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Regulated Expression of the c-*myb* and c-*myc* Oncogenes During Erythroid Differentiation

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We have investigated the expression of the genes c-*myb*, c-*myc*, and alpha globin in murine erythroid cells at different stages of development, in viral-induced erythroleukemias, as well as in two mouse erythroleukemia cell lines that can be induced to terminally differentiate when exposed to dimethylsulfoxide. We find that there is a reciprocal correlation between the cell's production of messenger RNA for c-*myb* and globin. c-*myc* message shows a similar but less dramatic decrease coincident with globin RNA production. Initially with the administration of an inducing agent, dimethylsulfoxide, there is a rapid decrease of *myc* and *myb* mRNA, which is followed by signs of differentiation in the induced culture. We conclude that these oncogenes function in early maturational stages of development of these cells. In the erythroleukemic state these genes are down-regulated by forced differentiation and may play a direct role in influencing the state of differentiation of these cells.

Key words: erythroleukemia, red cell maturation, DMSO

A dedifferentiation or block to normal differentiation often accompanies malignant transformation of cells. Quantitative or qualitative changes in oncogene expression in cancerous tissue has been implicated in causing the malignant phenotype. To understand the relationship of oncogenes to malignant transformation we must obtain a more detailed knowledge of the normal expression and functions of these genes during differentiation. Earlier studies on oncogene expression in human hematopoietic cells concluded that certain of these genes, c-*myb* and c-*myc*, were expressed in the less mature cells of the lymphoid, myeloid, and erythroid lineages, although the "erythroid" cell line screened was derived from a chronic myelogenous leukemia cell line [1,2]. Analysis of avian hematopoietic cells also suggests that these oncogenes are expressed differentially during development [3]. Furthermore, Westin et al dem-

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onstrated that in the case of an acute promyelocytic leukemia cell line, HL60, differentiation led to a diminution of c-*myc* expression. Changes in c-*myc* expression during differentiation have also been noted in murine B cells [4] and murine teratocarcinoma cells [5]. Decreased c-*myb* expression during differentiation has previously been reported by Gonda and Metcalf [6], Craig and Block [7], and Sheiness and Gardinier [8]. Recently a decrease in c-*myc* expression during induced differentiation in a murine erythroleukemia cell line has been observed [9].

In this article we report our analyses of oncogene expression in murine erythroid cells in various stages of maturation in vivo. Then, utilizing murine erythroleukemia cell lines that can be induced to differentiate in vitro we follow oncogene expression as cellular differentiation proceeds. We find a reciprocal relationship between the level of expression of the c-*myb* and c-*myc* oncogenes and extent of cellular maturation.

Futhermore, we report a provocative finding that suggests that sudden transient decreases in the level of transcript of these two oncogenes are either causally related or may serve as markers of movement of these cells into a pathway of progressive differentiation. As an outgrowth of this work, we are also able to arrive at an upper limit of the half-lives of these two oncogene messages in this system.

METHODS

Cell Lines and Induction

The inducible murine erythroleukemia cell lines F4-6 (a polycythemic variant) and 745 (an anemic variant) were kindly supplied to us by Dr. J. Billelo, University of Maryland. The cells were grown in Joklik's modified medium supplemented with 10% fetal calf serum. We observed that induction of the cells varied with the batch of fetal calf serum used. However, we obtained essentially identical results on different occasions with separate lots of serum from three different suppliers.

Induction was accomplished by the addition of dimethylsulfoxide (DMSO:SIGMA) to the culture (10^5 cells/ml) at 1.5% v/v. Measurement of induction is based on amounts of globin message produced but can also be assessed by changes in cellular morphology, hemoglobin, orseillein-aniline blue dye staining for stage of cellular development [10], and most simply by cell pellet color.

Spleen Cells

Spleens were obtained from normal, phenylhydrazine-treated, or erythroleukemic spleens from mouse strains NFS, Balb/c, C57B1/6, and DBA/2. No significant difference in strains was observed. Phenylhydrazine provocation of erythroid proliferation and splenomegaly was performed as previously decribed [11]. Precent of splenic cells of erythroid lineage was judged following Wright-Giemsa and nonspecific esterase [12] staining of splenic touch preps.

DNA Probes

The myb probe used was 1.2-kb linkered Bam HI-Bam HI DNA fragment containing the avian v-*myb* oncogene and was kindly given to us by Dr. E.P. Reddy, NIH, Bethesda, MD. The c-*myc* probe used was a 1.5-kb Cla I-EcoRI fragment containing the third exon of the human c-*myc* oncogene. The human *erb* A1, *erb* A2, and *erb* B probes used were the 2.4-kb EcoRI-Hind III fragment, the 1.9-kb EcoRI fragment, and the 2.5-kb EcoRI-Hind III fragment, respectively, given to us by Dr.

B. Vennstrom, EMBL, Heidelberg, FRG. The *ets* probe used was a 1.28-kb BglI insert in plasmid and was kindly given to us by Dr. M. Nunn, Salk Inst., LaJolla, CA. The murine α-globin probe was a 3-kb SstI fragment prepared from a plasmid kindly supplied to us by Y. Nisihoka, McGill University, Montreal. The $\beta_2$microglobulin probe was a cDNA clone kindly supplied to us by J. Seidman, Harvard University, Boston. The β-actin probe was a 1.9-kb Bam HI to Bam HI approximately full length cDNA for human scleroblast cytoplasmic β-actin kindly supplied to us by L. Kedes via J. Battey.

RNA Preparation

RNA was prepared from the spleens and cell lines essentially as described by Chirgwin et al [13]. Ten micrograms of the total RNA was loaded per lane for the "Northern" blots.

Oncogene Screen

We screened Friend virus induced murine erythroleukemia cells for the coincident expression of non-Friend-virus-related oncogenes [14–16]. The oncogenes analyzed, *myb, myc, erb A, erb B,* and *ets,* have all been cited as contributing to the transformed state of erythroid cells in avian systems when carried by acute transforming viruses [14–16]. Only c-*myc* and c-*myb* showed detectable transcription in this system, being seen in both Friend virus- alone and in Friend complex- (Friend helper plus spleen focus-forming virus, SFFV)-induced erythroleukemias.

In Vivo Studies

We then performed a comparative study of alpha-globin, c-*myb*, and c-*myc* gene expression in normal mouse spleen cells, as well as spleen cells from mice that had been treated with the hemolytic-anemia-promoting agent phenlhydrazine, and the Friend- and Friend complex-induced erythroleukemia mentioned above. The normal spleens, containing approximately 5% erythroid cells showed a strong globin hybridizing RNA species but barely detectable *myb* and *myc* transcripts (Fig. 1). We had chosen to study mice treated with phenylhydrazine because of the marked reactivity of their spleens subsequent to the administration of this drug and the anemia it produces [11]. Following injection of this agent, the spleens are noted to increase in weight five- to ten-fold over the next 4 days. At the time of this analysis, the mice manifested a reticulocytosis of 30–50%, and greater than 95% of the mononuclear cells in the spleen were of erythroid lineage. The pattern and amount of globin, *myb*, and *myc* transcripts in these mice did not vary significantly from that seen in the normal spleens. All strains of mice tested (NFS, Balb/c, C57B1/6, DBA/2) and both adult and 6-wk-old weanlings showed essentially this same pattern except for a slight increase in the amount of *myb* and *myc* transcript seen in the younger mice (data not shown).

The Friend-helper-virus-induced erythroleukemia also yielded a mouse with a massively enlarged spleen of greater than 98% erythroid lineage, but with a reticulocytopenia. In the spleens from these mice, a quite different pattern of expression was seen. Much less globin was produced by the erythroleukemic cells, but much more *myb* and *myc* transcript was present in comparison with either normal or phenylhydrazine-treated spleen cells (Fig. 1). By the criteria of spleen cell morphology, reticulocyte count, and globin RNA production, the normal and phenylhydrazine spleens

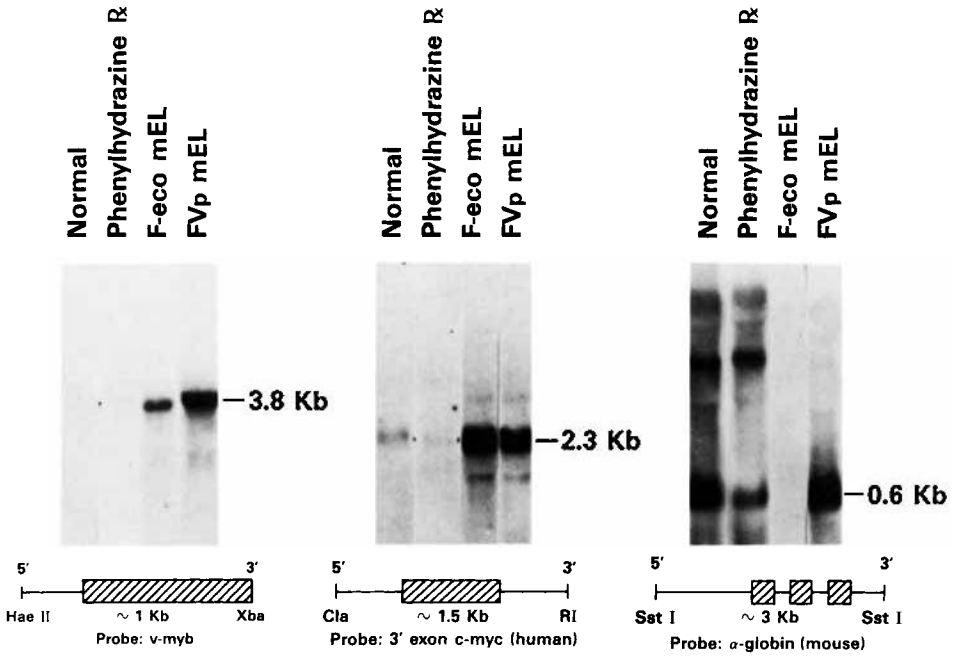


Fig. 1. RNA transcripts of the *myb* and *myc* oncogenes and a alpha-globin in normal and diseased mouse spleens.

were made up of developmentally more mature erythroid cells than the Friend-virus-induced erythroleukemic spleens. Thus, *myb* or *myc* expression was not increased over the course of the massive erythroid proliferation.

A murine erythroleukemia was also induced by injection of Friend complex, FVp, and RNA was extracted from the spleen cells. In this case, comparatively high amounts of both globin and oncogene transcripts were present (Fig. 1). It is important to note that histologically the Friend complex acute polyclonal erythroleukemic spleen shows a much greater spectrum of erythroid activity (from erythroblasts to reticulocytes) than does the monoclonal, long-latency erythroleukemia induced by Friend virus alone, which demonstrates a narrower spectrum of development dominated by less mature cells.

In Vitro Cell Line Induction

To clarify the relationship between oncogene expression and stage of erythroid development we turned to an inducible murine erythroid cell line system. These widely available cell lines transformed by Friend complex appear to represent cells blocked just prior to terminal differentiation [17,18]. Numerous agents (eg DMSO, hexamethylbisacetamide, hemin, purine, and pyrimidine derivatives) are capable of releasing this block to differentiation [17,18].

Data are shown for analyses performed on a murine erythroleukemia cell line F4-6 induced by a polycythemia (SFFVp) variant of Friend complex. Essentially identical results have been obtained with cell line 745 induced by an anemia (SFFVa) variant of Friend complex. The time course shown represents the results of single

experiments. The induction has been performed multiple times with both cell lines and has yielded identical results.

Time zero is the point at which the cells are first exposed to the inducing agent, DMSO, added to give a final concentration of 1.5%. The cells are maintained in the presence of this agent throughout the time course. By day 5, incorporation of ^{3}H-thymidine in the induced cultures was 60% that of control. Cell number in the induced culture was 95% that of control, and viability as measured by trypan blue exclusion was 90–95% in both induced and control cultures.

Alpha-globin messenger RNA, which is barely detectable throughout the experiment in the control culture, shows a steady increase in the induced culture starting at day 1 and reaching a 24-fold increase by day 5 (Fig. 2). In contrast to alpha globin expression, c-*myb* shows a biphasic pattern of expression in the DMSO-induced erythroleukemia cell cultures over the same time course (Figs. 3,4). There is a marked decrease (20-fold) in the amount of c-*myb* RNA by 14 hr after induction (Fig. 3). This result established an upper limit on the half-life of c-myb RNA of 3.5 hr in this system. c-*myb* RNA level begins to increase by 24 hr (greater than 50% of control), peaks around 48 hr (70% of control), then begins a second phase of decrease in the amount of transcript until by day 5 there is 20-fold less c-*myb* RNA than in the control (Fig. 4).

c-*myc* shows a similar dramatic reduction, falling greater than 20-fold by the 4th hr after addition of DMSO in the culture (Fig. 5). This rate of decrease establishes an upper limit on the half-life of c-*myc* RNA in this system to less than 1/2 hr. After this reduction, c-*myc* RNA begins to increase, reaching an amount equivalent to control by 24 hr. The level of c-*myc* RNA then falls gradually during the rest of the experiment, being reduced two-fold below control by day 5 (Fig. 6). These data are summarized graphically in Figure 7.

Detailed studies by others [19–22] have demonstrated that the steroid dexamethasone suppresses the DMSO-induced terminal differentiation of cells in this murine erythroleukemia system. These studies also suggest that this suppression is a posttranscriptional phenomenon. Essential messenger RNA species for terminal differentiation appear to be made in inducer-treated cells but not translated into protein in the presence of dexamethasone.

We examined the expression of the alpha-globin, *myc*, and *myb* messages in our system in the presence of DMSO and 4 μM dexamethasone. The accumulation of alpha-globin mRNA was essentially identical in the induced and "induced plus dexamethasone" treated cultures and not more than three-fold different at 120 hr (Fig. 8). Despite this, only the cells cultured with inducer alone progressed to terminal differentiation and hemoglobin synthesis. Thus, our data are consistent with a post-transcriptional event suppressing evidence of differentiation [19–22]. The messages for the oncogenes *myb* and *myc* showed an identical early shut off at 4 hr in the induced and induced-plus-dexamethasone cultures. However, the cells cultured in the presence of dexamethasone showed an approximately two-fold more rapid rebound accumulation of *myb* and did not show the terminal (hr 120) drop-off seen in the cells cultured with inducer alone.

We wished to determine whether the decrease in message seen from 4 to 14 hr after induction was specific for *myb* and *myc* messages. The baseline alpha-globin message is unaffected at this time, and the beta-actin message shows no decrease over this period relative to control (Fig. 9). Looking at steady-state transcript levels can be

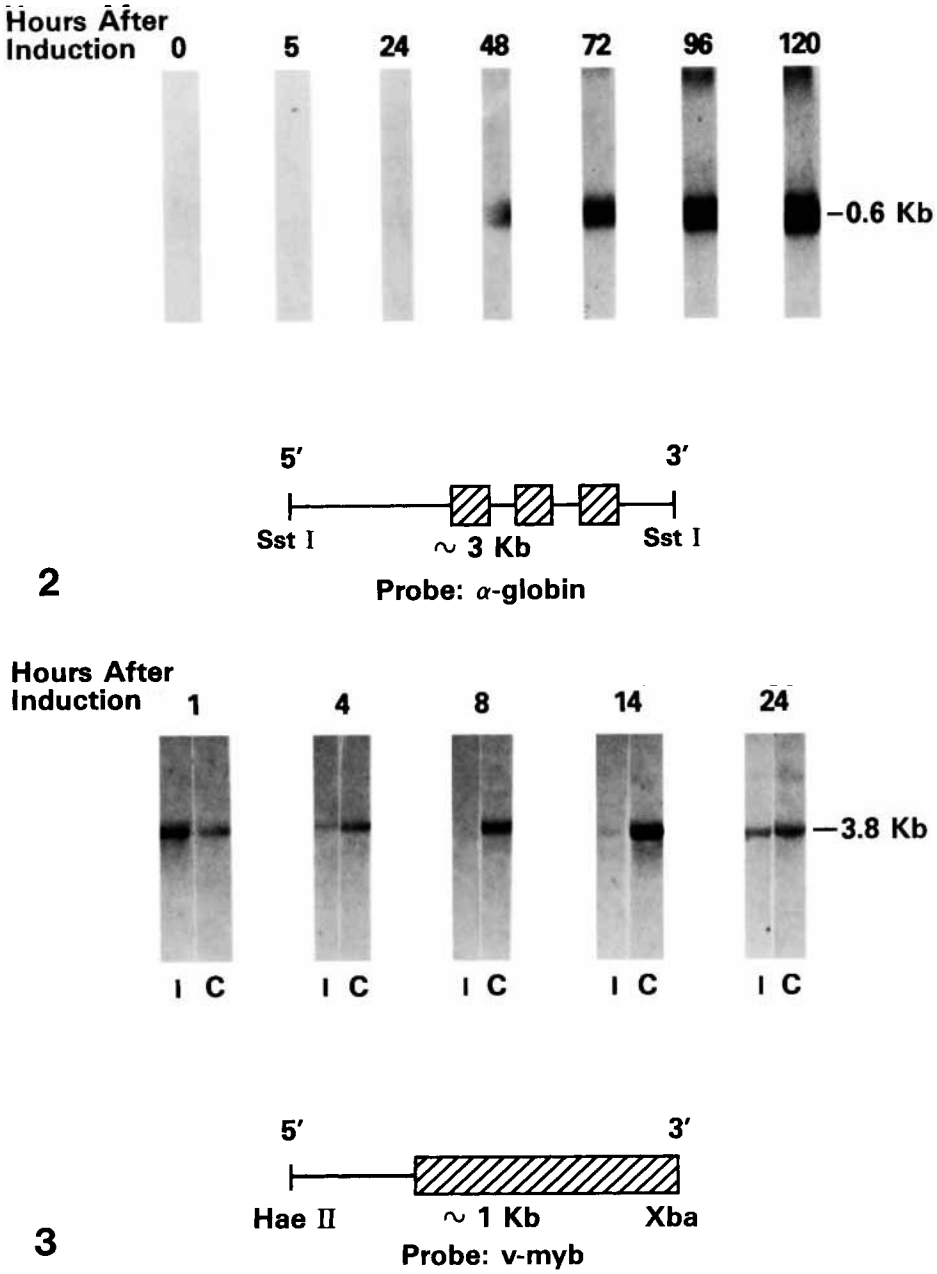
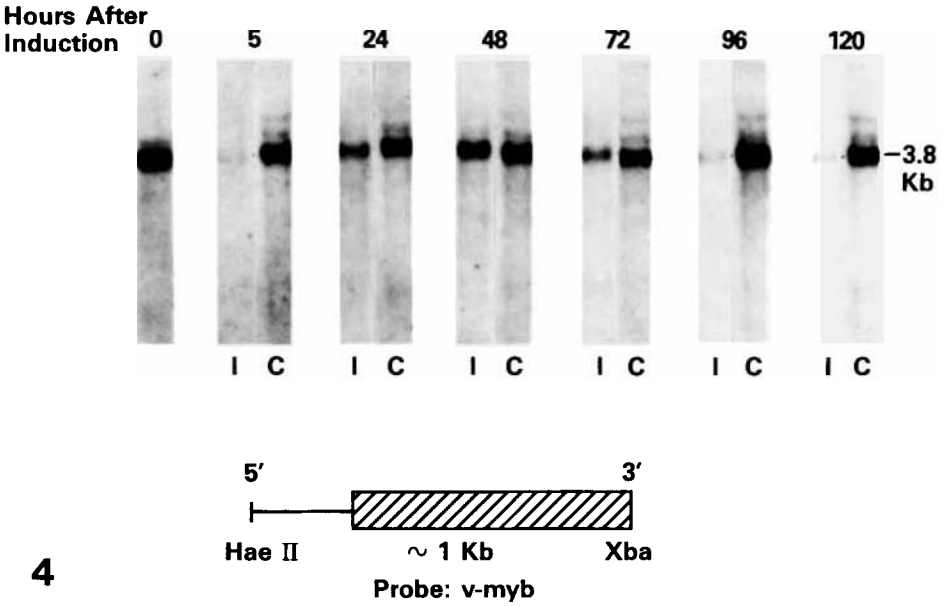


Fig. 2. Alpha-globin transcripts in a murine erythroleukemia cell line induced to differentiate with DMSO at t = 0. The mouse erythroleukemia cell line F4-6 was grown in Joklik modified medium, 20% fetal calf serum, to 5 × 10^5 cells/ml then cut back to 10^5 cells/ml and treated with 1.5% DMSO and allowed to continue to grow. Cells were harvested at 0, 5, 24, 48, 72, 96, and 120 hr, and RNA was prepared by the guanidine thicocyanate method [13]. Ten micrograms of total RNA was loaded on a 1% agarose formaldehyde gel and blotted. The blots were hybridized to the nick-translated probe indicated, washed in 15 mM NaCl/1.5 mM sodium citrate, pH 7.0, containing 0.1% $NaDodSO_4$ and visualized by autoradiography.

Fig. 3. c-*myb* transcripts in a murine erythroleukemia cell line induced to differentiate with DMSO at t = 0. Cells were grown and induced as in Figure 2 and harvested at 1, 4, 8, 14, and 24 hr following induction. Ten micrograms of total RNA was run per lane. The blot was hybridized to the v-*myb* probe that is diagrammed. Note the sharp decrease in a c-*myb* transcript occurring between hours 1 and 14. I, induced; C, control.



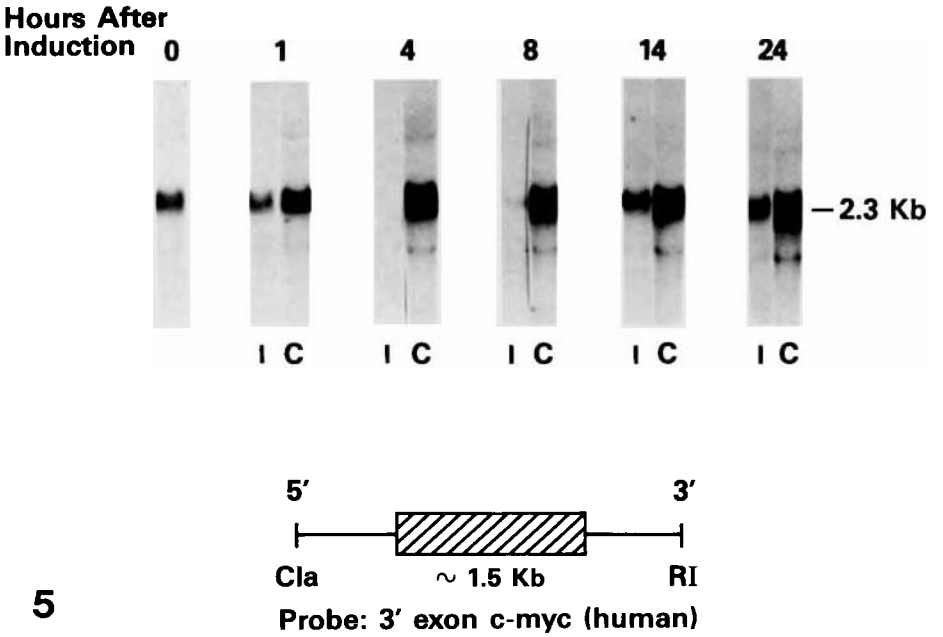
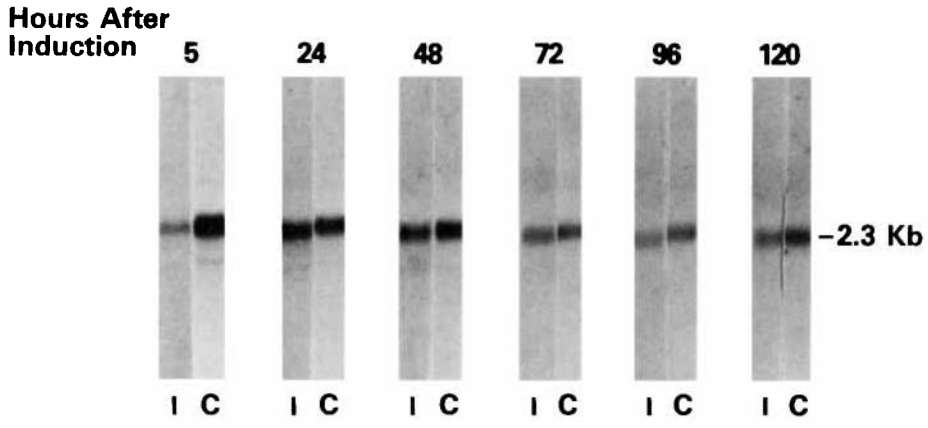


Fig. 4. c-*myc* transcripts in a murine erythroleukemia cell line induced to differentiate with DMSO at t = 0. Cells were grown, induced, and harvested and RNA was prepared as in Figure 2. Note the decrease in c-*myb* transcript in the induced culture at hr 5 and 120. I, induced; C, control.

Fig. 5. c-*myc* transcripts in a murine erythroleukemia cell line induced to differentiate with DMSO at t = 0. Cells were grown and induced, and RNA was prepared as in Figure 2. The blot was hybridized to the c-*myc* probe as described. Note the sharp decrease in c-*myc* transcripts in the induced culture of 4 hr following induction. I, induced; C, control.



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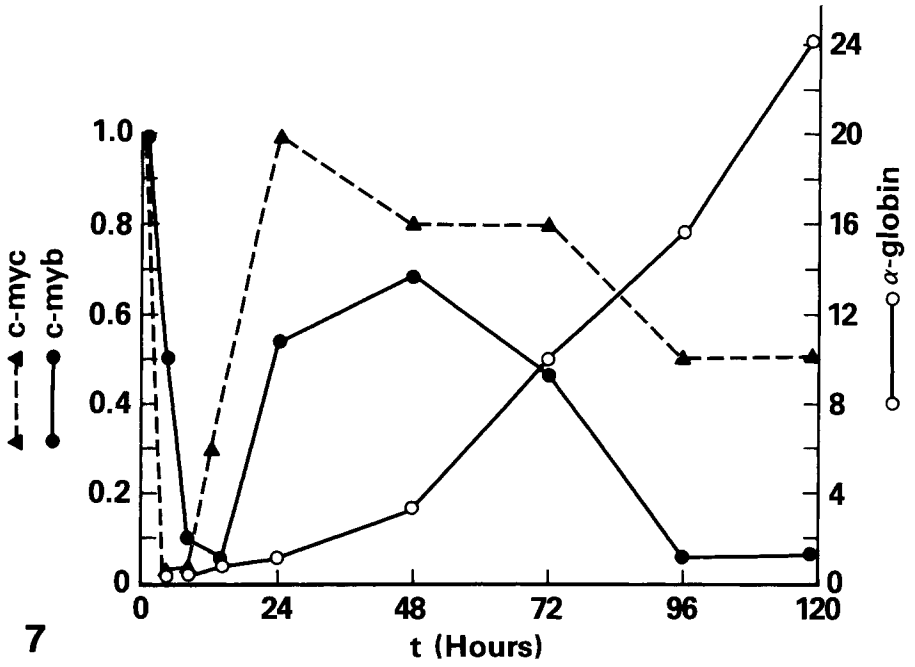

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Fig. 6. c-*myc* transcripts in a murine erythroleukemia cell line induced to differentiate with DMSO at t = 0. Cells were grown and induced, and RNA was prepared as in Figure 2. I, induced; C, control.

Fig. 7. Summary of expression of c-*myc*, c-*myc*, and alpha-globin transcripts in murine erythroleukemia cell line F4-6 induced to differentiate with DMSO at t = 0. Densitometric scans were performed on the bands (Fig. 2-6) for each time point. The points on the graph represent the ratio of the density of the band in the induced culture divided by the density of the band in the control culture at each time point.

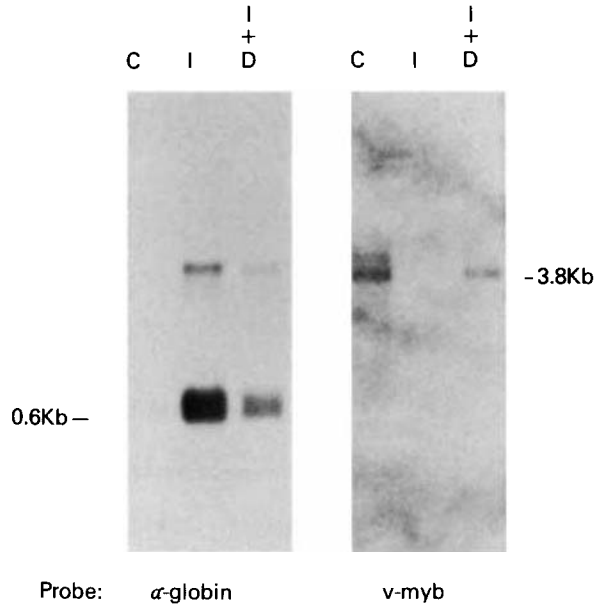

Fig. 8. The effect of dexamethasone on transcript level of alpha globin and c-*myb* in DMSO induced a murine erythroleukemia cell line. Four micromolar dexamethasone was added to an aliquot of the DMSO-induced cells at t = 0. The cells were grown and harvested at t = 120 as described in Figure 2. C, control; I, induced, I + D, induced plus dexamethasone.

deceiving because of differences in the half-lives of the various messages being analyzed. We therefore assessed the level of poly A mRNA transcription in our induced and control cultures during the early phase when the oncogene messages showed such a substantial decrease in the induced cells (hr 3.5–4.5), and then later when the level of transcript was almost that of the control (hr 23.5–24.5). ^{3}H-5 uridine (10μCi/ml) was added for the 1-hr pulses to the induced and control cultures. One hundred μg of labeled total RNA was supplemented with 4,900 μg of total unlabeled RNA. Poly A mRNA was obtained by three passages of each sample through an oligo dT column as previously described [23]. The RNA was followed by optical density and ethidium-bromide-staining characteristics of the fractions. The counts per minute per microgram of poly A RNA are shown in Table I. The 4 hr induced culture poly A RNA synthesis is at least 59% of the control culture. Given that amount of *myb* and *myc* transcript at 4 hr is less than 5% of the control culture, these data suggest either that DMSO selectively inhibits the RNA polymerase II transcription of certain genes, or that all transcription is shut off equally but that this transiently leads to the disappearance of more short-lived messages. In either case, the effect of this action is a more severe drop in the level of *myb* and *myc* messenger RNA than in messenger RNA with longer half-lives. Despite the continued presence of DMSO in the culture, transcripts of both c-*myb* and c-*myc* have returned practically to baseline level within 24–48 hr.

DISCUSSION

The c-*myb* and *c-myc* oncogenes when inappropriately expressed are capable of contributing to cellular malignant transformation. The normal function(s) of each of

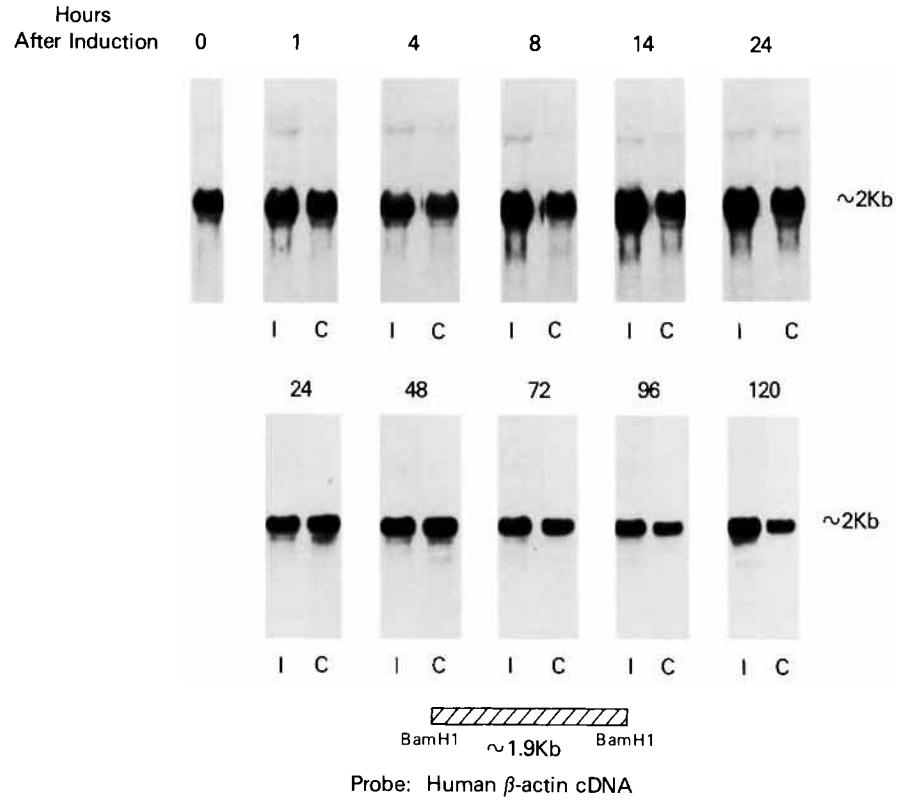

Fig. 9. Beta-actin transcripts in a murine erythroleukemia cell line induced to differentiate with DMSO at t = 0. Cells were grown, induced, harvested, and RNA was prepared as in Figure 2. The blot was prepared as in Figure 2 and hybridized to the human beta-actin probe described. In contrast to alpha-globin, c-*myb*, or c-*myc*, minimal if any changes in beta-actin transcript level is seen during the course of the experiment. I, induced; C, control.

TABLE I. mRNA Synthesis During DMSO Induction

Pulse (hr)	Sample	Oligo dT × 3 μg poly A RNA recovered	^{3}H cpm/μg	Average induced/control (range from two experiments)
3.5–4.5	Control (C)	10.87	1,727	0.59
	Induced (I)	11.66	1,022	(0.56–0.64)
23.5–24.4	Control (C)	14.1	805	1.43
	Induced (I)	5.6	1,155	(1.39–1.46)

these genes remains unclear. Transcripts from these genes are often seen in the presence of cellular proliferation such as that observed by us in the spleen cells of mice with Friend-induced erythroleukemias. But if the net effect of expression of these genes is to stimulate cellular proliferation, it must be a very tightly regulated stimulus as demonstrated by our study of the phenylhydrazine-treated mice. The spleens of these mice were as large on a weight-for-weight basis as their leukemic counterparts, and they had reached that size in a mere 5 days. The percentage of

mononuclear cells of erythroid lineage was approximately the same, yet as noted there was a marked discrepancy in oncogene message between these two spleen types.

This observation leads us to consider the possibility that *myc* and *myb* are playing a more fundamental role in determining the differentiated state of erythroid cells. Our data from the studies of the inducible murine erythroleukemic cell lines is consistent with this idea, particularly with respect to c-*myb* whose expression seems to be inversely correlated with the extent of globin production. Unexpectedly, we found a sharp drop in both *myc* and *myb* messages following addition of the inducing agent and prior to any evidence of cellular differentiation. Shortly after the decrease in *myb* and *myc* mRNA, morphologic evidence of differentiation and globin production commences. We cannot say whether DMSO momentarily inhibits all mRNA transcription. At the time of the trough levels of *myb* and *myc* message there is at least 60% the control amount of ^{3}H-uridine incorporation into poly A message. The net effect of any transient inhibition of transcription would be to lower the level of short half-lived messages more than long half-lived messages. Perhaps it will be found that many genes serving important functions for limited times during cellular differentiation will encode mRNAs with relatively short half-lives, making their levels more subject to changes in environmental stimuli.

On the basis of the observations reported in this study, we are currently examining whether the expression of *myb* and *myc* can block differentiation. Our approach is to try and reintroduce these oncogenes into the inducible murine erythroleukemic cells in such a way that we will be able to control the level of their transcription even when an inducing agent is added to the system.

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