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Changes in gene expression associated with induced differentiation of erythroleukemia: Protooncogenes, globin genes, and cell division

(hexamethylene bisacetamide/hemin/dexamethasone/oncogenes c-myb, c-myc, and c-fos)

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ABSTRACT **Hexamethylenebisacetamide (HMBA)-induced differentiation of murine erythroleukemia cells (MELC) is a multistep process involving an early latent period during which a number of metabolic changes have been detected, but the cells are not yet committed irreversibly to differentiate. Commitment is defined as the capacity of MELC to go on to express the program of terminal cell division and gene expression (such as the accumulation of globin mRNA) upon removal of the HMBA from the culture. In the presence of HMBA, a small proportion of MELC are committed by 10–12 hr and >90% by 48–60 hr. The present study shows that, during the initial 4 hr of culture, HMBA causes a marked decrease in c-myb and c-myc and an increase in c-fos mRNA levels. With continued culture, the decrease in c-myb and the increase in c-fos mRNA persists, while c-myc mRNA returns to control levels before the time that MELC begin to show irreversible differentiation. Dexamethasone, which blocks expression of HMBA-induced MELC differentiation, does not alter the early pattern of changes in protooncogene mRNA nor the sustained elevation of c-fos, but it does inhibit the continued suppression of c-myb allowing c-myb to return toward control levels. Hemin, which induces MELC to accumulate globins but does not initiate commitment to terminal cell division, does not alter these protooncogene mRNA levels. These studies suggest that, although the early decrease in c-myb and c-myc and increase in c-fos mRNAs may be involved in the multistep events leading to differentiation, the continued suppression of c-myb is critical for HMBA-induced MELC commitment to terminal cell division.**

Alteration in expression of a number of genes occurs during terminal differentiation of the eukaryotic cells (1, 2). Such genes include those responsible for products characteristic of the terminal differentiated phenotype, such as the globin genes; genes controlling metabolic functions in the cells, such as rRNA genes; and genes involved in regulating DNA replication and cell proliferation, among which certain protooncogenes have been implicated (3).

We have reported (4, 5) on the changes in α- and β-globin and rRNA gene expression during hexamethylenebisacetamide (HMBA)-induced murine erythroleukemia cell (MELC) differentiation to the erythroid phenotype. Induced differentiation of MELC is a multistep process (6). The inducer initiates a number of early metabolic changes (8–10 hr), which are not associated with commitment of MELC to irreversible differentiation (2, 6, 7). With continued culture with HMBA, MELC become irreversibly committed to differentiate in that they can be removed from the inducer and still go on to express the differentiated phenotype including hemoglobin accumulation and cessation of cell division (8).

The protooncogenes c-*myb*, c-*myc*, and c-*fos*, and the p53 gene control products that accumulate in the nucleus (9–12) and may play regulatory roles in gene expression related to cell replication. A decrease in c-myc (13–16) and c-myb (14, 17) mRNA levels and an increase in c-fos mRNA (16, 18) has been reported in several cell lines during induced differentiation. p53 protein levels decrease during HMBA-induced MELC differentiation (19). The present report examines the alterations in expression of the protooncogenes c-*myb*, c-*myc*, and c-*fos*, and protein p53 during both the early period of HMBA treatment of MELC before commitment and during the longer periods of culture that are associated with irreversible terminal differentiation. The relationship between inducer-mediated changes in protooncogene mRNA levels and commitment was also examined under two sets of conditions in which exposure to the inducer was not associated with loss of proliferative capacity. Hemin-treated MELC accumulate α- and β-globin mRNAs but do not initiate commitment to terminal cell division. Dexamethasone blocks the expression of HMBA-induced MELC differentiation (6).

Here we report that HMBA-induced MELC differentiation is associated with an early (within 1 hr) decrease in c-myb and c-myc mRNA levels at a time when MELC are not yet committed to differentiate. Unlike c-myc mRNA, which returns to control levels by 8–10 hr, c-myb mRNA levels remain suppressed throughout the period during which commitment is expressed during prolonged culture with HMBA. c-fos mRNA accumulates within 4 hr and remains elevated during commitment. Dexamethasone inhibits HMBA-induced MELC differentiation but does not block the decrease in c-myc or c-myb or the increase in c-fos mRNA levels that occurs during the initial 4 hr of culture. However, in culture with the steroid and HMBA, c-myb mRNA reaccumulates by 12 hr and is at levels comparable to that in uninduced cells at 48 hr. There is no change in c-myc, c-myb, or c-fos mRNAs induced by exposure to hemin, which, as noted above, mediates globin mRNA accumulation but does not induce commitment. The changes observed in c-myc, c-myb, and c-fos mRNA levels, which occur early during culture of MELC with HMBA, are not associated with irreversible terminal differentiation. The continued suppression of c-myb mRNA is associated with expression of differentiation and may be an important factor in the irreversible commitment of MELC to terminal cell division.

MATERIALS AND METHODS

Cell and Materials. MELC DS19-sc9, derived from 745A cells, are cells sensitive to dexamethasone suppression of HMBA-mediated commitment to terminal cell division.

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Abbreviations: HMBA, hexamethylenebisacetamide; MELC, murine erythroleukemia cells; kb, kilobase; IVS, intervening sequence.

MELC DS19-sc9 cells were maintained in α minimal essential medium containing 10% fetal calf serum (6). Cell cultures for all experiments were initiated with day 2 cultured cells (cells in logarithmic growth phase) at a cell density of 1–2 $\times$ 10^5 cells per ml in Corning 850-cm^2 roller flasks. Cell density and benzidine reactivity were determined as described (20). HMBA (20) (a gift of Pfizer) was added to medium at a final concentration of 5 mM; dexamethasone, at 4 μM; cycloheximide, at 1.5 μg/ml; and hemin, at 0.1 mM.

Cell Commitment Assay. Commitment to terminal cell differentiation characterized by limited cell division (colony size, <64 cells) and accumulation of hemoglobin (benzidine-reactive colonies) was assayed as described by Fibach *et al.* (8).

Separation of RNA by Gel Electrophoresis. MELC grown under the conditions described for each experiment were recovered from suspension culture by centrifugation. The pellet of cells was disrupted in the presence of guanidine thiocyanate (Fluka) by Polytron homogenization (21). Total RNA was collected as a pellet after CsCl gradient centrifugation. Poly(A)-enriched RNA was prepared by separation on an oligo(dT) column. Poly(A)$^+$ RNA was separated by electrophoresis in 1.2% agarose/formaldehyde gels (21) and transferred to GeneScreenPlus (New England Nuclear) for hybridization with the appropriate probe as described below.

RNA Synthesis in Isolated Nuclei. Assay of RNA synthesis in isolated nuclei was performed by the method of Hofer and Darnell (22) with the modifications as detailed in Sheffery *et al.* (4).

DNA Probes. The following probes were used in the course of these studies as indicated for each experiment: α-globin^c is a *Bam*HI 0.27-kilobase (kb) DNA fragment corresponding to a portion of the second exon and first intervening sequence (IVS); β-globin^d is a *Sau*AIII 0.39-kb DNA fragment corresponding to the 3′ end of the third exon; β-globin cDNA is a *Hha* I DNA fragment corresponding to the entire transcript of β-globin mRNA; β^{maj}-globin IVS-II is a *Hin*dIII/*Pst* I 0.3-kb DNA fragment corresponding to the second IVS of the β^{maj}-globin gene (4, 22, 23); and the murine α-tubulin cDNA probe. All were provided by Michael Sheffery. Human γ-actin plasmid (LK215 DHF1) (24) was a gift of Jeffrey Ravetch. pUC19 DNA was purchased from Pharmacia. Mouse-derived c-*myb* probes (gifts of E. P. Reddy) used were c-*myb1*, an *Xba* I–*Sac* I 0.6-kb DNA fragment that is 5′ to probe c-*myb2*, an *Xba* I 1.1-kb DNA fragment containing exons (25). c-*myb3* is a human *Eco*RI 1.9-kb DNA fragment corresponding to the fourth exon (26). c-*myc* probe, a human *Pst* I 0.5-kb DNA fragment within exon 2 and the viral v-*fos* gene (p-fos-1) (27) were gifts of Allen Oliff. p53 probe (P208) was a gift of Moshe Oren. The probes were made radioactive by labeling with ^{32}P by nick-translation (1–5 $\times$ 10^8 cpm/μg of DNA) (21). β-Globin IVSII DNA fragment was labeled by random primer DNA synthesis (2 $\times$ 10^9 cpm/μg of DNA) (28). Hybridization of DNA probes to RNA in filters and washing procedures were performed according to the manufacturer's instructions (New England Nuclear), radioautographed (21), and scanned.

RESULTS

Early Changes in Protooncogene mRNA Levels in MELC Cultured with HMBA. When MELC are cultured with HMBA, a number of biochemical changes occur during the initial 4 hr, but the cells do not become committed because, if the inducer is removed, the cells continue to proliferate and do not express their globin genes. The first detectable commitment to terminal differentiation requires 10–14 hr of culture with HMBA (8).

In the present studies, we first examined mRNA levels of c-myc, c-myb, c-fos, and p53 during the initial 240 min of culture with HMBA. As reported by others (13), c-myc mRNA level decreased by 30% by 15 min and by 90% by 120 min; by 240 min it began to reaccumulate (Fig. 1). c-myb mRNA increased 2-fold (within 15 min), then decreased by 30 min, and fell by 90% by 240 min. c-fos mRNA was low in uninduced cells, began to accumulate by 60 min, and increased 4-fold through 240 min. The level of p53 mRNA increased about 2-fold by 15 min and returned to the level in uninduced cells by 120 min. Throughout the initial 240 min of culture of MELC with HMBA, there was no detectable change in the level of γ-actin mRNA (Fig. 1). In MELC cultured with HMBA and dexamethasone, the patterns of change in c-myb, c-myc, and c-fos mRNA levels during the initial 240-min period were similar to those in cells cultured with HMBA alone (data not shown). Thus, dexamethasone, which blocks HMBA-induced expression of MELC differentiation, does not alter the early decrease in c-myb or c-myc mRNA levels.

Protooncogene mRNA Levels in MELC During Prolonged Culture with HMBA. HMBA-mediated commitment of MELC to irreversible differentiation begins to be detected after a latent period of 10–14 hr (8). By 48–60 hr of culture with HMBA, >90% of a population of MELC express irreversible commitment to differentiation (8). Dexamethasone suppresses commitment as measured by inhibition of both the onset of terminal cell division and of increased α- and β-globin gene transcription (6). Hemin, on the other hand, induces MELC α- and β-globin mRNA accumulation but does not commit the cells to terminal division (7, 29, 30).

We determined the mRNA levels of c-myb, c-myc, c-fos, and p53 in MELC after 48 and 60 hr of culture with no addition, with addition of HMBA alone, with HMBA and dexamethasone, or with hemin. In culture without inducer, the mRNA levels of these four protooncogenes remained essentialy unchanged at 48 and 60 hr (Fig. 2), whereas in MELC cultured with HMBA for 48–60 hr, c-myb mRNA was virtually undetectable. The levels of c-myb mRNA in cells

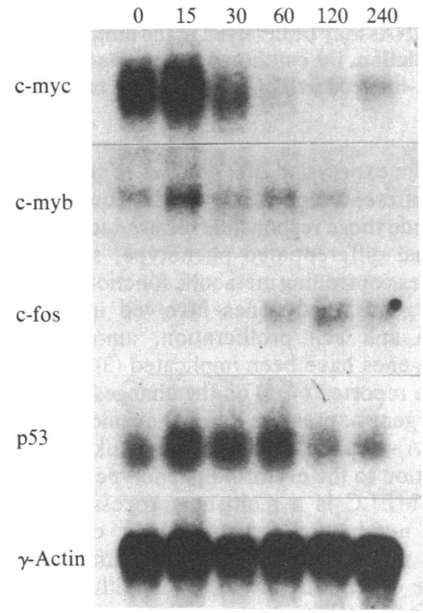

FIG. 1. Protooncogene mRNA levels in MELC cultured with HMBA. Cells were harvested at the time in minutes indicated at the top of the lanes. Poly(A)$^+$ mRNA was prepared and separated by electrophoresis. Blot-hybridization filters with 2 μg of mRNA per lane were hybridized overnight with nick-translated probes homologous to c-*myc*, c-*myb3*, c-*fos*, p53 gene, and γ-actin gene. The percentage of benzidine-reactive cells after 5 days of culture with HMBA in this experiment was 92%.

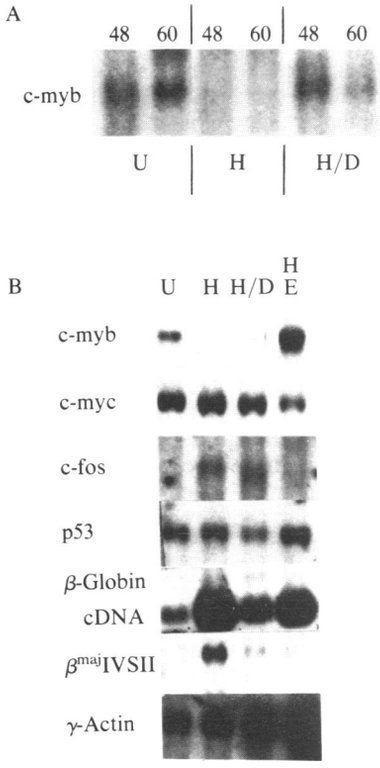

FIG. 2. Protooncogene and β-globin mRNA levels in MELC cultured without additions or with HMBA, with HMBA/dexamethasone, or with hemin. (*A*) Cells were harvested after 48 and 60 hr of culture (noted at tops of lanes) without inducer (lanes U), with HMBA (lanes H), or with HMBA/dexamethasone (lanes H/D). Total RNA (15 μg) was applied to each lane for electrophoresis; RNA then was transferred to a filter and hybridized overnight with nick-translated c-*myb3* probe. The proportion of benzidine-reactive cells after 5 days of culture were: uninduced, 0%; HMBA, 96%; and HMBA/dexamethasone, 30%. (*B*) Cells were harvested at 60 hr after treatment with HMBA (lane H), HMBA/dexamethasone, (lane H/D), or hemin (lane HE) for RNA preparation. Two micrograms of poly(A)$^+$ mRNA per lane was electrophoresed, and separate filters were hybridized overnight with ^{32}P-labeled DNA probes. The percentage of benzidine-reactive cells after 5 days of culture were: uninduced (lane U), 0%; HMBA, 98%; HMBA/dexamethasone, 32%; and hemin, 31%. MELC were also assayed for commitment to terminal cell division after 60 hr in culture: uninduced, 0%; HMBA, 85%; HMBA/dexamethasone, 28%; and hemin, 5%.

cultured with HMBA and dexamethasone returned toward control levels by 12 hr and remained at levels similar to that in control cells up to 48 hr. At 60 hr there was still substantially more c-myb mRNA than in HMBA-treated cells. c-myb mRNA levels were unaffected by culture with dexamethasone alone or with hemin (Fig. 2*B*). c-myc and p53 mRNA levels were essentially the same after 48 or 60 hr in culture with HMBA, with HMBA and dexamethasone, with dexamethasone, with hemin, or without inducer (Fig. 2*B*). c-fos RNA accumulated in MELC culture with HMBA or with HMBA and dexamethasone but was not detected either in uninduced cells or in cells cultured with hemin.

β-Globin mRNA levels, assayed with a β-globin cDNA probe, were increased >10-fold in MELC cultured with HMBA and about 7- or 8-fold in cells cultured with hemin but were only slightly increased in cells cultured with HMBA and dexamethasone. Use of a β^{maj} IVSII DNA probe (Fig. 2*B*) to assay for β-globin mRNA levels revealed an increase in MELC cultured with HMBA but not in cells cultured with HMBA and dexamethasone or with hemin (Fig. 2*B*). The β-globin mRNA induced by hemin was primarily β^{min}, whereas β^{maj} is primarily induced by HMBA (31). The γ-actin mRNA level was similar in uninduced, HMBA-, HMBA/dexamethasone- and hemin-treated cells.

Transcription of Protooncogenes in MELC Cultured with HMBA. The above results indicate that mRNA levels of c-myb, c-myc, and c-fos undergo substantial changes during HMBA-induced MELC differentiation because of either altered transcription or stability of protooncogene mRNAs. To distinguish between these possibilities, the effect of HMBA on the transcription of c-*myb*, c-*myc*, and c-*fos* genes was assayed at intervals during culture of MELC with HMBA (Fig. 3). Transcription of c-*myb* decreased 90% by 15 min, became undetectable by 60 min, and remained undetectable. Transcription of c-*myc* decreased 70% by 60 min; this decrease was transient, as the transcriptional activity of c-*myc* began to rise by 120 min (Fig. 3). Transcription of c-*fos* was at a low level in uninduced cells, increased 3-fold by 30 min, and then decreased. Transcription of p53 was also at a low level in uninduced cells, and no change was detected during the initial 240 min of culture with HMBA (data not shown). These data suggest that the early HMBA-induced suppression of c-*myb* and, possibly to a somewhat lesser extent, c-*myc* is due to suppression of transcription of these genes. The inducer-mediated increase in c-fos mRNA levels does not appear to reflect a change in the rate of transcription.

Effect of Cycloheximide on Protooncogene mRNA Levels. We next examined the effect of inhibition of protein synthesis by cycloheximide on the accumulation of c-myc, c-myb, and c-fos mRNA. Addition of cycloheximide to MELC cultured with HMBA for up to 240 min did not block the inducer-mediated decrease in c-myc or c-myb mRNA levels but did increase the level of accumulation of c-fos mRNA (Fig. 4). Treatment of MELC with cycloheximide in the absence of HMBA had no detectable effect on the level of c-myc or c-myb mRNA but did lead to an increase in c-fos mRNA and accumulation of a previously undetected c-myb-related RNA, perhaps a c-myb RNA precursor (Fig. 4). In all of these studies, cycloheximide caused a >90% decrease in total protein synthesis. These findings increase the evidence suggesting that c-fos mRNA may be post-transcriptionally regulated.

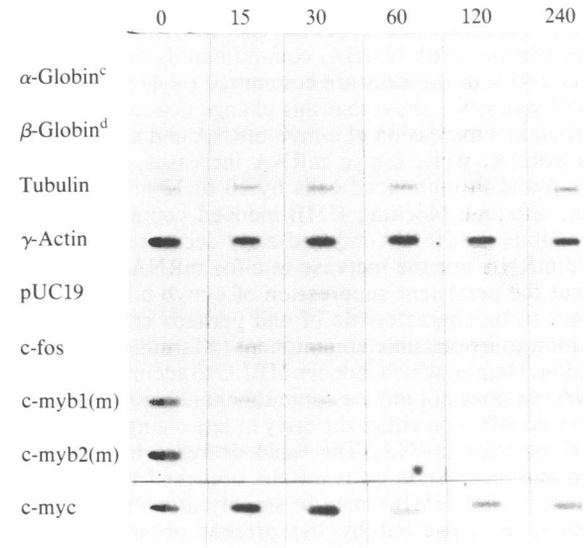

FIG. 3. Transcription of protooncogenes and globin genes in MELC cultured with HMBA. Nuclei were prepared from cells cultured in HMBA for the minutes indicated at the top of the lanes. The nuclei were labeled with [^{32}P]UTP for 30 min; RNA was isolated and hybridized to separate slot blots with 5 μg of plasmid DNA probes indicated at the left above. MELC from this culture with HMBA had 96% of cells benzidine-reactive after 5 days. The "m" in parentheses after c-myb1 and c-myb2 signifies mouse.

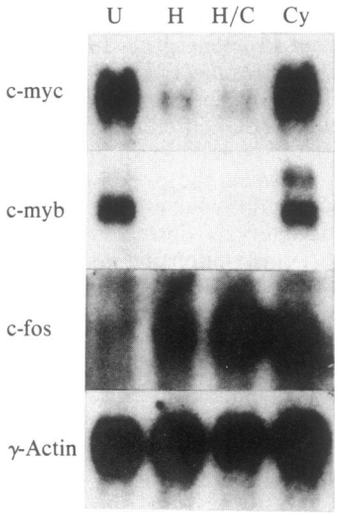

FIG. 4. Effect of cycloheximide on protooncogene mRNA levels in MELC cultured without additions (lane U), with HMBA (lane H), with HMBA/cycloheximide (lane H/C), and with cycloheximide (lane Cy). Cells were harvested after 4 hr in culture, and poly(A)$^+$ mRNA was prepared. Two micrograms of mRNA was electrophoresed per lane and transferred to filters, which were hybridized overnight with the ^{32}P-labeled DNA probes indicated. The percentage of benzidine-reactive cells after 5 days in culture were 0% for untreated cells and 91% for HMBA-treated cells.

DISCUSSION

HMBA-induced MELC differentiation is a multistep process that includes an early phase while the cells are not yet irreversibly committed to differentiate (6–8). In the present study, we show that during this early latent period, in the initial 1–4 hr, there is a marked decrease in the levels of c-myb and c-myc mRNAs and an increase in c-fos mRNA. No decrease is detected in p53 mRNA levels, though we have reported that p53 protein decreases in this time period (19). Prolonged culture of MELC with HMBA leads to irreversible commitment (8) as defined by the fact that, upon removal of HMBA, the cells can express the characteristics of terminal differentiation. With HMBA, commitment is detected by 12 hr and >95% of the cells are committed by 48 hr (7). In the present study, we show that this change is accompanied by a persistent suppression of c-myb mRNA and an increase in c-fos mRNA, while c-myc mRNA increases, returning to levels found in uninduced cells by 10 to 12 hr. Dexamethasone, although blocking HMB-induced commitment, neither inhibits the HMBA-induced early decrease in c-myb or c-myc mRNA nor the increase in c-fos mRNA, but it does prevent the persistent suppression of c-myb mRNA, which appears to be characteristic of and perhaps critical for the transition to irreversible commitment to terminal cell differentiation. Hemin, which induces MELC to accumulate globin mRNA but does not induce commitment (29, 30), was found to have no effect on either the early or late changes in c-myc, c-myb, or c-fos mRNA. The rapid decrease in c-myc and c-myb and increase in c-fos mRNA observed when MELC are treated with HMBA may be important events in differentiation, as suggested by the present observations and reports from others using MELC (13, 14, 32) and a number of other systems of induced cell differentiation (15–18). However, the demonstrated effect of dexamethasone suggests a complex pattern of regulation of c-*myb* expression involving both an early fall in response to HMBA and a persistent suppression. The persistent suppression of c-*myb*, which is blocked by the glucocorticoid, may be critical to the process of commitment to terminal differentiation. An association of increased levels of c-myb RNA and hematopoietic malignancies has been suggested by the finding that a variety of proliferating hematopoietic tumors have increased levels of this oncogene mRNA and that c-*myb* is a homologue of the v-*myb*-containing avian myeloblastosis virus that transforms avian myeloblasts (33, 34).

The mechanism of HMBA-induced suppression of c-myb and c-myc mRNA in MELC appears to involve inhibition of transcription of the corresponding genes, and this inhibition is not dependent on protein synthesis. On the other hand, the HMBA-induced increase in c-fos mRNA appears to involve primarily a post-transcriptional mechanism that is sensitive to modulation by a short-lived protein, since exposure to cycloheximide leads to a marked increase in c-fos mRNA in cells cultured with or without HMBA. Previous studies have reported evidence that c-myb (35), c-myc (36), and c-fos (18) may be principally regulated at a post-transcriptional level in proliferating cells. Perhaps, the mechanisms regulating commitment to terminal cell division during induced differentiation are different from those regulating the normal progression of cell division.

A possible extension of the present findings may be found in evaluating the biological effects of HMBA in inducing differentiation of neoplastic cells. Clinical studies already have been initiated to evaluate the effects of HMBA as a cytodifferentiation agent (37). In many human tumors, assay for induced differentiation by cytologic characteristics is difficult. Following the effects of administration of HMBA on protooncogene mRNA levels may provide useful indicators of the action of inducers on tumor cells.

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