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Synthesis and turnover of globin mRNA in murine erythroleukemia cells induced with hemin

(erythroid differentiation/Friend cells/dimethyl sulfoxide induction)

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ABSTRACT When murine erythroleukemia (MEL) cells are induced with hemin, they carry out several early functions of the erythroid program. However, they do not become committed to terminal differentiation nor do they become benzidine positive. This is in contrast to MEL cells induced with dimethyl sulfoxide (Me_2SO) which undergo a more complete program of erythroid differentiation. In order to determine the relationship between commitment and various events in the erythroid program, we compared the induction of MEL cells with hemin and with Me_2SO. The amount of globin mRNA accumulated in inducing MEL cells and the rate of its synthesis and turnover were quantitated. Although MEL cells induced with hemin accumulated significantly less globin mRNA than did cells induced with Me_2SO, the rate of synthesis of globin mRNA was the same in fully induced cells, irrespective of inducer. Therefore, there is no evidence that induction with hemin produces an early program that is different or altered from that which is part of Me_2SO induction. MEL cells induced with Me_2SO specifically destabilize their globin mRNA after 4 days of induction. This raises the question of whether this destabilization of globin mRNA is an independently programmed late event, as suggested by the time of its occurrence, or, alternatively, whether it might be the inevitable consequence of an early event(s). For instance, destabilization might be linked to the synthesis or translation of globin mRNA. Because MEL cells induced with hemin do not destabilize their globin mRNA, we have concluded that this turnover of globin mRNA is a late event, occurring only in a committed cell population.

In order to understand the program of timed sequential events that occur during differentiation, it is necessary to define independent steps and establish their temporal and causal order. The timing of certain events may be independent of preceding program events, whereas the expression of other late steps is dependent on and controlled by earlier steps. Many events in the erythroid program have been identified both in normal erythroid precursor cells and in murine erythroleukemia (MEL) cells. These events include the accumulation of globin mRNAs and their respective globins (1–3), changes in sensitivity to agglutination (4), cell surface antigens (5), changes in activity of heme biosynthetic enzymes (6), and changes in chromatin structure (7) (also reviewed in ref. 8). In addition, in MEL cells the terminal commitment event, when cells lose the ability to proliferate without limit and initiate the final four cell divisions, can be observed (9). The temporal order of the events in MEL cell differentiation has been determined and these events have been classified as *early* events (those that precede commitment) and *late* events (those that occur in fully committed cells) (8).

Although most inducers of MEL cell differentiation, including dimethyl sulfoxide (Me_2SO), induce the entire program of events, hemin appears to induce only early events. MEL cells exposed to 75–100 μM hemin accumulate spectrin (10) and synthesize increased amounts of globin mRNA (11–13) and globin (14); however, they do not cease cell division and become committed (15, 16). In the commitment assay, cultures induced with hemin give results identical to those with uninduced cultures. When cloned on semisoft agar, less than 1% of the clones contain fewer than 32 cells. This has been established for a number of MEL cell lines including 745, the line used in this study (15, 16). MEL cells induced with hemin do not show a positive benzidine reaction for hemoglobin (14). In addition, hemin does not induce IP_{25}, the chromatin-associated protein found in MEL cells induced with Me_2SO (7, 15), nor does it affect enzyme activities that have been identified as part of the late program (15). Because the induction potential of hemin appears to be limited to initiating the early program, whereas Me_2SO will induce both early and late events, comparison of the effects of the two inducers on MEL cells allows us to investigate the temporal and causal relationships between specific events in the developmental program and commitment.

In order to determine whether induction of globin mRNA accumulation in MEL cells treated with hemin is quantitatively similar to the induction in MEL cells treated with Me_2SO, we determined both the amount of globin mRNA accumulated in inducing MEL cells and the rate of its synthesis (appearance) and turnover. We propose that the synthesis and turnover of globin mRNA in inducing MEL cells are causally independent steps although each acts to modulate the final concentration of globin mRNA in the fully induced cell.

MATERIALS AND METHODS

Cell Growth and Induction. MEL cells of line 745 (Friend) were obtained from the Institute for Medical Research, Camden, NJ (GM86). Cultures were maintained on Ham's nutrient mixture F-12 (K/C) supplemented with 10% fetal calf serum (Hy clone). Cells were grown in 5% CO_2/95% air at 37°C in a humidified incubator. Cell number was determined by using a Coulter Counter (model F_N).

Inducer (Me_2SO or hemin) was routinely added to freshly subcultured cells at 1–2 × 10^5 cells per ml. Me_2SO stored at −20°C (under N_2) was added to 1.8% (vol/vol). A 10 mM hemin stock solution was prepared as described by Ross and Sautner (11) and frozen until just before use. Hemin stock was added to cells to a final concentration of 75 μM.

Pulse Chase. Labeling of RNA with [^{3}H]uridine and chase procedures were performed as described by Lowenhaupt and Lingrel (17).

Isolation of Total Cellular RNA. Total RNA was isolated by a modification of the procedure of Glisin *et al.* (18) and Ross and Sautner (11). Cells were harvested as described (3). They

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Abbreviations: MEL cells, murine erythroleukemia cells; Me_2SO, dimethyl sulfoxide.

were lysed and digested with proteinase K as described by Kwan *et al.* (19) except that up to 5×10^7 cells were lysed in a final volume of 0.5 ml. After digestion for 30 min at 37°C, 0.4 ml of 20% Sarkosyl was added along with an additional 1.8 ml of buffer without detergent or proteinase K. CsCl (1.5 g) was then dissolved in the digested lysate and this mixture was layered onto a 1.5-ml cushion of a filtered solution of CsCl (n_D = 1.400) in 1 mM EDTA contained in an SW 50.1 tube. After centrifugation in an SW 50.1 rotor (Beckman) at 32,000 rpm for 24 hr at 25°C, the tubes were drained and cut as described by Glisin *et al.* (18). The RNA pellet was dissolved in water and stored, either frozen or as an ethanol precipitate, at −20°C.

Analysis of RNA. Saturation hybridization was performed as described by Lowenhaupt *et al.* (3). Poly(A)-containing RNA was purified by using oligo(dT)-cellulose as described (20, 21). Globin mRNA was purified by using cDNA-cellulose as described by Wood and Lingrel (22).

RESULTS

Induction of MEL Cells with Hemin and Me_2SO. Committed MEL cells exhibit limited proliferative activity, dividing a maximum of four times (9). The majority of cells in a culture induced with Me_2SO are committed, whereas those in hemin-treated cultures are not and may divide indefinitely. Because cell viability is related to cell density, it was important to establish induction conditions that would promote continued cell viability. MEL cells that are induced with 1.8% Me_2SO at an initial density of 10^5 cells per ml will grow to a density of 10^6 cells per ml by day 3 of induction. (3). These cells will remain at 10^6 cells per ml for up to 6 additional days with no evidence of decreased viability (as determined by trypan blue exclusion); however, any dilution of the culture after day 2 of induction leads to a loss of total cell number and visible evidence of cell death. MEL cells induced with 75 mM hemin at the same density will also achieve 10^6 cells per ml, although there is approximately 50% increase in the doubling time as compared to uninduced or Me_2SO-induced cells. Actively dividing cells, such as uninduced or hemin-induced cells, cannot be maintained at 10^6 cells per ml for more than 2 days without loss of cell number and viability. A hemin-induced culture can and must be subcultured repeatedly to allow the cells to continue to divide. If a culture is diluted to 10^5 cells per ml at any time during induction, after 3 days, the culture will contain 10^6 cells per ml.

Accumulation of Globin mRNA during Hemin Induction. The accumulation of globin mRNA in hemin-induced cells was quantitated by using saturation hybridization (Fig. 1). A 4-fold increase in globin mRNA was observed during hemin induction, followed by a small additional increase in globin mRNA content as cells reached stationary phase (day 4). This is in agreement with the 4-fold increase in globin mRNA content of MEL cells of line 745 treated with 100 μM hemin observed by Ross and Sautner (11). The increase in globin mRNA content was already well established by day 1, the first time point, of induction. Marks and his coworkers (12) studied early times of induction and found that MEL cells respond to hemin by accumulating globin mRNA within 6 hr.

The pattern of accumulation of globin mRNA by MEL cells induced with hemin was strikingly different from the pattern of accumulation seen in the same cells with Me_2SO (Fig. 1). MEL cells induced with Me_2SO responded less rapidly and, after 4 days, a striking loss of globin mRNA was seen. This loss is the result of an abrupt change in the stability of globin mRNA (17), and its absence in MEL cells induced with hemin suggests that there is no change in the stability of globin mRNA. In addition, the extent of maximal accumulation was much less in

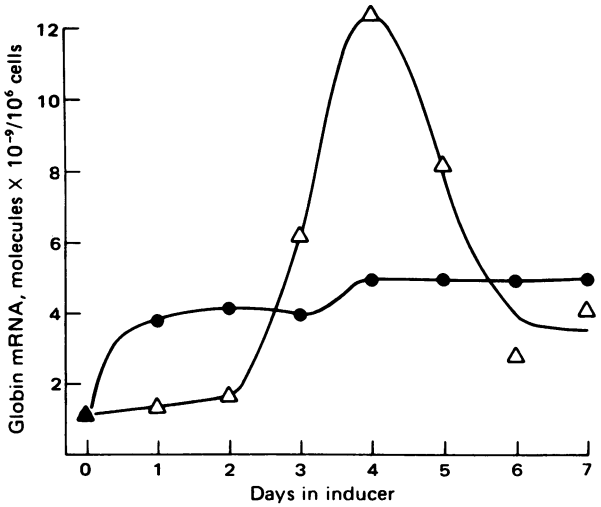

FIG. 1. Amount of globin mRNA accumulated in MEL cells induced with hemin (●) or Me_2SO (△). The amount of globin mRNA in the cytoplasm of MEL cells was quantitated by saturation hybridization (3). Globin cDNA was prepared and hybridized to total MEL cell RNA and, after 24 hr, the extent of hybridization was determined by using S1 nuclease.

cells induced with hemin than in those induced with Me_2SO. This difference may result from: (*i*) a lower rate of synthesis of globin mRNA in cells induced with hemin; (*ii*) an increased rate of turnover; (*iii*) continued cell division; or (*iv*) a lower proportion of cells affected by the inducer.

Synthesis of Globin mRNA. The rate of appearance of total RNA and globin mRNA in MEL cells induced with hemin was quantitated at various times of induction. Cells were incubated for 10 min or 2 hr in the presence of [^{3}H]uridine and the amount of radioactivity incorporated into total RNA and globin mRNA was determined. The incorporation of [^{3}H]uridine in 2 hr is a measure of the rate of appearance of mature, cytoplasmic RNA (3, 23). A 10-min labeling allows quantitation of the rate of synthesis of globin mRNA; under this condition, most of the [^{3}H]uridine is found in the precursor to globin mRNA (3). The rate of incorporation of [^{3}H]uridine into total RNA remained constant during hemin induction (Fig. 2*A*). This is not unexpected because the total amount of RNA in hemin-induced MEL cells is also constant (Fig. 3). In contrast, in Me_2SO-induced MEL cells there was a decrease in the rate of synthesis of total RNA (Fig. 2*A*) that resulted in a loss of total RNA (3, 23) (Fig. 3).

The incorporation of [^{3}H]uridine into globin mRNA was measured by selectively binding globin mRNA to a cDNA-cellulose column. A 4-fold increase over control levels in rate of synthesis of globin mRNA was seen at the earliest time point, day 1, of induction with hemin (Fig. 2*B*). A similar increase was seen with both 2-hr and 10-min labelings [the identity of values is a coincidental result of labeling conditions (3)]. Therefore, the efficiency of posttranscriptional processing remains the same. This 4-fold increase is sufficient to account for the 4-fold accumulation of globin mRNA (Fig. 1) aid indicates that there is no change in the stability of globin mRNA at early times of induction of MEL cells with hemin. The absolute incorporation of [^{3}H]uridine into globin mRNA per 10^6 fully induced MEL cells was the same whether hemin or Me_2SO was the inducer. Therefore, the difference in the accumulation of globin mRNA with the different inducers is probably not the result of differential synthesis.

Finally, in cells induced with hemin there was no change in

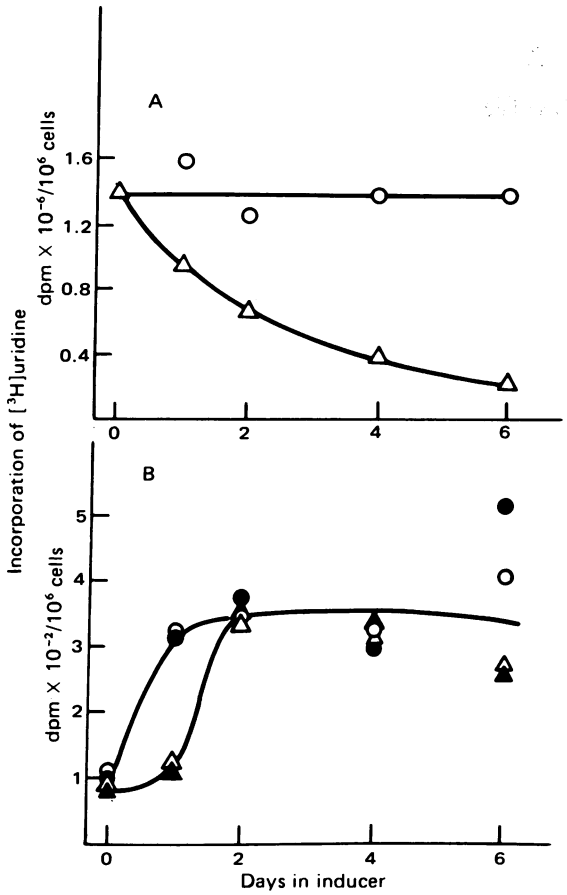

FIG. 2. The rate of synthesis of total RNA (*A*) and globin mRNA (*B*) was determined by measuring the incorporation of [^{3}H]uridine into RNA. Inducing cells were incubated for 10 min with 100 μCi of [^{3}H]uridine (●, ▲) or for 2 hr with 10 μCi of [^{3}H]uridine (○, △) per ml (3). Globin mRNA was isolated by hybridization to a cDNA-cellulose column. ○, Cells induced with hemin; △, cells induced with Me_2SO.

the stability of globin mRNA late in the induction process. We have verified this directly in the following section.

Turnover of Globin mRNA. The rate of turnover of globin mRNA in MEL cells induced with hemin was measured in pulse-chase experiments (Fig. 4). Cells were labeled with [^{3}H]uridine (10 μCi/ml; 1 Ci = 3.7×10^{10} becquerels) for 2 hr, the unincorporated uridine was removed by washing, and the cells were incubated in medium free from exogenous uridine.

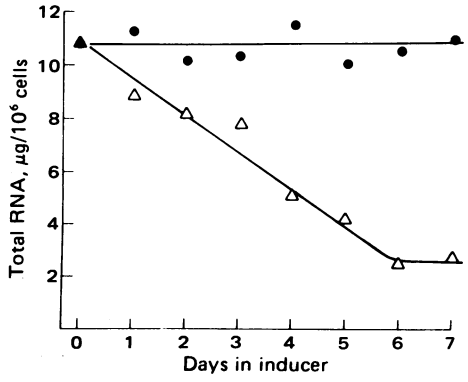

FIG. 3. Amount of total RNA in MEL cells induced with hemin or Me_2SO. Total RNA was isolated from inducing MEL cells and quantitated on the basis of the A_{260} of an aliquot.

After various times of chase, the cells were lysed and [^{3}H]RNA was fractionated to determine the incorporation into total RNA (95% rRNA), poly(A)-containing RNA, and globin mRNA. Pulse-chase experiments were initiated after 2, 4, and 6 days of induction. At all times of induction, the total RNA remained stable. This is predominantly rRNA and therefore is expected to have a long half-life (17, 24). Poly(A)-containing RNA turned over as a mixed population of RNAs that may be graphed as two groups of RNAs turning over with half-lives of 6 and 36 hr, respectively. Similar curves have been obtained with various cell types (17, 24, 25).

Globin mRNA has been shown to be very stable (half life >50 hr) in uninduced MEL cells as well as in cells early in induction with Me_2SO (17). This is in contrast to cells fully induced with Me_2SO and normal late erythroid cells, in which globin mRNA has a half-life of 17 hr (17, 26, 27). In MEL cells induced with hemin, globin mRNA remains stable for up to 8 days of induction (Fig. 4).

Although these data suggest that the destabilization of globin mRNA in inducing MEL cells is an independently regulated late event, an alternative is that hemin is acting to stabilize the globin mRNA late in hemin induction. This is supported by the observation that hemin is a potent inhibitor of the activity of reticulocyte RNase, both in cell lysates (28) and as a partially purified enzyme (29). In order to address this possibility, cells were induced with Me_2SO for 5 days, a time when globin mRNA should be degraded with a 17-hr half-life (17). Pulse labeling and chase were performed as usual except that 75 mM hemin was added to the medium throughout the chase (Fig. 5). There was no evidence that hemin was acting to stabilize the globin mRNA; the half-life was the same as that in cells induced by Me_2SO alone.

DISCUSSION

The accumulation of globin mRNA in MEL cells induced by hemin differs from that of cells induced by Me_2SO. This difference can be attributed to changes in the developmental programs supported by the two inducers. In each, a rapid accumulation of globin mRNA results from the 4-fold increase in the rate of transcription of globin mRNA. However, in cells induced with Me_2SO, the amount of globin mRNA per cell is affected by changes in the stability of the mRNA and in the proliferative capacity of the cells; hemin induces no such changes.

The fact that fully induced cells synthesize globin mRNA at the same rate, irrespective of inducer (Me_2SO or hemin), suggests that only two transcriptional levels are involved: a low level observed in uninduced cells, and a high level in induced cells. Although the primary actions of hemin and Me_2SO are different, as indicated by the difference in the rates of response to the two inducers, the change in transcriptional levels is not different.

Although the rate of synthesis of globin mRNA is the same in cells induced with hemin and with Me_2SO, it must be remembered that globin mRNA is actually a mixture of closely related mRNAs. The ratios between α- and β-globin mRNAs (12) and β^{maj} and β^{min} globin mRNAs (13) are different in MEL cells induced with Me_2SO and hemin. Therefore, the constant rate of synthesis of total ilobin mRNA must be the sum of varied rates of synthesis of individual globin mRNAs. It will be of interest to determine whether the different ratios between α, β^{maj}, and β^{min} globin mRNAs are regulated at the transcriptional or posttranscriptional level.

Two factors act to limit globin mRNA content: mRNA turnover, and cell division. When a cell divides, the mRNA is partitioned and the amount of mRNA per cell is halved. These

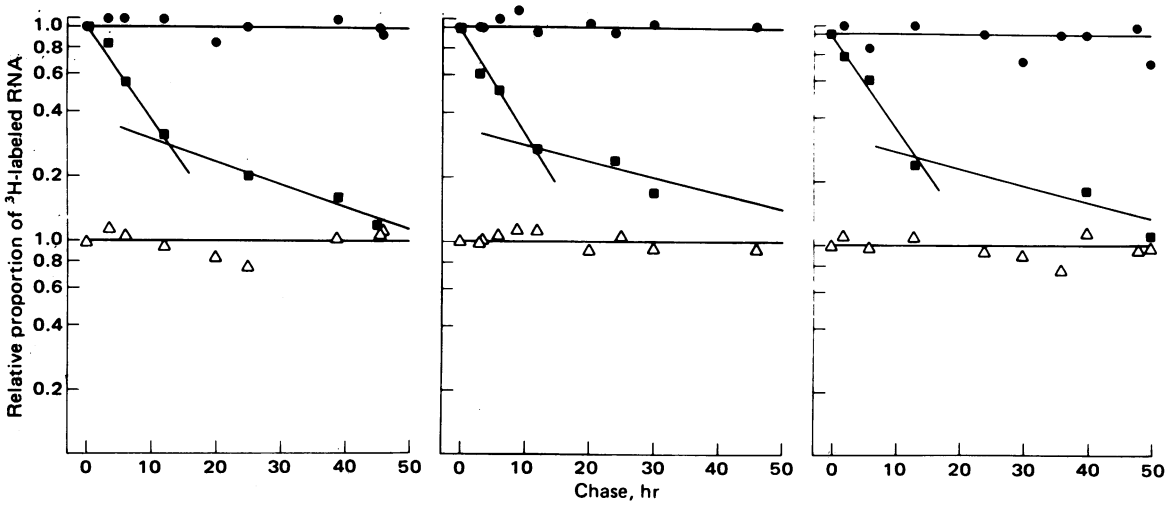

FIG. 4. Decay kinetics of [³H]RNA in MEL cells induced with hemin for 2 days (*Left*), 4 days (*Center*), or 6 days (*Right*). Values are expressed as a proportion of the amount of [³H]RNA isolated after 0 hr of chase in each experiment. (*Left*) Approximately 2×10^8 dpm of total [³H]RNA (●) was isolated from 50 ml of culture. At 0 hr of chase, 7% of the [³H]RNA was poly(A)-containing (■) and 0.04% was globin mRNA (△).

factors play different roles in the accumulation of mRNA in MEL cells induced with hemin and Me_2SO. In cells induced with Me_2SO, cell division ceases early in induction. This results in a 20- to 40-fold accumulation of globin mRNA. Late in induction, destabilization of globin mRNA results in a loss of globin mRNA. MEL cells induced with hemin have the potential to divide throughout induction and in growing cultures, the accumulation of globin mRNA is limited to 4-fold. Additional accumulation occurs if cells are not allowed to divide (e.g., Fig. 1, 4–5 days); however, this accumulation is limited, either by the reestablishment of cell division after subculture (Fig. 1) or by cell death (data not shown).

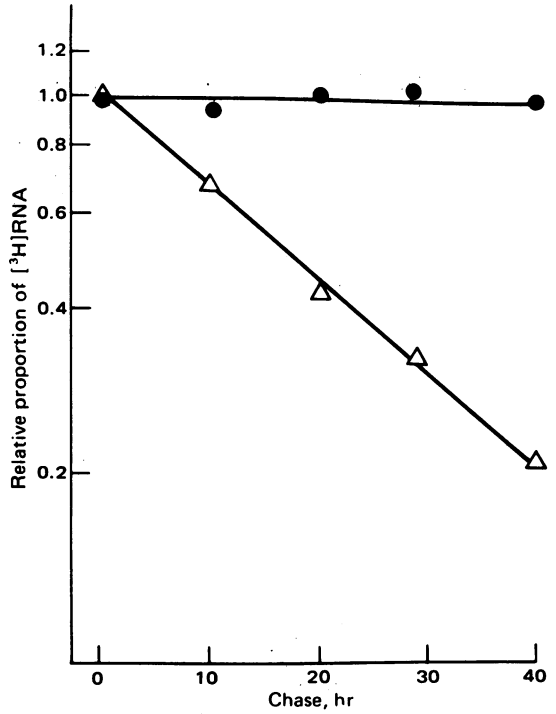

FIG. 5. Decay kinetics of [³H]RNA in MEL cells induced with Me_2SO for 5 days. Cells were labeled and resuspended for the period of chase in medium supplemented with 1.8% Me_2SO and 75 mM hemin. Values are expressed as a proportion of the amount of [³H]RNA isolated after 0 hr of chase in each experiment. Approximately 2×10^7 dpm of total [³H]RNA was isolated from 50 ml of culture. At 0 hr of chase, 0.15% of the total [³H]RNA (●) was globin mRNA (△).

This study identifies two independent, separable events in the program of erythroid differentiation which relate to globin mRNA: the increase in the rate of synthesis and the change in stability. The first is an early event and occurs with two different inducers. The destabilization of globin mRNA has been identified as a late function in the erythroid program and is independent of the accumulation, and probably the utilization, of the RNA. This is shown by the fact that hemin-induced MEL cells, which accumulate globin mRNA, do not destabilize their RNA. Globin mRNA is translated in hemin-induced MEL cells (14) although the efficiency has not been established.

In addition, a third event, the decrease in the rate of synthesis of total and poly(A)-containing RNA, has been identified as a late event, indicating that the time of occurrence of an event in Me_2SO-induction may not accurately reflect its position in the erythroid program. The decrease in the amount of total RNA synthesis and resulting loss of total RNA occurs similtaneously with the accumulation of globin mRNA in Me_2SO-induced cells. No such loss occurs in hemin-induced MEL cells, indicating that this event is late and is dependent on commitment, whereas the accumulation of globin mRNA is early and does not require commitment.

Speculation about the nature of developmental programs leads to testable hypotheses about the mechanisms by which events are controlled. For example, a transcriptional program may be postulated in which each event results from a primary change in the pattern or rate of transcription. In such a program, independent events must involve separate transcriptional alterations. Increased transcription of globin mRNA is a primary event; however, destabilization is a secondary event and must result from a change in transcription. Possible mechanisms could include the production of a nuclease or the inhibition of the synthesis of a nuclease inhibitor. In contrast, the program may involve changes in the state of the cytoplasm. In this case, destabilization of globin mRNA would require no new transcription or protein synthesis. Instead, changes in the composition [e.g., poly(A) length, capping] or subcellular location (mRNP vs. polysomal) of the mRNA might be essential.

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