed against p10 *gag* or gp70 *env* (data not shown). Thus, the new proteins had the appropriate precipitation characteristics for A-MuLV proteins. Analysis of 14 tumors from animals injected with A-MuLV-P90 revealed that only two animals still synthesized an A-MuLV protein that comigrated with the P90 molecule immunoprecipitated from extracts of A-MuLV-P90-transformed cells. Most of the tumors (9 of 14) synthesized A-MuLV proteins smaller than P90; proteins of 85,000 M_w were observed most frequently. The proteins synthesized included P80 (1 tumor), P83 (1), P85 (6), P87 (1), P90 (2), P118 (1), and P120 (2). The size of the A-MuLV proteins was estimated based on the migration of standard marker proteins. In contrast, all of the tumors induced by A-MuLV-P120 (20 of 20) and A-MuLV-P160 (17 of 17) expressed P120 and P160, respectively.

The virus harvested from the tumor cultures was capable of inducing transformation of NIH 3T3 cells, and these transformed cells synthesized A-MuLV proteins that were the same size as those found in the tumor cell cultures. A total of 31 clonally derived NIH 3T3 transformed nonproducer cell lines were generated from 6 representative variants. In 30 of these, the clones synthesized a protein that comigrated with that synthesized by the tumor cell culture from which the virus was originally derived (data not shown). In one instance, whereas six of seven nonproducer clones synthesized the P83 protein characteristic of the original tumor cell culture, clone 7 synthesized a protein that comigrated with P85 proteins synthesizd by other variants. Although this variant was not noted in the original tumor, several tumors analyzed in other experiments have contained more than one variant, indicating that the variants may arise at a high frequency. Thus, the altered A-MuLV proteins observed in the tumor cells are a stable property of the viruses in the tumors. Each of these new variants was designated according to the size of the A-MuLV protein produced. Independent isolates encoding proteins indistinguishable in size on SDS-PAGE analysis were designated both by size and by a letter.

The new variants are highly oncogenic in vivo. Four of the smaller variants (A-MuLV-P80, A-MuLV-P83, A-MuLV-P85A, A-MuLV-P85B) and one of the larger variants (A-MuLV-P120*A) were selected for further study. One of the NIH 3T3 nonproducer cell cultures transformed with each of these viruses was superinfected with M-MuLV-C12 to prepare virus stocks, and neonatal BALB/c mice were injected with approximately 10^4 FFU of each of the variant viruses. Animals were killed and autopsied when signs of disease were evident. In all cases, the gross pathology of the disease was typical of A-MuLV lymphoma and was indistinguishable among the various isolates. Mortality curves (Fig. 2) illustrate that each of the variant viruses induced disease at a high frequency after a short latent period. In fact, in this particular trial several of the variant viruses induced a higher frequency of disease than did the A-MuLV-P120 control. For comparison, two mortality curves generated in the A-MuLV-P90 experiment from which the variants were isolated are also illustrated. The curve labeled P90 represents the two animals in this experiment whose tumors still synthesized P90, and the unlabeled curve includes all of the animals analyzed in that experiment. Thus, not only did all of the variants induce a higher frequency of tumors after a shorter latent period than their A-MuLV-P90 parent, but the tumors arose more rapidly than those from which the viruses were originally isolated. This difference probably indicates that time was required to generate the variant viruses in the original animals from which they were isolated. Animals injected with a virus stock prepared from tumor cells that still synthesized P90 developed tumors at the same frequency and after a similar latent period as those injected with A-MuLV-P90 (data not shown), suggesting that this virus retained the properties of A-MuLV-P90.

Cell cultures were established from each of the tumors, and the size of the A-MuLV protein synthesized by the

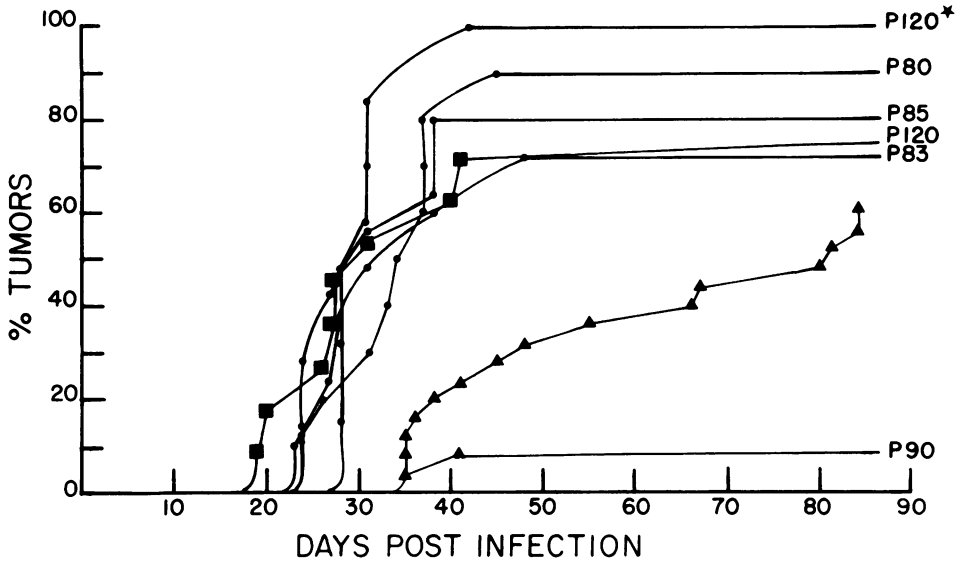

FIG. 2. Induction of A-MuLV disease by A-MuLV variants. Neonatal BALB/c mice were injected via an intraperitoneal route with approximately 10^4 FFU of the various viruses and were sacrificed as soon as tumors developed. Diagnosis of A-MuLV disease was based on the presence of enlarged peripheral lymph nodes, spinal or cranial tumors, a normal thymus, and the presence of A-MuLV protein in the tumor cells. Each point represents a single animal.

tumor cells was analyzed by immunoprecipitation and SDS-PAGE. In contrast to the results obtained with A-MuLV-P90, all of the tumors induced by the new variants synthesized A-MuLV proteins that were identical in size to those synthesized by the virus injected into the mice (Table 1). Of the four tumors from animals injected with A-MuLV-P90 recovered from a mouse tumor, two no longer synthesized P90, further supporting the notion that the A-MuLV-P90 found after initial injection of A-MuLV-P90 is similar to the parental virus.

A-MuLV-P90 does not generate variants upon in vitro passage. The appearance of variants within the clonally derived A-MuLV-P90 stock could relate to its reduced ability to transform lymphoid cells in vivo and in vitro (23) or could reflect selective pressures that operate on the virus in vivo. To distinguish between these two possibilities, we infected bone marrow cells in vitro with A-MuLV-P90, A-MuLV-P120, and variant virus A-MuLV-P85A and plated the cells in the standard agar transformation assay (20). At 9 to 10 days after plating, individual colonies were removed from agar and lysed in immunoprecipitation buffer. After clarification, the extracts were immunoprecipitated, and the immune complexes were reacted with [^{32}P]ATP by using the standard kinase assay (32). The size of the labeled proteins was analyzed on SDS-polyacrylamide gels and compared with that of A-MuLV proteins synthesized by a set of standard cell lines. Analysis of 65 A-MuLV-P90-induced colonies as well as 50 P120- and 42 P85A-induced colonies revealed that all of these synthesized proteins of the same size as the virus that induced the colony (Table 1). Thus, variant viruses are not generated at a high frequency simply by the process of lymphoid cell transformation.

TABLE 1. Stability of A-MuLV strains

Virus strain	No. of cell lines with parental protein size/no. examined	
	Derived in vivo	Derived in vitro
P160	17/17	60/60
P120	20/20	50/50
P90	2/15	65/65
P85A	24/24	42/42
P85B	4/4	NT[a]
P87	10/10	NT
P80	13/13	NT
P118	7/7	NT
P120*A	4/4	NT

[a] NT, Not tested.

The process of generating variant viruses may require extensive virus replication and spread, features that are important in disease induction in vivo. Because the agar transformation assay yields colonies that usually arise as a result of a single virus-cell interaction, we passaged virus through lymphoid cells in vitro in an effort to mimic in vivo conditions and examined the size of the A-MuLV protein at each passage. For these experiments, a series of five bone marrow cultures were infected with A-MuLV-P120, A-MuLV-P90, and A-MuLV-P85A, and the culture fluid from each of these cultures was harvested, filtered, and passaged to fresh bone marrow cells 10 days later, and then passaged to a third set of cultures 15 days later. At each passage, the cells in individual transformed cultures were extracted, and immunoprecipitates were analyzed by using the standard kinase assay (32). SDS-PAGE analysis revealed that each of the cultures synthesized A-MuLV proteins that were identical to those of the original infecting virus strain. Thus, the ability to generate variants appears to be an in vivo phenomenon.

The new variants do not transform lymphoid cells in vitro at high efficiency. A-MuLV-P90 induces 5- to 10-fold fewer lymphoid transformants in the standard agar assay than expected, based on the fibroblast focus-forming titer of the virus (23). To determine whether the variants generated from A-MuLV-P90 in vivo had an enhanced ability to interact with lymphoid cells in vitro, we infected bone marrow cultures with representative variants and evaluated the cells by using the agar transformation assay (20). Both A-MuLV-

P120* and A-MuLV-P118 induced large numbers of colonies, with the A-MuLV-P118 strain inducing a significantly higher frequency of colonies than the A-MuLV-P120 strain (Table 2). The P118 strain has a small deletion in the 3′ portion of v-*abl* (R. Huebner and N. Rosenberg, unpublished data), and experiments are in progress to determine whether this deletion is responsible for the enhanced transforming potential of this virus. The three smaller variants behaved like their immediate parent, A-MuLV-P90. Preliminary results indicate that other smaller variants behave similarly. Consistent with these data, the smaller variants transformed fewer cultures of bone marrow cells when assayed in the mass culture system (22) and induced fewer erythroid colonies when early-gestation fetal liver cells were infected (S. Ratnofsky, L. Keyes, and N. Rosenberg, unpublished data).

The variants differ from P90 in the v-*abl*-encoded portion of the A-MuLV protein. As described earlier, initial immunoprecipitation analyses indicated that the A-MuLV proteins encoded by the variant viruses showed the expected reactivity with various antiviral antibodies. To explore further the nature of the changes in these proteins, we analyzed the proteins after limited proteolysis with *S. aureus* V8 protease. Since a primary cleavage site for this enzyme exists in the amino-terminal portion of the v-*abl*-encoded sequences (34), this analysis distinguishes between changes that occur in the amino- and carboxy-terminal portions of the molecule. For this study, [^{35}S]methionine-labeled extracts prepared from NIH 3T3 transformed virus nonproducer cell clones were immunoprecipitated with goat anti-M-MuLV antiserum. The immunoprecipitates were collected on *S. aureus*, eluted by heating, and reacted with V8 protease or treated with control buffer. The reaction was stopped, and the products were analyzed by SDS-PAGE (Fig. 3). After digestion, all of the variants (lanes 4, 8, and 10) shared a common set of fragments of 45,000 M_w with both P120 (lane 2) and P90 (lane 6) previously shown to contain *gag*-derived determinants (34). Analysis of A-MuLV-P120*A (lane 4) revealed a second major fragment of 75,000 M_w which comigrated with the 75,000 M_w fragment containing v-*abl*-derived determinants observed after treatment of P120 (lane 2; 34). As observed in our previous study (34), the incubation in control buffer generated a set of degradation products from each of the proteins (odd-numbered lanes). The larger products, migrating slightly slower than the F75 v-*abl* fragment (lanes 2 and 4) and decreasing in size for the smaller proteins (lanes 5, 7, and 9), reflect the differences in v-*abl*-encoded determinants in the various proteins. Digestion of immunoprecipitates from two smaller variants, A-MuLV-P87 and A-MuLV-P85 (lanes 8 and 10), revealed the presence of unique v-*abl* fragments smaller than the 45,000 M_w v-*abl* fragment from A-MuLV-P90 (lane 6). The identification of the various fragments was confirmed by reprecipitation of the extracts after digestion (data not shown). These analyses confirm that the changes in size observed map to the v-*abl*-encoded portion of the molecule.

TABLE 2. In vitro transformation of lymphoid cells by variants

Virus strain	Foci/10^5 fibroblast FFU	
	Expt 1	Expt 2
P120	103	63
P90	11	10
P120*	217	104
P118	950	705
P85A	7	5
P85B	34	9
P80	8	4

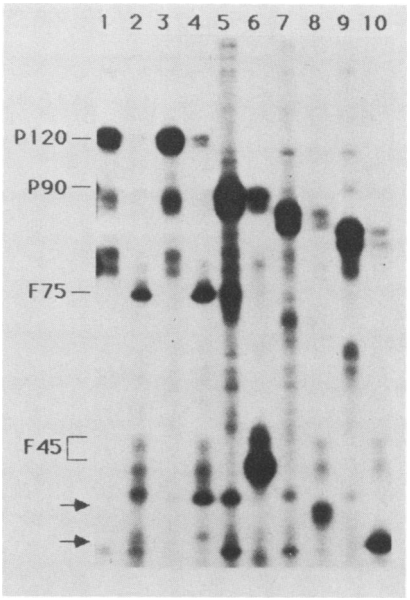

FIG. 3. *S. aureus* V8 mapping of the A-MuLV proteins encoded by the A-MuLV variants. Transformed virus nonproducer cell clones were labeled with [^{35}S]methionine for 1 h, extracted, clarified, and immunoprecipitated (23). The immune complexes were collected by using IgSorb, washed, and suspended in a Tris buffer containing 100 μg of *S. aureus* V8 protease per ml (even-numbered lanes) or buffer alone (odd-numbered lanes) and reacted for 30 min at 37°C (3, 31, 34). The reaction products were analyzed on a 10% SDS-polyacrylamide gel. Cells transformed by A-MuLV-P120 (lanes 1 and 2), A-MuLV-P120*A (lanes 3 and 4), A-MuLV-P90 (lanes 5 and 6), A-MuLV-P87 (lanes 7 and 8), and A-MuLV-P85A (lanes 9 and 10) were analyzed. Arrows indicate the altered carboxy-terminal fragments present in lanes 8 and 10.

A series of rabbit antibodies directed against specific regions of v-*abl* were also used to analyze the molecules encoded by the variant viruses. These antibodies, prepared against a series of synthetic peptides and *TrpE*-v-*abl* fusion proteins, are specific for the kinase region (anti-pEX-2), the carboxy-terminal 101 amino acids of region IV (anti-pEX-5), a portion of region III plus 42 amino acids at the region III-IV border (anti-pEX-4), and the A-MuLV-P160-unique region (anti-peptide 3) (11). An anti-p15 monoclonal antibody (Schiff-Maker and Rosenberg, submitted) that reacts with all of the A-MuLV proteins under study was also used. As expected, all of the antibodies precipitated P160 (Fig. 4, panel A), whereas all but the anti-peptide 3 antibody (panel B, lane 5) directed against P160-specific sequences precipitated P120 (panel B). Antibodies directed against the P160-specific region (lane 5) and the carboxy-terminal 101 amino acids shared by P120 and P160 (lane 4) did not precipitate P90 (panel C). Both P85 (panel E) and P80 (panel F) are similar to P90 in that each of these molecules was precipitated only by antibodies directed against the *gag* domain (lanes 1), the kinase domain (lanes 2), and the portion of the molecule proximal to the kinase domain (lanes 3). The absence of precipitation with the antibodies directed against the A-MuLV-P160-unique portion of the molecule (lanes 5) indicates that the viruses did not acquire these sequences in vivo. Furthermore, the absence of precipitation with the anti-pEX-5 reagent (lanes 4) indicates that these proteins lack all or most of the carboxy-terminal 101 amino acids of

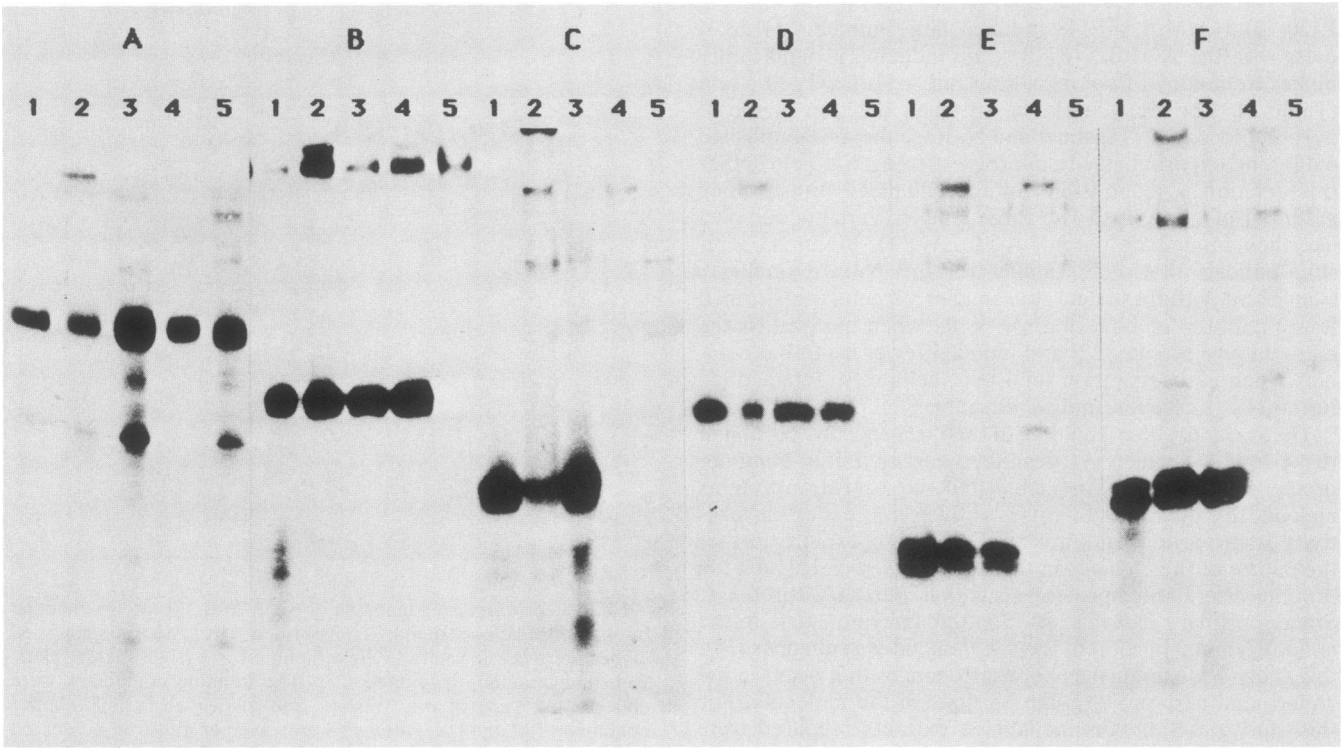

FIG. 4. Precipitation of A-MuLV proteins with site-directed antibodies. Transformed virus nonproducer cell lines were labeled for 1 h with [^{35}S]methionine and processed for immunoprecipitation (23). A monoclonal antibody directed against P15 *gag* determinants (lanes 1) (Schiff-Maker and Rosenberg, submitted), and polyclonal rabbit antisera directed against determinants in region II (lanes 2), determinants at the extreme amino-terminal portion of region IV (lanes 3), determinants at the extreme carboxy-terminal portion of region IV (lanes 4), and determinants in region III (lanes 5) (10) were used for immunoprecipitation. Cells transformed by A-MuLV-P160 (A), A-MuLV-P120 (B), A-MuLV-P90 (C), A-MuLV-P120*A (D), A-MuLV-P80 (E), and A-MuLV-P87 (F) were analyzed.

region IV but retain some or all of the 42 amino acids at the amino-terminal end of region IV. The protein encoded by A-MuLV-P120* (panel D) could not be distinguished from authentic A-MuLV-P120 (panel B), suggesting that the increase in size observed in this variant involves expression of determinants normally found at the carboxy terminus of the wild-type strain.

DISCUSSION

The rationale behind these experiments was that the strong selective pressure of tumor formation might allow the identification of a virus with enhanced oncogenic potential. By selection for transformation, new transductions of portions of c-*onc* genes in MC29 virus (16) and transformation-defective Rous sarcoma virus (10) have been observed. In both of these instances, the starting viruses contained deletions, and in the case of Rous sarcoma virus, analysis of a series of deletion mutants has shown that a small portion of the 3′ domain of v-*src* is required for transduction to occur (9, 27). In our case, the starting virus has the same genome size as A-MuLV-P120 (Huebner and Rosenberg, unpublished data), indicating that the premature termination of the A-MuLV protein probably results from a point mutation or a small deletion. Thus, recombination with c-*abl* was not expected, and the high frequency and variety of new strains obtained was not anticipated.

Although the A-MuLV-P90 strain studied here arose from A-MuLV-P120 during in vitro passage and several other independent isolates of A-MuLV-P90 have been observed (8), wild-type strains of A-MuLV seem fairly stable in vivo and in vitro. One group (29) has reported difficulty in recovering viruses generated from A-MuLV-P160 that are deleted near the *Sal*I site at base 3071, and a second group (14) has been unable to recover viruses with deletions in the *gag* portion of the A-MuLV-P160 genome that retain complete v-*abl* genes. In each of these cases, viruses were found with deletions in sequences encoding parts of region IV. Both of these groups were using an NIH 3T3 cell transfection system to recover the viruses, and they have suggested that a lethal function associated with region IV (29, 38) is responsible for this problem. Similar instability was not found in our analysis of lymphoid cells infected with A-MuLV-160 and A-MuLV-P120 in vivo or in vitro. This fact, coupled with the observation that a series of weakly transforming A-MuLV strains truncated in region III (15) do not generate variants in vivo (unpublished data) and the fact that the smaller variants generated from A-MuLV-P90 are stable in vivo, argues that the tendency of A-MuLV-P90 to generate variants in vivo seems somewhat unique.

The A-MuLV-P90 stocks used in these experiments were prepared from a clonal NIH 3T3 transformed virus nonproducer cell line that synthesizes only P90, and infectious virus recovered from a molecular clone of A-MuLV-P90 gives rise to variants in vivo (Huebner and Rosenberg, unpublished data). These data strongly suggest that the variants are generated in the animals. In vivo growth may favor these variants because a large number of rounds of replication occur in the mice or because A-MuLV-P90 is extremely defective in its ability to transform cells in vivo. Several tumors in A-MuLV-P90-injected mice have synthesized P90 and passage of virus from one of these resulted in

tumors containing variants. Thus, that original tumor was probably induced by A-MuLV-P90, indicating that cells infected with A-MuLV-P90 in vivo can occasionally give rise to tumors. Some of the viruses from the other tumors that synthesize 90,000 M_w A-MuLV proteins may be variants with changes too subtle to be distinguished by SDS-PAGE analysis.

Although A-MuLV-P90 transforms a lower frequency of lymphoid cells in vitro, the transformed cells isolated in this way all synthesize P90. The observation that three in vitro-derived lymphoid cell lines transformed by A-MuLV-P90 can form tumors that still synthesize P90 (data not shown) indicates that, once transformed, cells expressing P90 can grow in vivo. However, because in vitro-derived A-MuLV-P90-infected transformants are difficult to adapt to culture and are weakly tumorigenic early in their passage history (unpublished data), changes secondary to virus infection may be required for a fully transformed state. Similar conclusions have been reached with more stringent in vitro growth conditions and wild-type A-MuLV (31).

Two types of variants have been generated from A-MuLV-P90, both of which have changes in region IV of the A-MuLV protein. All of the variants grow to equal titer in vitro, as do both A-MuLV-P90 and A-MuLV-P120 (23; data not shown), suggesting that the change in the carboxy-terminal portion of the protein may be the only change important in the biology of the variants. The first type encodes A-MuLV proteins that are significantly larger than P90 and behave like A-MuLV-P120 in transformation assays. The A-MuLV proteins that are encoded by the two variants of this type can be precipitated by polyclonal antibodies directed against determinants at the extreme carboxy terminus of wild-type P120. Thus, it is likely that these viruses use most of the same coding sequences as A-MuLV-P120. The genomes of the larger variants are the same size as that of A-MuLV-P120 (Huebner and Rosenberg, unpublished data), and therefore these variants probably arose by repair of the mutation in A-MuLV-P90 rather than by recombination with c-*abl*. The ability to recover variants similar to those described here from animals injected with virus recovered from a molecular clone of A-MuLV-P90 (Huebner and Rosenberg, unpublished data) argues that the A-MuLV-P120* isolates recovered here did not arise from contaminating A-MuLV-P120.

The second type of variant generated is unique in that these viruses induce a high frequency of tumors but transform a low frequency of lymphoid cells in vitro. Each of these viruses encodes an A-MuLV protein that is smaller than that of its immediate parent, A-MuLV-P90. Both the proteolysis experiment and the immunoprecipitation data suggest that the A-MuLV proteins encoded by these variants have lost determinants normally present at the carboxy terminus of P90. Because this region of the protein is not glycosylated (29) and because other modifications that would affect the mobility of the protein in SDS-polyacrylamide gels have not been reported to occur in this region of the genome, it seems likely that the changes in size observed reflect changes in the primary structure of the protein.

The smaller variants are the first viruses that separate the ability of an A-MuLV strain to transform hematopoietic cells in vitro from the ability of the virus to induce lymphoma in vivo. Because multiple, distinct variants can be isolated, it seems likely that loss of specific sequences rather than retention of a particular set of sequences is important in the derivation of the variants. This model predicts that the sequences at the extreme carboxy terminus of P90 are detrimental to the function of the virus in vivo. Significant differences in the ability of the various A-MuLV proteins to phosphorylate angiotensin in an immune complex assay have not been detected (L. Keyes and N. Rosenberg, unpublished data) suggesting the gross changes in protein kinase activity are not involved. Experiments are in progress to determine whether cells infected with the variants are less immunogenic than cells infected with either A-MuLV-P120 or A-MuLV-P90. Such a model would be consistent with the impaired in vitro transformation potential of the smaller variants.

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