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Induced Differentiation of Avian Myeloblastosis Virus-Transformed Myeloblasts: Phenotypic Alteration Without Altered Expression of the Viral Oncogene

G. SYMONDS,* K.-H. KLEMPNAUER,† G. I. EVAN,‡ AND J. M. BISHOP

Department of Microbiology and Immunology and The G. W. Hooper Foundation, University of California, San Francisco, California 94143

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Cells of a clone of avian myeloblastosis virus-transformed myeloblasts were induced to differentiate to adherent myelomonocytic cells by treatment with lipopolysaccharide. These adherent cells were subcultured and maintained as a line for more than 6 months with lipopolysaccharide present. Cells of this line were induced to differentiate to nondividing macrophage-like cells by the addition of the tumor promoter 12-O-tetradecanoylphorbol-13-acetate. In this way, the following homogeneous cell populations representing three distinct stages of myeloid differentiation were obtained: I, actively dividing myeloblasts that grew in suspension: II, actively dividing adherent cells; and III, fully differentiated nondividing cells resembling macrophages. When the expression of v-*myb* (the oncogene of avian myeloblastosis virus) was examined in cells of these three differentiation stages, it was found that the protein encoded by v-*myb* ($p45^{v\text{-}myb}$) continued to be synthesized in similar quantities and showed no obvious alteration (assessed by partial proteolytic digestion and two-dimensional gel electrophoresis) during differentiation. These results show that cells transformed by v-*myb* can be induced to differentiate without affecting the expression of v-*myb* and imply that, during differentiation, the effect of v-*myb* is suppressed by a mechanism other than altered expression of the oncogene.

A variety of cells can be transformed by retroviruses in vitro and in vivo (1). Whereas in vivo transformation can be caused by retroviruses possessing or lacking an oncogene, only retroviruses possessing an oncogene have been shown to transform cells in vitro. Transformation by retroviruses possessing an oncogene is thought to be effected by the protein encoded by the oncogene (1). Very little is known, however, about the way in which these proteins act. We have approached this question by asking, how can the action of the protein be phenotypically suppressed? One way to do this is to attempt to induce the transformed cells to differentiate. An extensive literature exists concerning induced differentiation of various cells types including normal and transformed hematopoietic cells (7, 15, 18, 21–24, 35, 36, 38).

Avian myeloblastosis virus (AMV) specifically transforms cells of the myelomonocytic lineage, resulting in the unrestrained growth of immature blastlike cells standardly termed AMV-transformed myeloblasts (1, 5, 11, 14, 25, 26, 28, 30). The oncogene of this virus, v-*myb* (1, 9), encodes a 45,000-M_r protein, $p45^{v\text{-}myb}$ (6, 16). In the present study we have employed three distinct homogeneous populations of myeloid cells to analyze a means by which the effect of $p45^{v\text{-}myb}$ may be obviated. These stages consist of (I) myeloblasts, (II) an intermediate stage of myeloid differentiation, and (III) mature macrophages. We have found that AMV-transformed myeloblasts are able to differentiate without a decrease in the synthesis or obvious alteration of $p45^{v\text{-}myb}$. In an extension of the present studies (17), however, there appears to be an altered subcellular location of the protein during differentiation—from predominantly nuclear in the first two stages to predominantly cytoplasmic (perinuclear) in mature macrophages.

* Corresponding author.

† Present Address: ZMBH, Universitat Heidelberg, Im Neuenheimer Feld 364, D-69, Heidelberg, West Germany.

‡ Present Address: Ludwig Institute for Cancer Research, MRC Centre, Cambridge CB2 2QH, United Kingdom.

MATERIALS AND METHODS

Cells and cell culture. BM2 is a line of chicken nonproducer myeloblasts (30) obtained as previously described (26, 28, 30, 33). The clone used in the present experiments was BM2 clone 3 obtained from G. and C. Moscovici. The growth medium employed was BT-88 (25, 30) (GIBCO Laboratories; medium formulation 78-5440), to which 10% (vol/vol) tryptose phosphate broth, 160 μg of folic acid (Sigma Chemical Co.) per ml, 50 μg of gentamycin (Irvine Scientific, Santa Ana, Calif.) per ml, 5% heat-inactivated (56°C, 30 min) calf serum, and 5% heat-inactivated chicken serum (GIBCO) were added. Cells were split 1:3 or 1:4 every 2 or 3 days and were grown in 60-mm petri dishes (Corning Glass Works) to densities of 4×10^6 to 7×10^6 cells per ml.

The BM2+LPS line was obtained by seeding 2×10^6 BM2 cells per 60-mm dish plus 10 μg of lipopolysaccharide (LPS) (endotoxin from *S. typhimurium* B; Difco Laboratories) per ml. The majority of cells (approximately 80%) adhered to the plate with extended processes by day 2 after seeding. The remainder of the cells were blasts (approximately 15%) and macrophages (approximately 5%). Adherent cells were removed and subcultured by removing the medium, rinsing with a solution of trypsin-versene (0.05% trypsin, 0.02% EDTA) and then incubating for 5 to 10 min in trypsin-versene at 37°C. They were split 1:4 into fresh growth medium with LPS present. By the second passage a relatively homogeneous population of adherent cells was obtained. These were maintained for over 6 months in culture by splitting as above 1:4 to 1:8 every 2 or 3 days. Cells of the BM2+LPS line could also be frozen with 10% dimethyl sulfoxide at −90°C and successfully recovered.

The third stage of differentiation was obtained by seeding 1×10^6 to 2×10^6 BM2+LPS cells per 100-mm dish in growth medium plus LPS and adding the tumor promoter 12-O-tetradecanoylphorbol-13-acetate (TPA; CMC Research Chemicals Inc., Brewster, N.Y.) at 250 ng/ml.

Differentiation-associated properties. The differentiation-associated properties of Fc and C3 receptors, Fc immune phagocytosis, and cellular morphology were examined. Fc receptors were assayed by rosette formation with sheep erythrocytes (SRBCs) sensitized with the immunoglobulin G (IgG) component of anti-SRBC serum (12, 20) (kindly supplied by G. and C. Moscovici). SRBCs were first washed three times with phosphate-buffered saline (PBS) and then sensitized by incubating for 30 min at 37°C with a 1:800 dilution of the anti-SRBC serum containing IgG. After washing three times with PBS, 10^8 sensitized SRBCs were incubated for 30 min at 37°C with 10^6 cells (previously washed in PBS) in PBS, and the percentage of cells with a rosette (10 or more erythrocytes) was determined by counting in a hemacytometer.

The presence of C3 receptors was determined as for Fc receptors. In sensitizing the SRBCs (12, 20) in this case, however, the IgM component of anti-SRBC serum was used—generally at the dilution recommended by the manufacturer (Cordis Laboratories)—although this was checked by titrating the endpoint agglutination. The SRBCs were then washed three times with PBS and incubated for 30 min at 37°C with a 1:50 dilution of fresh chicken serum as the source of complement. The SRBCs were then again washed with PBS and incubated with cells under the same conditions as for the Fc receptor assay.

Fc immune phagocytosis (12, 20) was assayed by incubating cells as for the Fc receptor assay plus an additional 60 min at 37°C. The percentage of cells phagocytosing erythrocytes via the Fc receptor was determined by centrifuging the cells in a Shandon Southern Cytospin and staining with May-Grunwald-Giemsa. Over 200 cells were counted in each case, and the percentage of cells with one or more ingested erythrocytes was determined.

Cellular morphology was determined (12, 45) by using a Cytospin for myeloblasts and by growing adherent cells on cover slips. In each case, cells were then stained with May-Grunwald-Giemsa, and more than 200 cells were counted for each treatment.

[^{35}S]methionine labeling of cells and immunoprecipitations. Cells of the three differentiation stages were radiolabeled with [^{35}S]methionine as follows. BM2 cells were washed and suspended at approximately 4×10^6 cells per ml of serum-free Dulbecco modified Eagle medium lacking methionine; 5 ml was used per 60-mm petri dish. BM2+LPS cells and BM2+LPS cells plus TPA were washed on the plate, and 3 ml of the above medium was added per 100-mm dish. In each case cells were incubated for 30 min in a 37°C 5% CO_2 incubator, and [^{35}S]methionine (specific activity, >600 Ci/mmol; Amersham Corp.) was added at 100 μCi/ml for an additional 60 min. To terminate the labeling and to remove most of the unincorporated [^{35}S]methionine, BM2 cells (in suspension) were washed once by centrifugation with PBS, whereas cells of the other two stages were washed once on the plate with PBS. Cells were then lysed at 0°C by the addition of 1 ml of lysis buffer (10 mM Tris-hydrochloride [pH 7.5], 50 mM NaCl, 0.5% sodium deoxycholate, 0.5% Nonidet P-40, 0.1% sodium dodecyl sulfate [SDS]) followed by the addition of NaCl to 0.5 M to ensure nuclear lysis (16). The procedure for immunoprecipitation has been described previously (16).

Partial proteolytic peptide mapping. [^{35}S]methionine-labeled proteins immunoprecipitated with anti-*myb* serum were isolated by preparative gel electrophoresis (19). They were then subjected to partial proteolytic peptide mapping with *Staphylococcus aureus* V8 protease (Miles Laboratories) (16) as described by Cleveland et al. (8).

Two-dimensional gel electrophoresis. Two-dimensional gel electrophoresis was performed by the method of O'Farrell (32), with the following modifications. After immunoprecipitation of [^{35}S]methionine-labeled extracts, the *S. aureus*-containing pellet was suspended in 9 μl of distilled water, 3 μl of 10% SDS, and 1.5 μl of 0.5 M Tris-hydrochloride (pH 6.8). After 30 min at 37°C, urea crystals were added to a final concentration of 9.5 M, followed by 2 volumes of lysis buffer containing 9.5 M urea, 8% (vol/vol) Nonidet P-40, 2% (vol/vol) ampholines (Pharmacia) (pH 5 to 8), and 5% (vol/vol) 2-mercaptoethanol. The samples were then run on isoelectric focusing gels, the only difference to O'Farrell's method being the use of the ampholine pH range of 5 to 8 and running the proteins from acidic to basic pH. The proteins were separated by size in the second dimension by electrophoresis on an SDS–12.5% polyacrylamide gel. Radioactively labeled spots were visualized by fluorography with sodium salicylate as described previously (16).

RESULTS

Production from AMV-transformed myeloblasts of two distinct stages of myeloid differentiation. The BM2 line used in the present studies was obtained from chicken bone marrow cells transformed by AMV (26, 28, 30, 33). It is a line of nonproducer myeloblasts (Fig. 1a) that, under certain conditions, exhibits spontaneous differentiation in culture. A clone of this line (BM2 clone 3) was used. To avoid spontaneous differentiation in the present experiments, the cells were kept at high density (4×10^6 to 7×10^6/ml), subcultured every 2 to 3 days at approximately 1:4, and washed by centrifugation at the time of passaging. The degrees of expression of certain markers of the myeloid lineage by the BM2 line are summarized in Table 1. Approximately 50% of the cells were positive for Fc rosette formation, indicating functional Fc receptors, although there was no detectable C3 rosette formation or Fc immune phagocytosis (the phagocytosis of the SRBC via Fc receptors). This degree of expression of myeloid properties is characteristic of cells at an early stage of the myeloid lineage. The majority of the cells were morphologically blasts, with a small percentage somewhat more morphologically advanced (Table 1). In the experiments reported here there were no detectable mature cells in cultures subcultured every 2 or 3 days.

The partially differentiated line (termed BM2+LPS) was obtained by treating BM2 cells in culture with 10 μg of LPS per ml and subculturing these cells every 2 or 3 days (see above). LPS has been shown to induce myeloid differentiation in certain mouse myeloid leukemic cells (39, 41). A homogeneous population of adherent cells was obtained after the second passage and displayed similar growth and morphology characteristics (Fig. 1b) over a period exceeding 6 months in culture. The differentiation-associated properties of this line were tested and are shown in Table 1. Whereas the level of Fc rosettes was similar to BM2 cells, unlike BM2 cells, these partially differentiated cells were also positive for C3 rosette formation and Fc immune phagocytosis (Table 1). Staining confirmed that the BM2+LPS cells were at a more mature stage of differentiation—the ratio of cytoplasm to nucleus was greater (Fig. 1b,

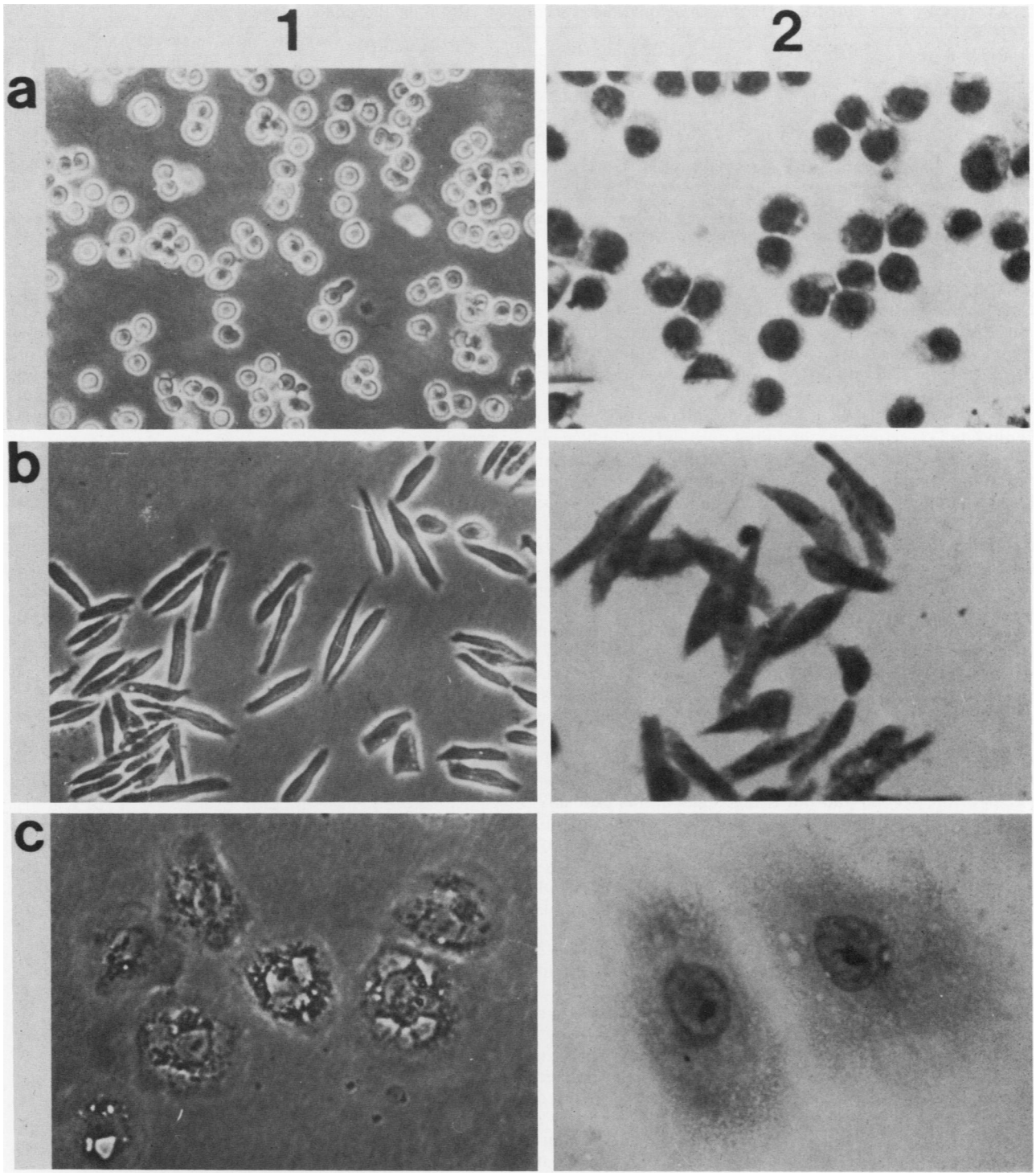

FIG. 1. Morphology of cells of the three differentiation stages. Panels 1 show gross morphology of cells of the three stages. Cells were grown in plastic petri dishes and photographed with a Wild inverted-phase microscope. Panels 2 show cellular morphology after staining with May-Grunwald–Giemsa. BM2 cells growing in suspension were spun onto slides with a Cytospin; adherent cells (BM2+LPS cells with or without TPA) were grown on cover slips before staining. Cells were then photographed with a Zeiss Photomicroscope. (a) BM2 cells; (b) BM2+LPS cells; (c) BM2+LPS cells treated with 250 ng of TPA per ml for 2 days. The degree of magnification of cells within each panel is the same; the degree of magnification of cells in panels 2 is approximately double that seen in panels 1.

panel 2) and the cytoplasm was paler in color (data not shown). The degree of differentiation of the BM2+LPS cells was maintained only as long as LPS was present; 2 days after removing LPS, the cells were no longer adherent, and by 4 days they were again morphologically classified as myeloblasts.

Thus, two homogeneous populations of replicating cells were obtained: myeloblasts and a line of cells intermediate in

TABLE 1. Expression of myeloid properties by cells of the three differentiation stages[a]

Stage no.	Cell type or treatment	Rosettes		Fc immune phagocytosis	Morphology		
		Fc	C3		Blast	Intermediate	Mature
I	BM2	52 ± 5	0	0	95 ± 2	5 ± 2	0
II	BM2+LPS	55 ± 4	15 ± 2	33 ± 3	3 ± 2	97 ± 2	0
III	BM2+LPS + TPA	85 ± 7	71 ± 6	27 ± 4	0	1 ± 1	99 ± 1

[a] Myeloid-associated properties of the three cell populations: I, BM2 cells; II, BM2+LPS cells; and III, BM2+LPS cells treated with the tumor promoter TPA (250 ng/ml) for 2 days. The assays used were as follows. Rosettes (Fc or C3) refers to the binding of appropriately coated SRBCs and measures the percentage of cells in a population with functional Fc or C3 receptors. Fc immune phagocytosis refers to the ingestion of SRBCs via the Fc receptor. Morphology of cells was determined by May-Grunwald–Giemsa staining—cells were divided into blast, intermediate, or mature categories according to the ratio of nucleus to cytoplasm and the color and appearance of the nucleus and cytoplasm. Mature cells were all macrophages. The numbers shown are the means of at least three independent experiments ± standard deviations.

the myelomonocytic lineage. After 2 months in culture, cells of the BM2+LPS line were exposed to the tumor promoter TPA, a compound known to induce terminal differentiation in BM2 cells (33) and other transformed mouse and human myeloid cells (18, 20).

TPA has previously been shown to induce BM2 cells to differentiate (33). However, an initial dose did not induce all cells to differentiate, and only by continually adding TPA for 6 days could a relatively homogeneous population of mature cells be obtained (33). These results with BM2 cells were confirmed here (data not shown). In contrast to BM2 cells, the partially differentiated cells of the BM2+LPS line differentiated rapidly to mature macrophages in response to TPA. Cells morphologically resembling macrophages were first seen approximately 6 h after adding the inducer and, by 36 to 48 h after the addition of TPA, virtually all of the cells in culture morphologically resembled macrophages (see below) and ceased division. Furthermore, no additional TPA was required after the initial dose. By 2 days, approximately 90% of the cells were positive for Fc rosettes, 70% were positive for C3 rosettes, and approximately 30% were positive for Fc immune phagocytosis (Table 1). The finding that the percentage of cells exhibiting Fc immune phagocytosis was similar for cells of the second and third stage (Table 1) is probably due to the fact that TPA has an inhibitory effect on this parameter (33). When TPA was removed after 2 days, at day 3 50% of cells exhibited Fc immune phagocytosis. By May-Grunwald–Giemsa staining, approximately 100% of cells of the third stage were morphologically classified as macrophages (Table 1, Fig. 1c). The effect of TPA, in contrast to that of LPS, was not reversible. The removal of TPA after 2 days did not cause the macrophage-like cells to revert to earlier stages in differentiation or to replicate.

The 45,000-M_r protein encoded by v-*myb* is synthesized to a similar extent during differentiation. The product of v-*myb* has previously been shown to be a 45,000-M_r protein ($p45^{v\text{-}myb}$) (6, 16). To examine this protein during induced differentiation of AMV-transformed myeloblasts, we used the three stages of differentiation described above.

When the amount of this protein was examined by immunoblotting cell extracts (40), it was found to be similar at each stage (data not shown). It was also found by [^{35}S]methionine labeling that the rate of synthesis of this protein was similar at each stage of differentiation (Fig. 2). Furthermore, pulse-chase experiments indicated that the half-life of the protein was similar (approximately 1 h; data not shown) in each of the three differentiation stages. That this 45,000-M_r protein present during differentiation was indeed the authentic v-*myb* product was verified (i) by showing the absence of a 45,000-M_r band in immunoprecipitates with preimmune rabbit serum (data not shown) and in antigen-blocked immune serum (data not shown) and (ii) by comparing V8 partial digestions of the 45,000-M_r protein in undifferentiated and differentiated cells (Fig. 3). We conclude that the same 45,000-M_r polypeptide ($p45^{v\text{-}myb}$) is present in each of the three differentiation stages, and that differentiation (assessed morphologically and functionally) proceeds without a decrease in the synthesis or steady-state amounts of this protein.

Modification of $p45^{v\text{-}myb}$ during differentiation. SDS-polyacrylamide gel electrophoresis does not, however, allow detection of various charge modifications in proteins. To determine whether there are different molecular forms of $p45^{v\text{-}myb}$ in BM2 cells and whether these forms are altered during differentiation, immunoprecipitates of the first and

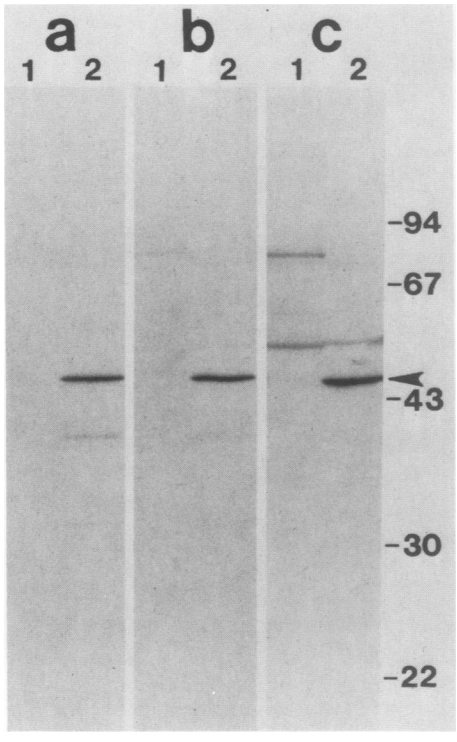

FIG. 2. Synthesis of $p45^{v\text{-}myb}$ in cells of the three differentiation stages. Cells were radiolabeled with [^{35}S]methionine, lysed, and immunoprecipitated with anti-*myb* serum. Cell extracts from approximately 2×10^6 cells were run on a 10% SDS–polyacrylamide gel and visualized by autoradiography. (a) BM2 cells; (b) BM2+LPS cells; (c) BM2+LPS cells treated with 250 ng of TPA per ml for 2 days. Lanes: 1, anti-p19 *gag* serum; 2, anti-*myb* serum. The arrow marks the position of $p45^{v\text{-}myb}$. The numbers on the right-hand side refer to the positions of molecular weight markers.

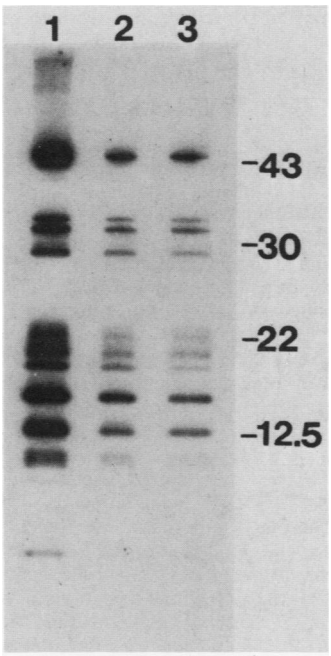

FIG. 3. Partial proteolytic peptide mapping of v-*myb*-encoded proteins. Radiolabled p45$^{v\text{-}myb}$ from immunoprecipitates of cells of the three differentiation stages was applied to a 15%–SDS polyacrylamide gel with 50 ng of *S. aureus* V8 protease per lane. Lanes: 1, extract from approximately 20 × 10^6 BM2 cells; 2, extract from approximately 5 × 10^6 BM2+LPS cells; 3, extract from approximately 5 × 10^6 BM2+LPS cells treated with 250 ng of TPA per ml for 2 days. The numbers on the right-hand side refer to the positions of molecular weight markers.

last differentiation stages were examined by two-dimensional gel electrophoresis. In extracts of AMV-transformed myeloblasts three polypeptides of 45,000 M_r were found by isoelectric focusing in the range of pH 7.1 to 7.6 (Fig. 4). These polypeptides presumably represent different post-translational modifications of p45$^{v\text{-}myb}$. This protein has been found to be phosphorylated (Klempnauer, unpublished results), and certain of these modifications may be different degrees of phosphorylation of the protein. Three polypeptides were found at the same position in extracts from mature macrophages (Fig. 4), indicating, at this degree of resolution, no obvious alteration in the p45$^{v\text{-}myb}$ during differentiation.

DISCUSSION

Cells transformed by v-*myb* can be induced to differentiate without affecting the expression of v-*myb*. The oncogene of AMV, v-*myb*, encodes the transforming protein, p45$^{v\text{-}myb}$ (1, 6, 16). In the present studies, we have used induced differentiation as a means of obviating the effects of v-*myb*. The results have shown that, during induced differentiation of AMV-transformed myeloblasts, the synthesis and steady-state levels of p45$^{v\text{-}myb}$ do not alter. Thus, the transformed phenotype is not suppressed by a reduction in the synthesis or level of the transforming protein, nor does there appear to be an alteration in charge modification of p45$^{v\text{-}myb}$ during differentiation. Therefore, the transforming protein may be necessary, but is not sufficient, for transformation—its effects may be suppressed.

There are at least two possible explanations for the suppression of the effects of v-*myb*: (i) alteration of the transforming protein's putative targets and (ii) alteration of the protein's subcellular location during differentiation. The targets of this protein are not yet known and therefore cannot be analyzed during differentiation. In an extension of the present study, however, it has been shown that the subcellular location of p45$^{v\text{-}myb}$ does appear to alter during differentiation. By subcellular fractionation and immunofluorescence, it was found (17) that the protein is predominantly located in the nucleus in the first two stages of differentiation, but assumes a cytoplasmic (perinuclear) location in macrophage-like cells. It can therefore be envisaged that, during differentiation, the action of the transforming protein is suppressed by an alteration of subcellular location. This altered subcellular location is not produced by the inducing agent (TPA) per se, since AMV-transformed myeloblasts

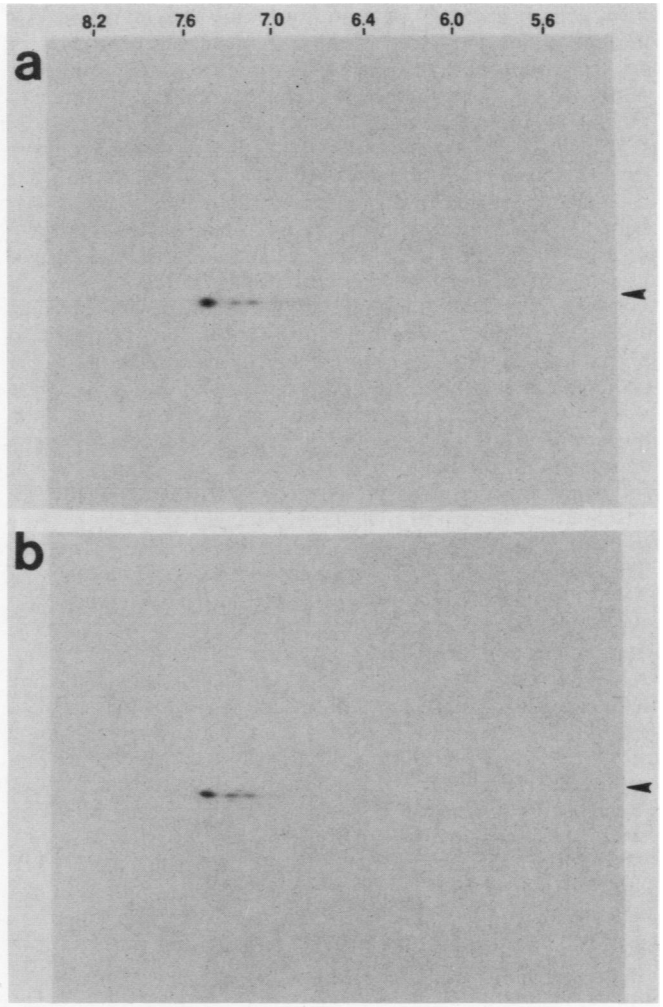

FIG. 4. Two-dimensional gel electrophoresis of p45$^{v\text{-}myb}$. (a) BM2 cells or (b) BM2+LPS cells treated for 2 days with TPA (250 ng/ml) were radiolabeled for 1 h with [^{35}S]methionine, and the cell extracts were immunoprecipitated with anti-*myb* serum. Immunoprecipitations from 3 × 10^6 cells of each cell type were analyzed by autoradiography after equilibrium pH gradient electrophoresis. In the isoelectric focusing gel, samples were run from acidic to basic pH with an ampholine range of 5 to 8. The pH gradient was measured by cutting slices and incubating in distilled water for 2 to 3 h. The arrow shows the position of the polypeptides representing p45$^{v\text{-}myb}$.

that have spontaneously differentiated also exhibit the same altered subcellular location of the transforming protein (17).

***myb* oncogene and differentiation.** It has been shown that the cellular homolog of v-*myb*, c-*myb*, is expressed primarily in immature hematopoietic cells (12a, 13, 42). It has also been shown that, in human myeloid leukemic cells (not known to have been virally transformed), the transcription of c-*myb* decreases when the cells are chemically induced to differentiate (10, 12a, 42, 43). These results indicate that the protein encoded by c-*myb* may be involved in the replication of myeloid cells.

The cells used in the present experiments were transformed by v-*myb* and express the protein encoded by this gene, $p45^{v\text{-}myb}$. We have shown that during induced differentiation, v-*myb*, unlike the cellular counterpart c-*myb* (10, 12a, 42), continues to be transcribed. The normal cellular machinery responsible for regulating the expression of c-*myb* during differentiation does not appear to function for v-*myb*. This is probably because the transcription of v-*myb* is under the control of a viral rather than cellular promoter; or the RNA may be more stable. No matter what the means, our results indicate the cell is unable to regulate the level of the v-*myb*-encoded protein during differentiation.

It has been postulated (14) that at least some oncogenes (including v-*myb*) function by blocking differentiation. It is clear that the normal linkage between growth and differentiation (36) has been lost in the cases of transformation by v-*myc*, v-*erb*-B, and v-*myb*. Here we show that, in the case of v-*myb*, this linkage may be restored by a mechanism not involving the switching off of the synthesis or obvious alteration of the transforming protein (present results) and appears to involve an altered subcellular location of the protein (17). In other cases (either involving or not involving v-*myb*) where transformed cells are induced to or spontaneously differentiate, any one of several mechanisms may apply: (i) decrease in synthesis of the transforming protein, (ii) structural alteration of the protein to inactivate it, (iii) alteration of targets, or (iv) altered subcellular location of the transforming protein. For example, in the case of myeloblasts transformed by a temperature-sensitive mutant of AMV (29), the induction of differentiation by temperature shift appears to be accompanied by a decreased synthesis of the transforming protein (Klempnauer, unpublished results). A similar result was found with erythroblasts transformed by temperature-sensitive mutants of avian erythroblastosis virus (3). Synthesis of the final form of the transforming protein was blocked by temperature shift to the nonpermissive temperature (2). To date, studies of transforming proteins during chemically induced differentiation of avian erythroblastosis virus-transformed erythroblasts (2–4, 12, 37), E26-transformed myeloblasts and erythroblasts (27, 34), and Abelson virus-transformed lymphocytes (44) have not been reported. It will be of interest to determine in these cases which mechanisms operate to suppress the action of the transforming protein. It will also be of interest to analyze the expression of c-*myb* during normal development (31).

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