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Increased Concentration of an Apparently Identical Cellular Protein in Cells Transformed by Either Abelson Murine Leukemia Virus or Other Transforming Agents

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Abelson murine leukemia virus (A-MuLV)-transformed cells, simian virus 40 (SV40)-transformed cells, and chemically transformed cells all have increased levels of a 50,000-molecular-weight host cell protein. The protein was detected with sera raised to the A-MuLV-transformed and chemically transformed cells and was tightly bound to T-antigen in extracts of SV40-transformed cells. Partial protease digests showed that the proteins from all three sources were indistinguishable. The three proteins were phosphorylated in cells, and the linkage of phosphate to the A-MuLV-associated P50 was to a serine residue. By immunofluorescence methods, P50-related protein was found on the surface of both normal lymphoid cells and A-MuLV-transformed lymphoid cells, but cell fractionation showed that the majority of P50 was free in the cytoplasm of the transformed cells. Immunofluorescence also showed that P50 was found in granules in the cytoplasm of both untransformed and SV40-transformed fibroblasts. Other cells gave indistinct patterns. Cocapping experiments showed that the A-MuLV-specified P120 protein is weakly associated with the surface P50-related protein of lymphoid cells, but no association of P120 and P50 could be demonstrated by immunoprecipitation methods. Although a monoclonal antiserum to P50 was used in many of these studies, the identity of the bulk P50 protein with the molecules that are reactive at the cell surface requires further study.

Mice bearing an Abelson murine leukemia virus (A-MuLV)-induced syngeneic tumor often make antibodies against a 50,000-molecular-weight phosphorylated host cell protein (23). This protein, called P50, is abundantly present in A-MuLV-transformed lymphoid cells and is at higher concentration in A-MuLV-transformed fibroblastic cells (NIH/3T3) than it is in normal NIH/3T3 cells. The monoclonal antibody, RA3-2C2, reacts with P50 and also reacts with the surface of normal B-lymphocytes (23; R. Coffman and I. Weissman, submitted for publication).

Accentuation of the concentration of specific phosphorylated host cell proteins of approximately 50,000 molecular weight has been reported for cells transformed by the oncogenic virus simian virus 40 (SV40), in the methylcholanthrene-induced fibrosarcoma called Meth A, and in a variety of other tumor cells (3, 5, 6, 8, 11, 13, 15-19, 25, 26). In SV40-transformed cells, the P54 protein is recovered in a complex with the nuclear SV40 T-antigen (15, 16, 18), and fluorescent-antibody detection of the protein has usually localized it to the nucleus (8, 19).

We have examined whether the 50,000-molecular-weight proteins in cells transformed by A-MuLV, SV40, and methylcholanthrene are related proteins. We have found apparent identity between the proteins found in these three systems. We have also shown that phosphate is linked to a serine residue in P50. Using fluorescence methods, we have found P50-related antigen on the surface of pre-B-lymphoid tumor cells but not on the surface of transformed fibroblasts. The bulk of P50, however, is in the cytosol of lymphoid cells. Fibroblasts often show antigen in a granular pattern in the cytoplasm.

MATERIALS AND METHODS

Mice and cell lines. BALB/cN female mice from our colony, 8 to 12 weeks old, were used.

A-MuLV-transformed lymphoid cell lines of BALB/c origin, 2M3 and its Moloney MuLV-superinfected derivative 2M3/M (30), as well as the C57L-derived L1-2 line (29), were grown in RPMI-1640 plus 10% heat-inactivated fetal calf serum and 2×10^{-5} M β-mercaptoethanol. Other cell lines were grown in Dulbecco-modified Eagle medium plus 10% calf serum. They included 70Z, a BALB/c-derived, chemically transformed pre-B-lymphoid cell line (20); NIH/3T3

cells; ANN-1, an NIH/3T3-derived line transformed by A-MuLV (24); SVT2, an SV40-transformed BALB/3T3-derived line (1) obtained from A. Levine, State University of New York, Stony Brook; and Meth A, a chemically-induced fibrosarcoma from BALB/c mice (6) obtained from A. DeLeo, Sloan-Kettering Institute, New York, N.Y.

Immune sera. Ab-TB serum was obtained from BALB/c mice bearing a 2M3/M tumor. Tumor-bearing mice were bled 3 to 4 weeks after tumor appearance. Sera that contained high titers of anti-P50 antibodies were pooled and used in these experiments (23). Anti-SV40 serum was a gift of A. Levine. Anti-Meth A serum was provided by A. DeLeo. RA3-2C2 were monoclonal rat antibodies that immunoprecipitated P50 from A-MuLV-transformed cell lysates (23). Anti-AbT serum, containing antibodies that immunoprecipitated the P120 of A-MuLV-transformed cell lines, was prepared as described previously (29). Goat anti-Moloney MuLV serum reacting with all of the virion proteins was provided by the Division of Cancer Cause and Prevention, National Cancer Institute. Fluorescein-labeled anti-rat immunoglobulin, rhodamine-labeled anti-mouse immunoglobulin, and fluorescein-labeled anti-goat immunoglobulin were prepared from sera made in rabbits and were purchased from Cappel Laboratories. The rabbit anti-mouse rhodamine serum was purified on mouse immunoglobulin-Sepharose 4B, and the other fluorescent sera were all cross-absorbed on mouse immunoglobulin-Sepharose 4B. Mouse anti-SV40 large T-antigen was a gift of A. Levine.

Immunofluorescence analysis. Surface staining of lymphocytes in suspension by using an indirect immunofluorescent assay was carried out as described (21); the fibroblastic cells were grown on glass cover slips and labeled as living monolayers.

For cocapping studies, the cells were incubated with the first antiserum for 25 min at 37°C, then washed twice and suspended in fresh medium with the appropriate fluorescent rabbit antiserum for about 60 min at 37°C. The cells were again washed twice, suspended in medium containing 0.2% sodium azide at 4°C, and labeled at 4°C for the presence of the second protein.

For intracellular staining, fibroblastic cells were grown on glass cover slips, or, in some instances, Meth A and ANN-1 cells were allowed to settle onto cover slips coated with poly-L-lysine (Sigma type V) to induce the cells to spread out. Lymphocytic cells were smeared by using a Shandon-Elliott cytospin, and all cell types were washed in cold phosphate-buffered saline (PBS). Cells were fixed for 20 min in 3.7% formaldehyde in PBS at room temperature, washed in cold PBS, and then permeabilized in acetone-PBS (1:1) for 2 min at 4°C, in acetone for 5 min at 4°C, and finally in acetone-PBS (1:1) for 2 min at 4°C, before being washed in cold PBS. The fixed cells were incubated with each serum for 25 min at room temperature in a damp chamber. After washing, 1 drop of solution containing 1 part glycerol, 1 part PBS, and 3.5% formaldehyde was added to each smear, which was then mounted over a microscope slide. Cells were examined under a standard 18 Zeiss fluorescence microscope.

Cell labeling and immunoprecipitation. Radioactive chemicals were purchased from New England Nuclear Corp., Boston, Mass. 2M3 cells were washed several times with PBS and suspended in labeling medium at a concentration of 10×10^6 to 15×10^6 cells per 2 ml. The fibroblastic cell lines were grown to almost confluence in 10-cm sterile plates. Monolayers were washed several times with PBS, and 3 ml of labeling medium was added. When labeled with [^{35}S]methionine, cells were incubated with Dulbecco-modified Eagle medium without methionine, supplemented with 5% dialyzed fetal calf serum and 125 μCi of [^{35}S]methionine per ml for 1 h at 37°C. When labeled with $^{32}PO_4$, cells were resuspended in Dulbecco-modified Eagle medium without PO_4 and enriched with 5% fetal calf serum; 300 μCi of $^{32}PO_4$ was added, and the mixture was incubated for 5 to 16 h at 37°C. Labeled cells were washed in PBS, and either the pellets of cells or the monolayers were extracted into 5 ml of lysis buffer: 10 mM $NaHPO_4$-Na_2HPO_4 (pH 7.5), 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS) at 0°C; 1 mM phenylmethylsulfonyl fluoride (Sigma) was then added. Cell lysates were clarified at 150,000 × *g* for 2 to 3 h as previously described (27). From 0.5 to 1 ml of cell lysate was immunoprecipitated with 5 to 10 μl of normal or immune serum. Antigen-antibody complexes were collected by binding to *Staphylococcus aureus* (12). SDS-polyacrylamide gel electrophoresis was performed on the discontinuous stacking system of Laemmli (14).

Partial proteolytic digestion. Proteolytic digestion was performed essentially as described by Cleveland et al. (4). The immunoprecipitated products were separated by electrophoresis through SDS-polyacrylamide gels, and the bands of interest were cut out from the wet gels. The fragments were washed several times in 0.1% SDS–1 mM EDTA–0.125 M Tris buffer (pH 6.8) and then applied to a second gel. *S. aureus* V8 protease (Miles) and α-chymotrypsin at several concentrations were added. The samples were concentrated at the interphase between the stacking and running gel and stopped for 30 min at room temperature, and then electrophoresis was continued.

High-voltage electrophoresis of phosphorylated amino acids. Phosphorylated proteins were immunoprecipitated and further purified by precipitation with trichloroacetic acid and washing in absolute alcohol. About 5,000 cpm of phosphate-labeled protein was hydrolyzed for 2 to 6 h in 6 N HCl at 100°C. Hydrolyzed samples were suspended in 5 μl of unlabeled phosphoserine, phosphothreonine, or phosphotyrosine at a concentration of 1 mg/ml each. Samples were analyzed by high-voltage electrophoresis in pyridine-acetic acid (pH 3.5) on Whatman 3MM paper at 2,400 V for 1 h (28).

RESULTS

To compare the 50,000-molecular-weight protein found in A-MuLV-transformed cells with that of SV40- or chemically transformed cells, we studied the following cell lines: 2M3, an A-MuLV-transformed lymphoid cell line; SVT2, a BALB/3T3-derived, fibroblastic, SV40-transformed cell line; and Meth A, a chemically induced fibrosarcoma of BALB/c origin. The three cell lines were labeled for 1 h with [^{35}S]methio-

nine, and the cell lysates were immunoprecipitated with a variety of antisera.

It was shown previously that mice carrying an A-MuLV-induced tumor often make antibody to the P50; we have called such P50-reactive sera Ab-TB sera (23). When labeled cell extracts were precipitated with an Ab-TB serum, the three tested cell lines showed a strong band of a 50,000-molecular-weight protein (Fig. 1, lanes c). Normal serum precipitated very little or no protein of this size (Fig. 1, lanes a). We show below that these three 50,000-molecular-weight proteins are closely related and probably identical; they will henceforth be referred to as P50.

An antiserum made to the SV40-induced tumor cell line precipitated a faint P50 band from 2M3, a strong band from the homologous tumor, and a weak band from Meth A cells (Fig. 1, lanes d). This behavior indicates that the majority of the reactivity of this serum with the SV40-transformed cell is a consequence of its reactivity with the 95,000-molecular-weight T-antigen that is known to be complexed to P50 in these cells (15, 16, 18).

When an anti-Meth A serum was used to precipitate extracts of the three cell lines, strong bands of P50 were found (Fig. 1, lanes e). This result confirms the serological identity of the P50 in the three cell lines. The anti-Meth A serum precipitated a strong band of T-antigen from SVT2 cells, as did the Ab-TB serum, demonstrating again the tight association of T-antigen with P50 in the SVT2 extracts.

Judging from the intensity of the bands, Meth A cells have the most P50, and SVT2 and 2M3 have about equal amounts. Virtually all of the P50 in SVT2 cells is complexed with T-antigen because the anti-SVT2 serum, which is mainly anti-T-antigen, brings down only slightly less P50 than Ab-TB serum, which reacts directly with P50. The different shape of the bands precipitated by the various sera and the apparent differences in migration rate of the P50 proteins are a consequence of the physical behavior of the specific sera during electrophoresis and are not differences intrinsic to the proteins.

As a control, extracts of the L1-2 cell line were precipitated by the various sera. L1-2 is an A-MuLV-transformed cell line of C57L origin that lacked detectable P50 when assayed previously with Ab-TB sera. It also lacked reactivity with anti-SVT2 and anti-Meth A sera (Fig. 1).

Further tests of the identity between the three P50s were performed by serially precipitating with one serum and then a second. Once one serum cleared P50 from an extract, the other sera could not precipitate further P50 (data not shown).

In previous studies the anti-P50 monoclonal serum RA3-2C2 did not coprecipitate the A-MuLV-encoded P120 protein (23). This result was confirmed in the present work by the lack

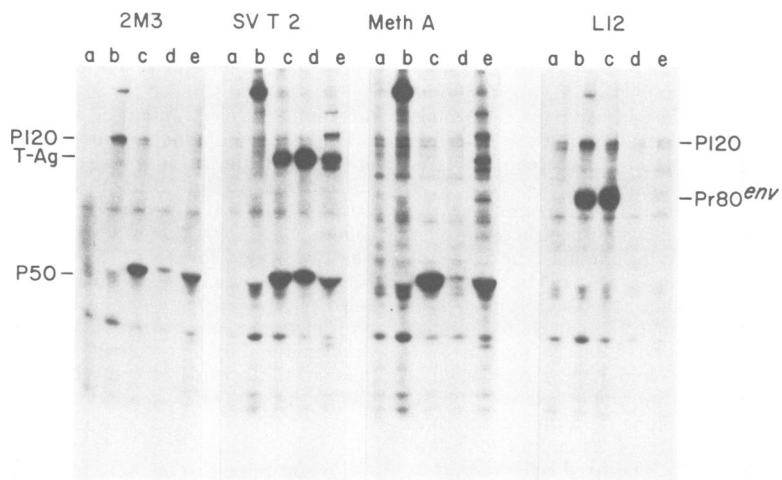

FIG. 1. *Immunoprecipitation of P50 host protein from 2M3, SVT2, Meth A, and L1-2 [^{35}S]methionine-labeled cell lysates. Equal numbers of counts per minute of ^{35}S-labeled cell lysates from 2M3, SVT2, Meth A, and L1-2 cells were immunoprecipitated with the following sera: normal mouse serum (lanes a); anti-AbT serum shown to immunoprecipitate the P120 of A-MuLV (lanes b); Ab-TB serum obtained from BALB/c mice bearing a syngeneic A-MuLV-induced tumor (lanes c); anti-SVT2 serum (lanes d); and anti-Meth A serum (lanes e). Samples were processed as described in the text, and the products were analyzed by electrophoresis through an SDS-polyacrylamide gel.*

of P120 in the anti-Meth A precipitate of the 2M3 cell extract. An anti-P120 serum also precipitated very little P50 from any of the cell lines and, specifically, did not precipitate more P50 from 2M3 than from the other cells (Fig. 1, lanes b). The anti-P120 serum had reactivity with a high-molecular-weight protein in the fibroblast transformed lines; this has been previously observed with other such lines and represents a protein that has not been identified. The anti-P120 serum, as well as the Ab-TB serum, precipitated a band of $Pr80^{env}$ from the L1-2 cells because they were coinfected with helper Moloney MuLV.

Comparison of the partial digestion products of P50 host protein obtained from the different cell lines. Further comparison between the P50s of 2M3, SVT2, and Meth A cells was carried out by analyzing the partial proteolytic digestion products of P50 obtained from the different sources. [^{35}S]methionine-labeled extracts were immunoprecipitated with Ab-TB serum, the P50 proteins were resolved by SDS-polyacrylamide gel electrophoresis, and the P50 bands were excised from the wet gels. The bands were applied to a second gel and digested with various concentrations of *S. aureus* V8 protease. The three P50s gave identical partial digestion patterns (Fig. 2). Identical patterns of proteolytic products were also observed when P50 of 2M3, SVT2, or Meth A cells, immunoprecipitated with Ab-TB serum, was treated with α-chymotrypsin (Fig. 3). When each of the cell lines was immunoprecipitated with the serum specific to that line, the P50 molecules presented the same pattern of proteolytic products when digested with V8 protease (results not shown). Thus the various P50s appear identical at this level of resolution.

Phosphorylation of P50 host protein and characterization of the phosphate-protein bond. We have shown previously that the P50 in 2M3 cells is a phosphoprotein (23). To examine whether the P50 found in SVT2 and Meth A cells was also phosphorylated, the lines were labeled for 5 h with $^{32}PO_4$, and lysates were immunoprecipitated with either normal mouse serum (Fig. 4, lanes A), RA3-2C2, a monoclonal anti-P50 antibody (23) (lanes B), Ab-TB serum (lanes C), or anti-P120 serum (lanes D). Phosphorylated P50 was present in all of these cell lines. These antibodies were also able to immunoprecipitate the phosphorylated form of large T-antigen from SVT2 cells, reflecting the complex of this protein with P50. Although anti-P120 precipitated a heavy band of phosphorylated P120 from 2M3 cells, the monoclonal anti-P50 precipitated no more P120 than did normal serum. From Meth A cells, no protein other than P50 was evident in the immunoprecipitates. Thus, no other phosphorylated proteins are tightly bound to P50 in either the A-MuLV-transformed cells or the Meth A cells.

Recently it was shown that the transforming proteins of various viruses are phosphorylated on a tyrosine residue (10, 28). To examine the

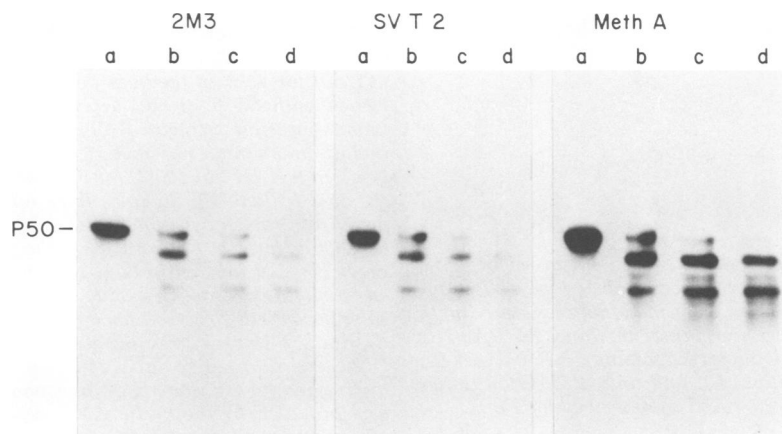

FIG. 2. *Comparison of partial proteolytic products, digested with S. aureus V8, of P50 obtained from 2M3, SVT2, and Meth A cell lysates. The different cell lysates were immunoprecipitated with Ab-TB serum, and the P50 bands were cut out from the wet gel, washed in 0.1% SDS–125 mM Tris buffer (pH 6.8)–1 mM EDTA, and loaded on a second SDS-polyacrylamide gel. The individual lanes were treated with no enzyme (lanes a), or 10 ng (lanes b), 20 ng (lanes c), or 40 ng (lanes d) of V8 protease. Samples were collected at the interphase between the stacking and the running gel, the current was stopped for 30 min to let digestion occur, and then electrophoresis was continued.*

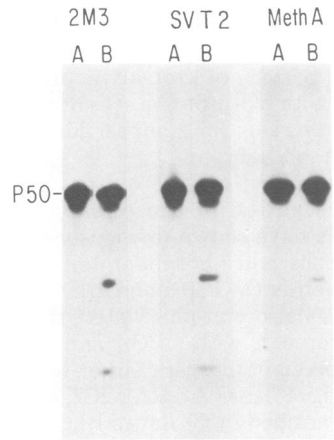

FIG. 3. *Comparison of partial proteolytic products of P50, obtained from 2M3, SVT2, and Meth A cell lysates and digested with α-chymotrypsin. No enzyme (lanes A); 100 μg of α-chymotrypsin per lane (lanes B). The samples were handled as described for Fig. 2.*

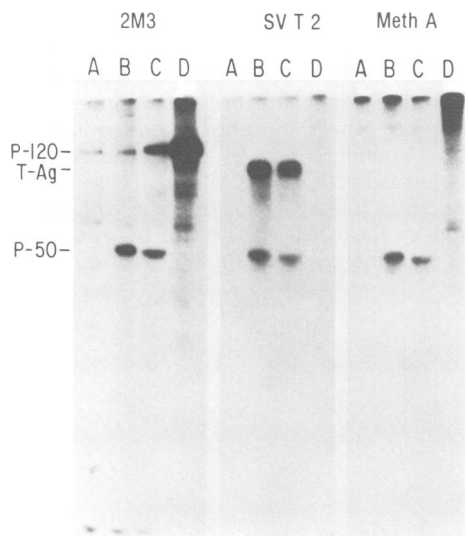

FIG. 4. *Phosphorylation of P50 in vivo. 2M3, SVT2, and Meth A cells were labeled with $^{32}PO_4$ for 5 h, and cell lysates were immunoprecipitated with the following sera: normal serum (lanes A); RA3-2C2 monoclonal antibodies recognizing the P50 (lanes B); Ab-TB serum (lanes C); and anti-AbT serum (lanes D). Samples were treated as described for Fig. 1.*

site of phosphorylation in P50, P50 from ^{32}P-labeled 2M3 cells was immunoprecipitated with the monoclonal RA3-2C2 antibody, acid hydrolyzed, and analyzed by high-voltage electrophoresis at pH 3.5. As controls, we examined the phosphoamino acids released from in vivo-labeled P120, in vitro-labeled P120 (a phosphotyrosine marker; 28), and the product of a cyclic AMP-dependent kinase (a phosphoserine marker). The different phosphorylated proteins were precipitated with trichloroacetic acid, washed with absolute alcohol, and hydrolyzed. After various times of hydrolysis, the major phosphoamino acid released from P50 comigrated with phosphoserine (Fig. 5, lanes A, B, and C). In addition, a heavy spot of free radioactive orthophosphate was also detected. A minor spot of phosphothreonine was also detected, but no phosphotyrosine was found. The spot migrating just ahead of the phosphotyrosine marker was UMP (2, 22); a spot of CMP was also present near the origin. These either were

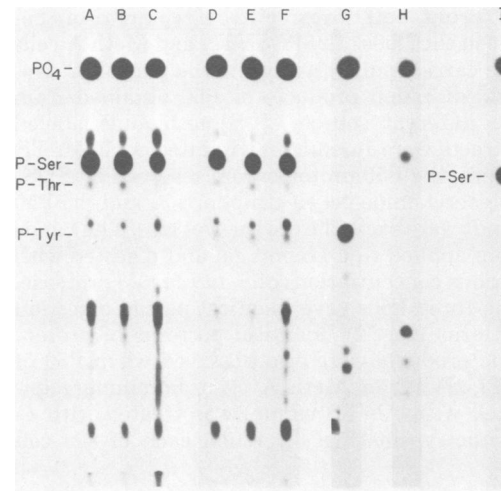

FIG. 5. *Characterization of the phosphoamino acids in P50. 2M3 cells were labeled with $^{32}PO_4$ (300 μCi/ml) for 16 h in medium devoid of PO_4 and enriched with 5% fetal calf serum. Cell lysates were immunoprecipitated with RA3-2C2 monoclonal antibodies, and samples of about 5,000 cpm were hydrolyzed in 6 N HCl at 100°C for 6 h (lane A), 4 h (lane B), or 2 h (lane C). Samples were applied to 3MM paper and run in pyridine-acetic acid buffer (pH 3.5) at 2,400 V for 1 h. For analyzing the phosphoamino acids of P120 labeled in vivo, the above cell lysates were immunoprecipitated with anti-AbT serum, and samples were hydrolyzed for 6 h (lanes D), 4 h (lane E), and 2 h (lane F). For a phosphotyrosine marker, P120 was allowed to label itself in vitro (28) and hydrolyzed for 2 h (lane G). A phosphoserine marker was provided by the cyclic AMP-dependent kinase acting under in vitro conditions (lane H). A purified P50 was obtained by electroelution from an SDS-polyacrylamide gel. About 800 cpm of purified P50 was hydrolyzed for 2 h, and the autoradiogram was exposed for 8 days (lane I). Each of the hydrolyzed samples was mixed with unlabeled phosphoserine, phosphothreonine, and phosphotyrosine marker, and the electrophoresis paper was stained with ninhydrin for the location of markers.*

contaminants from RNA or were released from protein by the acid.

In agreement with our previous report (28), the major phosphoamino acid from in vivo-labeled P120 was phosphoserine, but some phosphotyrosine and phosphothreonine was observed (Fig. 5, lanes D, E, and F). As expected, P120 phosphorylated in vitro yielded only phosphotyrosine (Fig. 5, lane G), and cyclic AMP-dependent kinase yielded phosphoserine (Fig. 5, lane H).

To confirm that P50 immunoprecipitated by RA3-2C2 monoclonal antibody gave identical results to that immunoprecipitated by Ab-TB serum, $^{32}PO_4$-labeled 2M3 cell lysates were immunoprecipitated with Ab-TB serum. In this case, the specific proteins were separated by electrophoresis, and the radioactive P50 was excised and electroeluted. Hydrolysis of the purified P50 protein yielded a single spot of radioactive phosphoserine and free orthophosphate (Fig. 5, lane I), further indicating the P50 is phosphorylated in vivo only at a serine residue. We have not been able to phosphorylate the P50 under in vitro conditions either in a soluble reaction or when immunoprecipitated with Ab-TB serum and adsorbed onto *S. aureus* particles.

Localization of P50. To localize P50 in the cells, a number of lines were examined using immunofluorescence with both an Ab-TB serum and the monoclonal anti-P50 (Fig. 6). When tested on live cells for surface P50 and on fixed cells for intracellular P50, both reagents produced the same staining patterns (Table 1). Consistent with previous determinations (Coffman and Weissman, submitted for publication), a small percentage of thymus cells (<3%) and 30% of spleen cells had a P50-related surface protein (Fig. 6, A and B). By double labeling with an anti-mouse immunoglobulin reagent, the P50-positive cells were shown to have surface immunoglobulin and thus were B-lymphocytes. No intracellular fluorescence could be detected in these cells. Coffman and Weissman originally chose the monoclonal anti-P50 because it recognized a B-lymphocyte-specific antigen (see reference 23), and the present work thus confirms their data.

When lymphoid, A-MuLV-transformed cells were examined, surface staining was evident (Fig. 6, C and D), but the rounded morphology of the cells prevented detection of intracellular fluorescence. Because the A-MuLV-transformed lymphoid cells are related to pre-B-lymphocytes, a similar cell line derived by chemical treatment of mice, 70Z, was examined and found to have surface fluorescence with the monoclonal antibody (Table 1). Immunoprecipitation studies had previously shown that 70Z had a very low level of P50; in this way, 70Z is like the A-MuLV lymphoid transformant L1-2, which has very little immunoprecipitable P50 (23) but a strong surface fluorescence with the monoclonal antibody (Table 1).

To examine whether the majority of the P50 in A-MuLV-transformed lymphoid cells might be in the cytosol rather than attached to membranes, a [^{35}S]methionine-labeled 2M3 extract was fractionated into nucleus, total membranes, and cytosol. The various fractions were then immunoprecipitated with either normal serum (Fig. 7, lanes A), the RA3-2C2 monoclonal anti-P50 (lanes B), or anti-Moloney MuLV serum (to precipitate the A-MuLV P120) (lanes C). The P50 protein was largely in the cytosol, with minor amounts in the other cell fractions. By contrast, P120 was mainly recovered in the nuclear and membrane fractions, consistent with its previous localization in the plasma membrane (29).

When fibroblastic, A-MuLV transformants were examined by immunofluorescent techniques, a different picture emerged. No surface antigen was detectable on any fibroblastic cells (Table 1). When uninfected NIH/3T3 cells were fixed and stained, however, normal serum showed no reactivity (Fig. 6, E and F), but the anti-P50 reagent showed a granular, cytoplasmic fluorescence (Fig. 6, G and H). The SV40-transformed line SVT2 showed a much more brightly staining pattern of cytoplasmic, granular fluorescence (Fig. 6, I and J). In the SV40-transformed cells, the possibility of nuclear antigen cannot be excluded because the bright cytoplasmic staining makes it difficult to judge whether the nucleus has background staining or a real signal. Others have described the occurrence of P50 in the nucleus of SV40-transformed cells (8, 19), and we have confirmed that in an SV40-transformed CV-1 monkey cell line (7), there is preponderant nuclear staining. Furthermore, we have confirmed that in SVT2 the T-antigen staining was mainly nuclear but that some granular cytoplasmic staining was also evident. Thus, we conclude that in normal fibroblasts, P50 can be a cytoplasmic protein concentrated in vesicles; in SV40-transformed cells it is apparently often carried into the nucleus probably because of its association with T-antigen (8, 19).

The A-MuLV-transformed fibroblastic line, ANN-1, and the Meth A cell line did not stain for P50 in our hands, although it is clear that both lines possess P50. A possible explanation for this observation is that both lines have a very transformed morphology and do not spread out well compared to untransformed fibroblasts. When cells display a rounded-up morphology, the P50 protein might become so dispersed

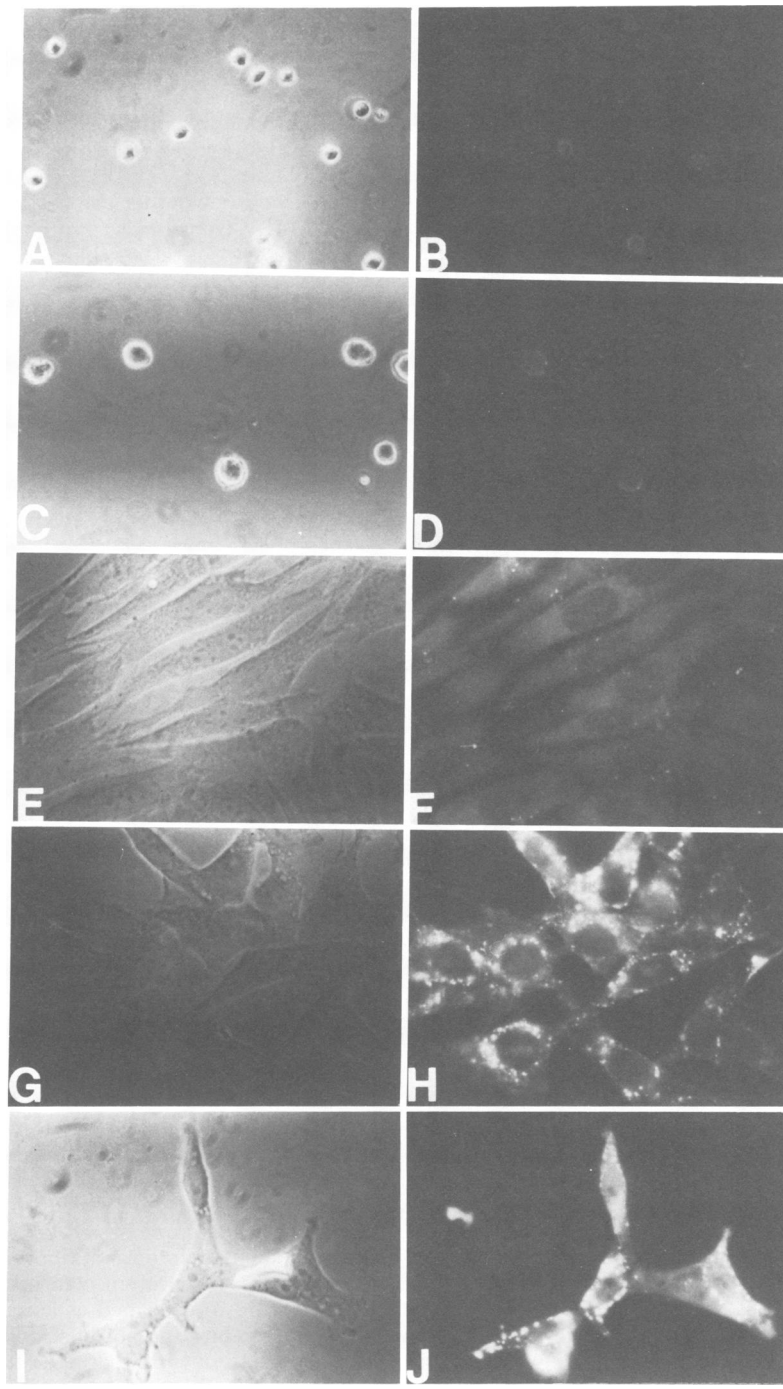

FIG. 6. *Localization of P50 by immunofluorescence. Cells were labeled for the presence of P50 either as living suspensions or as fixed monolayers by using the monoclonal or murine reagents. (A) Phase contrast of BALB/c spleen cells labeled in suspension with monoclonal anti-P50. (B) Spleen cell fluorescent profile with anti-P50. (C) Phase contrast of 2M3 cells labeled in suspension with monoclonal anti-P50. (D) 2M3 fluorescent profile with anti-P50. (E) Phase contrast of fixed NIH/3T3 fibroblasts labeled with normal mouse serum and rhodamine-labeled rabbit anti-mouse immunoglobulin serum. (F) NIH/3T3 normal mouse serum fluorescent profile. (G) Phase contrast of NIH/3T3 fibroblasts labeled with Ab-TB serum. (H) NIH/3T3 fibroblast profile with Ab-TB serum. (I) Phase contrast of SVT2 fibroblasts labeled with Ab-TB serum. (J) SVT2 fluorescent profile with Ab-TB serum.*

TABLE 1. *Distribution of P50 as assessed by immunofluorescence*

Cell type	Location of fluorescence					
	Surface		Cytoplasm		Nucleus	
	Ab-TB[a]	2C2[b]	Ab-TB	2C2	Ab-TB	2C2
BALB/c thymus	ND[c]	+ (1-3%)	ND	–	ND	–
BALB/c spleen	ND	+ (30%)	ND	–	ND	–
2M3	+	+	–	–	–	–
70Z	ND	+	ND	ND	ND	ND
L1-2	+	+	ND	ND	ND	ND
NIH/3T3	–	–	+	+	–	–
SVT2	–	–	+++	+	–	–
Meth A	–	–	–	–	–	–
ANN-1	–	–	–	–	–	–

[a] Ab-TB serum is the serum from a BALB/c mouse carrying a transplanted A-MuLV-induced syngeneic tumor. Such sera are selected for a high anti-P50 reactivity.
[b] 2C2 is the monoclonal anti-P50 rat serum RA3-2C2.
[c] ND, Not done.

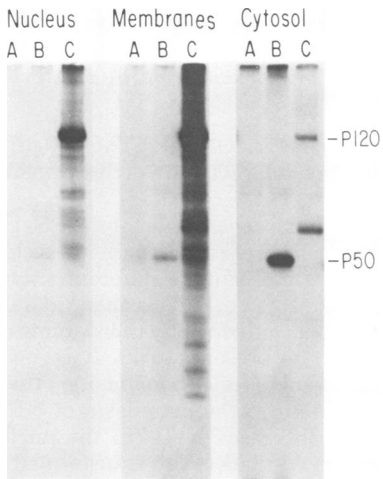

FIG. 7. *Distribution of P50 and P120 in different cell fractions. [^{35}S]methionine-labeled 2M3 cells were fractionated according to the method of Hay (9). Briefly, cells were allowed to swell for 10 min at 4°C in 10 mM Tris (pH 7.4)-1 mM $MgCl_2$, followed by Dounce homogenization. Nuclei and large cell fragments were removed by centrifugation at 1,000 × g for 5 min (nucleus), and the supernatant was further clarified at 100,000 × g for 60 min to yield a pellet (membranes) and the supernatant (cytosol). Each of the fractions was immunoprecipitated with normal (A), RA3-2C2 anti-P50 (B), and goat anti-Moloney MuLV (C) sera.*

through the cytoplasm that the immunofluorescent staining would not be clearly distinct from background staining. Melero et al. (19) reported that Meth A cells had a nuclear 48,000-molecular-weight, T-antigen-associated protein; the relationship of their data to the present observations is uncertain.

Association of the surface P50-related protein and P120. We approached the question of whether the surface P50-related protein and P120 were associated by investigating whether the two proteins would cocap on A-MuLV-transformed lymphoid cells. Using the 2M3 cell line, we first demonstrated that the fluorescent second antibodies used contained no cross-reacting activities. The fluoresceinated rabbit anti-rat immunoglobulin serum was found to label only cells previously incubated with the monoclonal anti-P50 reagent. Similarly, the rhodaminated rabbit anti-mouse immunoglobulin serum only labeled cells previously incubated with mouse anti-AbT serum (anti-P120; 29) and not anti-P50 or normal serum. In addition, the fluorescent antibodies did not cross-react with each other.

When 2M3 cells were separately capped for either P50 or P120 proteins and restained, using a different fluorochrome for the other protein under noncapping conditions, an identical fluorescence pattern was observed irrespective of which protein was first capped. A fraction of the two proteins was found to be localized to the same parts of the cell surface, and a fraction was found to be independent (Fig. 8). This finding suggests that some of both the P120- and P50-related proteins is associated with the other protein, and some is not associated or so weakly associated that the two do not cocap. As a control, cocapping of Moloney MuLV gp70 and the P50-related protein was studied on an A-MuLV-transformed, producer cell line, 2M3/M. No association at all of the two antigens was evident in either direction. Thus, the partial cocapping of P120 and the P50-related protein is meaningful because it is distinguishable from a case where no association is evident.

DISCUSSION

It has previously been shown by others that an approximately 50,000-molecular-weight pro-

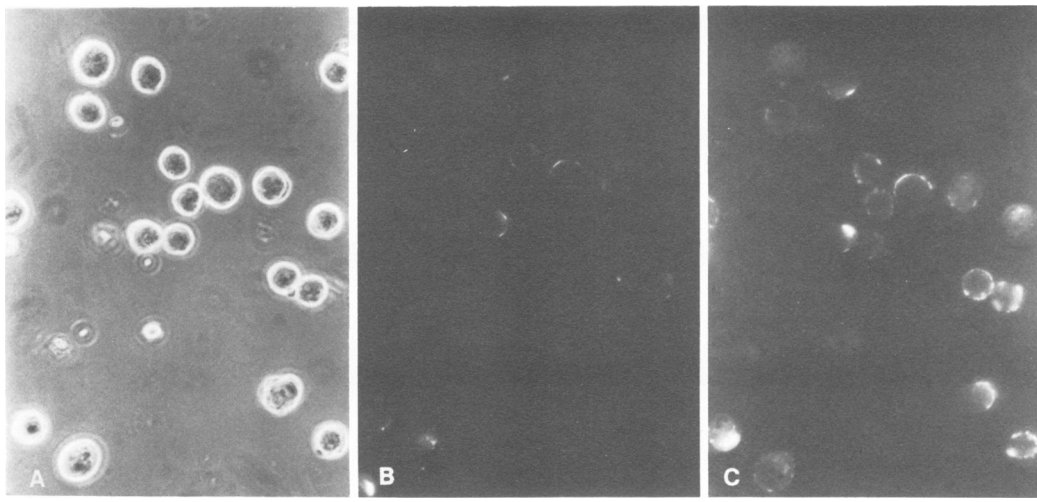

FIG. 8. *Association of P50 and P120 proteins showing partial cocapping of the two proteins. P50 proteins on 2M3 cells were capped by using monoclonal rat anti-P50 followed by fluorescein-labeled rabbit anti-rat immunoglobulin serum. Cells were then restained under noncapping conditions for the presence of P120, using mouse anti-AbT serum and rhodamine-labeled rabbit anti-mouse immunoglobulin serum. (A) Phase contrast. (B) P50 fluorescein staining. (C) P120 rhodamine staining.*

tein has a greatly increased concentration in cells transformed by a variety of means (5, 11). This protein is one that associates tightly with T-antigen of SV40 and has been called nonviral T-antigen (15, 16, 18). We had previously shown that mice carrying or rejecting an A-MuLV-induced lymphoid tumor make high concentrations of anti-P50 antibody (23). That antibody reacts with a prominent cell surface antigen of the A-MuLV-induced lymphoid tumor cells. The present evidence shows that the P50 of A-MuLV-transformed lymphoid cells, which is also increased in A-MuLV-transformed fibroblastic cells, has the same pattern of partial protease digest products as the nonviral T-antigen of an SV40-transformed cell line and the 50,000-molecular-weight antigen of a methylcholanthrene-transformed BALB/3T3 cell line. Furthermore, antibodies made to the A-MuLV-, SV40-, and methylcholanthrene-transformed cell lines each precipitated P50 proteins from all three types of transformed cells. Also, a monoclonal antibody to the A-MuLV-associated P50 protein reacted with the P50s from the other cell lines. Even Rous sarcoma virus-transformed mouse cells have a significant level of the same P50 found in other transformed cells, but the 50,000-molecular-weight protein that coprecipitates with the $pp60^{src}$ transforming protein of Rous sarcoma virus (10) appears to differ from the P50 protein described here (Rotter, unpublished data). Thus, closely related and presumably identical P50 proteins are found at increased concentration in many tumor cells, including both lymphoid and fibroblastic cell types. The P50 probably has some key relationship to the transformed state.

The subcellular localization of the P50 protein in different cells presents a puzzle. It is clearly a B-lymphocyte cell surface differentiation antigen (R. Coffman and I. Weissman, manuscript in preparation; confirmed by the staining of normal cells in the present work). It is, however, evident in thymocytes although not on them (11, 23). It is present in the cytoplasm of NIH/3T3 cells and at increased levels in the nucleus or cytoplasm of various SV40-transformed fibroblastic cells. Its localization by fluorescence methods in A-MuLV-transformed cells or chemically transformed cells was not possible in our studies because intracellular proteins in these rounded cells were difficult to examine by immunofluorescence; had the protein been in the nucleus it should probably have been visible, and therefore it is probably not concentrated in the nucleus. Cell fractionation studies on lymphoid cells showed P50 to be a soluble protein of the cytosol. It would seem that the protein is a cell surface protein in normal B-lymphoid cells, is greatly increased in the cytosol of certain transformed lymphoid cells, is a cytoplasmic protein in fibroblastic cells, and can be drawn into the nucleus in SV40-transformed cells probably because of its association with SV40 T-antigen. Its localization to cytoplasmic granules requires further study to determine the nature of the granules. The protein has a short half-life in cells (Rotter and Baltimore, unpublished

data), and it could be that the granules are lysosomes. The readily detectable fluorescence in NIH/3T3 cells, although less than that in SV40-transformed cells, seems more than might have been expected from the low level of immunoprecipitable protein in such cells; possibly, the antibody is detecting fragments of the protein in the process of degradation.

It is clear that P50 in lymphoid cells is not all surface localized; in fact, the vast majority was found free in the cytosol. Because of the difficulty of visualizing P50 in rounded cells, there must be quite high concentrations of P50 in the cytoplasm of lymphoid cells that are not detected by fluorescence methods. The discordance of surface antigen and total P50 is best shown by the lack or low level of immunoprecipitable P50 in 70Z and L1-2 cells, lines that have a brightness of surface fluorescence equivalent to those of 2M3 and other high-P50 A-MuLV transformants. This suggests that the proteins with P50 antigenicity in different locales and different cells may be different molecules. In fact, the possibility exists that the antigen on the B-lymphocyte surface is not the P50 at all but is a cross-reactive protein. The strong reason for saying that the P50 is related to the surface antigen is their common antigenicity as revealed both by a monoclonal serum and by a series of sera from tumor-bearing animals.

A-MuLV transformation clearly enhances P50 concentration in fibroblastic cells (23). It could be that A-MuLV transformation also enhances cytoplasmic P50 in lymphoid transformants and that 70Z has so little P50 because it was not transformed by A-MuLV. L1-2 was transformed by A-MuLV but does not have an enhanced P50 level; however, its lack of P50 is a unique observation among many A-MuLV transformants we have examined, and it is also unique in being rejected by syngeneic animals.

P50 is a phosphorylated protein in both fibroblastic and lymphoid cells (3, 11, 16, 18; present studies). The phosphate is linked to a serine residue, at least in the A-MuLV-transformed lymphoid cell P50. Thus, the kinase activity associated with the A-MuLV transforming protein, which makes only phosphotyrosine bonds in the A-MuLV protein itself, either is not active on the P50 or has a different amino acid specificity when acting on P50; most likely P50 is not a substrate for the A-MuLV kinase. Also, no labeling of P50 in vitro could be shown in immunoprecipitates of P50.

The partial linkage of the surface P50-related protein to the A-MuLV protein revealed by the cocapping experiments suggests a weak association of the two proteins. Immunoprecipitation experiments, done under the fairly harsh conditions of the immunoprecipitation buffer solution, have not shown any indication of an association. We conclude that the proteins weakly interact at the cell surface, but the significance of the interaction is unclear. It is equally unclear what the tight association of T-antigen and P50 signifies. P50 is an unusual protein in that it has a half-life of only a few hours in the cell (Rotter and Baltimore, unpublished data). This raises the possibility that some of the differences in steady-state concentration may be a consequence of changes in half-life of the protein.

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LITERATURE CITED

1. **Aaronson, S. A., and G. J. Todaro.** 1968. Basis for the acquisition of malignant potential by mouse cells cultivated *in vitro*. Science **162**:1024–1026.
2. **Ambros, V., and D. Baltimore.** 1978. Protein is linked to the 5′ end of poliovirus RNA by a phosphodiester linkage to tyrosine. J. Biol. Chem. **253**:5263–5266.
3. **Carroll, R. B., K. Muello, and J. A. Melero.** 1980. Coordinate expression of the 48K host nuclear phosphoprotein and SV40 T Ag upon primary infection of mouse cells. Virology **102**:447–452.
4. **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**:1102–1106.
5. **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**:2420–2424.
6. **DeLeo, A. B., H. Shiku, T. Takahoshi, M. John, and L. J. Old.** 1977. Cell surface antigens of chemically induced sarcomas of mouse. I. Murine leukemia virus related antigens and alloantigens on cultured fibroblasts and sarcoma cells: description of a unique antigen on BALB/c Meth A sarcoma. J. Exp. Med. **146**:720–734.
7. **Gluzman, Y., J. F. Sambrook, and R. J. Frisque.** 1980. Expression of early genes of origin-defective mutants of simian virus 40. Proc. Natl. Acad. Sci. U.S.A. **77**:3898–3902.
8. **Gurney, E. G., R. O. Harrison, and J. Fenno.** 1980. Monoclonal antibodies against simian virus 40 T antigens: evidence for distinct subclasses of large T antigen and for similarities among non-viral antigens. J. Virol. **34**:752–763.
9. **Hay, A. J.** 1974. Studies on the formation of the influenza virus envelope. Virology **60**:398–418.
10. **Hunter, T., and B. H. Sefton.** 1980. Transforming gene product of Rous sarcoma virus phosphorylates tyrosine. Proc. Natl. Acad. Sci. U.S.A. **77**:1311–1315.
11. **Jay, G., A. B. DeLeo, E. Appella, G. G. Dubois, L. W.**

Law, G. Khoury, and L. J. Old. 1980. A common transformation related protein in murine sarcomas and leukemias. Cold Spring Harbor Symp. Quant. Biol. **44:** 659–664.

12. **Kessler, S. W.** 1975. Rapid isolation of antigens from cells with a staphylococcal protein A antibody adsorbent: parameters of the interaction of antibody-antigen complex with protein A. J. Immunol. **115:**1617–1624.
13. **Kress, M., E. May, R. Cossingena, and P. May.** 1979. Simian virus 40-transformed cells express new species of proteins precipitable by anti-simian virus 40 tumor serum. J. Virol. **31:**472–483.
14. **Laemmli, U. K.** 1975. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) **227:**680–685.
15. **Lane, D. P., and L. V. Crawford.** 1979. T antigen is bound to a host protein in SV40 transformed cells. Nature (London) **278:**261–263.
16. **Linzer, D. I. H., 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:**43–52.
17. **Luka, J., H. Jornvall, and G. Klein.** 1980. Purification and biochemical characterization of the Epstein-Barr virus-determined nuclear antigen and an associated protein with a 53,000-dalton subunit. J. Virol. **35:**592–602.
18. **McCormick, F., and E. Harlow.** 1980. Association of murine 53,000 dalton phosphoprotein with simian virus 40 large-T antigen in transformed cells. J. Virol. **34:** 213–224.
19. **Melero, J. A., S. Tur, and R. B. Carroll.** 1980. Host nuclear proteins expressed in simian virus 40 transformed and infected cells. Proc. Natl. Acad. Sci. U.S.A. **77:**97–101.
20. **Paige, C. J., P. Kincade, and P. Ralph.** 1978. Murine B cell leukemia line with inducible surface immunoglobulin expression. J. Immunol. **121:**641–647.
21. **Raff, M. C., M. Feldman, and S. de Petris.** 1973. Monospecificity of bone marrow-derived lymphocytes. J. Exp. Med. **137:**1024–1030.
22. **Rotenberg, P. G., T. J. R. Harris, A. Nomoto, and E. Wimmer.** 1978. O^4-(5′-uridylyl)tyrosine is the bond between the genome-linked protein and the RNA of poliovirus. Proc. Natl. Acad. Sci. U.S.A. **75:**4868–4872.
23. **Rotter, V., O. N. Witte, R. Coffman, and D. Baltimore.** 1980. Abelson murine leukemia virus-induced tumors elicit antibodies against a host cell protein, P50. J. Virol. **36:**547–555.
24. **Scher, C. D., and R. Siegler.** 1975. Direct transformation of 3T3 cells by Abelson murine leukemia virus. Nature (London) **253:**729–731.
25. **Simmons, D. T., M. A. Martin, P. T. Mora, and C. Chang.** 1980. Relationship among tau antigens isolated from various lines of simian 40-transformed cells. J. Virol. **34:**650–657.
26. **Smith, A. E., R. Smith, and E. Paucha.** 1979. Characterization of different tumor antigens present in cells transformed by simian virus 40. Cell **18:**335–346.
27. **Witte, O. N., and D. Baltimore.** 1978. Relationship of retrovirus polyprotein cleavages to virion maturation studied with temperature-sensitive murine leukemia virus mutants. J. Virol. **26:**750–761.
28. **Witte, O. N., A. Dasgupta, and D. Baltimore.** 1980. Abelson murine leukemia virus protein is phosphorylated *in vitro* to form phosphotyrosine. Nature (London) **283:**826–831.
29. **Witte, O. N., N. Rosenberg, and D. Baltimore.** 1979. Preparation of syngeneic tumor regressor serum reactive with the unique determinants of the Abelson MuLV encoded P120 protein at the cell surface. J. Virol. **31:** 776–784.
30. **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 fibroblasts and lymphoid cells. Proc. Natl. Acad. Sci. U.S.A. **75:**2488–2492.