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Expression of the c-*myb* and c-*myc* Genes Is Regulated Independently in Differentiating Mouse Erythroleukemia Cells by Common Processes of Premature Transcription Arrest and Increased mRNA Turnover

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The mechanisms that modulate c-*myb* mRNA levels in mouse erythroleukemia cells induced toward erythroid differentiation were compared with those that act on c-*myc*. Both genes exhibited regulation at the levels of premature transcription arrest and RNA turnover. However, these common processes allowed temporally distinct control of gene expression.

The chicken and mouse c-*myb* genes have been identified as targets for retrovirus-mediated insertion or transduction events leading to the induction of hemopoietic malignancies primarily of myeloid origin (3, 16, 21). Transcription of c-*myb* is found predominantly in hemopoietic cells of all lineages (5, 13, 20, 25, 26) and is subject to regulation during cell differentiation. Hence, induction of monocyte differentiation in both mouse WEHI-3B (12) and human ML-1 cells (7) results in a marked decrease in c-*myb* mRNA levels with kinetics that precede suppression of cell proliferation. In some contrast, Friend cells that are induced to differentiate along the erythroid pathway demonstrate a biphasic mode of c-*myb* mRNA abundance regulation, and nadirs in concentration occur during the early period of commitment and following the loss of cell proliferation (14, 19).

It has been demonstrated recently that the control of constitutive c-*myb* mRNA abundance in mouse cells is effected primarily at the level of transcription arrest (1, 24). Thus, the 20-fold difference in c-*myb* mRNA levels observed between myelomas and more immature hemopoietic cells could be accounted for largely by the propensity with which transcription arrested prematurely near the middle of the first gene intron. It was of interest, then, to determine whether this mechanism could account also for the modulation of c-*myb* mRNA levels in differentiating Friend cells, particularly in light of previous observations that c-*myc* mRNA levels are regulated in part by transcription arrest in these cells (17) and in other differentiating hemopoietic cells (2, 9, 10, 22).

The effects of dimethyl sulfoxide (DMSO) induction of Friend cell differentiation on the steady-state levels of c-*myb* mRNA were reexamined. The Friend cell line F4-12B2 (23) was induced at an initial density of 10^5 cells per ml with 1.5% DMSO in Dulbecco modified Eagle medium supplemented with 10% fetal calf serum. At various times from 0 to 96 h after induction, cytoplasmic RNAs were prepared from these cells and resolved on denaturing agarose gels as described previously (25). After RNAs were transferred to nylon membranes by blotting (25), they were hybridized simultaneously with nick-translated c-*myb* and c-*myc* probes and subsequently with a β-actin probe. Autoradiography (Fig. 1) revealed a biphasic modulation of c-*myb* and c-*myc* mRNA levels, as described previously (14, 15, 19). Levels of c-*myb* declined eightfold by 4 h postinduction, increased subsequently as that by 16 h levels were twofold lower than those in untreated cells, and declined again to a level that was sixfold less than those of the controls at 96 h. The regulation of c-*myc* mRNA levels paralleled that of c-*myb* to some extent (Fig. 1). However, the lowest levels were obtained by 2 h postinduction, and this was followed by a gradual increase in expression which peaked at about 24 h. Moreover, c-*myc* levels did not decline by 96 h postinduction to as great an extent as that observed for c-*myb*.

To determine whether transcription arrest was implicated in this regulation of c-*myb* mRNA levels, nuclear run-on experiments were performed as described previously (24) by using nuclei isolated from F4-12B2 cells treated with 1.5% DMSO for 0, 1, 4, 16, and 96 h. Equal amounts of the labeled products of run-on transcription were then hybridized with

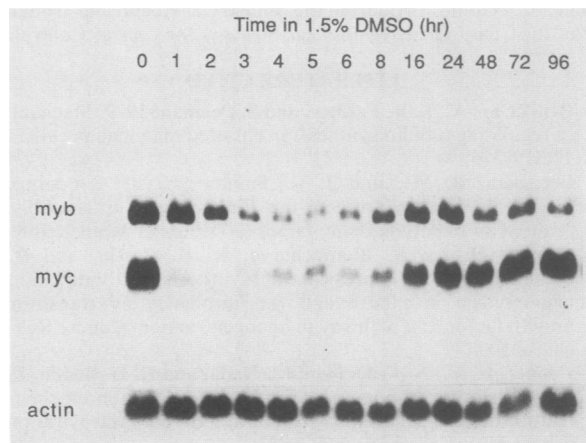


FIG. 1. Northern blots of DMSO-induced Friend cell RNAs. Amounts of 12 μg of cytoplasmic RNA extracted from F4-12B2 cells treated with 1.5% DMSO for 0, 1, 2, 3, 4, 5, 6, 8, 16, 24, 48, 72, and 96 h were resolved by electrophoresis on 1% agarose gels containing 2.2 M formaldehyde (25). After the RNAs were blotted onto a nylon membrane, they were hybridized with equivalent amounts of nick-translated mouse c-*myb* and c-*myc* probes and subsequently with a mouse β-actin cDNA probe. After 96 h of DMSO treatments, 55% of the cells stained positive with benzidine (11), indicating the accumulation of hemoglobin.

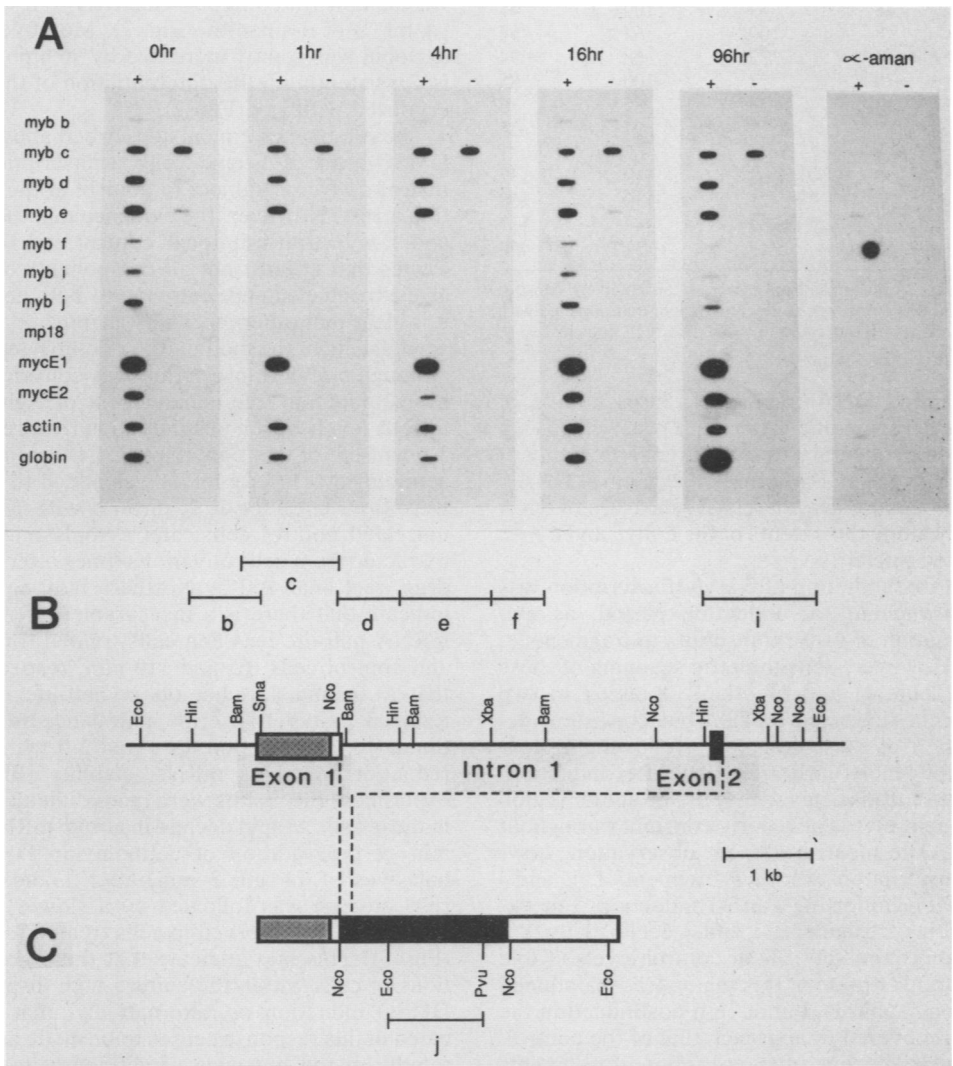


FIG. 2. Run-on analyses of DMSO-induced Friend cells. (A) Equal amounts (10^7 cpm ml^{-1}) of ^{32}P-labeled run-on products extracted from nuclei of F4-12B2 cells treated with 1.5% DMSO for 0, 1, 4, 16, and 96 h and from untreated F4-12B2 nuclei incubated in the presence of 2 μg of α-amanitin per ml were hybridized to slot blots containing the indicated DNAs. The map locations of the various *myb* fragments, cloned in mp18 or mp19 M13 vectors, are indicated in panels B and C. A total of 5 μg of each M13 DNA was applied to each lane. Lanes: +, M13 clones containing inserts complementary to sense transcripts of c-*myb* and c-*myc*; −, fragments of the opposite sense. Only M13 clones complementary to sense transcripts of *myb* j, *myc* E1, and *myc* E2 were applied. The mouse c-*myc* M13 clones contained a 0.4-kilobase (kb) *Bam*HI-*Xho*I fragment that was representative of the first exon (*myc* E1) and a 1.1-kilobase *Xba*I-*Sac*I fragment that was representative of the second exon (*myc* E2). Controls consisted of 5 μg of the mp18 vector, 2 μg of a plasmid containing mouse β-actin cDNA, and 5 μg of a plasmid containing mouse α-globin cDNA. (B) Restriction map of the 7.8-kilobase *Eco*RI fragment containing the first and second exons of c-*myb* for the enzymes *Hin*dIII, *Bam*HI, *Sma*I, *Nco*I, and *Xba*I. The hatched area of the first exon denotes the variable-sized noncoding sequence, and the open area denotes the nonvariable noncoding region. Coding regions are indicated by a closed box. (C) Restriction map of the c-*myb* cDNA for enzymes *Nco*I, *Eco*RI, and *Pvu*II. Restriction fragments cloned in M13 vectors are indicated above and below these maps.

TABLE 1. Densitometry of run-on hybridization data

Fragment	Hybridization (%) at the following times (h) of DMSO induction[a]:			
	1	4	16	96
myb b	48	59	100	45
myb c	78	100	62	92
myb d	64	72	68	94
myb e	70	77	100	85
myb f	13	23	85	36
myb i	7	13	97	25
myb j	4	7	68	31
myc E1	90	102	119	132
myc E2	5	25	130	168
Actin	94	82	144	137
Globin	62	45	131	450

[a] The slot blots of Fig. 2 (plus strand only) were quantitated by densitometry, and hybridization was expressed as a percentage of that obtained with the corresponding fragment with untreated (0 h) F4-12B2 cell nuclei.

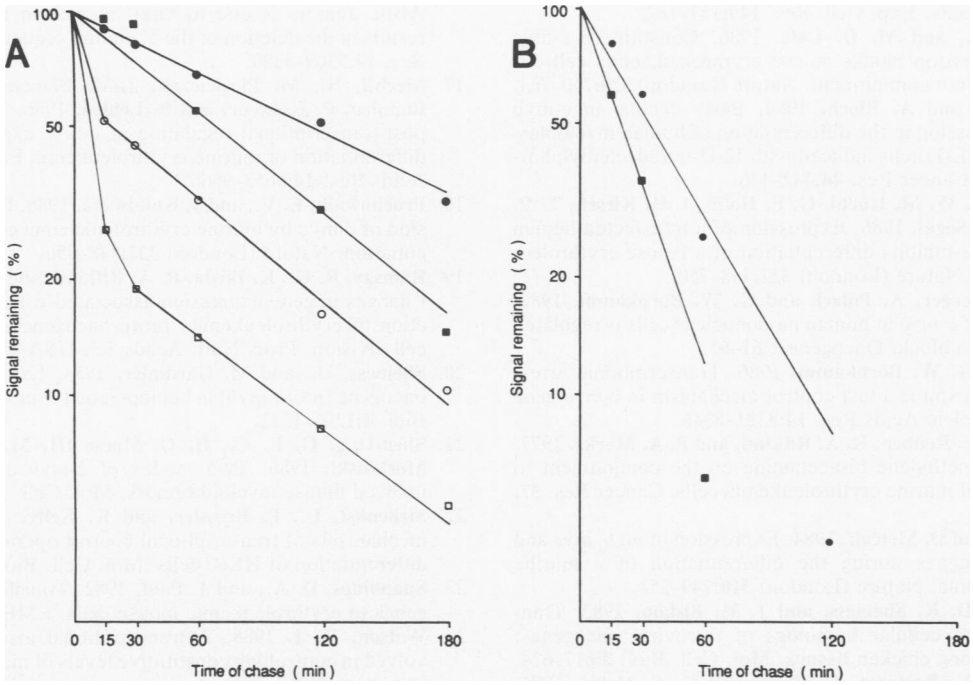
M13 clones containing DNA fragments representative of either strand of the 5′ region of c-*myb* (Fig. 2), as well as with DNA fragments complementary to sense transcripts only of the first and second exons of c-*myc* and other control DNAs. In principle, this procedure gives a relative measure of RNA polymerase density along the extents of the c-*myb* and c-*myc* genes in the isolated nuclei.

It was observed that initiation of c-*myb* transcription was fairly constant throughout the induction period, as evidenced by hybridization of sense transcripts to fragments c, d, and e (Fig. 2). However, densitometric scanning of these autoradiographs (Table 1) and of others obtained in two separate experiments suggested a slight but consistent decline in transcription of these fragments following DMSO treatment, most noticeably early (i.e., 1 h) after induction. Initiation of antisense transcripts within the promoter region (contained in fragment c) was also fairly constant throughout induction (Fig. 2). The most prominent observation, however, was that transcription of c-*myb* fragments f, i, and j fluctuated appreciably following DMSO induction. For example, transcription of fragments i and j declined by 1 h postinduction to approximately 5% of control levels (Table 1). A low level of transcription of this region was maintained at 4 h postinduction (Table 1), but at 16 h postinduction the transcription rate recovered to approach that of the control. However, a secondary decline of transcription of fragments i and j (to 25 to 31% of control levels) was observed 96 h postinduction. These data indicate that a block to enlongation moderates c-*myb* transcription in DMSO-induced Friend cells and locate the arrest site within fragment f. This region has previously been implicated as the site of transcription arrest that acts in the control of constitutive c-*myb* expression (24). It is apparent, then, that the observed biphasic modulation of c-*myb* mRNA abundance (Fig. 1) is consequent to changes in the transcription rate effected at the level of elongation.

Transcription rates along the c-*myc* gene roughly paralleled those observed with c-*myb*. Hence, transcription of the c-*myc* first exon was fairly constant throughout induction, although a slight but significant increase (to 132% of control levels) was found at 96 h postinduction (Table 1). Most apparent, however, was the sharp decline in transcription of the c-*myc* second exon to 5% of control levels at 1 h postinduction. In contrast to c-*myb*, transcription of c-*myc* regions downstream of the arrest site showed a more rapid and substantial recovery following DMSO treatment. Hence, at 4 h postinduction transcription of the c-*myc* second exon was already back to 25% of control, and by 16 and 96 h it exceeded that found in control nuclei (130 and 168% of control rates, respectively). That the transcription observed in this assay was directed by RNA polymerase II was demonstrated by its sensitivity to low concentrations (2 $\mu g\ ml^{-1}$) of α-amanitin (Fig. 2). Moreover, transcription of α-globin was greatly increased by 96 h postinduction, which is consistent with the differentiation of these cells along the erythroid pathway (Fig. 2).

The run-on experiments described above indicated that in DMSO-induced Friend cells, transcription of c-*myb*, like that of c-*myc*, is subject to control at the level of transcription arrest. However, the evidence that the kinetics of c-*myb* and c-*myc* transcriptional control are somewhat different argues that at least not all components of the transcription arrest mechanism are common to both genes. Furthermore, it is clear that other mechanisms must be invoked to explain some facets of the modulation of c-*myb* mRNA levels. Thus, although c-*myb* transcription was considerably ablated by 1 h postinduction, the achievement of eightfold lower c-*myb* mRNA levels by 4 h postinduction requires that the reported 3-h half-life of this transcript (1, 24) is reduced. To test this, actinomycin D (5 $\mu g\ ml^{-1}$) was added to F4-12B2 cells that were pretreated for 1, 2, or 96 h with 1.5% DMSO and to untreated control cells; and cytoplasmic RNAs that were extracted from cells at various times after the addition of the drug were analyzed by Northern blotting. The data (Fig. 3) indicate that there is a measurable difference in the c-*myb* mRNA half-life between cells treated for 96 h with DMSO and control cells (65 and 110 min, respectively), indicating that a sustained reduction in half-life contributes to the reduced c-*myb* transcript abundance following induction. Similarly, cells treated for 1 and 2 h with DMSO showed a reduction in c-*myb* mRNA stability (Fig. 3), but in this experiment the results were more difficult to interpret. Thus, in these cells a rapid decline in c-*myb* mRNA levels within 15 min of the addition of actinomycin D (corresponding to half-lives of 14 and 8 min, after 1 and 2 h of induction, respectively) was followed by a slower turnover at a rate similar to that observed in cells treated for 96 h with DMSO (Fig. 3). This may indicate that there are different populations of cells within the culture with disparate responses to DMSO induction or, alternatively, that the RNase implicated in this response ceases to be made and itself turns over rapidly in the presence of actinomycin D. It can be concluded that the addition of DMSO reduces the c-*myb* mRNA half-life, that this contributes to the rapid decline in mRNA levels, and that an effect on mRNA stability is retained for 96 h into DMSO induction. A rapid effect of DMSO induction on the c-*myc* half-life has been reported previously (17), and was also apparent in this study (Fig. 3). Moreover, it was observed that a reduction in the c-*myc* half-life was retained after 96 h of DMSO treatment (20 min in comparison with 33 min in control cells).

In summary, it was found that control of c-*myb* gene expression at the level of arrest of transcript elongation, like that for c-*myc* (17), plays a major role in regulating the levels of the mRNA during DMSO-mediated induction of Friend cell differentiation. The kinetics of c-*myb* gene regulation at this level, however, differ from those found with c-*myc*, suggesting that this process may be mechanistically distinct for each gene. Fluxes in the mRNA half-life also contribute to the attainment of a rapid decline in c-*myb* transcript levels. That this observed down-regulation of c-*myb* mRNA levels is a necessary prerequisite for erythroid differentiation



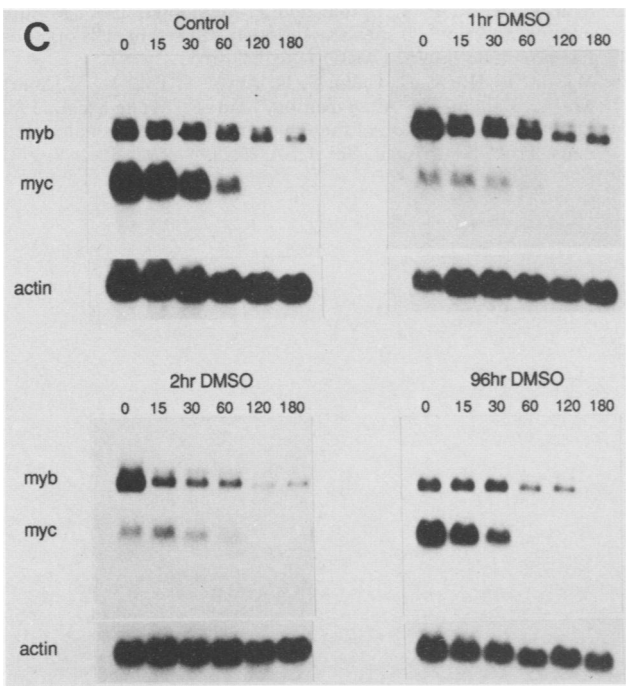


FIG. 3. c-*myb* mRNA half-lives. Actinomycin D (5 μg/ml) was added to F4-12B2 cells that were either untreated or treated with 1.5% DMSO for 1, 2, or 96 h; and cytoplasmic RNAs were extracted at 0, 15, 30, 60, 120, and 180 min following the addition of the drug. Approximately 12 μg of each RNA sample was analyzed (C) by Northern blotting, as described in the legend to Fig. 1. RNA turnover was plotted (A and B) as a function of the signal remaining (determined by densitometry and adjusted with respect to the actin signal) with the time of chase in actinomycin D. (A) Half-life of c-*myb* mRNA in control cells (●) and cells treated for 1 h (○), 2 h (□), and 96 h (■) with DMSO. (B) Half-life of c-*myc* mRNA in control cells (●) and cells treated for 96 h with DMSO (■).

has been indicated from studies in which constitutively high levels of this gene product (achieved by transfection with an exogenous c-*myb* gene) were found to block DMSO-induced differentiation of Friend cells (4; J. McMahon, K. Howe, and R. J. Watson, manuscript in preparation). In similar experiments (6, 8, 18), c-*myc* has also been implicated in erythroid differentiation. It is apparent, then, that both common and unique components of the transcription arrest mechanisms that control the expression of c-*myb* and c-*myc* may be of fundamental importance in governing hemopoietic cell differentiation. It is now of interest to study the *cis*- and *trans*-acting elements of this regulatory mechanism and to determine whether this process is implicated in growth factor-induced differentiation of hemopoietic cells.

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