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Early Decline in *c-myb* Oncogene Expression in the Differentiation of Human Myeloblastic Leukemia (ML-1) Cells Induced with 12-*O*-Tetradecanoylphorbol-13-acetate[1]

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ABSTRACT

The relationship of oncogene expression to proliferation and differentiation has been examined in a line of human myeloblastic leukemia (ML-1) cells. Proliferating leukemic cells were found to express a 4.3-kilobase cellular homologue (*c-myb*) of the transforming sequence of avian myeloblastosis virus. A rapid decline in the expression of this transcript was seen in cells induced to differentiate with 12-*O*-tetradecanoylphorbol-13-acetate. The level of *c-myb* RNA was decreased by >50% as early as 3 hr after 12-*O*-tetradecanoylphorbol-13-acetate exposure, and at 8 to 72 hr the reduction was ≥4-fold. Subsequent to the decrease in oncogene expression at 3 hr, DNA synthesis began to decline; by 24 hr, cell proliferation had ceased. At this time, monocyte- and macrophage-like cells were beginning to emerge. These findings demonstrate that *c-myb* is expressed during ML-1 cell proliferation and declines prior to the loss of DNA synthesis that accompanies the differentiation process.

INTRODUCTION

Oncogenic retroviruses have been demonstrated to cause cell transformation *in vitro* and tumor formation *in vivo* through the expression of specific sequences known as oncogenes (3). Cellular homologues (*c-onc*)[2] of these viral genes may participate in maintaining the proliferative cycle in normal tissues, as well as in cancers of nonviral origin. If this is the case, decreased expression of specific *c-onc* genes might be expected to translate into decreased cell proliferation.

We have examined oncogene expression in relation to cell proliferation and differentiation, using a line of human myeloblastic leukemia (ML-1) cells. These cells differentiate to nondividing monocyte- and macrophage-like cells upon treatment with TPA and other agents, such as antineoplastic drugs and conditioned medium factors (20–22). The expression of *c-myb*, the cellular homologue of the transforming sequence (*v-myb*) of avian myeloblastosis virus, was assayed during the growth and TPA-induced differentiation of this cell line. The *c-myb* gene was selected for study because avian myeloblastosis virus produces myeloblastic leukemia in chickens (13), and the ML-1 cells are myeloblastic in nature (20). Our findings demonstrate that decreased *c-myb* expression is an early event in the loss of proliferation that accompanies TPA-induced ML-1 cell differentiation.

MATERIALS AND METHODS

Materials. The ML-1 cell line was generously provided by Dr. J. Minowada, of the Hines Veteran's Administration Medical Center, Hines, IL. Recombinant plasmids containing the *v-myb* (10) and *v-myc* (27) sequences were kindly supplied by Dr. J. M. Bishop of the University of California at San Francisco. RPMI 1640 medium and FBS were purchased from Grand Island Biological Co., Grand Island, NY. TPA was obtained from Chemicals for Cancer Research, Inc., St. Louis, MO, and stored as described previously (20). [α-^{32}P]TTP (800 Ci/mmol) was obtained from Amersham Corp., Arlington Heights, IL. [*methyl*-^{3}H]Thymidine (30 to 38 Ci/mmol) and [5-^{3}H]uridine (14 Ci/mmol) were purchased from ICN Pharmaceuticals, Inc., Chemical and Radioisotopes Division, Irvine, CA. DNase I and DNA polymerase I were supplied by Worthington Biochemical Corp., Freehold, NJ, and Boehringer Mannheim, Inc., West Germany, respectively. Oligodeoxythymidylic acid-cellulose was obtained from Collaborative Research, Lexington, MA, and nitrocellulose membrane filters were from Schleicher and Schuell, Keene, NH.

Cell Culture. ML-1 cells were maintained in suspension culture in RPMI 1640 medium supplemented with 7.5% heat-inactivated FBS, as described in a prior report (20). For isolation of RNA and other assays, cells were harvested from logarithmically growing cultures, and inocula of 3×10^5 viable cells/ml were incubated at 37° for 1 to 72 hr in the presence or absence of 5×10^{-10} M TPA, using the same medium containing 10% FBS, penicillin (100 units/ml), and streptomycin (100 μg/ml). Cell growth and viability were assayed by hemocytometer using trypan blue dye exclusion.

Any adherent cells (generally ≤30% in TPA-treated cultures) were harvested using the method of Schon-Hegrad and Holt (19). A modification of this method, in which washed cells were agitated at 37° in 0.02% EDTA, was used to reduce cell adhesion for cell count determinations.

To assess the stability and/or reversibility of the TPA effect, cells were centrifuged from the drug-containing medium and washed with drug-free medium. The cell pellet was incubated at 37° for 30 min in two 25-ml aliquots of drug-free medium and then was resuspended in fresh medium.

Assay of DNA and RNA Synthesis. DNA and RNA synthesis were evaluated by measuring the incorporation of radiolabeled thymidine or uridine into acid-insoluble material using established methods (26). After 3 hr exposure to the labeled precursors (0.032 μCi/ml), cells were washed with phosphate-buffered 0.9% NaCl solution and extracted with ice-cold 5% trichloroacetic acid. Acid-precipitable material from 10^6 viable cell equivalents was collected on glass microfiber (GF/C) discs, which were successively rinsed with 5% trichloroacetic acid, absolute ethanol, and acetone. After drying, radioactivity was quantitated by liquid scintillation counting. This procedure was found to remove >98% of the unincorporated precursors, and quenching by the disc was found to be minimal (about 10%). Precursor incorporation in untreated ML-1 cells was linear with time and cell concentration.

Assessment of Morphological Differentiation. Morphological differentiation was monitored by examining fixed slide preparations, which

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[2] The abbreviations used are: *c-onc*, cellular oncogene; FBS, fetal bovine serum; *c-myb*, cellular homologue of the transforming sequence of avian myeloblastosis virus; *c-myc*, cellular homologue of the transforming sequence of avian myelocytomatosis virus; poly(A), polyadenylated; SDS, sodium dodecyl sulfate; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; *v-myb*, transforming sequence of avian myeloblastosis virus; *v-myc*, transforming sequence of avian myelocytomatosis virus; *v-onc*, viral transforming sequence.

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were stained as described previously (20). Differentiation was scored using established criteria (11), macrophage-like cells manifesting a reniform nucleus and decreased nucleocytoplasmic ratio (20), monocyte-like cells exhibiting a more centrally located maturing nucleus and less cytoplasmic enlargement than did macrophage-like cells, and intermediate-stage (promonocyte-like) cells typically displaying an immature nucleus in the presence of moderate cytoplasmic enlargement.

Assay of Oncogene Expression. Labeling of isolated (23) plasmid DNA was accomplished by standard nick translation procedures (16), using [α-^{32}P]TTP (0.1 Ci/0.1 ml reaction mixture). The labeled DNA was separated by column chromatography with SP-Sephadex using an elution buffer of 0.01 M sodium acetate (pH 5.0) containing 0.3 M NaCl. The PVM2 recombinant probe containing the *v-myb* sequence (10) was used either before or after isolation (12) of a *v-myb*-containing fragment obtained by restriction enzyme cleavage of the intact plasmid with *Kpn*I. The probe containing the *v-myc* sequence (27) was used either before or after isolation of a *v-myc*-containing fragment obtained by cleavage with *Pst*I.

RNA isolation was performed using the guanidine-HCl method, as described by Hastie *et al.* (9), except that Dounce homogenization was used. Poly(A)-enriched RNA was fractionated by passage through an oligodeoxythymidylic acid-cellulose column, using established methods (1). Non-poly(A) RNA was eluted with 0.1 M Tris-HCl (pH 7.6), containing 0.5 M KCl, prior to elution of poly(A)-RNA with 0.01 M Tris-HCl (pH 7.6). RNA was precipitated with ethanol and quantitated spectrophotometrically.

Gel electrophoresis was carried out in 1.5% agarose containing 10 mM sodium phosphate (pH 6.5) and 2.2 M formaldehyde. Samples were electrophoresed for 450 V-hr using 10 mM sodium phosphate (pH 6.5) as the running buffer. Transcript sizes were estimated by comparison with rRNA markers, which were detected by ethidium bromide staining.

RNA blotting analysis was accomplished using modifications of established methods (25). RNA samples, denatured by incubation at 50° for 15 min in 12 mM sodium phosphate buffer (pH 7.4) containing 60% deionized formamide and 1.8 M formaldehyde, were either dot-blotted onto nitrocellulose filters presoaked in 1.5 M NaCl-0.15 M trisodium citrate or subjected to electrophoresis and transferred to nitrocellulose filters.

Prehybridization of the filters was carried out at 62–65° for approximately 3 hr using a buffer consisting of 0.75 M NaCl-0.075 M trisodium citrate, 0.1% SDS, 0.1% sodium pyrophosphate, denatured salmon sperm DNA (150 μg/ml), and 10× Denhardt's solution (0.2% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll). The filters were annealed with radiolabeled DNA probes under the same conditions, except that a concentration of 1× Denhardt's solution was used in the hybridization buffer. After hybridization, the filters were rinsed at 62–65° at least 6 times with 2× Benton-Davis solution (50 mM Tris-HCl, pH 7.9, containing 1 M NaCl, 0.1% SDS, 0.1% sodium pyrophosphate, and 1× Denhardt's solution); then washed 3 times for 30 min in 0.3 M NaCl-0.03 M trisodium citrate containing 0.1% SDS and 0.1% sodium pyrophosphate. The filters were exposed to XAR-5 film at −70° for up to 2 weeks using Optex High Plus intensifying screens. Relative changes in oncogene expression were quantitated by densitometric scanning of the autoradiographs.

RESULTS

A dot-blot hybridization analysis of the relative levels of *c-myb* RNA in control and TPA-treated ML-1 cells is shown in Fig. 1*a*. In untreated ML-1 cells, RNA sequences homologous to the *v-myb* probe were readily detectable. The expression of these sequences did not change substantially from 8 to 72 hr of incubation, the variation between independent RNA preparations being ≤23%. In contrast, sequences homologous to the *v-myb* probe were appreciably decreased in cells treated with TPA, this reduction being observed in cells exposed for 8, 15, or 72 hr. Decreased levels of *c-myb* sequences were seen in samples of both total and poly(A)-selected RNA. Some variation in the extent of hybridization was noted in different RNA preparations from TPA-treated cells.

An analysis of the size of the *c-myb* transcript using gel blot hybridization is shown in Fig. 1*b*. In untreated ML-1 cells, a RNA species of approximately 4.3 kilobases was detected in total and poly(A)-enriched (Fig. 1*c*) RNA preparations, in agreement with reports of a transcript of similar size (4 to 4.5 kilobases) in other hematopoietic tissues (5, 29). A decrease in the level of this transcript was seen in cells exposed to TPA, validating the dot-blot hybridization analysis. As determined by quantitative densitometry, a small decrease (approximately 16%) was noted as early as 1 hr. A >50% reduction was consistently observed at 3 hr, with a 4- to 5-fold decrease being seen at 8 to 72 hr.

The reduction in *c-myb* expression in TPA-treated cells did not appear to result from nonspecific inhibition of RNA transcription. As measured by [^{3}H]uridine incorporation, RNA synthesis was not decreased at 3 hr (when a >50% decrease in *c-myb* expression was evident) and declined by only about 6% at time points up to 11 hr.[3] As measured by the orcinol reaction (28), no change in cellular RNA content was noted at the 24-hr time point, a 1.7-fold increase being observed in TPA-treated samples at 72 hr.[4] The expression of another cellular oncogene, *c-myc*, was not substantially affected by TPA treatment. Using the intact plasmid as a probe, this oncogene was only very faintly detectable; with the *Pst*I *v-myc*-containing fragment as a probe, expression was detectable only after prolonged exposure of the autoradiographs. Under these conditions, *c-myc* expression was decreased by only about 15.5% in TPA-treated cells at time points up to 15 hr. In addition, several of the *in vitro* RNA translation products from TPA-treated samples were found to be increased above control levels.[5]

The changes in *c-myb* expression and ML-1 cell proliferation that occurred during TPA treatment are shown in Chart 1. Decreased oncogene expression was seen before a significant decline in cell proliferation was noted. Within 3 hr after exposure of the leukemic cells to TPA, the level of *c-myb* RNA had decreased by more than 50% (Chart 1*c*), although at this time incorporation of [^{3}H]thymidine into acid-insoluble material had not declined appreciably (Chart 1*b*). At 8 hr, *c-myb* expression in TPA-treated cells had reached a nadir (≥75% reduction), and incorporation of [^{3}H]thymidine had begun to decline. By 24 hr, incorporation of [^{3}H]thymidine had decreased to nearly undetectable levels, and cell proliferation had ceased (Chart 1*a*). As determined by flow cytometry (8, 24), entry of cells into S phase had also come to a halt, with only about 3.1% of cells remaining in S phase at 24 hr, as compared to 42.9% in controls.

The time course of accrual of differentiation-associated characteristics in TPA-treated cells is shown in Chart 2. At 10 hr, TPA caused only a minor increase in ML-1 cell differentiation. At 24 hr, about 27% of the treated cell population had entered the differentiation sequence, with 10% of the cells exhibiting monocyte-like or macrophage-like characteristics (as compared to

[3] R. W. Craig, O. S. Frankfurt, H. Sakagami, and A. Bloch. Macromolecular and cell cycle effects of different classes of agents inducing the maturation of human myeloblastic leukemia (ML-1) cells, manuscript in preparation.

[4] R. W. Craig and A. Bloch, unpublished data.

[5] R. W. Craig, R. A. Hromchak, and A. Bloch, manuscript in preparation.

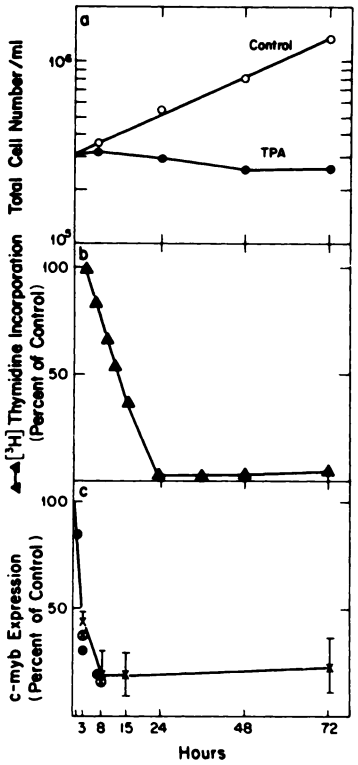

Chart 1. Time course of cell proliferation, DNA synthesis, and *c-myb* expression in TPA-treated ML-1 cells relative to untreated controls. Untreated or TPA-treated ML-1 cells were assayed for cell proliferation (*a*), incorporation of [^{3}H]thymidine into acid-insoluble material (*b*), and expression of *c-myb* (*c*). Total cell number (*a*) was determined in 4 separate experiments, using an inoculum of 3×10^5 viable cells/ml. The percentage of viable cells in control and in TPA-treated samples averaged, respectively, 95.5 and 88.1% on Day 1 and 95 and 79% on Day 3. Incorporation of [^{3}H]thymidine into acid-insoluble material (*b*) was calculated as a percentage of the incorporation in concurrent controls. Each *point* represents the mean of at least 2 determinations. The level of *c-myb* RNA (*c*), relative to untreated controls, was determined by dot-blot hybridization of serial dilutions of the poly(A)-selected RNA (⊗), or by densitometric scanning of autoradiographs in which either the intact PVM2 probe (×) or a *KpnI myb*-containing fragment of this probe (●) was used. *Bars*, S.D. of at least 2 preparations. In independent control preparations hybridized simultaneously, the level of *c-myb* RNA was not found to vary by more than 28%. The first *time point* shown in *c* was assayed at 1 hr.

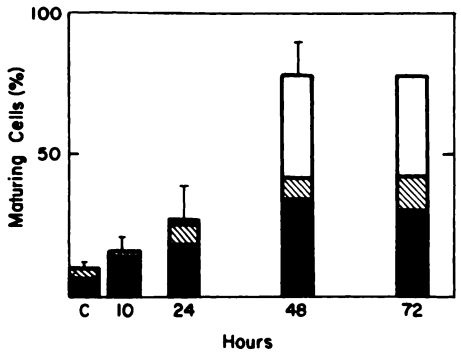

Chart 2. Time course of TPA-induced morphological differentiation of ML-1 cells. ML-1 cells were incubated in the presence or absence of 5×10^{-10} M TPA and assessed for morphological differentiation at intervals of 10 to 72 hr. □, macrophage-like cells; ■, monocyte-like cells; ■, intermediate-stage (promonocyte-like) cells. *Bars*, S.D.s of total maturing cell population derived from 2 replicate samples. Because the number of maturing cells in control cultures did not vary significantly over the 72-hr incubation period, the mean of all concurrent controls is shown on the *origin*.

approximately 3% in controls). Removal of the TPA-containing medium at this time did not reverse the commitment to differentiation (Table 1). After 24 hr, maturing cells emerged rapidly in

Table 1

Irreversibility of the effect of TPA on morphological differentiation

ML-1 cells were incubated in the presence or absence of 5×10^{-10} M TPA for 24 hr, and one portion of the culture was washed and recultured in drug-free medium. Differentiation was assessed after an additional 72 hr of incubation. Reculturing did not significantly affect control cells, and the average control value is listed.

	Maturing cells (%)[a]			
Treatment	Intermediates	Monocyte-like cells	Macrophage-like cells	Total
Control	3.5 ± 1.0[a]	5.2 ± 1.7	0.2 ± 0.5	9.0 ± 0.8
96 hr TPA	32.0 ± 7.1	5.0 ± 0.0	43.5 ± 5.5	80.5 ± 2.1
24 hr TPA/72 hr drug-free medium	28.0 ± 7.8	1.7 ± 0.5	63.5 ± 9.3	92.7 ± 2.1

[a] Mean ± S.D. of 2 replicate determinations.

TPA-treated cultures (Chart 2); at 72 hr nearly 80% of the population displayed maturing phenotype. A comparison of these results with the data in Chart 1 indicates that the decline in *c-myb* expression largely precedes the acquisition of differentiated phenotype.

DISCUSSION

Since sequences homologous to *v-myb* are highly conserved in the genome of vertebrates (2), this gene is thought to code for a vital cellular function. This study examined the temporal relationship between changes in *c-myb* expression, DNA synthesis, and differentiation in ML-1 cells. A considerable decline in *c-myb* expression was observed at 3 hr, followed by a decrease in DNA synthesis between 3 and 24 hr. An increase in morphological differentiation became manifest at about 24 to 48 hr. This sequence of events demonstrates that the *c-myb* gene is expressed during ML-1 leukemic myeloblast proliferation and that its decline precedes the loss of proliferation that accompanies differentiation.

Gonda *et al.* (5) and Chen (4) have postulated that *c-myb* expression may be involved in cell proliferation in immature hematopoietic tissues, since the expression of this gene is restricted to such tissue types in chickens. The demonstrated association between decreased *c-myb* expression and cessation of myeloblastic ML-1 cell proliferation tends to support this hypothesis. A correlation between oncogene expression and proliferation is further suggested by reports demonstrating elevated *c-myb* expression in some neoplastic cells (15, 17).

The expression of another cellular oncogene (*c-ras*) has been studied (6) in regenerating rat liver, where an increase in *c-ras* expression occurs at the time of activation of DNA synthesis associated with regenerative growth. Conversely, the decrease in *c-myb* expression in ML-1 cells occurs at the time of inactivation of DNA synthesis, during the onset of differentiation. Thus, these reports link oncogene expression to cell growth processes.

An association between oncogene expression and differentiation has been provided by several investigators. Westin *et al.* (29, 30) have observed decreases in the expression of *c-myb* and *c-myc* in human promyelocytic leukemia (HL-60) cells induced to differentiate with dimethyl sulfoxide or retinoic acid. Muller *et al.* (14) have demonstrated that fluctuations in the expression of *c-abl* and *c-fos* occur during mouse prenatal and/or early postnatal development. A correlation between *v-onc* gene expression and differentiation has been observed in avian erythroblastosis virus-transformed cells (7), where continuous

expression of a viral gene is "necessary to maintain the undifferentiated state," inhibition of the viral product allowing cells to undergo partial differentiation. A similar relationship may exist in ML-1 cells, where the *c-myb* gene is expressed during myeloblast proliferation, this expression declining prior to the loss of proliferation that accompanies the differentiation process.

The facts that the *c-myb* transcript is quite labile and that its decline is followed by decreased ML-1 cell entry into S phase represent a parallel to the lability of the mediators of proliferation proposed to control transit from G_1 to S phase (18). This coincidence may indicate the existence of a functional relationship between the endogenous mediators of cell proliferation and the product of an oncogenic sequence.

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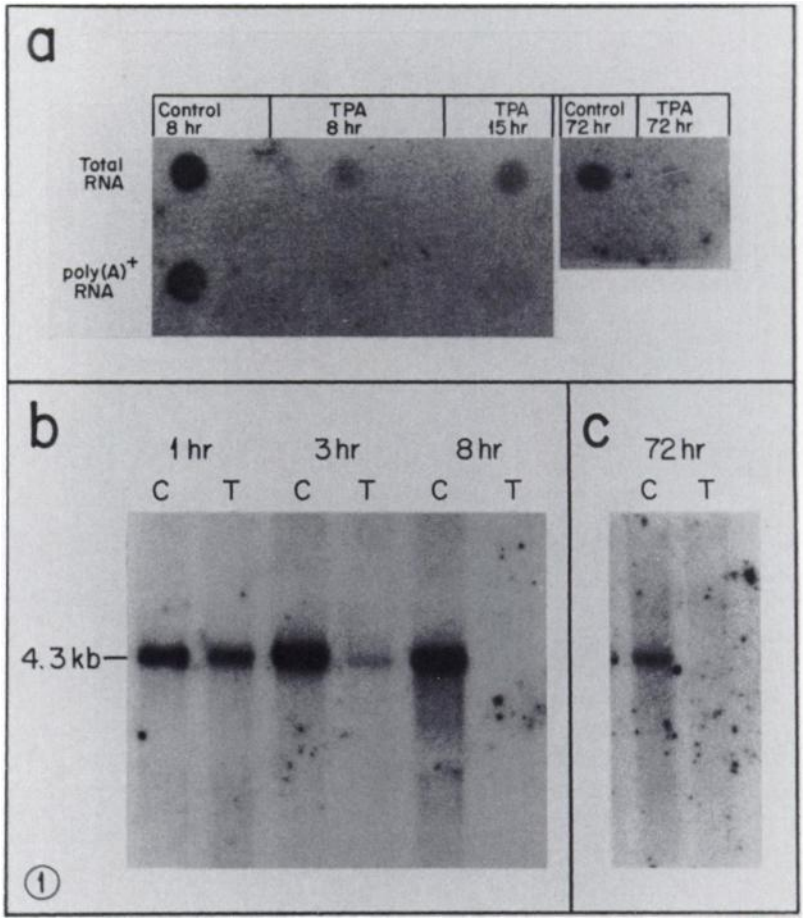

Fig. 1. Expression of *c-myb* in control and in TPA-treated ML-1 cells. RNA was isolated from ML-1 cells cultured in the presence or absence of 5×10^{-10} M TPA for 1 to 72 hr and either (*a*) dot-blotted onto nitrocellulose filters or (*b* and *c*) transferred to filters after agarose gel electrophoresis. Filters were hybridized with the labeled intact probe containing the *v-myb* sequence (*a* and *c*) or a *Kpn*I *myb*-containing fragment of the plasmid (*b*). *a*, dot-blot hybridization of 20 μg total RNA or 5 μg poly(A)-selected RNA. The 15-hr control sample did not differ substantially from the other controls; *b*, gel blot hybridization of 20 μg total RNA; *c*, gel blot hybridization of 5 μg poly(A)-selected RNA. Pairs of samples (control and treated) from different *time points* were isolated from separate cell inocula. *C*, control; *T*, TPA treated.