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Down-regulation of c-*myb* gene expression is a prerequisite for erythropoietin-induced erythroid differentiation

(nuclear oncogene/transcription/*fos*/*myc*/erythroleukemia)

KAZUO TODOKORO*†, ROGER J. WATSON‡, HIROMI HIGO*, HIROSHI AMANUMA*, SATOMI KURAMOCHI*, HIROMI YANAGISAWA*, AND YOJI IKAWA*

*Tsukuba Life Science Center, The Institute of Physical and Chemical Research, 3-1 Kohyadai, Tsukuba, Ibaraki 305, Japan; and ‡Imperial Cancer Research Fund Laboratories, Saint Bartholomew's Hospital, Dominion House, Bartholomew Close, London EC1A 7BE, United Kingdom

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ABSTRACT **The role of nuclear protooncogenes during erythroid cell differentiation was examined by transfecting exogenous c-*fos* and c-*myb* genes into mouse erythroleukemia cells, which can be induced to differentiate either with erythropoietin (Epo) or dimethyl sulfoxide. Expression of exogenous c-*myb* or c-*fos* oncogene completely inhibited Epo-induced erythroid differentiation but only partially inhibited dimethyl sulfoxide-induced differentiation. Normally Epo-induced differentiation leads to a drastic decline of c-*myb* mRNA levels and an increase of c-*myc* transcripts in the early stage of differentiation. Cells expressing exogenous c-*fos* gene, however, maintained high levels of c-*myb* mRNA after Epo treatment. This high level of c-*myb* transcripts was found to be due to block of transcription shutoff (or transcriptional activation) rather than to mRNA stabilization. It is concluded that the down-regulation of endogenous c-*myb* gene expression is a prerequisite for commitment of Epo-induced erythroid differentiation and that expression of c-*myb* gene may be indirectly regulated by c-*fos* gene product. We also concluded that early down-regulation of c-*myc* gene expression is not essential for erythroid differentiation and that gene regulation of chemically induced erythroid differentiation may differ from that of Epo-induced differentiation.**

Hemopoiesis is regulated by a set of specific glycoprotein factors that affect the program for proliferation and differentiation of progenitor cells (1). The interaction of a polypeptide hormone with specific cell-surface receptors on progenitor cells may induce a signal (a cascade of biochemical events) that is transmitted from the cell membrane to the nucleus. This signal may involve activation of a set of specific regulatory genes that determine ordered expression of specific genes during the transition of cells to a proliferating or differentiating state (2). The protooncogenes c-*myb*, c-*fos*, c-*myc*, c-*jun*, and c-*ski* and the p53 gene, which encode nuclear proteins, have been suggested to play regulatory roles in specific gene expression related to DNA replication and cell differentiation (3–7). Thus, these nuclear protooncogenes are specifically modulated in their expression during cell growth and differentiation. For instance, the expression of c-*myc*, c-*fos*, and c-*jun* genes is rapidly and transiently induced in response to mitogens and growth factors in various cell types (5, 8–10). In contrast, expression of c-*myc* and c-*myb* genes decreases during differentiation of myeloid precursors to macrophages concurrent with an increase in c-*fos* gene expression (11–13). Furthermore, erythroid differentiation induced by chemicals causes rapid and transient c-*fos* gene expression, followed by a biphasic decline of c-*myb* and c-*myc* transcripts (14–16). It has not yet been fully established whether modulated expressions of nuclear protooncogenes represent obligate events in regulation of hemopoietic cell differentiation. This possibility has been investigated by using established hematopoietic cell lines capable of undergoing differentiation when exposed to various inducers as models. For example, it has been reported that maintenance of high levels of c-*myc* expression inhibits chemically induced differentiation of erythroleukemia cells (16–19). We have described the characterization of mouse erythroleukemia SKT6 cells (20), which can be induced to differentiate with either natural inducer erythropoietin (Epo) (1, 21) or the chemical inducer dimethyl sulfoxide (DMSO).

To investigate the molecular mechanism of Epo-induced erythroid differentiation and to gain insight into the possible roles of nuclear protooncogenes during Epo-induced differentiation, we have investigated the regulation of their expression in SKT6 cells in response to this hormone. Moreover, by transfecting exogenous nuclear oncogenes c-*fos* and c-*myb* into SKT6 cells, we have studied the implications of dysregulation of the normal controls of their gene expression on erythroid differentiation.

MATERIALS AND METHODS

Cell Culture and DNA Transfection. Mouse erythroleukemia SKT6 cells have been described (20). Where appropriate, recombinant human Epo (>70,000 units/mg; Kirin Brewery, Tokyo) and DMSO with or without $CdCl_2$ (5 μM) were added to cell culture at final concentrations of 0.5 unit/ml and 1.5%, respectively, and hemoglobin-positive cells were stained by 0.2% benzidine (20) after 4 days of induction. Transfections into SKT6 cells were performed by electroporation (22) in 0.8 ml of Dulbecco's phosphate-buffered saline containing 1–2 × 10^7 cells per ml, 11 mM glucose, and 20 μg of DNA. Cells were pulsed at 500 V and 25 μF twice on ice (Bio-Rad Gene Pulser). Stable transformants resistant to antibiotic G418 (1 mg/ml) were cloned by limiting dilution.

DNA Construction and Probe DNAs. To construct pMTfosSVneo, the *Bam*HI site of pSV2neo (23) was filled in by the Klenow fragment, and the DNA was digested with *Eco*RI. Similarly, the *Hin*dIII–*Bam*HI DNA fragment containing human metallothionein IIA promoter region (24), of which the *Hin*dIII site was blunt-ended, was prepared. The *Nae* I site at position +41 in pc-fos(mouse)3 DNA (25), which contains whole mouse c-*fos* genomic DNA, was replaced by a *Bam*HI site, and an *Eco*RI site was added at the *Bam*HI site at the 3′ end of the c-*fos* gene. This *Bam*HI–*Eco*RI fragment was ligated with the above two DNA fragments to generate pMTfosSVneo. To construct pMTmybSVneo, the plasmid pQ24/10.4, which contains the entire mouse c-*myb* cDNA

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Abbreviations: DMSO, dimethyl sulfoxide; CAT, chloramphenicol acetyltransferase; Epo, erythropoietin.

†To whom reprint requests should be addressed.

coding sequence within a *Bam*HI linker-adapted *Sma* I site at position 1 to the *Sac* I site at position 2449 (26), was digested with *Bgl* II, filled in by the Klenow fragment, and digested with *Bam*HI. The *Sal* I site of pMTfosSVneo, which is located between the stop codon and poly(A) signal, was filled in and partially digested with *Bam*HI. The resulting *Bam*HI–*Sal* I and *Bam*HI–*Bgl* II fragments were ligated to generate pMTmybSVneo. Probe DNAs used for hybridization were *fos*, 4.8 kilobases (kb) of the *Hin*dIII–*Bam*HI fragment of pc-fos(mouse)3 (25); *myb*, 2.1 kb of the *Bam*HI–*Bgl* II fragment of pQ24/10.4 (26); *myc*, 4.8 kb of the *Xba* I–*Bam*HI fragment of pSVc-myc-1 (27); *jun*, pJUN plasmid DNA, which contains 0.95 kb of the *Bam*HI–*Eco*RI fragment of v-*jun* DNA (28); *ski*, pCCL*ski*5 plasmid DNA (29); and p53, pLTRp53cG plasmid DNA (30).

RNA Analysis. Total cellular RNA was isolated as described (31). Ten micrograms of poly(A) RNA was fractionated on 1% agarose gels containing 2.2 M formaldehyde (32). Hybridizations with nick-translated probes were carried out for 36 hr at 40°C, and the filters were washed as described (14). When the half-life of c-*myb* mRNA was measured, actinomycin D was added to the cell culture medium to a final concentration of 5 μg/ml and RNA was extracted at the specified time.

Nuclear Run-On Transcription Assay. Preinitiated transcripts in 2×10^7 nuclei were elongated, labeled with [^{32}P]UTP *in vitro*, and extracted as described (33). The labeled run-on transcripts (10^7 cpm/ml) were hybridized to 5 μg of oncogene DNA fragments cloned in mp18 and mp19 M13 vectors, to 5 μg of mp18 vector DNA, and to 2 μg of β-actin DNA on a slot blot. Hybridizations and washing were carried out as described (33). Autoradiographs were exposed for 3 days with intensifying screens.

RESULTS

Relative mRNA Levels of Nuclear Protooncogene During Epo- or DMSO-Induced Erythroid Differentiation. The relative transcriptional levels of nuclear protooncogene following Epo- or DMSO-induced SKT6 cell differentiation were analyzed by RNA blot hybridization. Fig. 1*A* shows the changes in relative mRNA levels of c-*myb*, c-*fos*, c-*myc*, c-*jun*, and c-*ski* protooncogenes and the p53 gene following Epo induction. c-*myb* mRNA levels rapidly decreased: by 80% in 1 hr, and declining further to much lower levels by 24 hr. In contrast, the levels of c-*myc* mRNA increased about 3-fold in 30 min and remained high during the initial 24 hr. No c-*fos*, c-*jun*, or c-*ski* mRNA was detected in this RNA blot analysis. High levels of p53 transcripts were observed, but there was no detectable change in concentration.

DMSO-induced SKT6 cells, on the contrary, showed a rapid and transient increase in c-*fos* gene expression, associated with a biphasic deline of c-*myb* and c-*myc* mRNA levels (Fig. 1*B*). These profiles of mRNA levels are similar to those of other chemically induced mouse erythroleukemia cells as reported previously (14, 15). No c-*jun* or c-*ski* mRNA was detected, and high and constant levels of p53 mRNA were observed. These data suggest that the molecular mechanism and/or pathway of chemically induced erythroid differentiation in the nucleus may differ from those of Epo-induced differentiation. In particular, an increase in c-*myc* expression in Epo-induced SKT6 cells indicates that early reduction in c-*myc* levels is not an obligate event in erythroid differentiation.

Effects of Transfected Oncogenes on Erythroid Differentiation. To study the potential relationship between c-*myb* and c-*fos* expression and Epo (or DMSO)-induced erythroid differentiation, plasmids (pMTfosSVneo and pMTmybSVneo) that contain pSV2neo and human metallothionein promoter-regulated mouse c-*fos* (or c-*myb*) gene were constructed (Fig. 2*E*) and transfected into SKT6 cells by electroporation. More than 20 independent stable transformants were cloned from each transfection. Of these, two clones, each of which expressed the highest level of exogenous oncogene mRNA in the presence of $CdCl_2$ among the cloned transformants, were selected for further studies. All clones examined, however, expressed exogenous oncogene constitutively without heavy metals, and they showed only 2- to 3-fold induced expression with $CdCl_2$ (data not shown). No changes in cell growth rate or in morphology were observed in these oncogene transfected cells.

To determine the differentiation potential of these transformants, the selected transformants (c-*fos* transformants clones 1 and 7 and c-*myb* transformants clones 3 and 8) were induced with Epo or DMSO, and hemoglobin-positive cells were stained by bendizine. Strikingly, all of these transformants failed completely to differentiate above background levels with Epo (Table 1). Control SKT6 cells to which pSV2-neo was transfected showed >70% bendizine positivity with Epo (Table 1), which was about the same as the level shown by untransfected SKT6 cells. Thus, the observed inhibition of differentiation by exogenous c-*fos* and c-*myb* expression was not due to a transfection artifact. Furthermore, the mixture of c-*fos* (or c-*myb*) stable transformants (>100 clones) similarly exhibited a differentiation block (Table 1), indicating that these effects are not a specific character of the particularly cloned transformants. DMSO-induced differentiation of c-*fos*- or c-*myb*-transfected cells was inhibited >50% (Table 1) but not completely to the basal level as observed in Epo-induced differentiation.

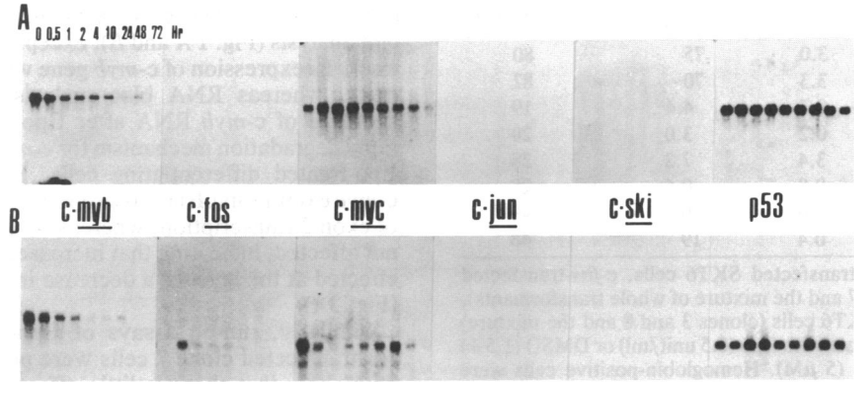

FIG. 1. Relative levels of protooncogene mRNA during Epo- or DMSO-induced SKT6 cell differentiation. Cells were harvested at the indicated times following Epo (*A*) or DMSO (*B*) induction. RNA blots were hybridized with labeled c-*myb*, c-*fos*, c-*myc*, c-*jun*, c-*ski*, or p53 probe.

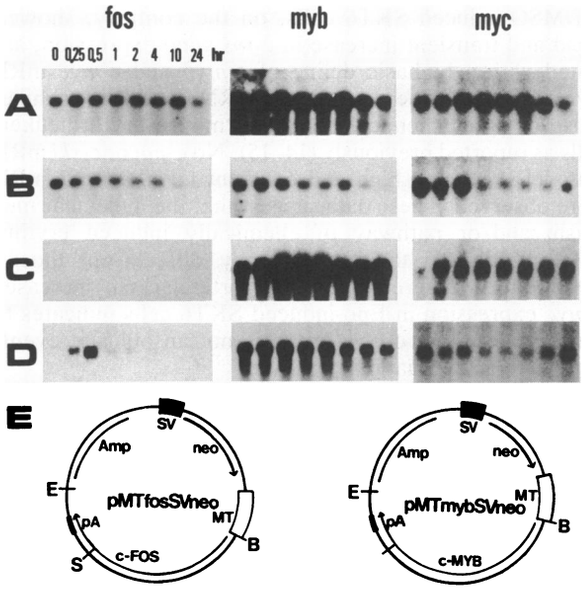

FIG. 2. Changes of c-*fos*, c-*myb*, and c-*myc* mRNA levels in differentiation-induced transformants. c-*fos* transformant clone 7 (*A* and *B*) or c-*myb* transformant clone 3 (*C* and *D*) were induced to differentiate with Epo (*A* and *C*) or with DMSO (*B* and *D*) in the presence of $CdCl_2$, and cells were harvested at the indicated time. RNA blot analyses were performed with c-*fos*, c-*myb*, and c-*myc* probes. (*E*) Construction of c-*fos* and c-*myb* expression vector. pMTfosSVneo consists of pSV2neo, human metallothionein IIA promoter (MT, open box), and complete mouse c-*fos* genomic DNA. B, *Bam*HI; E, *Eco*RI; S, *Sal* I; pA, poly(A) signals. In pMTmybSV-neo, *Bam*HI–*Sal* I c-*fos* fragment was replaced by mouse c-*myb* cDNA.

Expression of Nuclear Protooncogenes in Transfected Cells. To examine the effects of transfected oncogenes on the expression of endogenous nuclear oncogenes (c-*fos*, c-*myb*, and c-*myc*) during Epo- or DMSO-induced differentiation, a c-*fos* transformant (clone 7) and a c-*myb* transformant (clone 3) were induced with Epo or DMSO, and RNA blot analyses were carried out. In c-*fos*-transfected clone 7 cells, the exogenous c-*fos* gene was expressed constantly during the initial 24 hr of Epo treatment (Fig. 2*A*) in the presence of $CdCl_2$. Since no endogenous c-*fos* mRNA was detected in Epo-induced SKT6 cells (Fig. 1*A*), all c-*fos* transcripts observed here are likely to be exogenous mRNA. Interestingly, endogenous c-*myb* mRNA levels were maintained at quite high levels in c-*fos*-transfected clone 7 cells after Epo treatment (Fig. 2*A*), whereas Epo-induced differentiation normally leads to a rapid decline in c-*myb* transcripts (Fig. 1*A*). This may be due to the inhibition of transcription shutoff (or the transcriptional activation) of the c-*myb* gene or, alternatively, may result from mRNA stabilization. Levels of c-*myc* mRNA increased in c-*fos*-transfected clone 7 cells in response to Epo as they did in parental SKT6 cells (Figs. 1*A* and 2*A*), indicating that the regulation of this gene is not altered by exogenous c-*fos* expression.

Table 1. Effects of transfected c-*fos* and c-*myb* oncogenes on Epo- or DMSO-induced SKT6 cell differentiation

Cells	% hemoglobin-positive cells		
	None	With Epo	With DMSO
SKT6	3.0	75	80
SKT6 (neo), mix	3.3	70	82
SKT6 (fos), clone 1	0.7	4.4	19
SKT6 (fos), clone 7	0.2	3.0	20
SKT6 (fos), mix	3.4	7.2	28
SKT6 (myb), clone 3	0.8	0.6	28
SKT6 (myb), clone 8	2.6	0.2	40
SKT6 (myb), mix	0.4	19	48

SKT6 cells, pSV2neo-transfected SKT6 cells, c-*fos*-transfected SKT6 cells (clones 1 and 7 and the mixture of whole transformants), and c-*myb*-transfected SKT6 cells (clones 3 and 8 and the mixture) were induced to differentiate with Epo (0.5 unit/ml) or DMSO (1.5%) in the presence of $CdCl_2$ (5 μM). Hemoglobin-positive cells were stained after 4 days of culture. Values represent the percentage of stained cells in total cell suspension and are means of three experiments. Mix stands for the mixture of whole transformants (>100 clones).

In DMSO-treated c-*fos*-transfected clone 7 cells, the c-*fos* mRNA levels were not as high as those in Epo-treated cells. The endogenous c-*myb* mRNA was maintained at relatively high levels for 4 hr but thereafter decreased (Fig. 2*B*). These findings suggest that the low levels of c-*fos* expression did affect the c-*myb* mRNA levels but not efficiently. The profile of c-*myc* mRNA levels was a biphasic decline (Fig. 2*B*), which is similar to that of DMSO-induced parental cells (Fig. 1*B*).

In c-*myb*-transfected clone 3 cells, extraordinarily high levels of c-*myb* mRNA were observed (Fig. 2 *C* and *D*), since these cells contain endogenous and exogenous mRNAs that are not distinguishable by RNA blot analysis. These high levels were maintained upon Epo (Fig. 2*C*) or DMSO (Fig. 2*D*) treatment, and no obvious effects of c-*myb* transfection on endogenous c-*fos* and c-*myc* gene expression were detected—i.e., the profiles of these mRNA levels in Epo- or DMSO-induced c-*myb*-transfected clone 3 cells (Fig. 2 *C* and *D*) are very similar to those of SKT6 cells (Fig. 1 *A* and *B*).

Data obtained with c-*fos*- and c-*myb*-transfected cells (Table 1 and Fig. 2), therefore, implicate down-regulation of c-*myb* (but not c-*myc*) as a prerequisite for Epo-induced erythroid differentiation.

Nuclear Run-On Transcription Assay of Epo- or DMSO-Induced Cells. To investigate transcriptional control of endogenous c-*myb* and c-*myc* genes, run-on transcription assays were carried out by using nuclei from Epo (and DMSO)-induced SKT6 cells (Fig. 3*A*). Fig. 3*C* shows the various mouse c-*myb* genomic (c, d, e, f, and i) and cDNA (j) fragments used as probes. The data show a decrease of c-*myb* transcription and an increase of c-*myc* transcription immediately following Epo induction of SKT6 cells (Fig. 3*A*). Down-regulation of c-*myb* transcription is at the level of transcript initiation (see hybridization to fragments c, d, and e) as well as at the level of transcript elongation (fragments i and j) (Fig. 3*A*). Initiation of the c-*myb* antisense transcript, which probably originates with the promoter region (fragment c) (33), was found to coregulate with initiation of c-*myb* sense transcription in response to Epo (Fig. 3*A*). The DMSO track indicates that down-regulation of c-*myb* expression is effected only at the level of a block to elongation (fragments i and j) (Fig. 3*A*). These data are consistent with those of RNA blot analysis (Fig. 1 *A* and *B*), except for the data of the 18-hr track. Reexpression of c-*myb* gene was observed in the 18-hr track, whereas RNA blot analysis showed a monotonic decrease of c-*myb* RNA after Epo treatment (Fig. 1*A*). A rapid degradation mechanism for c-*myb* mRNA might exist in Epo-treated differentiating cells. Hybridization to mouse c-*myc* exon 1 (mycE1) and exon 2 (mycE2) shows an increase of exon 2 transcription, whereas transcription of exon 1 was not affected, indicating that increased expression of c-*myc* is effected at the level of a decrease in the block to elongation (Fig. 3*A*).

Similarly, run-on assays of Epo- or DMSO-induced c-*fos*-transfected clone 7 cells were performed (Fig. 3*B*). The data show that there is little effect of Epo on c-*myb* transcription in c-*fos*-expressing SKT6 cells. Transcription initiation is not affected (fragments c, d, and e), nor is elongation (fragments i and j) (Fig. 3*B*). However, DMSO still ablates

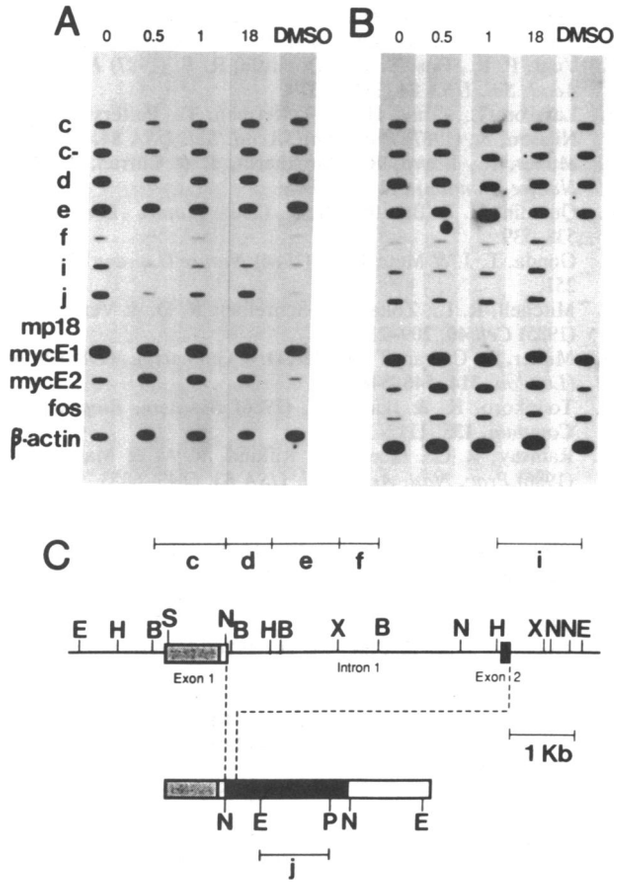

FIG. 3. Run-on transcription analysis of SKT6 cells and c-*fos* transformants following Epo or DMSO induction. Preinitiated transcripts in nuclei isolated from SKT6 (*A*) and c-*fos* transformant clone 7 (*B*) induced with Epo for 0, 0.5, 1, and 18 hr or with DMSO for 1 hr were labeled with [^{32}P]UTP. These run-on products were hybridized to slot blots containing the indicated probes. The various mouse c-*myb* genomic (c, d, e, f, and i) and cDNA (j) fragments used here are shown in *C*. c- indicates the probe complementary to antisense transcripts of c-*myb*. Other M13 clones contained a *Bam*HI–*Xho* I fragment of mouse c-*myc* exon 1 (mycE1), an *Xba* I–*Sac* I fragment of c-*myc* exon 2 (mycE2), or a *Bgl* II–*Pvu* II v-*fos* fragment (fos). (*C*) Restriction map of mouse c-*myb*. The shaded area denotes the noncoding sequence. The coding region is indicated by a closed box. Restriction fragments used as probes are indicated above and below the maps. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; N, *Nco* I; X, *Xba* I.

c-*myb* elongation. Moreover, c-*myc* transcription still increases in response to Epo but not to DMSO. All of these data are consistent with those of RNA blot analyses (Fig. 2 *A* and *B*). The conclusion is that c-*fos* transfection blocks the down-regulation of endogenous c-*myb* expression upon Epo induction but has no effect on endogenous c-*myc* expression. The observed modulations in level of c-*myb* and c-*myc* mRNA upon Epo or DMSO induction disclosed by RNA blot analyses (Figs. 1 and 2) were therefore found to be, at least in part, regulated at a transcriptional level.

Half-Life of c-*myb* mRNA in SKT6 Cells and c-*fos* Transfected Cells. The half-life of endogenous c-*myb* mRNAs in SKT6 cells and c-*fos*-transfected SKT6 cells was determined by RNA blot analyses of the cells treated for various times with the RNA synthesis inhibitor actinomycin D. Fig. 4 shows that c-*myb* mRNA turned over with a half-life of about 90 min in c-*fos*-transfected cells as well as in SKT cells, although we still cannot rule out the possibility that in Epo-treated cells c-*fos* expression prevents a decrease in c-*myb* mRNA stability.

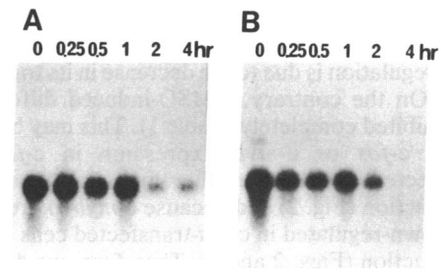

FIG. 4. Half-life of c-*myb* mRNAs. RNAs were isolated from SKT6 cells (*A*) and c-*fos*-transfected clone 7 cells (*B*) at the indicated times following addition of actinomycin D (5 μg/ml), and the RNA blots were hybridized with labeled c-*myb* probe.

DISCUSSION

Nuclear protooncogenes such as c-*myb*, c-*fos*, and c-*myc* are considered to play an important regulatory role in cell differentiation and proliferation (3–6). Therefore, it is critical to understand the molecular mechanism controlling the expression of these protooncogenes during cell differentiation.

We examined the changes of nuclear protooncogene expression during the early stage of Epo- and DMSO-induced erythroid differentiation when commitment for differentiation is determined. Although the natural differentiation factor and the chemical inducer lead to the same terminal erythroid cell differentiation, their effects upon expression patterns of nuclear oncogenes during early stage of differentiation appeared to be entirely different. This suggests that chemically induced differentiation bypasses the normal receptor-mediated signaling pathway—that is, although the effects of DMSO on nuclear protooncogene expression must be sufficient for differentiation, they may not truly reflect events that occur in response to Epo. Therefore, down-regulation of c-*myc* expression, which was demonstrated to be essential for chemically induced erythroid differentiation (16–19), appeared not to be required for Epo-induced differentiation, since, as shown in Figs. 1 and 3, transcriptional activation of c-*myc* was observed in the Epo-induced differentiation process. We cannot exclude a requirement in Epo-induced differentiation for a late down-regulation of c-*myc* expression, as the levels of this transcript eventually decline at the late stage of terminal differentiation (Fig. 1). It is likely, however, that the late down-regulation of c-*myc* expression is associated with cessation of cell proliferation, which is a necessary step for terminal differentiation. It has been shown that c-*myc* product promotes DNA replication and cell growth (34). Aberrant expression of exogenous c-*myc* during the late stage of terminal differentiation process may thus lead to unlimited cell proliferation and consequently to inhibition of terminal cell differentiation. Rapid and transient c-*fos* expression observed in DMSO-induced cells (but not in Epo-induced cells) is similar to that observed in mitogen- or growth factor-stimulated cells (8, 9, 11, 13, 35–38), indicating that c-*fos* induction may not be related to the cell differentiation process. In monocyte–macrophage differentiation, it has been shown that c-*fos* induction is neither sufficient nor obligatory for differentiation (39, 40). As chemical inducers have been used in studying the mechanism of cell growth/differentiation, such systems may not always represent the cell growth/differentiation events occurring *in vivo*.

To investigate the function of c-*myb* and c-*fos* gene expression on erythroid differentiation, we transfected expression vectors specifying these oncogenes into SKT6 cells. We found that gene expression leads to complete inhibition of Epo-induced differentiation (Table 1), and we concluded that down-regulation of c-*myb* (but not c-*myc*)

expression is a prerequisite for Epo-induced differentiation commitment (Figs. 1, 2*A*, 2*C*, and 3*A* and Table 1) and that this down-regulation is due to the decrease in its transcription (Fig. 3*A*). On the contrary, DMSO-induced differentiation was not inhibited completely (Table 1). This may be because exogenous c-*fos* or c-*myb* expression in c-*fos*- or c-*myb*-transfected cells was neither high nor constant after DMSO induction (Fig. 2) and because c-*myb* expression was partially down-regulated in c-*fos*-transfected cells 10 hr after DMSO induction (Figs. 2 and 3). Therefore, we decided not to draw any conclusions from the data of DMSO-induced differentiation.

Expression of the exogenous c-*fos* gene maintained high levels of c-*myb* mRNA even after Epo treatment (Fig. 2*A*), and this effect was found to be due to a block in down-regulation of c-*myb* expression rather than to its mRNA stabilization (Figs. 3*B* and 4). Evidence obtained above that c-*fos* expression may influence c-*myb* transcription prompted us to examine the effect of the c-*fos* product on transcription from the c-*myb* promoter. A plasmid, pmybCAT, in which 1.7 kb of the mouse c-*myb* promoter region (41) was linked to chloramphenicol acetyltransferase (CAT) gene, was transfected into fibroblast L929 cells as well as into L929 cells expressing the exogenous c-*fos* gene using the DEAE-dextran method (42), and CAT assays were performed (43). The result was that CAT activity in c-*fos*-transfected cells was slightly (two to five times) higher than that in untransfected cells (data not shown). Since the enhancement was not remarkably high, we cannot definitively conclude whether the c-*fos* product (or other nuclear proteins activated by c-*fos*) positively trans-activates the c-*myb* transcription or this effect resulted from inhibition of the Epo-induced down-regulation of c-*myb* expression. There are 11 octanucleotide sequences similar to an AP-1 consensus sequence in mouse c-*myb* promoter region (between −250 and −1030), and, in fact, we detected several DNA binding proteins in this region by gel retardation assay (unpublished data). There is no evidence, however, that these AP-1-like sequences function as regulatory elements for c-*myb* gene expression. On the other hand, the c-*fos* product does not represent an obligate activation mechanism for c-*myb* gene expression, since SKT6 cells normally do not exhibit detectable levels of c-*fos* mRNA during Epo-induced differentiation, but they express rather high levels of c-*myb* mRNA (Fig. 1*A*). Further analyses are necessary to clarify these phenomena.

It remains to be clarified how c-*myb* gene expression is regulated in hematopoietic cells. The nuclear extracts from SKT6 cells before and after Epo induction can be used to identify the positive and negative regulatory proteins for c-*myb* gene expression and the regulatory sequences in c-*myb* promoter. The identification of the signal that down-regulates c-*myb* expression may lead to the clarification of the molecular mechanism of Epo receptor-mediated erythroid differentiation.

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