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Constitutive c-*myb* Expression in K562 Cells Inhibits Induced Erythroid Differentiation but Not Tetradecanoyl Phorbol Acetate-Induced Megakaryocytic Differentiation

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K562 cells were stably transfected with a plasmid vector constitutively expressing a full-length human c-*myb* gene. Parental cells possess the dual potential of inducibility of cellular differentiation along two lineages, i.e., erythroid and megakaryocytic. The resulting lineage is dependent on the inducing agent, with a number of compounds being competent to various degrees for inducing erythroid differentiation, while the tumor promoter tetradecanoyl phorbol acetate (TPA) induces a macrophage-like morphology with enhanced expression of proteins associated with megakaryocytes. Exogeneous expression of c-*myb* in transfected cell lines abrogated erythroid differentiation induced by cadaverine or cytosine arabinoside as assessed by hemoglobin production. However, TPA-induced megakaryocytic differentiation was left intact, as assessed by cell morphology, cytochemical staining, and the expression of the megakaryocytic antigens. These results indicate that c-Myb and protein kinase C play important roles in cellular differentiation of K562 cells and suggest that agents which directly modulate protein kinase C can induce differentiation in spite of constitutively high levels of c-Myb.

The v-*myb* oncogene originally arose on two transforming avian retroviruses, avian myeloblastosis virus and avian erythroblastosis virus E26 (1, 23). Both viruses contain transduced cellular sequences which are an internal subset of the c-*myb* proto-oncogene (9, 31). v-*myb* has thus acquired both 5′ and 3′ deletions that are associated with its transforming activity.

The implied role of c-*myb*, as with all proto-oncogenes, lies in the proliferation-differentiation pathway of the cell. Deregulation of the differentiation program of the cell appears to be a major step in tumor development (34). Consistent with this concept is the action of v-*myb*, which induces uncontrolled proliferation with loss of differentiation within its target cell population, i.e., myelomonocytic cells in embryonic bone marrow (24). The c-*myb* gene is expressed most abundantly in immature cells of the hematopoietic lineage (40); hence, the role of c-*myb* is most probably as a positive regulator of bone marrow stem cell proliferation and/or a negative regulator of differentiation.

Direct experimental evidence that c-*myb* expression blocks differentiation was first provided by Clarke et al. (3) in the model system of Friend murine erythroleukemia (F-MEL) cells. In this and similar cell lines, cellular differentiation can be induced by dimethyl sulfoxide (DMSO), resulting in hemoglobin synthesis (21), a process which is accompanied by a down-regulation of c-*myb* expression (10, 30). Enforced expression of c-*myb*, which is accomplished by stable transfection of the cells with a c-*myb* cDNA clone under the control of a strong constitutive promoter, prevented the chemically induced differentiation of the resulting cell lines. This finding was extended to the myeloid lineage by Selvakumaran et al. (35) in a similar manner by constitutive expression of c-*myb* in the mouse M1 myeloid cell line, which can be induced to differentiate into macrophage-like cells by the growth factors interleukin 6 or leukemia inhibitory factor.

In addition to the c-*myb* gene product, protein kinase C (PKC) has been implicated in the proliferation-differentiation program of cells in the hematopoietic as well as in other lineages. Again, the F-MEL cell line was used to provide early evidence of this when, in 1977, it was noted that prior treatment of F-MEL cells with the potent PKC modulator tetradecanoyl phorbol acetate (TPA) prevented erythroid differentiation of the cells (22, 32). It is now known that PKC is actually a family of enzymes with at least 11 different isoforms (α, β, γ, δ, ε, ζ, η, θ, ι, λ, and μ) (5), each encoded by a different gene but all having certain structural features in common (13, 25, 26). The pattern of expression of each of these isoforms is a characteristic of the individual cell type. Modulation of PKC activity can occur both by changes in subcellular location as well as protein levels. Such modulations in the differentiation of a number of cell types, including hematopoietic cells, have been described elsewhere (4, 6, 11, 12, 16, 20, 29, 37, 41). It is, thus, the differential modulation of individual PKC isoforms that is thought to be the mechanism of signal transduction via the PKC pathway. However, in spite of the recent advancements in many areas of PKC research, much about the target proteins involved and the points in the differentiation pathway in which the isoforms might act remains undetermined.

In order to corroborate and extend the findings of c-*myb*'s role in cellular differentiation, we have constitutively expressed c-*myb* in the K562 cell line. K562 is a useful model for the studies of cellular differentiation because of its potential to differentiate along two cell lineages (15). Erythroid differentiation of the cell line can be induced by cytosine arabinoside (araC), cadaverine, or a number of other compounds (33). However, TPA treatment induces a macrophage-like morphology while inducing the expression of proteins associated with megakaryocytes (2, 15). Our data indicate that constitutive expression of c-*myb* prevents induced erythroid differentiation of the cell line, while the TPA-induced megakaryocyte differentiation potential of the cell line is left intact.

MATERIALS AND METHODS

Cell culture and DNA transfections. K562 cells were purchased from the American Type Culture Collection and cultured in RPMI medium supplemented

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with 10% fetal bovine serum plus antibiotics. The murine F-MEL and F-MEL-*myb* cell lines have been described elsewhere (3). F-MEL-*myb* is a transfectant of F-MEL containing the c-*myb* expression vector pMbMI/DHFR. All were generously provided by Michael F. Clarke and Edward Prochownik. pMbMI/DHFR contains a full-length human c-*myb* cDNA clone under control of the simian virus 40 (SV40) promoter. The plasmid also contains another transcription unit of dihydrofolate reductase, also under control of the SV40 promoter, which allows amplification of copy number by culturing of transfected cells in the presence of increasing levels of methotrexate. Stable transfectants were produced by electroporating 25 μg of pMbMI/DHFR along with 5 μg of pSV2*neo* in a suspension of phosphate-buffered saline using a Bio-Rad Gene Pulser equipped with a Capacitance Extender. One pulse of 300 V at 250 μF was used. After 10 min, the cells were suspended in culture medium, incubated for 1 day, adjusted to 1 mg of G418 per ml, and transferred to microtiter plates. At 3 to 5 weeks later, when G418-resistant clones appeared, the cells were transferred out of the microtiter wells, expanded, and analyzed for DNA content, protein expression, and differentiation ability.

Nucleic acid analysis. Cellular DNA was isolated by lysing cell pellets in a solution of 10 mM Tris-Cl (pH 7.5)–1 mM EDTA (TE) containing 1% sodium dodecyl sulfate (SDS) and 100 μg of proteinase K per ml. After an overnight incubation at 37°C, DNA was isolated by phenol extraction of the cell lysate and then by ethanol precipitation. DNA was dissolved in TE, and 1 μg was used in PCR analysis. PCR solutions contained 2.5 U of *Taq* polymerase per 100 μl (Boehringer Mannheim), buffer supplied by the enzyme's manufacturer, 10% DMSO, and primers. The 3′ primer was CCCATCATAGTCATGGTCACA CATC, which is complementary to sequences 172 to 197 of c-*myb* cDNA (19). The 5′ primer was GTACCTTCTGAGGCGGAAAGAAC, which is in the SV40 promoter region (27) of the plasmid approximately 550 bp upstream of the 3′ primer. PCR was performed, with 25 cycles of 30 s at 90°C, 30 s at 55°C, and 60-s extensions at 72°C. Cytoplasmic RNA was isolated by lysing the cells in hypotonic buffer containing 0.5% Nonidet P-40. After spinning out of nuclei, the supernatant containing RNA was extracted with a 1:1 mixture of phenol-chloroform-isoamyl alcohol and then ethanol precipitated. Five micrograms of total cytoplasmic RNA was utilized for the following reverse transcription-PCR (RT-PCR). In a 40-μl volume of RNA, the 3′-*myb* primer described above, 10 U of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim), buffer supplied by the enzyme manufacturer, and deoxyribonucleotides were incubated at 42°C for 30 min. The solution was then phenol extracted and ethanol precipitated, and half of the resulting cDNA was used in a PCR as described above.

Protein analysis. Cell pellets were lysed in Tris-Cl buffer containing 0.1% SDS and proteinase inhibitors. DNA was sheared to reduce viscosity, and protein concentration was determined with Bio-Rad protein assay dye reagent. For c-*myb* analysis, 50 μg of total protein was mixed with an equal volume of 2× sample buffer (0.125 M Tris-Cl [pH 6.8], 4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.002% bromophenol blue). Samples were heated in a boiling water bath for 5 min and then loaded onto a denaturing 6% polyacrylamide electrophoresis gel.

After separation, proteins were electrophoretically transferred to a nitrocellulose membrane. The filters were first treated with Tris-buffered saline (50 mM Tris-Cl [pH 7.5], 0.15 M NaCl) with 0.05% Tween 20 (TBST) containing 3% nonfat milk. This was followed by hybridization in 5 ml of TBST with 1% bovine serum albumin and 1 μg of anti-c-Myb antibody type I (Upstate Biotechnology, Inc.) or anti-α_2 integrin antibody (Chemicon) per ml for 5 h at room temperature. The blots were subsequently washed in TBST and then incubated with the appropriate immunoglobulin peroxidase-conjugated antibodies (Amersham) for 1 h. After washing in TBST, the blots were then developed using the enhanced chemiluminescence Western blotting (immunoblotting) system (Amersham). Analysis of integrins IIb-IIIa was performed similarly, except that 15 μg of total protein was used for electrophoresis and 2-mercaptoethanol was omitted from the sample buffer. Antibodies to the platelet fibrinogen receptor complex IIb-IIIa were polyclonal rabbit serum (14) provided by Karen Knudsen, and blots were developed colorimetrically using 3,3′-diaminobenzidine in hydrogen peroxide.

Analysis of cellular differentiation. Erythroid differentiation was induced by adjusting logarithmically growing cells to 0.36 μM araC or to 5 mM cadaverine. After 5 days, the cells were stained with a 1/4 volume of benzidine solution (0.04% benzidine, 2% hydrogen peroxide, 12% acetic acid). Positive (blue-staining) cells were counted under a hemocytometer. At least 200 cells were counted. Megakaryocytic differentiation was assessed by cellular morphology, cytochemical staining, cellular adherence, Western analysis of the differentiation antigens IIb-IIIa and the α_2 integrin, and flow cytometric analysis of CD15(LeuM1) and glycophorin A. All results presented are representative of at least three experiments.

RESULTS

In order to document that c-*myb* is down-regulated in K562 upon induced differentiation as it is in induced differentiation of F-MEL and M1 cells, we performed Western analysis on cellular extracts of K562 before and after differentiation induced by TPA, araC, and cadaverine. Figure 1 shows the results of this experiment. Untreated K562 cells express the 75-kDa c-Myb protein, p75^{myb}. This protein becomes undetectable upon differentiation of the cells by all three of the inducers. K562 cells were subsequently cotransfected with the plasmids pMbMI/DHFR and pSV2*neo* by electroporation as described above. After G418 selection and expansion, 50 clones were collected from two transfection experiments and subjected to Southern and Western analysis. More than 90% of the G418-resistant clones also contained the c-*myb* expression vector. However, only a relatively few seemed to exhibit substantially increased c-*myb* expression as assessed by Western analysis. Therefore, the cells were transferred into medium containing 0.25 μM methotrexate for 2 weeks in order to amplify the plasmid copy number. Surviving subclones were reanalyzed by Western blotting, and 10 lines expressing high levels of c-Myb proteins were selected for further analysis.

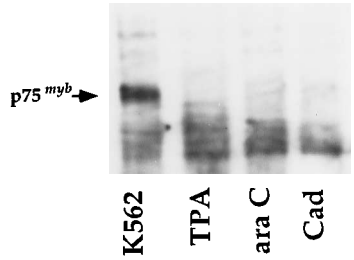

FIG. 1. Western blot analysis of protein extracts before and after treatment with differentiation inducers. Fifty-microgram samples of extracts from cells treated as described in Materials and Methods were analyzed with an anti-c-Myb antibody. The position of p75^{myb} is shown in the first lane, which contains untreated K562. The other lanes were of extracts of cells treated with the indicated inducers. Cad, cadaverine.

Integration and transcriptional analysis of the c-*myb* expression vector. To verify the stable integration of the c-*myb* expression vector in the G418 resistance clones, we isolated total nucleic acid from approximately 10^6 cells of each clone. In order to distinguish endogenous from exogenous c-*myb*, both of which are human, we used PCR analysis with a 3′ primer complementary to sequences in the 5′ end of c-*myb* and a 5′ primer homologous to promoter sequences of the SV40 promoter. PCR analysis of the pMbMI/DHFR plasmid amplifies a single band of approximately 550 bp. Figure 2A shows the results of a typical PCR experiment using genomic DNAs from various cell lines. Lanes 1 and 2 show the results of PCRs using genomic DNAs from F-MEL and F-MEL-*myb* cells. As expected, no band is amplified from F-MEL DNA, while DNA from the subclone F-MEL-*myb* (which contains the pMbMI/DHFR plasmid and was used in the previously cited study [3]) results in amplification of the expected 550-bp band. Similarly, DNA from parental K562 cells does not result in the amplification of c-*myb* sequences (lane 3), while DNAs from the 10 G418-resistant transfectants result in amplification of the same 550-bp band (lanes 4 to 13). The authenticity of this band was verified by Southern blotting of the gel and then by hybridization to a ^{32}P-labelled probe derived by excising the c-*myb* sequences from pMbMI/DHFR by *Xho*I digestion. Identical results were also achieved in another PCR analysis using primers which span several exon-intron borders within the 3′ portion of c-*myb* (data not shown).

Transcriptional expression from the exogenous c-*myb* was qualitatively assessed in the same clones in a similar manner using PCR analysis of cDNA produced in reverse transcriptase reactions with corresponding RNAs. Thus, total cytoplasmic RNA was isolated and reverse transcribed using the 3′ c-*myb*

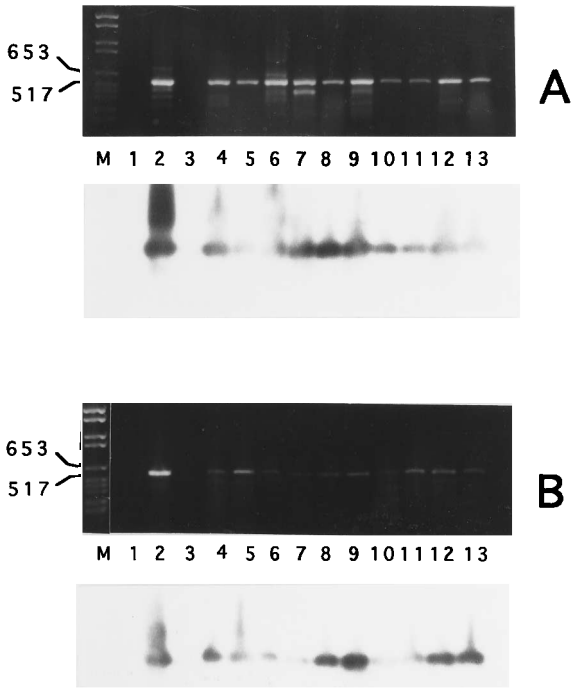

FIG. 2. Nucleic acid analysis of K562-*myb* clones. (A) PCR analysis of genomic DNA. PCR was preformed as described in Materials and Methods. One-half of the reaction mixture was loaded onto a 1.5% agarose gel and subjected to electrophoresis. Lane M contains molecular weight markers, pBR328 cleaved with *Bgl*I and *Hin*fI. The 653- and 517-bp fragments are indicated. Lanes and sources of genomic DNAs are as follows: 1, F-MEL; 2, F-MEL-*myb*; 3, K562; 4, K562-*myb* no. 1; 5, K562-*myb* no. 5; 6, K562-*myb* no. 7; 7, K562-*myb* no. 15; 8, K562-*myb* no. 20; 9, K562-*myb* no. 24; 10, K562-*myb* no. 30; 11, K562-*myb* no. 32; 12, K562-*myb* no. 39; 13, K562 *myb* no. 40. After photography, the DNA from the gel was blotted onto nitrocellulose that was hybridized to a ^{32}P-labelled c-*myb* probe. The autoradiogram of the resulting blot is shown beneath the ethidium bromide-stained gel. (B) RT-PCR analysis of total cytoplasmic RNA of K562-*myb* clones. RT-PCR was performed as described in Materials and Methods. Samples were subjected to electrophoresis and then to Southern hybridization as described above. The sources of RNAs in each lane are the same as those for genomic DNAs described for panel A.

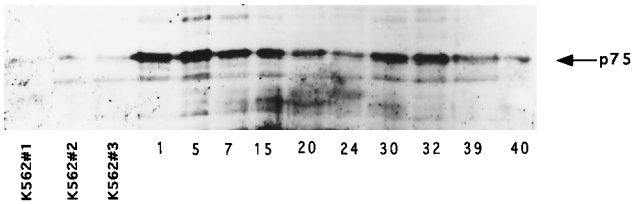

FIG. 3. Western blotting analysis of protein extracts of K562 and K562-*myb* cells. Fifty-microgram samples were loaded onto each lane and analyzed as described in Materials and Methods. The source of protein extract in each lane is as follows: 1 to 3, three independent subclones of K562; 4, K562-*myb* no. 1; 5, K562-*myb* no. 5; 6, K562-*myb* no. 7; 7, K562-*myb* no. 15; 8, K562-*myb* no. 20; 9, K562-*myb* no. 24; 10, K562-*myb* no. 30; 11, K562-*myb* no. 32; 12, K562-*myb* no. 39; 13, K562 *myb* no. 40. The position of p75^{myb} is indicated by the arrow.

primer described above and the resulting cDNA used in the PCRs described above in place of genomic DNA. Figure 2B shows RT-PCR analysis of the same cell lines. RNAs from F-MEL and F-MEL-*myb* gave the expected results of a specifically amplified fragment only in the pMbMI/DHFR transfectant, F-MEL-*myb*. The band had the same molecular weight as that amplified from genomic DNA, indicating that the major transcript was unspliced. Again, K562 RNA was blank (lane 3), while K562 transfectants showed the presence of exogenous transcripts from the input expression vector (lanes 4 to 13). The authenticity of the amplified band was also verified by Southern analysis. No band was amplified in controls from which reverse transcriptase was omitted.

Protein expression in K562 transfectants. In order to assess the relative levels of c-*myb* gene expression in the various cell lines, we analyzed relative c-Myb protein levels by Western blot analysis. Figure 3 shows typical results from extracts from each of the K562-*myb* clones, showing substantially increased amounts of c-Myb protein compared with that for the parental K562. Because variants of a cell line which differ in a particular characteristic from the parent can arise spontaneously, we subcloned several sublines of K562, three of which are shown. We noted no significant variation in these clones, as well as in cells transfected with pSVS*neo* alone or other *neo* gene-containing expression vectors in use in other studies. Densitometric analysis of the enhanced chemiluminescence-developed X-ray films, using as standards increasing volumes of K562 extracts, indicated that the K562-*myb* clones expressed three to eight times more protein than parental K562.

Induced erythroid differentiation of the K562-*myb* cell lines. Inhibition of erythroid differentiation by c-*myb* in each of the K562-*myb* transfectants as well as in several subclones of K562 was assessed by inducing erythroid differentiation with araC and cadaverine. Figure 4 shows the effects of exogenous c-*myb* expression on induced hemoglobin synthesis in each of the clones as well as five independent subclones of parental K562. The results are expressed as the percentage of benzidine-positive cells 5 days after induction. Parental K562 cells can be induced to a level of 50 to 65% differentiated cells by araC, a potent cell division inhibitor. c-*myb*-transfected cells, however, exhibit only 1 to 10% induction of hemoglobin synthesis. Cadaverine, a less potent differentiating agent, induces hemoglobin synthesis in 10 to 20% of the cells. Transfectants display markedly less induction by this agent as well. These results are consistent with the experience of others who have studied F-MEL (3) and M1 differentiation (35) and support the conclusion based on Western analysis which showed that elevated c-*myb* expression has been achieved in the transfected lines.

TPA-induced differentiation of the K562-*myb* cell lines. The PKC modulator TPA induces a macrophage-like morphology in treated cells while inducing some antigens associated with cells of the megakaryocyte lineage (2, 15). K562 cells treated for 2 days with 0.17 μM TPA develop a spindle-shaped morphology in 15 to 20% of the cells, while the remaining cells adhere more tightly to the dish. Adherence of the various K562-*myb* cell lines as well as the parent is shown in Table 1. Figure 5 shows the morphology of the TPA-treated c-*myb*-transfected clones, and cytochemical analysis is shown in Fig. 6. All lines showed marked morphological and cytochemical differentiation features upon treatment with TPA. Morphologically, all treated cultures contained cells which assumed the characteristic spindle or macrophage-like shape, which has always been a reliable indicator of differentiation. Cytochemically, untreated K562 and K562-*myb* cells show a relatively homogenous population of immature blast-like cells characterized by a small size with many cytoplasmic protrusions, a high (>1) nuclear-to-cytoplasmic ratio, and round nuclei. About 2 to 3% of the cells are mitotic. Upon differentiation, the population progresses to one with more mature cells with a megakaryocyte morphology characterized by a lower (≥ 1) nuclear-to-cytoplasmic ratio, a lobulated nucleus resulting from endomitosis, and a more acidophilic cytoplasm. Also present are cells with a histiocytoid morphology. These are cells which

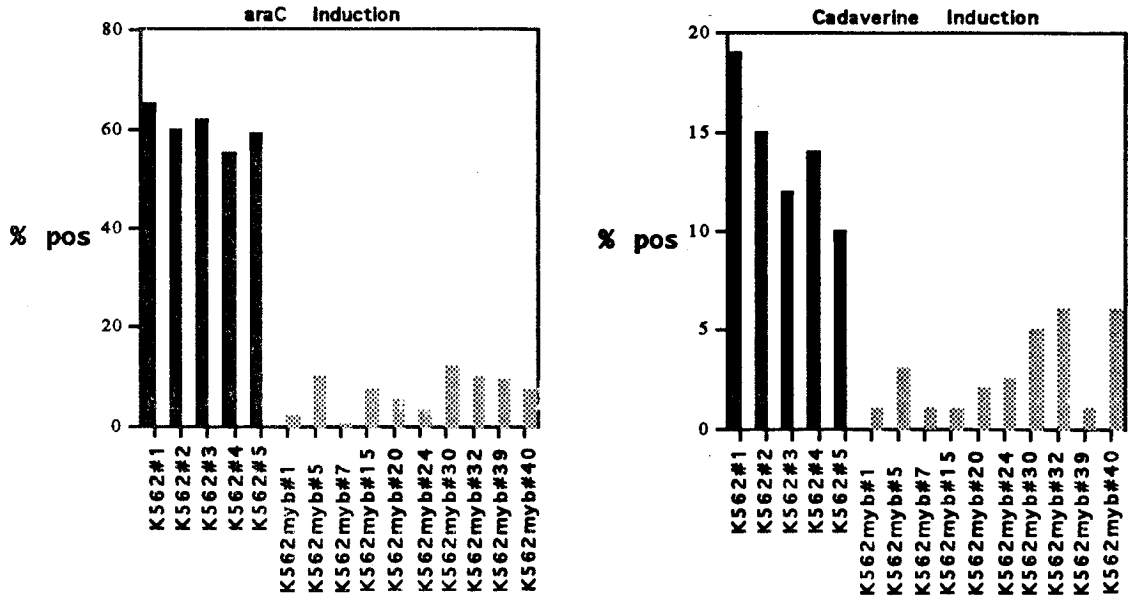

FIG. 4. Induced differentiation of K562 and K562-*myb* cells by araC and cadaverine. Induction and assaying with benzidine reagent were as described in Materials and Methods. Results of five independent subclones of K562, as well as of the 10 transfectants presented in Fig. 1 and 2, are shown. Data are expressed as percentages of benzidine-positive (blue-staining) cells (pos).

appear macrophage-like under light microscopy. They are characterized by a large acidophilic nucleus and lightly staining cytoplasm. All of these cells are more highly vacuolated and exhibit fewer cytoplasmic protrusions. No mitotic cells are evident. Table 1 shows a statistical analysis of this morphology. The analysis shows that some variation exists between clones in regard to the distribution of mature and immature cells. Cellular adherence after TPA treatment was also varied. However, the results for all cell lines were consistent, in that adherence, as well as the proportion of morphologically mature cells, increased markedly.

TABLE 1. Cytological analysis of K562 and K562-*myb* cells before and after treatment with TPA[a]

Cell line	% Adherence	% Megakaryoblasts	% Megakaryocytes	% Histiocytoids	% Mitotic cells
K562 (−)	<1	91	6	1	2
K562 (+)	34	12	55	33	
K562-*myb* no. 1 (−)	<1	70	23	5	2
K562-*myb* no. 1 (+)	50	8	60	32	
K562-*myb* no. 5 (−)	<1	66	24	7	3
K562-*myb* no. 5 (+)	79	12	56	31	
K562-*myb* no. 7 (−)	<1	85	9	2	4
K562-*myb* no. 7 (+)	60	15	75	10	
K562-*myb* no. 15 (−)	<1	85	11	1	3
K562-*myb* no. 15 (+)	62	10	70	20	
K562-*myb* no. 20 (−)	<1	91	4	2	3
K562-*myb* no. 20 (+)	61	11	66	23	
K562-*myb* no. 24 (−)	<1	71	25	1	4
K562-*myb* no. 24 (+)	52	7	71	22	
K562-*myb* no. 30 (−)	<1	91	5		4
K562-*myb* no. 30 (+)	57	5	60	35	
K562-*myb* no. 32 (−)	<1	93	4		3
K562-*myb* no. 32 (+)	62	3	69	28	
K562-*myb* no. 39 (−)	<1	92	4		4
K562-*myb* no. 39 (+)	19	3	65	32	
K562-*myb* no. 40 (−)	<1	92	5		3
K562-*myb* no. 40 (+)	19	1	62	37	

[a] Adherence of cells was measured as described elsewhere (17). Cytochemical analysis was assessed by classifying cells into one of four categories based on criteria described in the text. + and −, cells treated with TPA and untreated, respectively.

To carry the analysis of megakaryocytic differentiation further, the modulation of several markers was investigated. The platelet fibrinogen receptor complex IIb-IIIa antigen is a glycoprotein complex associated with platelets and megakaryocytes and is considered an early marker of megakaryocytic differentiation (2). Figure 7 shows Western blot analysis of K562 cells and the K562 transfectants before and after 2 days of treatment with TPA. Consistent with the observations described above, a marked increase in IIb-IIIa occurs in all cell lines after treatment with TPA. Similarly, the α_2 integrin protein was examined. The $\alpha_2\beta_1$ integrin complex serves as a cell surface receptor for collagen on platelets and other cells. Its elevated expression accompanies megakaryocytic differentiation of K562 cells (see reference 2 and references therein). Figure 8 shows the results of Western blot analysis of α_2 expression on cell extracts before and after TPA treatment as in the preceding experiment, again with the same results; elevated expression of the differentiation marker is seen in parental as well as in the transfected cell lines.

Two other markers whose modulation is associated with megakaryocytic differentiation of K562 were also examined. Because the antibodies did not work in Western analysis, flow cytometry was used as previously described (2). The levels of glycophorin, a marker for erythroid cells, and CD15(LeuM1), a myelomonocytic marker, expression are decreased as a result of TPA-induced differentiation (2). Figure 9 shows the results of flow cytometric analysis of the cell lines, which are consistent with the data above. Fluorescence intensity decreased five- to eightfold in parental K562 cells in studies with these markers. K562-*myb* cells showed similar decreases. In all of the marker studies, some variation existed between individual lines in regard to the level of antigen present in untreated cells. In

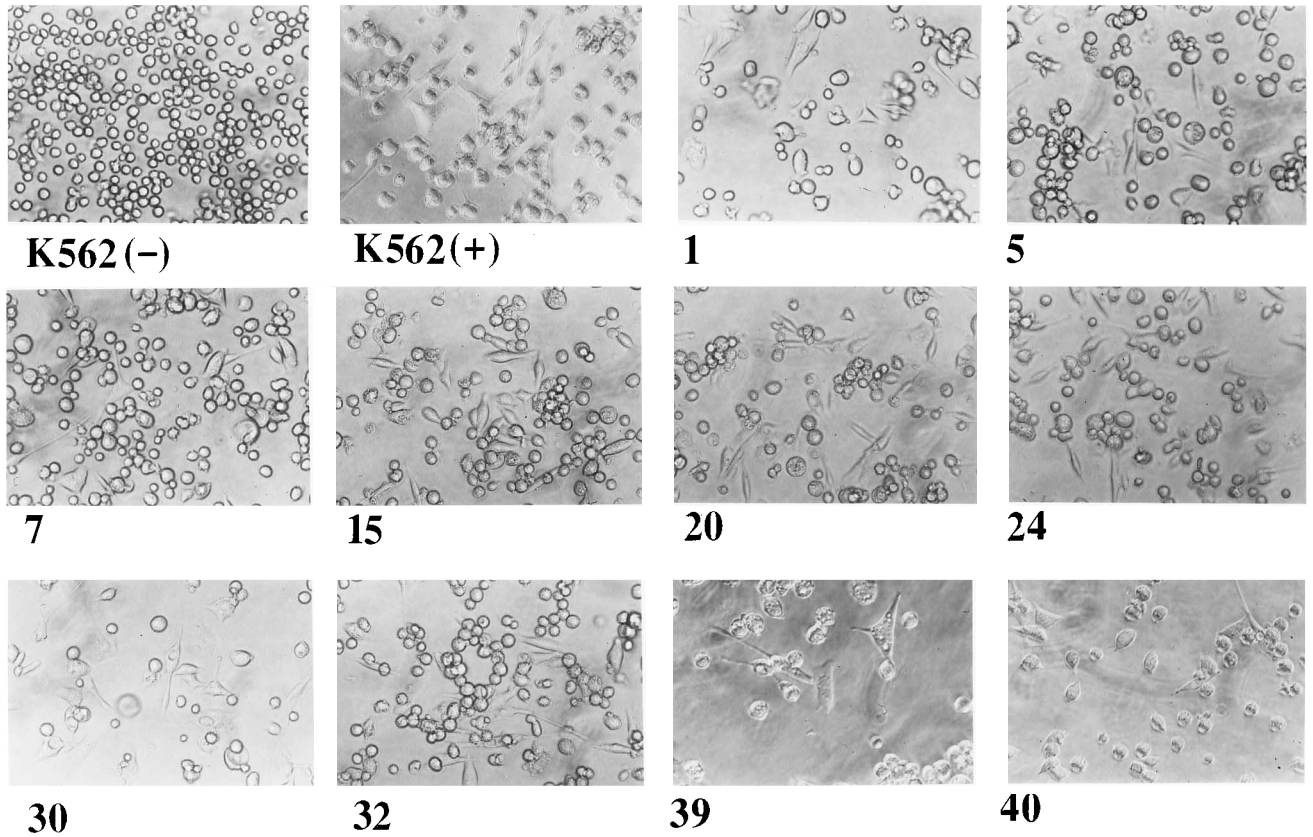

FIG. 5. Morphology of K562 and K562-*myb* cells treated with TPA. Logarithmetically growing cells were adjusted to 0.17 μM TPA for 2 days and then photographed. −, untreated K562 cells; +, TPA-treated K562 cells. All other panels are K562-*myb* cells treated with TPA. K562-*myb* cell line numbers are given beneath the picture.

K562-*myb* cells, the modulation of the marker with TPA treatment was consistent with that of the parental K562 and consistent with those previously reported by others (2). The one exception to this was an increase in CD15 expression in K562-*myb* no. 32 upon TPA treatment. However, taken together, the totality of measurement on cellular differentiation indicates that K562 cells expressing elevated c-*myb* are indeed competent for TPA-induced differentiation. c-*myb* is thus able to prevent the induced erythroid differentiation by cell division inhibitors but unable to negate the influences of the potent PKC activator, TPA.

In order to examine the mechanism of induced differentiation as it relates to c-Myb protein levels, we measured $p75^{myb}$ levels in extracts of inducer-treated cells. As seen in Fig. 10, TPA was able to down-regulate c-Myb protein in K562-*myb* cells. Levels did not become undetectable as in the case of TPA treatment of parental K562 (Fig. 1) but were reduced to such an extent as to suggest that the amounts of both the endogenous and the exogenous proteins were decreased. This phenomenon is also seen in DMSO-treated F-MEL-*myb* cell extracts. These results suggest that enhanced degradation is at least partially responsible for decreased c-Myb protein levels upon inducer treatment. This may be a direct action of the inducers or may be a result of a different panel of proteolytic enzymes that are present in differentiated cells. The fact that DMSO-treated F-MEL-*myb* cells also show degradation of exogenous c-Myb protein might still be explained by a partial differentiation of F-MEL-*myb* cells. In these studies (3), differentiation was assessed only by hemoglobin production; other properties of differentiation which were not recognized might have occurred. Further research will be needed to answer these questions.

DISCUSSION

The fact that c-*myb* plays a role in the signal transduction pathway of cellular differentiation in at least some hematopoietic cells has been firmly established (3, 35, 39). When probes became available to investigate the expression of proto-oncogenes in various tissues, it quickly became noted that c-*myb* is a gene whose expression is primarily associated with hematopoietic tissue and is highest in cells that are relatively immature (40). Further investigations of cell lines that could be induced to undergo differentiation revealed that c-*myb* expression was down-regulated during the maturation process (10, 30). Clarke et al. (3) first used the model system of Friend leukemia virus-immortalized erythroleukemia cells to establish that c-*myb* down-regulation was a necessary event in DMSO-induced erythroid differentiation. Similarly, Selvakumaran et al. (35) used the murine M1 cell line to show that constitutive expression of c-*myb* prevented growth factor-induced differentiation in myeloid cells. Recently, these latter results have been extended to v-*myb* (28).

We have similarly investigated the effect of constitutive expression of c-*myb* on the K562 cell line. This is a line established from the hematopoietic cells of a patient with chronic myelogenous leukemia (CML) (18) and, consistent with the presumed characterization of CML as a disease of pluripotent

FIG. 6. Cytochemical analysis of K562 and K562-*myb* cells treated with TPA. Cells were treated as described in the legend to Fig. 5, cytocentrifuged, and stained with Wright-Giemsa stain.

stem cells (34), this line can be made to undergo differentiation along at least two different lineages, depending on the inducing agent. We have established K562-derivative cell lines expressing higher levels of c-*myb* under a constitutive promoter. Because of the fact that variants can arise from any cell line which display altered properties from the parent, leading to misleading interpretations, we have been careful to examine a large number of transfectants as well as subclones of the parent. Cell transfectants contain and transcribe the exogenous sequences. Elevated expression is indicated by both Western analysis of c-Myb protein levels as well as the altered properties of the cell line, which are consistent with previous studies. Just as DMSO-induced erythroid differentiation and growth factor-induced myeloid differentiation were inhibited by c-Myb, so was cadaverine and araC-indicated erythroid differentiation in K562.

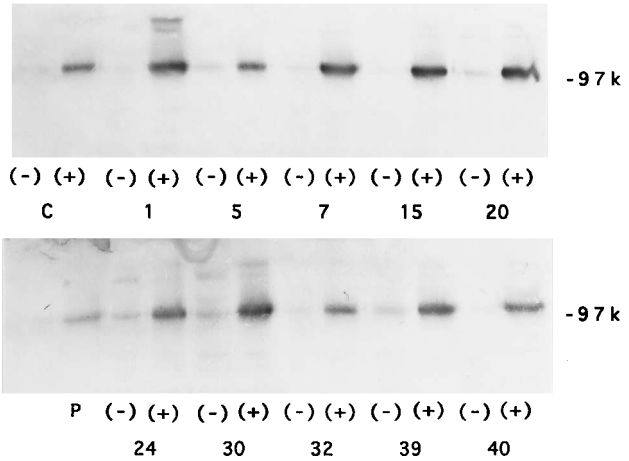

FIG. 7. Western blot analysis of the megakaryocyte-associated antigen IIb-IIIa in K562 and K562-*myb* cells with and without TPA treatment. Cells were treated with 0.17 μM TPA for 2 days as described in the legend to Fig. 5. Cell extracts were made, quantitated, and subjected to Western gel analysis as described in Materials and Methods. The position of the 97-kDa molecular mass marker is shown. Lanes representing control K562 cell extracts from untreated (−) and treated (+) cells are labelled C. An extract made from purified human platelets is labelled P. Extracts from the 10 K562-*myb* clones are labelled with their corresponding numbers.

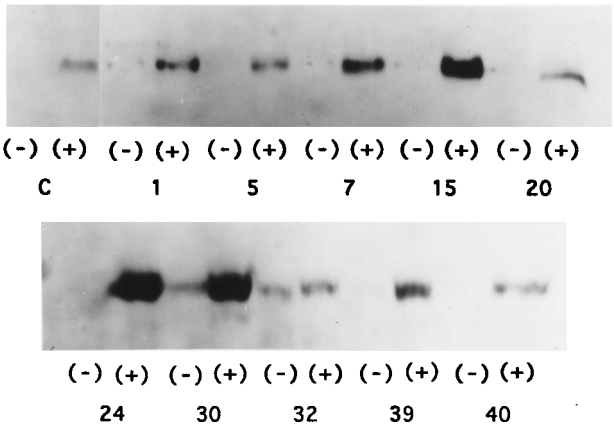

FIG. 8. Western blot analysis of the α_2 integrin. Treatment and analysis are as described in the legend to Fig. 7.

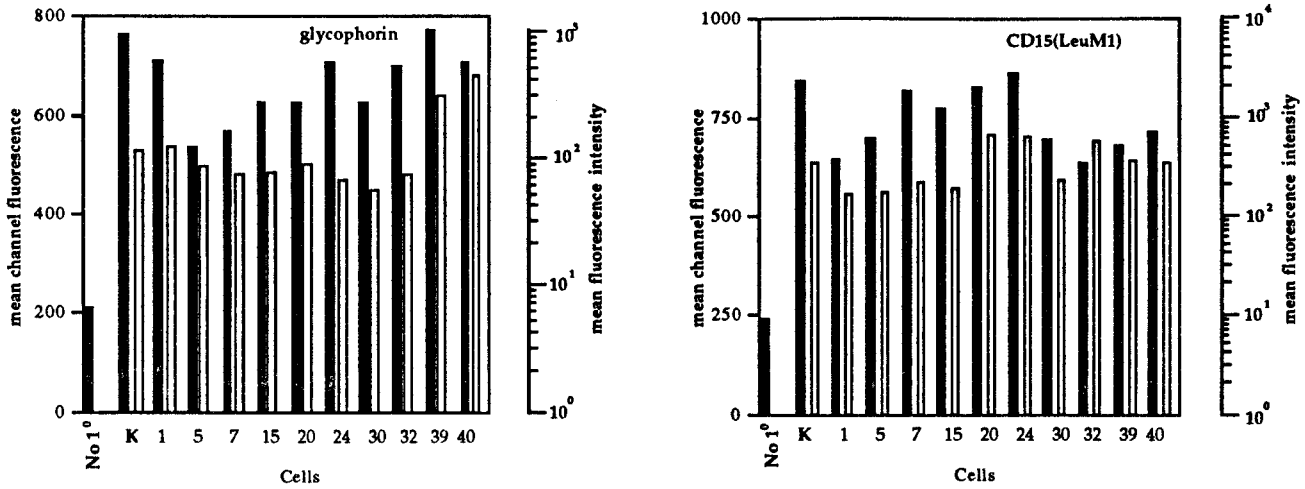

FIG. 9. Flow cytometry analysis of K562 and K562-*myb* cells. Flow cytometry was performed on a Becton Dickinson FACScan flow cytometer, and the results were analyzed as described elsewhere (2). Data are presented as mean channel fluorescence and mean fluorescence intensity. The bar labelled No. 1^0 represents the fluorescence of cells which were treated with no first antibody. Bars labeled K are untreated K562. K562-*myb* cells are indicated by their K562-*myb* cell line numbers. Open bars, TPA-treated cells; solid bars, untreated cells. The left panel shows the results of analysis with the anti-glycophorin A antibody (Sigma), and the right panel shows the results of analysis of the anti-CD15 antibody (Becton Dickinson).

This in itself represents an extension of the previous studies. The mechanism of action of cadaverine is conjectural but might be thought to somehow act through modulation of transglutaminase, an enzyme whose activity is associated with crosslinking of the erythrocyte membrane during maturation. However, the mechanism of araC on growth inhibition is more definitive; it simply destroys the nascent DNA strand's ability to be extended, thus preventing the cell from replicating. Constitutive c-*myb* expression was able to prevent erythroid differentiation of K562, even though the gene product had to, in effect, uncouple proliferation inhibition from differentiation induction.

We also examined the ability of the tumor promoter TPA to induce megakaryocytic differentiation of the c-*myb*-transfected cell lines. TPA is a compound whose principle, if not only, metabolic effect is as a modulator of PKC. Thus, PKC is apparently involved in megakaryocytic differentiation of K562 cells. Similarly, PKC was implicated in erythroid differentiation when Rovera et al. (33) demonstrated that DMSO-induced differentiation of F-MEL cells is prevented by treatment with TPA. Recently, Durkin et al. (8) showed that the PKC inhibitor H-7 blocks induced differentiation of F-MEL cells. Unlike erythroid differentiation, constitutive c-*myb* expression was found to have no effect on TPA-induced megakaryocyte differentiation. This was assessed by morphological, cytochemical, and cell adhesion changes as well as four cellular proteins whose expression is modulated as a result of TPA-induced differentiation. While one explanation for this might be that c-*myb* does not play a role in megakaryocytic differentiation, this seems unlikely, since it is down-regulated, as it is in erythroid and myeloid differentiation. Another explanation could be that TPA is able to bring about differentiation because it is a stronger inducer of down-regulation of c-*myb* than are the erythroid inducers. Western blot analysis shown in Fig. 10 suggests that TPA treatment of K562-*myb* cells results in down-regulation of c-Myb protein expressed from both the endogenous and the exogenous genes. This is also seen in DMSO treatment of F-MEL-*myb* cells, which do not differentiate. In both pMbMI/DHFR-transfected lines, inducer treatment fails to reduce c-Myb protein to undetectable levels. The fact that TPA-treated K562-*myb* cells differentiate and that DMSO-treated F-MEL-*myb* cells do not might be explained by different levels of exogenous c-*myb* expression obtained in the two lines. In other words, F-MEL-*myb* cells may have high enough levels of $p75^{myb}$ to prevent differentiation because greater expression has been obtained or because less is needed to prevent differentiation, compared with TPA-induced differentiation of K562 cells. Alternatively, these results are also consistent with the interpretation that c-*myb* exerts its influence on the cell's growth-differentiation pathway via PKC; that is, PKC lies downstream of c-*myb* in the differentiation pathway. According to this hypothesis, TPA, acting as a direct activator of PKC, bypasses the effects of c-*myb*, whereas the erythroid inducers cadaverine and araC presumably act on the signal transduction pathway at a point before c-*myb*. This finding was presaged by the work of Symonds et al. (38), who studied lipopolysaccharide-induced and TPA-induced differentiation of BM2 cells, which are avian myeloblastosis virus-transformed monoblasts. In this system, differentiation occurred without a concomitant down-regulation of v-*myb*, suggesting the agents act at a point downstream of *myb*. Also consistent with this interpretation is the fact that F-MEL cells

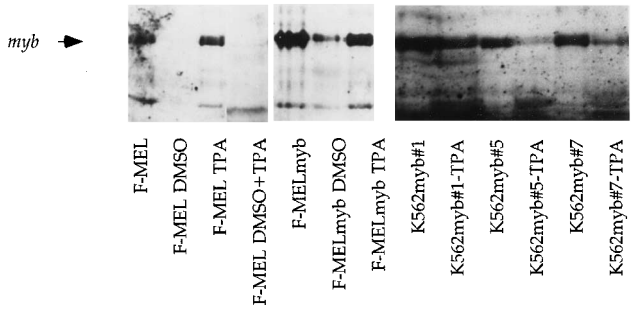

FIG. 10. c-Myb protein levels in protein extracts of inducer-treated cells. Western analysis was performed as described in Materials and Methods. Identities of cell extracts and treatment with DMSO and/or TPA are indicated below each lane.

treated with both DMSO and TPA still down-regulate c-*myb*, even though the cells do not differentiate (Fig. 10).

Interestingly, Smarda and Lipsick (36) have recently reported that exogenous c-*myb* expression inhibits TPA-induced myeloid differentiation of BM2 cells. The explanation for this presumably lies in the different cell lines expressing the exogenous c-*myb* and probably in the complication added by the presence of v-*myb* in the BM2 line. The authors point out that cotransfection experiments have shown that the C terminus of c-Myb can act as a dominant inhibitor of transcriptional activation of v-Myb protein (7). At this point, the divergent effects of c-*myb* in the two cell lines are difficult to interpret more specifically. However, with this in mind, it might be interesting to express v-*myb* in K562 as well as in one of the K562-*myb* clones to determine if v-*myb* can block the TPA induction that c-*myb* cannot.

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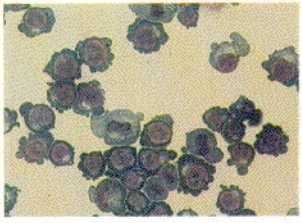
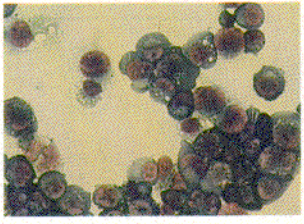
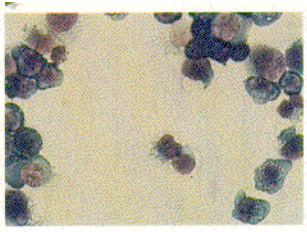


K562(−)



K562(+)



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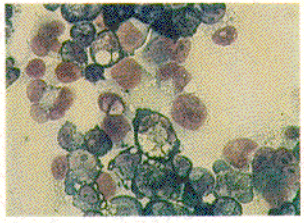

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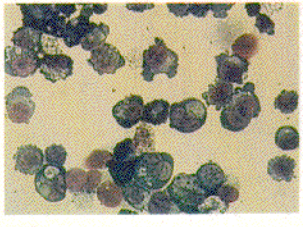

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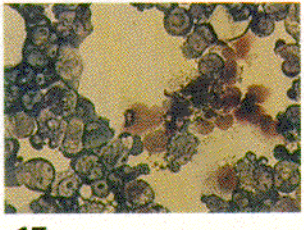

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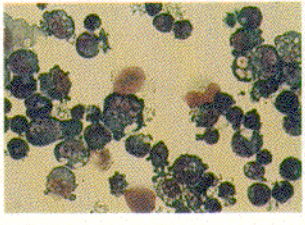

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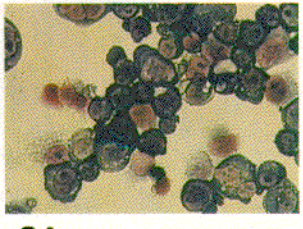

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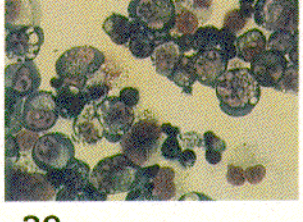

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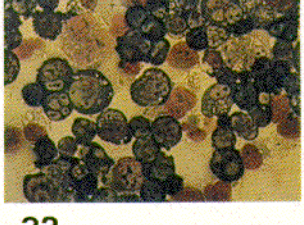

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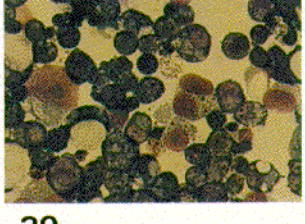

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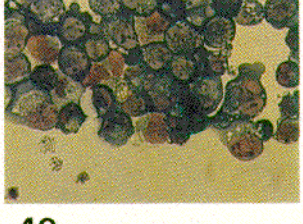

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