# P80: A TUMOR-RELATED PROTEIN FOUND IN MANY LYMPHOMAS OF MICE*

By DAVID J. GRUNWALD,‡ KENNETH MILLER, and REX RISSER§

*From the McArdle Laboratory for Cancer Research, University of Wisconsin, Madison, Wisconsin 53706*

Biochemical identification of tumor-specific antigens has, with rare exceptions, proved to be a difficult goal. Even the thymus leukemia (TL)[1] antigen, which is clearly tumor specific in some strains of mice, has been found as a normal constitutent of thymus cells in other strains of mice (1, 2). Tumor-specific antigens encoded by transforming viruses have proved far more amenable to analysis (3), and genetic experiments have demonstrated (4, 5) that these proteins play essential roles in in vitro transformation. These proteins, which include the avian sarcoma virus $pp60^{src}$ (3), the Abelson murine leukemia virus (A-MuLV) *gag*-fusion proteins p120 or p160 (6–8), and the feline sarcoma virus protein (9), are usually found phosphorylated in vivo, and immunoprecipitates of these proteins catalyze the phosphorylation of tyrosine residues on other proteins (10–13). Aside from these virally encoded proteins, a phosphoprotein(s) of ~53,000 mol wt, encoded by a cellular gene(s), has been found in elevated amounts in a wide variety of tumors and transformed cells (14–16). Although expression of both viral and cellular phosphoproteins has been correlated with transformation, the precise molecular rate of such proteins in transformation remains to be elucidated.

It has usually been found that, when cells transformed by retroviruses cease expression of the virally encoded transforming protein, the cells also revert to normal morphology and growth properties. Thus, morphological revertants of cells, initially transformed by A-MuLV strains encoding the p120 (17) and p160 (18) *gag*-fusion proteins, no longer synthesized these proteins. Recently, we observed an apparent exception to this generalization. At least one A-MuLV lymphoma, cloned repeatedly in vitro and found to express the A-MuLV *gag*-fusion protein p160, gives rise to tumor clones that no longer express the A-MuLV *gag*-fusion protein after growth in vivo in genetically distinguishable host mice. These novel cells are clearly of donor cell origin and are highly malignant in adult syngeneic mice (19). Tumor regressor sera prepared to such p160-negative tumors, as well as tumor regressor sera prepared to several other mouse lymphomas, immunoprecipitate three previously unidentified proteins, the properties of which we report here.

* Supported in part by grants MV-49D from the American Cancer Society and grant CA-07175 from the National Cancer Institute.

‡ Present address is the Molecular Biology Institute, University of Oregon, Eugene, Oregon 97403.

§ Scholar of the Leukemia Society of America.

[1] *Abbreviations used in this paper:* A-MuLV, Abelson murine leukemia virus; B6, C57BL/6; E, early; FBS, fetal bovine serum; *Gpi-1*, glucose phosphate isomerase-1; L, late; M-MuLV, Moloney murine leukemia virus; SDS, sodium dodecyl sulfate; TL, thymus leukemia.

## Materials and Methods

*Animals.* Mice were bred in our colony at the McArdle Laboratory or purchased from The Jackson Laboratory, Bar Harbor, ME.

*Tumor Cells.* The transplantable tumors EL4, ERLD, EδG2, RLδ1, Meth A, ASL1, RADA-1, AKSL2, and K36 were obtained from Dr. E. Stockert and Dr. L. J. Old, Memorial Sloan-Kettering Cancer Center, New York, the origin and maintenance of these tumors has been described (20). RBL-5, a transplantable B6 Rauscher MuLV lymphoma, was obtained from Dr. F. Bach, University of Minnesota, Minneapolis, MN, and was maintained by weekly ascites transfer of $10^7$ cells in B6 mice. BALENTL 9, a transplantable BALB/c lymphoma induced by ethylnitrosourea (21), was obtained from Dr. M. Potter, Laboratory of Cell Biology, National Cancer Institute, National Institutes of Health, Bethesda, MD, and was maintained by ascites transfer of $10^7$ cells in pristane-primed BALB/c mice.

The derivation of A-MuLV lymphoma lines that differ in their expression of the A-MuLV *gag*-fusion protein has been described recently (19). The lymphomas B6T1, B6T4, and B6T6 were induced in neonatal B6 mice by infection with the NIH strain of A-MuLV and transplanted intraperitoneally in semi-syngeneic irradiated hosts. Tumors transferred for a few transplants (0–5) are designated early (E), and tumors transferred for several transplants (12–45) are designated late (L). The clonal lines E2 and E3 were derived from early B6T1 cells after adaptation to tissue culture. The cells were cloned once in methylcellulose suspension, and E2 cells were cloned a second time by terminal dilution at 0.1 cells/well. The cloning efficiency in the second cloning experiment was 40%. E2 cells were then used to initiate a new tumor line by transplanting $10^7$ cells into irradiated (BALB/c × B6)$F_1$ mice. After five in vivo transplants, the cells, now called E2$F_1$, were placed back into culture and examined for expression of H-2 antigens and glucose phosphate isomerase (*Gpi-1*) isoenzyme markers, which differ between donor (B6) and host (BALB/c × B6)2 animals. E2$F_1$ cells were found to be of B6 parental origin (19). A similar protocol was used to derive the line $F_1$1A from E3 cells. $F_1$1A cells were recovered from transplanted E3 cells after seven in vivo passages and were shown to be of B6 origin (19).

*Cell Cultures.* The origins of cell lines NIH3T3, NRK, RAT-1, the A-MuLV nonproducer cells ANN-1 and AbNRK, the SFFV nonproducer cells SFFV-NIH and SFFV-NRK, a SV40-transformed rat embryo fibroblast line SVRE 17, and an A-MuLV-transformed BALB/c lymphoid line 18-4 have been described elsewhere (22–25). Cell cultures were also prepared from 14–16-d BALB/c and B6 fetuses.

*Mixed Lymphocyte Cultures.* B6 spleen cells were incubated either alone or with an equal number of mitomycin C-treated BALB/c spleen cells in Mishell-Dutton medium supplemented with 2% fetal bovine serum (FBS) and $5 \times 10^{-5}$ M 2-mercaptoethanol. After 70, 96, and 120 h, six replicate cultures of stimulated and nonstimulated spleen cells were each incubated at 37°C for 6 h with 1 μCi of [methyl-$^3$H]thymidine (80 Ci/mmol). Uptake of [$^3$H]thymidine was maximum at 96 h, at which time stimulated cells incorporated 16-fold more thymidine (135,790 ± 5305 cpm) than did unstimulated cells (8,488 ± 1865 cpm).

*Antisera and Cytotoxicity Tests.* Goat antisera directed against Rauscher murine leukemia virus structural proteins p15, pr65, and gp70 were obtained from R. Wilsnack through the offices of the Special Virus Cancer Program, NCI, NIH. The antisera B6 anti-B6T1, (C3H × B6)$F_1$ anti-B6T1, B6 anti-B6Mo3, and BALB/c anti-EL4 were prepared as described (22, 26). The antiserum B6 anti-A-MuLV was prepared according to the method described for B6 anti-FV (23). Monoclonal Thy-1 antibody was purchased from New England Nuclear, Boston, MA, and used in direct cytotoxicity tests (22) at dilutions of $10^{-3}$ to $10^{-5}$ or in absorption tests at a dilution of $10^{-2}$.

*Radioisotopic Labeling of Cells.* Subconfluent monolayers of fibroblasts or $10^7$ nonadherent cells were incubated at 37°C for 2–4 h with 200–400 μCi of [$^{35}$S]methionine (700–1,300 Ci/mmol) in RPMI 1640 medium lacking methionine and supplemented with 10% dialyzed FBS. Cells were also labeled for 2.5 h with 500 μCi [$^{32}$P]orthophosphate in phosphate-free RPMI 1640 medium supplemented with 10% dialyzed FBS. For experiments with tritiated sugar precursors, cells were incubated at 37°C for 2 h with 250 μCi of [$^3$H]glucosamine (38 Ci/mmol) or [$^3$H]mannose (7.2 Ci/mmol) in glucose-free minimum essential medium. Incoporation of radiolabel was terminated by the addition of 0.25 volumes of 5× lysis buffer (0.05 M $NaHPO_4$,

pH 7.2, 4.5% NaCl, 5% Triton X-100, 2.5% sodium deoxycholate, 0.5% sodium dodecyl sulfate (SDS), and 5% Trasylol (protease inhibitor)). Cell extracts were incubated with nonimmune mouse serum for 1 h, and then, after the addition of formalin-fixed *Staphylococcus aureus* bacteria, Cowan strain I (27), the lysates were centrifuged at 120,000 *g* for 90 min. The resulting supernatant was used in subsequent immunoprecipitation reactions as described.

Cells in phosphate-buffered saline were radioiodinated by the lactoperoxidase method (28), washed in 10 mM KI-phosphate-buffered saline, and resuspended in 0.8 ml hypotonic buffer (10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 1.5 M $MgCl_2$). Lysates were then prepared as above.

*Immunoprecipitation and SDS-Polyacrylamide Gel Analysis.* Cell extracts (1–20 × $10^6$ cpm) were incubated with 1–10 μl of goat or mouse antiserum at 4°C for at least 1 h. Immune complexes were collected by the addition of 200 μl of a 10% vol/vol solution of the *S. aureus* bacteria in RIPA buffer (3) followed by centrifugation. After four washes with RIPA buffer, immune complexes were solubilized by heating at 100°C for 5 min in sample buffer (26).

Immunoprecipitated proteins were electrophoresed through 10% SDS–polyacrylamide slab gels as described (29). After fixation and staining, gels that contained either $^{35}S$- or $^{3}H$-labeled proteins were prepared for fluorography (30), dried, and exposed to X-omat R Kodak film (Eastman Kodak Co., Rochester, NY). Gels containing $^{32}P$- or $^{125}I$-labeled proteins were dried and exposed to X-omat R Kodak film in the presence of an intensifying screen. All gels contained the standard marker proteins RNA polymerase subunits (160K, 150K, 90K, 36.5K), β-galactosidase (116K), phosphorylase A (96K), bovine serum albumin (68K), and ovalbumin (43K).

*Tryptic Peptide Analysis.* Immunoprecipitated proteins were resolved on preparative cylindrical gels. Proteins were eluted from appropriate gel slices and trypsinized according to the method of Kew et al. (31). Peptides were applied to a column of Technicon Chromobeads type P cation exchange resin, and the chromatogram was developed with a linear gradient of pyridine acetate, increasing in pH and concentration (0.2 M, pH 3.1, to 2 M, pH 5.0) for the first 200 fractions. The column was then washed with 2 M pyridine acetate (pH 5.0) for the final 50 fractions (31).

## Results

*Identification of Novel Proteins in A-MuLV Lymphomas That Cease Expression of the A-MuLV Gag-Fusion Protein.* We recently observed that continued in vivo propagation of some A-MuLV lymphomas results in the appearance of tumor cells that are different from most A-MuLV lymphoma cells in the expression of the A-MuLV-encoded *gag*-fusion protein (19). Tumor cells recovered after several in vivo passages (p12–p45) no longer synthesized detectable amounts of the A-MuLV-encoded *gag*-fusion protein p160, although this protein was readily detectable in earlier transplants (0–5) of the same tumors (32).

To detect other tumor-specific proteins synthesized by these unusual p160-negative cells, B6 mice were immunized with tumor cells from late transplant generations (p15–p45) of the A-MuLV-induced lymphoma, B6T1, referred to as B6T1(L) cells. Extracts of B6T1(L) cells, which had been metabolically labeled with [$^{35}S$]methionine, were mixed with tumor regressor serum and the immunoprecipitated proteins resolved by electrophoresis through SDS-polyacrylamide gels and visualized by fluorography. Six protein species of molecular mass 115K, 85K, 80K, 65K, 32K, and 15K mol wt were observed in this experiment (Fig. 1, lane b). The two proteins of 85K and 15K comigrated with the *env* gene products $pr85^{env}$ and p15E (Fig. 1, lane c). The proteins of 85K, 65K, and 15K bear only viral antigenic determinants because the precipitation of these proteins was blocked when unlabeled disrupted M-MuLV virions were added to the immunoprecipitation reaction (Fig. 1, lane a). Because the proteins of 115K, 80K, and 32K were precipitated in reactions containing exogenous unlabeled M-MuLV, these proteins must bear antigenic determinants not shared by M-MuLV

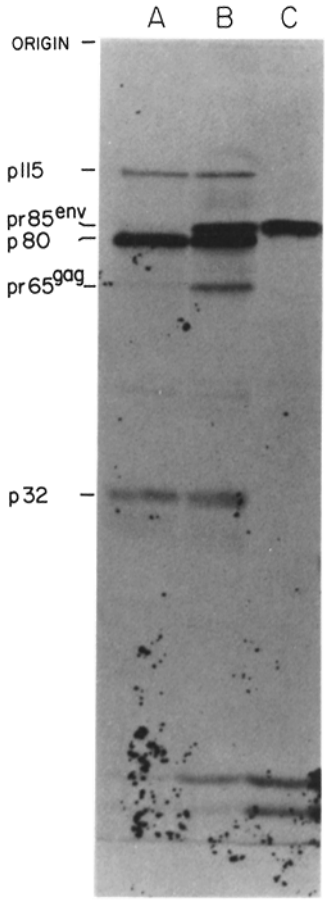


FIG. 1. Recognition of nonviral proteins by regressor serum prepared against the B6T1(L) tumor. $^{35}$S-labeled extracts of B6T1(L) cells were prepared and analyzed as in Materials and Methods. Proteins were immunoprecipitated with B6 anti-B6T1(L) antiserum (lane B) with B6 anti-B6T1(L) antiserum that had been preincubated with disrupted M-MuLV virions (lane A) or with goat anti-gp70 serum (lane C).

structural proteins.

To determine whether expression of p115, p80, and p32 was also observed in clonal lines that no longer expressed the A-MuLV fusion protein p160, the following experiment was performed. E2 cells derived by repeated cloning (40% cloning efficiency) of early passage B6T1 tumor cells were used to initiate a new tumor line in genetically distinguishable host mice. After five in vivo transplants, the tumor cells, termed $E2F_1$, were placed back into culture and checked for the presence of *H-2* or *Gpi-1* markers to determine whether the cells were derived from the donor cell population or from the host. $E2F_1$ cells expressed only the $H\text{-}2^b$ antigens and $Gpi\text{-}1^b$ isoenzyme characteristic of the donor (19). The initial E2 and derivative $E2F_1$ cells were then radiolabeled and examined for synthesis of the A-MuLV *gag*-fusion protein p160 using anti-gag p15 serum or the p115, p80, p32 molecules detected with anti-p80 tumor regressor serum (Fig. 2). Under these conditions, p160 and $pr65^{gag}$ were readily detected in E2 cells (Fig. 2, lane b), whereas no protein was specifically

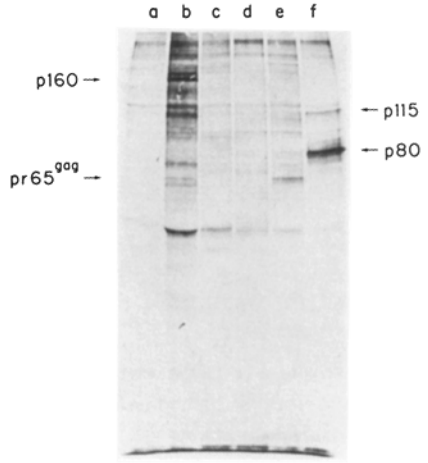


FIG. 2. Expression of p80 and the A-MuLV protein p160 in clonal lines. Lysates of E2 (a–c) and $E2F_1$ (d–f) cells (see text), radiolabeled with [$^{35}$S]methionine, were immunoprecipitated with normal mouse serum (a, d), anti-gag p15 serum (b, e), or with anti-p80 serum (c, f).

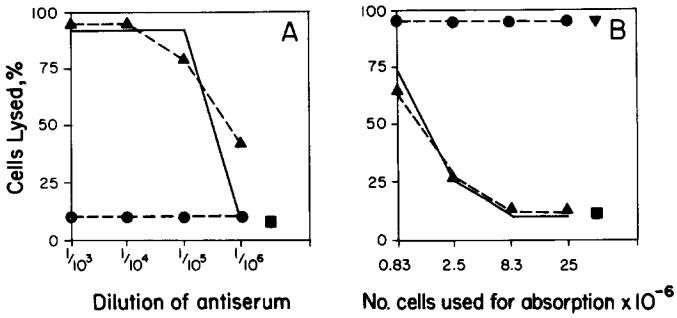


FIG. 3. Expression of the Thy-1 antigen by $p80^-$ and $p80^+$ A-MuLV lymphoma cells. Monoclonal Thy-1.2 antibody was used in (A) direct cytotoxic tests with thymocytes from B6 mice (—), E3 cells (●), and $F_1$1A cells (▲) or (B) was absorbed with B6 thymocytes (—), E3 cells (●) or $F_1$1A (▲) and tested on B6 thymocyte target cells. The cytotoxic activity of unabsorbed serum used in absorption tests is indicated (▼), as is lysis in the presence of complement alone (■).

immunoprecipitated from E2 cells with anti-p80 serum (Fig. 2, lane c). Conversely, $pr65^{gag}$ but not p160 was detected in $E2F_1$ cells (Fig. 2, lane e), and p80 was readily detected in these cells (Fig. 2, lane f). Moreover, nucleic acid hybridization experiments indicated that most, if not all, of the A-MuLV proviral genome was lost from $E2F_1$ cells (19). A similar result was observed when E3 cells were compared with $F_1$1A cells, a line derived from E3 after seven in vivo passages. Thus, it is apparent that the A-MuLV-encoded protein p160 and the p80 protein, identified with tumor regressor sera, bear non-cross-reactive antigenic determinants.

An additional feature that distinguished p160-positive and p160-negative cells was the expression of the Thy-1 differentiation antigen. When the p160-positive A-MuLV lines E2 and E3 were examined in direct cytotoxic tests with monoclonal Thy-1 antibody, the cells were refractory to lysis, whereas $F_1$1A or $E2F_1$ cells, derived from E3 or E2 cells, respectively, showed 95% lysis (Fig. 3 A and data not shown). To confirm the cell surface phenotype of these lines, absorption analysis was performed.

The results of that experiment demonstrate that neither E3 nor E2 cells absorbed cytotoxic activity, whereas $F_1$1A cells, $E2F_1$ cells, and thymocytes absorbed all cytotoxic antibody (Fig. 3 B and data not shown). Therefore, we conclude that E2 and E3 cells have the phenotype $p160^+$, $p80^-$, Thy-$1^-$ and that $F_1$1A and $E2F_1$ cells have the phenotype $p160^-$, $p80^+$, Thy-$1^+$.

*Comparison of p80 and the A-MuLV-encoded Protein p120.* Serologically, the A-MuLV-encoded *gag*-fusion protein appeared distinct from p80 (Fig. 2). To confirm the lack of relatedness of p80 to A-MuLV-encoded *gag*-fusion proteins, the tryptic peptides of p80 were compared with those of the A-MuLV-encoded protein p120 (P120 was selected because larger amounts of it could be recovered and because p120 and p160 share 15 tryptic peptides) (19). After immunoprecipitation with anti-pr65 antiserum from $^{35}$S-labeled lysates of 18-4 cells, the *gag*-fusion product, p120, was isolated from preparative SDS-polyacrylamide gels. Similarly, p80 was purified by gel electrophoresis of immunoprecipitates from B6T1(L) cells. The two purified proteins were trypsinized in parallel, and their peptides were applied to a sulfonated ion exchange column and eluted with a linear gradient of pyridine acetate (31). The profile of the methionine-containing peptides of p120 (Fig. 4, panel C) is quite different from the pattern of the p80 peptides (panel B). Comparison of the methionine-containing peptides of p160 (19) and p80 also demonstrated very different patterns. Thus, from both serological and biochemical analyses, there appears little, if any, homology between p80 and the *gag*-fusion products of A-MuLV.

The relationship of the three nonviral proteins, p80, p32, and p115, precipitated from the B6T1(L) cells, was examined. Under nonreducing denaturing conditions, p115 did not appear to be complexed with other proteins (data not shown). Pulse-chase analyses indicated that, whereas p115 was not a precursor polyprotein for the smaller proteins, p32 might represent a stable proteolytic product derived from p80 (data not shown). The relatedness of p80 and p32 was confirmed by comparison of their methionine-containing tryptic peptides. Lysates of $^{35}$S-labeled B6T1(L) cells were prepared and immunoprecipitated with anti-p80 antiserum. The immune complexes were collected and resolved in preparative SDS-polyacrylamide gels from which p32 and p80 were purified. After trypsinization, p32 (Fig. 4, panel A) and p80 (panel B) were chromatographed in parallel. The two proteins share several peptides, five of which are noted in Fig. 4. Because of the difficulty of obtaining sufficient material of p115, a similar comparison with this protein has not been performed. At present the relationship, if any, between p115 and p80 has not been elucidated.

*P80 Is a Nonglycosylated Phosphoprotein.* B6T1(L) cells were incubated in phosphate-free medium supplemented with [$^{32}$P]orthophosphate. Extracts of these cells were then immunoprecipitated with either anti-p80 serum (Fig. 5, lane a) or goat anti-p12 serum (lane b), and the reactive phosphorylated proteins were resolved by gel electrophoresis and visualized by autoradiography. Under these conditions of metabolic labeling, both p80 and $pr65^{gag}$ appear phosphorylated in these cells; however, p32 does not appear to be.

Although antibodies against p80 are frequently produced after hyperimmunization with many tumor lines, we have no evidence that p80 was expressed at the cell surface. Although the viral glycoprotein (gp70) present at the surface of B6T1(L) cells was readily iodinated by the lactoperoxidase method (Fig. 5, lane d), a technique that specifically radiolabels exposed tyrosine residues of cell surface proteins (33), no

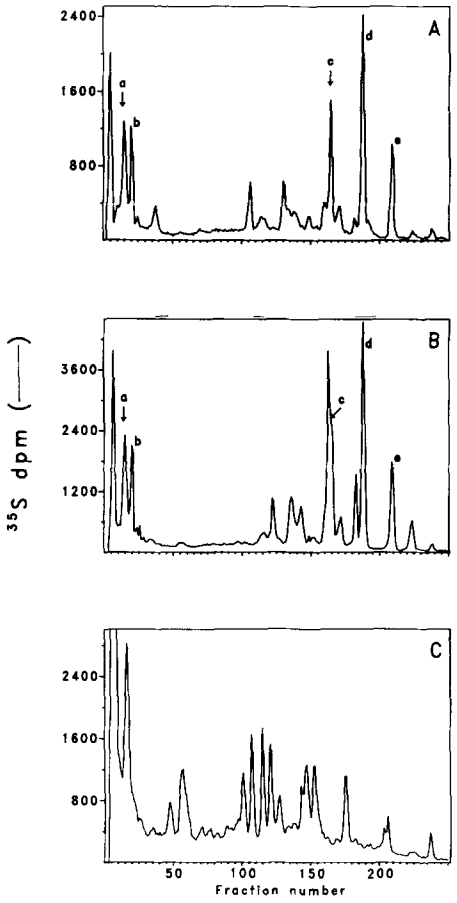


FIG. 4. Comparison of the methionine-containing tryptic peptides of p32, p80, and p120. After immunoprecipitation, the proteins were purified by elution from preparative SDS-polyacrylamide gels. P32 (A), p80 (B), and p120 (C) were trypsinized in parallel, and their peptides were resolved by chromatography. Labeled peaks represent peptides shared by p80 and p32.

radioactive material was precipitated by either nonimmune B6 or anti-p80 serum (Fig. 5, lanes c and e). Similarly, the M-MuLV *env* gene products pr85$^{env}$ and gp70 could be metabolically labeled in B6T1(L) cells with [$^3$H]glucosamine (Fig. 5, lane g) or [$^3$H]mannose (lane i), but p80 was not labeled with these sugar precursors (Fig. 5, lanes f and h). A further indication that p80 was not glycosylated came from experiments in which B6T1(L) cells were incubated with the antibiotic tunicamycin, which inhibits glycosylation. Increasing concentrations of tunicamycin in the medium had a marked effect on the apparent mobility of the *env* gene products of B6T1(L) cells but did not alter the rate of synthesis nor the mobility of p80 (data not shown).

*P80 Is Synthesized by Many T Cell Lymphomas of Mice.* To determine whether the expression of p80 was a property limited to A-MuLV-induced tumors that no longer expressed the *gag*-fusion protein, several murine tumors were examined for synthesis of this protein. Each tumor line was metabolically labeled with [$^{35}$S]methionine, lysed, and incubated with either nonimmune mouse or anti-p80 serum in the presence of disrupted M-MuLV virions. The results of such direct immunoprecipitation experi-

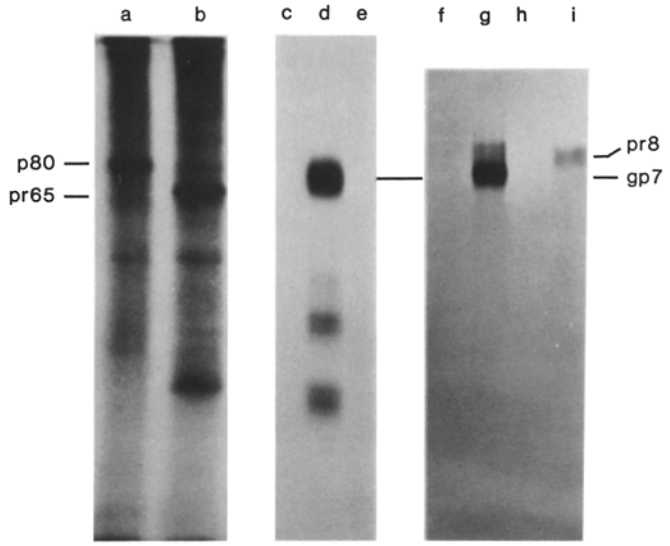


FIG. 5. Physical characterization of p80. B6T1(L) cells were either labeled with [$^{32}$P]orthophosphate (lanes a and b), radioiodinated by the lactoperoxidase method (c–e), or labeled with the tritiated sugar precursors, [$^{3}$H]glucosamine (f and g) and [$^{3}$H]mannose (h and i). Extracts of these cells were immunoprecipitated as in Materials and Methods with anti-p80 antiserum in the presence of M-MuLV virions (a, e, f, and h), goat anti-pr65 serum (b), nonimmune mouse serum (c), and goat anti-gp70 serum (d, g, and i).

ments (Fig. 6, Table I) indicate that P80, p115, and p32 were observed in many, but not all, T cell lymphomas of B6, BALB/c, and A/J mice. This group of p80-positive tumors included Thy-1-positive transplanted lymphomas that arose spontaneously or were induced by the Friend, Moloney, or Rauscher MuLV strains by a chemical carcinogen, dimethylbenzanthracene, or by radiation. Several B6 A-MuLV-induced lymphomas that had ceased expression of their *gag*-fusion proteins upon in vivo propagation (32) also synthesized p80.

Among those tumors in which p80 was not detected, were four A-MuLV-induced lymphomas that synthesized A-MuLV *gag*-fusion proteins. Several other lymphomas of B6, BALB/c, A/J, and AKR mice, some of which expressed the Thy-1 antigen, as well as a transplanted BALB/c sarcoma, did not synthesize detectable amounts of p80.

The 80,000 mol wt species detected in three different tumors were compared according to the method of Cleveland et al. (34). Extracts of B6T1(L) cells, EL4 cells, a chemically-induced B6 tumor, and RL♂1 cells, a radiation-induced BALB/c tumor, were immunoprecipitated with anti-p80 antiserum. The p80 molecules were partially purified by gel electrophoresis and then enzymatically digested with varying amounts of *S. aureus* V8 protease (Fig. 7). The pattern of the proteolytic products generated from the p80 proteins of EL4 and RL♂1 tumor cells was similar to that of the p80 protein from B6T1(L) cells.

The distribution of murine tumors with p80 suggested that this protein did not serve as a generalized marker of transformed cells. Among in vitro cultured cells, p80 has only been detected in cell lines derived from p80-positive tumor cells (Table I). NIH3T3 and NRK cells, either uninfected or harboring the replication defective

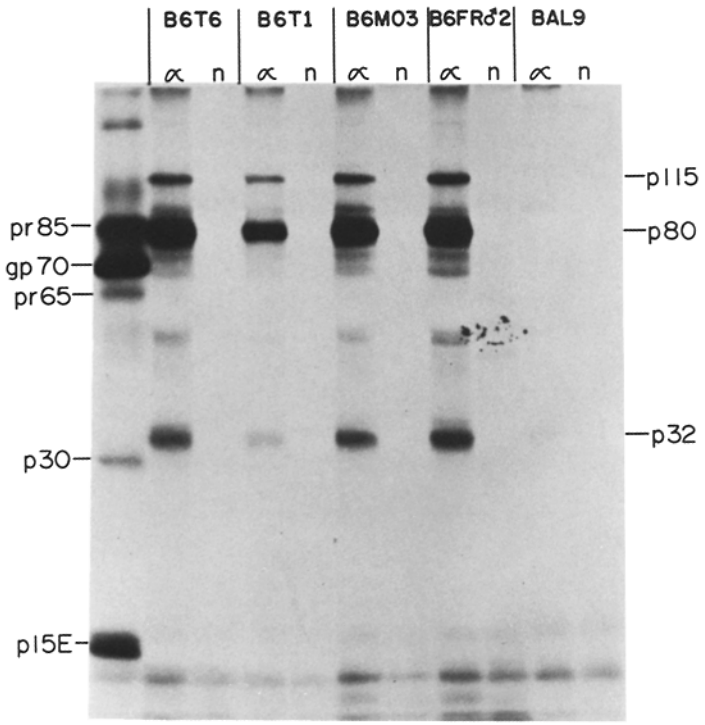


FIG. 6. Several murine lymphomas synthesize p80. $^{35}$S-labeled extracts of tumor cells were immunoprecipitated and analyzed in SDS-polyacrylamide gels. Proteins were immunoprecipitated with nonimmune mouse serum (n) or anti-p80 antiserum (α) in the presence of M-MuLV virions. The marker lane represents known MuLV proteins synthesized by B6T6 tumor cells and immunoprecipitated with goat antiserum prepared against disrupted M-MuLV virions.

genomes of A-MuLV or SFFV, did not synthesize detectable levels of p80 (data not shown). In competition experiments for the $^{35}$S-labeled p80 of B6T1(L) cells, nonradioactive lysates of these cells also failed to inhibit precipitation of p80. Similarly, p80 was not detected in RAT-1 cells, SV40 transformed rat embryo fibroblasts, nor secondary cultures of mouse embryo fibroblasts from B6 and BALB/c mice (data not shown). The A-MuLV-transformed lymphoid line 18-4 that synthesized the p120 *gag*-fusion protein also did not synthesize p80 (data not shown).

Syngeneic and semisyngeneic hyperimmunization with B6T1(L) cells invariably led to the production of p80-reactive antibodies (Table II). However, syngeneic tumor regressor antisera that were prepared against p160-positive E2 cells and recognized the A-MuLV-specific portion of the virally encoded *gag*-fusion protein, did not immunoprecipitate p80 from B6T1(L) cells. Antisera prepared by syngeneic immunization with M-MuLV tumor cells and by allogeneic immunization with EL4 cells were capable of immunoprecipitating p80 from B6T1(L) cells. Thus, p80 appears to be an immunogenic protein expressed by several T cell lymphomas of different strains of mice.

*P80 Is Not Detectable in Normal Lymphoid Tissue.* The presence of p80 in normal lymphoid tissue was examined by metabolic labeling of normal cells and by competition experiments with lysates of nonradioactive lymphoid cells. Cells from the bone

TABLE I

*Synthesis of p80 in Mouse Tumors and Cell Lines*

| Tumor line | Strain | Inducing Agent | Thy-1* |
|---|---|---|---|
| p80-positive tumors | | | |
| $F_1$1A (p160$^-$) | B6 | A-MuLV | + |
| E2$F_1$ (p160$^-$) | B6 | A-MuLV | + |
| B6T1(L) (p160$^-$) | B6 | A-MuLV | + |
| B6T4(L) (p160$^-$) | B6 | A-MuLV | + |
| B6T6(L) (p160$^-$) | B6 | A-MuLV | + |
| B6FRδ2 | B6 | Friend MuLV | + |
| B6Mo3 | B6 | M-MuLV | + |
| RBL-5 | B6 | Rauscher MuLV | + |
| EL4 | B6 | Dimethylbenzanthracene | + |
| ERLD | B6 | Radiation | +‡ |
| RLδ1 | BALB/c | Radiation | +‡ |
| ASL1 | A/J | Spontaneous | + |
| p80-negative tumors | | | |
| E2 (p160$^+$) | B6 | A-MuLV | − |
| E3 (p160$^+$) | B6 | A-MuLV | − |
| B6T4(E) (p160$^+$) | B6 | A-MuLV | − |
| B6T6(E) (p160$^+$) | B6 | A-MuLV | − |
| EδG2 | B6 | Gross MuLV | +‡ |
| Meth A | BALB/c | 3-methylcholanthrene | −‡ |
| BALENTL 9 | BALB/c | Ethylnitrosourea | +§ |
| RADA1 | A/J | Radiation | +‡ |
| AKSL2 | AKR | Spontaneous | + |
| K36 | AKR | Spontaneous | −‡ |

* Unless noted otherwise, the expression of the Thy-1 antigen was determined in our laboratory in direct cytotoxicity tests with monoclonal anti-Thy-1.2 antibody.

‡ The expression of Thy-1, as reported in reference 20.

§ The expression of Thy-1, as reported in reference 21.

marrow, lymph nodes, spleen, and thymus of B6, BALB/c, SWR, and DBA/2 mice were collected and labeled with [$^{35}$S]methionine for incubation periods ranging from 2–16 h. In no instance did anti-p80 antiserum specifically immunoprecipitate a protein of 80,000 mol wt or one serologically related to it from lysates of any of these tissues. Under conditions in which the precipitating activity of anti-p80 antiserum for the radioactive p80 of B6T1(L) cells was limiting, antiserum was incubated with nonradioactive lysates of cells from B6 lymph nodes, spleen, and thymus before the addition of radioactive tumor cell lysates. The lymphoid extracts were generated from 500–1,000 times the number of B6T1(L) cells ($2 \times 10^5$) used as a source of radioactive p80 in these competition experiments. The amount of p80 precipitated was not measurably affected by preincubation of the antiserum with lymphoid extracts (data not shown).

Two populations of rapidly dividing lymphoid cells were examined for synthesis of p80. Regenerating bone marrow cells taken 10 d after reconstitution of lethally irradiated B6 mice did not synthesize p80 (data not shown). Similarly, p80 was not detected in B6 spleen cells that were proliferating (16-fold stimulation of [$^3$H]thymidine uptake) in response to mitomycin C-treated BALB/c spleen cells (Fig. 8 A).

Reconstruction experiments were performed to determine the synthetic rate of p80

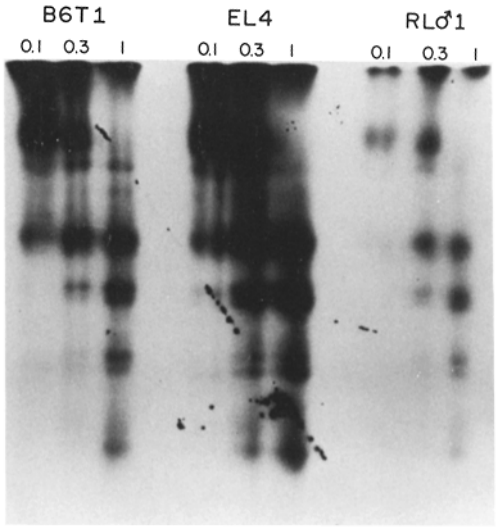


FIG. 7. The 80,000-mol wt proteins present in three different tumors are related. After immunoprecipitation and preparative gel electrophoresis, gel slices containing the p80 proteins from B6T1(L) cells, EL4 cells, and RL♂1 cells were cut out. The gel slices were mixed with 0.1 μg, 0.3 μg, or 1.0 μg *S. aureus* V8 protease, and the proteins were subjected to partial enzymatic digestion during electrophoresis through a 15% SDS-polyacrylamide gel.

TABLE II

*Sera Tested for the Ability to Precipitate p80 from B6T1(L) Cells*

| Immunization* | Precipitation of p80‡ |
|---|---|
| B6 anti-B6T1(E) | 0/2§ |
| B6 anti-B6T1(L) | 4/4 |
| (C3H × B6)$F_1$ anti-B6T1(L) | 1/1 |
| B6 anti-A-MuLV | 0/3 |
| B6 anti-B6Mo3 | 1/1 |
| BALB/c anti-EL4 | 1/1 |

* 8–10-wk-old mice were injected subcutaneously with $10^2$–$10^5$ tumor cells. After these tumors had regressed, 10-fold increasing numbers of tumor cells were injected intraperitoneally every 2 wk until a dose of $10^8$ cells per mouse was reached. Thereafter, mice were immunized with $10^8$ cells. B6 anti-A-MuLV was prepared by intravenous inoculation of mice with $10^4$ focus-forming units of A-MuLV every 2 wk. With the exception of (C3H × B6)$F_1$ anti-B6T1(L) antiserum, which was obtained from one mouse, each immunization reflects the pooled sera of 5–10 mice.

‡ Data are expressed as the number of independent immunization experiments leading to the production of antibodies that precipitated p80 per the total number of independent immunizations.

§ These two antisera do, however, precipitate the A-MuLV *gag*-fusion proteins through recognition of non-M-MuLV antigenic determinants.

in normal tissues that would have been detected by our procedures (Fig. 8 B). Parallel cultures of thymocytes were labeled alone or after mixing with varying amounts of p80-positive $F_1$1A cells. P80 and p32 were readily visualized in immunoprecipitates from lysates of cultures in which p80-positive cells comprised 0.5% of the total cell population (Fig. 8 B, lane i). However, p80 was not detected in normal thymocytes that had been labeled alone (lanes f and g). Only one protein was precipitated from

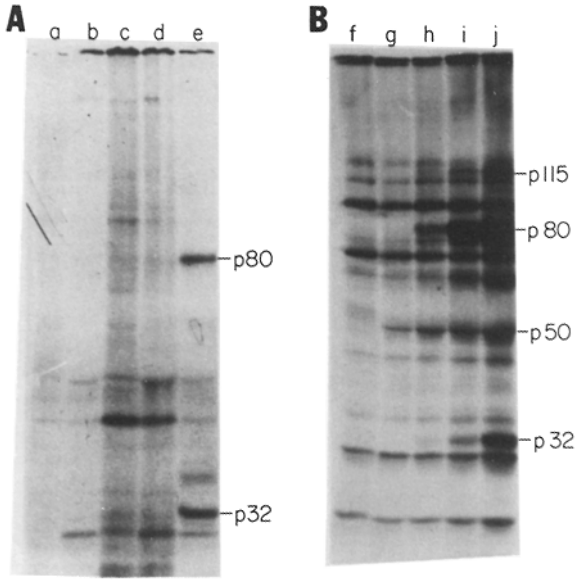


FIG. 8. Absence of detectable p80 in normal lymphoid cells. (A) B6 spleen cells were cultured for 4 d, either alone (lanes a and b) or in the presence of mitomycin C-treated BALB/c stimulator spleen cells (c and d). $10^7$ viable spleen cells from each culture were metabolically labeled with [$^{35}$S]methionine, lysed, and analyzed, as in Material and Methods. Protein were immunoprecipitated with nonimmune mouse serum (a and c) or anti-p80 antiserum antiserum (b and d). Lane e is a parallel immunoprecipitation from B6T1(L) cells. (B) Parallel cultures of $10^8$ thymocytes from B6 mice were incubated for 2.5 h in the presence of 400 $\mu$Ci [$^{35}$S]methionine. Thymocytes were incubated either alone (f and g) or with $5 \times 10^5$ (h), $2 \times 10^6$ (i), or $5 \times 10^6$ (j) $F_1$1A cells. Proteins were immunoprecipitated with nonimmune mouse serum (f) or anti-p80 antiserum (g–j) in the presence of M-MuLV virions.

thymus extracts by anti-p80 antiserum but not nonimmune B6 serum. Reactivity for this 50,000 mol wt species, present in B6T1(L) cells, has been observed in several pools of anti-p80 antisera. This result is analogous to that of others (15, 16) who also found that p53, a common tumor antigen, was synthesized by normal thymocytes. Longer exposures of this fluorogram permit the conclusion that, if p80 were synthesized by normal B6 thymocytes, its synthetic rate in this population of cells would have to have been <0.05% that of its synthetic rate in the p80-positive tumor cells.

## Discussion

Studies of Abelson lymphoma cells that no longer expressed the A-MuLV transforming protein led to the identification of a protein of ~80,000 mol wt that appears to be coordinately expressed with two minor species of 115,000 and 32,000 mol wt in many murine T cell lymphomas. P80 is phosphorylated but not glycosylated in vivo and is not accessible to lactoperoxidase iodination. Among A-MuLV-induced lymphoma cells, p80 has been detected only in those tumors and cell lines that have ceased synthesis of *gag*-fusion proteins and have acquired a marker usually associated with T lymphocyte differentiation. Several other molecular and phenotypic changes have been observed in these cells (J. Dudley, M. Scheid, C. Spellman, D. J. Grunwald, N. Warner, and R. Risser, unpublished observations), and these will be reported in due course.

The association of p80 expression with lymphomas of the T lymphocyte lineage was borne out in the investigation of several murine tumors and cell lines. The 80,000-mol wt proteins synthesized by tumors induced by different agents and from different strains of mice were serologically and biochemically similar. However, not all Thy-1-positive lymphomas synthesized detectable levels of p80. In addition, p80 was not detected in established fibroblastic cell lines, whether uninfected, infected with SFFV, or transformed with A-MuLV or SV40. Thus, synthesis of p80 is not a universal feature of transformed murine or rat cells, but rather it is a common characteristic of murine T cell lymphomas.

The finding that syngeneic immunization with $p80^+$ lymphomas frequently elicited the production of anti-p80 antibodies suggests that this protein is either not expressed at all or only at very low levels in normal tissues. Competition experiments, designed to detect molecules that might have low synthetic rates in normal tissues, failed to reveal proteins with p80 antigenic determinants in B6 lymph node, thymus, or spleen tissue. In direct labeling experiments, we never observed immunoprecipitation of p80 from extracts of normal B6, DBA/2, SWR, nor AKR lymphoid tissues. Reconstruction experiments indicated that the level of synthesis of p80 in a population of adult B6 thymocytes must be $<0.05\%$ that of tumor cells.

P80 appears to be unrelated to known or endogenous MuLV-encoded proteins. Serological and biochemical analyses showed that p80 is not related to the A-MuLV-specific *gag*-fusion proteins. In most experiments, p80 was detected in immune reactions performed in the presence of an exogenous source of MuLV, such that known MuLV determinants of the tumor cells were successfully competed. Antisera directed against each of the internal structural proteins of Rauscher MuLV failed to immunoprecipitate p80, and neither p80 nor p32 included the methionine-containing tryptic peptides of the major M-MuLV structural protein, p30 (unpublished observations).

Although p80 is distinguishable from other transformation-related proteins, it shares some features with the A-MuLV-encoded *gag*-fusion protein and with the recently described series of tumor-associated proteins of ~53,000 mol wt (14–16). These three sets of proteins have similar physical properties, and all are unglycosylated immunogenic components of the cells in which they are found. Unlike the A-MuLV-specific protein, the presence of p80 does not correlate with an increased cellular abundance of the amino acid phosphotyrosine. Although E2 cells do have elevated levels of this unusual amino acid, p160-negative transplanted A-MuLV cells do not (T. Hunter, personal communication). The 53K transformation-related proteins are synthesized in a wide variety of transformed and tumor cells, and these proteins can be readily detected in some normal tissue. The absence of p80 from normal lymphoid cells and its detection in lymphoid tumors might indicate that p80 is truly a tumor-specific antigen, or, more likely, that p80 is normally expressed in some other cell type and frequently activated in lymphoid tumors.

## Summary

Examination of syngeneic tumor regressor sera prepared by immunization of mice with several different lymphomas revealed a common pattern of reactivity to proteins expressed in these tumors. Antibodies present in these sera immunoprecipitate a triplet of proteins of 115,000 mol wt (p115), 80,000 mol wt (p80), and 32,000 mol wt

(p32) from many but not all T cell lymphomas of mice. P80, the predominant molecular species immunoprecipitated with these sera, is a nonglycosylated, phosphoprotein that does not appear to be expressed at the cell surface. Comparison of the tryptic peptides of p32 and p80 indicated that the peptides found in p32 are a subset of those found in p80. Comparison of the tryptic peptides of p80 with those of the p120 *gag*-fusion protein of Abelson murine leukemia virus demonstrated that p80 and p120 did not share tryptic peptides. Comparison of the partial proteolytic products generated by treatment of p80 molecules from different tumors with V8 protease did not reveal heterogeneity in p80 among tumors of different strains of mice. Direct labeling and competition blocking experiments with lysates from normal cells failed to provide evidence of p80 synthesis in normal thymus, spleen, or bone marrow. Thus, p80 is a biochemically identified tumor-related antigen of mouse lymphomas.

## References


1. Old, L. J., E. A. Boyse, and E. Stockert. 1963. Antigenic properties of experimental leukemias. I. Serological studies *in vitro* with spontaneous and radiation induced leukemias. *J. Natl. Cancer Inst.* **31:**977.
2. Rothenberg, E. 1980. Expression of differentiation antigens in subpopulations of mouse thymocytes: regulation at the level of de novo synthesis. *Cell.* **20:**1.
3. Brugge, J. S., and R. L. Erikson. 1977. Identification of a transformation-specific antigen from avian sarcoma virus-transformed cells. *Nature (Lond.).* **269:**346.
4. Toyoshima, K., and P. K. Vogt. 1969. Temperature-sensitive mutants of an avian sarcoma virus. *Virology.* **39:**930.
5. Martin, G. S. 1970. Rous sarcoma virus: a function required for the maintenance of the transformed state. *Nature (Lond.).* **227:**1021.
6. Reynolds, F. H., T. L. Sacks, D. N. Deobagkar, and J. R. Stephenson. 1978. Cells nonproductively transformed by Abelson murine leukemia virus express a high molecular weight polyprotein containing structural and nonstructural components. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3974.
7. Witte, O. N., N. Rosenberg, M. Paskind, A. Shields, and D. Baltimore. 1978. Identification of an Abelson murine leukemia virus-encoded protein present in transformed fibroblast and lymphoid cells. *Proc. Natl. Acad. Sci. U. S. A.* **75:**2488.
8. Rosenberg, N., D. Clark, and O. Witte. 1980. Abelson murine leukemia virus mutants deficient in kinase activity and lymphoid cell transformation. *J. Virol.* **36:**766.
9. Stephenson, J. R., A. S. Khan, A. H. Sliski, and M. Essex. 1977. Feline oncornavirus-associated cell membrane antigen: evidence for an immunologically cross-reactive feline sarcoma virus-coded protein. *Proc. Natl. Acad. Sci. U. S. A.* **74:**5608.
10. Collett, M. S., and R. L. Erikson. 1978. Protein kinase activity associated with the avian sarcoma *src* gene product. *Proc. Natl. Acad. Sci. U. S. A.* **75:**2021.
11. Levinson, A. D., H. Opperman, L. Levintow, H. E. Varmus, and J. M. Bishop. 1978. Evidence that the transforming gene of avian sarcoma virus encodes a protein kinase associated with a phosphoprotein. *Cell.* **15:**561.
12. Witte, O. N., A. Dasgupta, and D. Baltimore. 1980. Abelson murine leukemia virus protein is phosphorylated *in vitro* to form phosphotyrosine. *Nature (Lond.).* **283:**826.
13. Reynolds, F. H., W. Van de Ven, and J. R. Stephenson. 1980. Abelson murine leukemia virus transformation-defective mutants with impaired P120-associated protein kinase activity. *J. Virol.* **36:**374.
14. Lane, D. P., and L. V. Crawford. 1979. T antigen is bound to a host protein in SV40-transformed cells. *Nature (Lond.).* **278:**261.
15. Linzer, D., and A. J. Levine. 1979. Characterization of a 54K dalton cellular SV40 tumor

antigen present in SV40-transformed cells and uninfected embryonal carcinoma cells. *Cell.* **17:**43.

16. DeLeo, A. B., G. Jay, E. Appella, G. C. Dubois, L. W. Law, and L. J. Old. 1979. Detection of a transformation-related antigen in chemically induced sarcomas and other transformed cells of the mouse. *Proc. Natl. Acad. Sci. U. S. A.* **76:**2420.
17. Sacks, T. L., E. J. Hershey, and J. Stephenson. 1979. Abelson murine leukemia virus-infected cell lines defective in transformation. *Virology.* **97:**231.
18. Ziegler, S., C. A. Whitlock, S. Goff, A. Gifford, and O. Witte. 1981. Lethal effect of the Abelson murine leukemia virus transforming gene product. *Cell.* **27:**477.
19. Grunwald, D. J., B. Dale, J. Dudley, W. Lamph, B. Sugden, B. Ozanne, and R. Risser. 1982. Loss of viral gene expression and retention of tumorigenicity by Abelson lymphoma cells. *J. Virol.* **43:**92.
20. Old, L. J., and E. Stockert. 1977. Immunogenetics of cell surface antigens of mouse leukemia. *Ann. Rev. Genet.* **11:**127.
21. Mathieson, B. J., P. S. Campbell, M. Potter, and R. Asofsky. 1978. Expression of Ly-1, Ly-2, Thy-1 and TL differentiation antigens of mouse T cell tumors. *J. Exp. Med.* **147:**1267.
22. Risser, R., E. Stockert, and L. J. Old. 1978. Abelson antigen: a viral tumor antigen that is also a differentiation antigen of BALB/c mice. *Proc. Natl. Acad. Sci. U. S. A.* **75:**3918.
23. Risser, R. 1979. Friend erythroleukemia antigen. A viral antigen specified by spleen focus-forming virus (SFFV) and differentiation antigen controlled by the *Fv-2* locus. *J. Exp. Med.* **149:**1152.
24. Rosenberg, N., and D. Baltimore. 1976. A quantitative assay for transformation of bone marrow cells by Abelson murine leukemia virus. *J. Exp. Med.* **143:**1453.
25. Risser, R., and R. Pollack. 1974. A non-selective analysis of SV40 transformation of mouse 3T3 cells. *Virology.* **59:**477.
26. Risser, R., and D. Grunwald. 1981. Production of anti-self H-2 antibodies by hybrid mice immune to a viral tumor. *Nature (Lond.).* **283:**563.
27. Kessler, R. 1975. Rapid isolation of antigens from cells with a staphylococcal protein A-antibody adsorbent: parameters of the interaction of antibody-antigen complexes with protein A. *J. Immunol.* **115:**1617.
28. Marchalonis, J. J. 1969. An enzymic method for the trace iodination of immunoglobulins and other proteins. *Biochem. J.* **113:**299.
29. Laemmli, U. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond.).* **227:**680.
30. Bonner, W. M., and R. W. Laskey. 1974. A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. *Eur. J. Biochem.* **46:**83.
31. Kew, O. M., M. A., Pallansch, D. R., Omilianski, and R. R. Rueckert. 1980. Changes in three of the four coat proteins of oral polio vaccine strain derived from type 1 polioviruses. *J. Virol.* **33:**256.
32. Risser, R., D. Grunwald, C. Sinaiko, and P. Jelen. 1980. Cell-surface antigens of Abelson and Friend murine leukemia viruses and of hematopoietic differentiation. *Cold Spring Harbor Symp. Quant. Biol.* **44:**1195.
33. Phillips, D. R., and M. Morrison. 1970. The arrangement of proteins in the human erythrocyte membrane. *Biochem. Biophys. Res. Commun.* **40:**284.
34. Cleveland, D., S. Fisher, M. Kirshner, and U. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. *J. Biol. Chem.* **252:**1102.