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# Inducer-mediated commitment of murine erythroleukemia cells to differentiation: A multistep process

**(Friend virus/chromatin protein/dexamethasone/globin mRNA/hemin)**

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**ABSTRACT** There are a number of agents which, when added to cultures of murine erythroleukemia cells (MELC), markedly increase the probability of commitment to express the characteristics of terminal erythroid differentiation, including loss of proliferative capacity and increased accumulation of globin mRNA and hemoglobin. Some characteristics of inducer-mediated commitment of MELC to terminal erythroid differentiation were examined by determining the effects of dexamethasone (an inhibitor of inducer-mediated MELC differentiation) and of hemin (an inducer of globin mRNA accumulation). Previously, it was shown that exposure of MELC to hexamethylene-bisacetamide (HMBA) leads to commitment, detectable within 12 hr. MELC cultured with both HMBA and dexamethasone do not express commitment. MELC transferred from culture with HMBA and dexamethasone to cloning medium without these agents express commitment to terminal erythroid differentiation, indicating that MELC retain a "memory" for some early HMBA-mediated changes leading to commitment which occur even in the presence of the inhibitory steroid. The kinetics of commitment in experiments in which exposure to HMBA is interrupted, or dexamethasone is added to the culture with HMBA, suggest that there is a rate-limiting step early in the commitment process. The memory for this step persists for more than one cell cycle. Addition of hemin to cultures with HMBA and dexamethasone initiates accumulation of globin mRNA but does not reverse the steroid-mediated inhibition of terminal cell division (that is, the cells retain their proliferative capacity). Inducer-mediated MELC commitment is associated with accumulation of the chromatin protein $IP_{25}$; dexamethasone does not inhibit this accumulation. Accumulation of $IP_{25}$ may be inducer-related, but it is not sufficient to cause expression of terminal erythroid differentiation.

Murine erythroleukemia cells (MELC) are virus-transformed erythroid precursor cells that retain the capacity, under *in vitro* conditions, for self-renewal and for terminal differentiation upon exposure to any of a number of inducing agents (1–5). Till *et al.* (6), studying mouse hematopoietic stem cells, suggested that the kinetics of loss of the capacity for self-renewal and terminal differentiation are compatible with a stochastic process involving a random-event component. The kinetics of inducer-mediated MELC commitment to terminal erythroid differentiation are also compatible with a stochastic process (7, 8). The mechanism determining the probability of commitment to terminal differentiation is unknown.

Inducing agents, such as dimethyl sulfoxide ($Me_2SO$) (1) and hexamethylenebisacetamide (HMBA) (9), markedly increase the probability of the event(s) that commits MELC to terminal erythroid differentiation. Induced differentiation of MELC is characterized by the coordinated expression of the program of erythroid differentiation, including initiation of terminal cell division (7, 8), increased accumulation of $\alpha$- and $\beta$-globin mRNA (10, 11), of $\alpha$-, $\beta_{major}$-, and $\beta_{minor}$-globins and hemoglobins (12, 13), of heme synthesizing enzymes (14), and of spectrin (15). Commitment is defined operationally as the capacity of cells that have been exposed to inducer to initiate their terminal cell divisions and to express other differentiated characteristics in the absence of inducer (8) and is detectable after 12 to 16 hr of culture with inducer (8, 16, 17); the lag period in onset and the rate of commitment vary with the type and concentration of inducer.

We (8, 18) and others (19) have obtained evidence to suggest that there are inducer-mediated effects in MELC, associated with commitment, for which the cells retain a "memory" through more than one cell cycle, even if the expression of commitment is suppressed. For example, the tumor promoter, 12-*O*-tetradecanoylphorbol 13-acetate (TPA), is a potent inhibitor of HMBA-mediated MELC differentiation. MELC transferred from culture with HMBA and TPA, in which they do not express commitment, to medium without either agent will subsequently display the characteristics of terminal erythroid differentiation (18).

The present studies were designed to define further some aspects of HMBA-mediated commitment of MELC to terminal erythroid differentiation. These studies, using dexamethasone, an inhibitor of inducer-mediated differentiation (20–24), provide evidence that commitment is a multistep process. Inducer-dependent initial steps in the commitment process are not sensitive to the steroid; dexamethasone inhibits later steps associated with the initiation of terminal cell division and globin mRNA and hemoglobin accumulation. Commitment to terminal cell division involves steps distinct from the expression of globin genes; addition of hemin to MELC in culture with HMBA and dexamethasone permits the accumulation of globin mRNA but does not reverse the steroid-mediated suppression of commitment to terminal cell division.

## METHODS

MELC cell line DS19, subcloned from line 745A (provided by Charlotte Friend), was maintained in culture as described (8). Cells were grown in liquid suspension culture and diluted every 2 days to $10^5$ cells per ml in fresh medium. HMBA (9) was a gift of Charles Pfizer & Co. Cell density and benzidine-reactive cells were assayed as detailed elsewhere (25).

Commitment, characterized by limited cell division (small colony size) and accumulation of hemoglobin (benzidine-reactive cells), was assayed by transfer of MELC from liquid sus-

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Abbreviations: MELC, murine erythroleukemia cells; TPA, 12-*O*-tetradecanoylphorbol 13-acetate; HMBA, hexamethylenebisacetamide; $IP_{25}$, 25,000-dalton chromatin protein.

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pension culture with HMBA to medium containing 1.8% methylcellulose without inducer (8). Hemoglobin accumulation was measured spectrophotometrically at 535 nm. MELC ($10^5$ in 1.0 ml of saline) were mixed with 0.5 ml of 0.2% benzidine dihydrochloride and 0.5 ml of 1% $H_2O_2$ and then kept at room temperature for 30 min. Hemoglobin concentration was calculated by using a standard cyanmethemoglobin curve (Diagnostic Technology, Great Neck, NY).

Newly synthesized RNA was isolated from MELC cultured with [$^3$H]uridine (50 $\mu$Ci/ml; 1 Ci = $3.7 \times 10^{10}$ becquerels) for 2 hr at 37°C, extracted as described (26, 27), and measured by hybridization with $\alpha$-globin cDNA immobilized on nitrocellulose filters (26). The $\alpha$-globin cDNA was prepared from plasmid pCR1$\alpha$M10, the generous gift of B. Mach, and grown and prepared as described by Rougeon and Mach (28). National Institutes of Health guidelines for work with recombinant DNA were followed. The 25,000-dalton chromatin protein $IP_{25}$ was assayed in MELC nuclear protein by the method described by Keppel *et al.* (29).

## RESULTS

**Dexamethasone Suppresses HMBA-Mediated MELC Differentiation.** In preliminary experiments, it was shown that dexamethasone inhibited the accumulation of benzidine-reactive cells, hemoglobin, and $\alpha$-globin mRNA and the commitment to terminal cell division (Table 1). These findings are consistent with previous reports (21–24, 30).

**Dexamethasone Suppresses Expression of HMBA-Mediated Differentiation but Not Early Changes in the Commitment Process.** MELC were cultured in liquid suspension medium with 4 mM HMBA and, at intervals up to 72 hr, aliquots were transferred to inducer-free semisolid (methylcellulose) cloning medium without or with 4 $\mu$M dexamethasone. After 20 hr of culture with HMBA, 21% of the cells were committed; but, when dexamethasone was included in the cloning medium, <5% of the cells actually expressed their commitment (Fig. 1). After 30 hr in culture with HMBA, 52% of the cells were committed, but in the presence of dexamethasone only 6% showed that commitment. After 40 hr with HMBA, 78% of the cells were committed, but only 32% expressed it in the presence of dexamethasone. After 50 and 72 hr with HMBA, 97% and 100% of MELC were committed, respectively, and, at these times, dexamethasone had little or no inhibiting effect on expression of HMBA-mediated commitment.

In a parallel series of experiments, MELC were cultured in liquid suspension with both 4 mM HMBA and 1 $\mu$M dexamethasone; at intervals up to 72 hr, aliquots were transferred to inducer-free semisolid medium either without or with 4 $\mu$M dexamethasone (Fig. 1). Cells transferred to semisolid medium without steroid showed a progressive increase in proportion of cells expressing commitment. Those cells transferred to semisolid medium with steroid alone failed to express that commitment. These findings suggest that HMBA-induced effects that initiate the commitment process are detectable within 20 hr and, based upon earlier findings (9, 16), probably within 12 hr. Dexamethasone inhibited the expression of commitment—that is, the limitation of proliferative capacity and the accumulation of hemoglobin. That HMBA-treated cells whose expression of commitment has been suppressed by simultaneous exposure to dexamethasone do express commitment upon transfer to semisolid medium without either agent suggests that MELC acquire a "memory" for some inducer-mediated effects leading to commitment. The process of commitment in the total cell population appears to require up to 40 or 50 hr for its expression to become resistant to the inhibitory effect of dexamethasone. Taken together, those findings suggest that HMBA-mediated commitment involves multiple steps.

**Comparison of the Effect of Interrupted Exposure to HMBA with That of Adding Dexamethasone.** MELC were cultured with 5 mM HMBA for 12 hr. After this initial exposure to inducer, the cultures were interrupted, for progressively longer periods (12, 24, 36, 48, and 72 hr), by exposure either to fresh medium without inducer or to fresh medium with HMBA and dexamethasone. At the end of the interruption period, the cells were returned to fresh medium containing HMBA. All cultures were assayed for commitment at intervals throughout the experiment (Figs. 2 and 3). After the initial 12 hr in culture with inducer, 20–25% of the cells were committed. During the ensuing interruption, the proportion of cells expressing commitment decreased to <10%. This decrease in percentage of committed cells may be explained, in part, by the increase in cell number without further recruitment of cells to express terminal differentiation (Figs. 2 and 3; data for 72 hr not shown). Upon transfer of cells from medium with inducer and inhibitor into fresh medium with inducer alone, the proportion of committed cells increased within 2–4 hr. The longer the interval in culture with HMBA and dexamethasone, the greater the magnitude of this rapid increase in proportion of cells expressing commitment. This increase approached, but did not quite achieve the level of commitment in cells cultured continuously with HMBA. This rapid increase in proportion of cells expressing commitment required transfer of MELC from medium with HMBA and dexamethasone to fresh medium with HMBA; when transfer was into fresh medium without HMBA (Fig. 3), the rapid increase was not observed.

In the case of cells whose culture in HMBA was interrupted by transfer to fresh medium with neither inducer nor inhibitor, for 12, 24, 36, 48, or 72 hr, there was little rapid increase in expressed commitment during the 2–4 hr upon transfer to fresh medium after 12 and 24 hr, and none after 48 or 72 hr (Figs. 2 and 3). In these cultures, after transfer of MELC from medium without HMBA to medium with inducer, the increase in

Table 1. Effect of dexamethasone on HMBA-induced MELC differentiation

| Addition | Cell density,* no. × $10^{-6}$/ml | Benzidine reactive cells,* % | Hemoglobin,* $\mu$g/$10^6$ cells | Commitment,† % | $\alpha$-Globin mRNA,‡ % |
|---|---|---|---|---|---|
| None | 1.92 | 0.5 | 0.6 | 1 | 0.004 |
| HMBA (5 mM) | 2.12 | 99.0 | 6.7 | 98 | 0.148 |
| HMBA (5 mM) and dexamethasone (1 $\mu$M) | 2.04 | 3.0 | 1.1 | 28 | 0.027 |

* Cell density, benzidine reactive cells, and hemoglobin were determined after 120 hr of culture.

† Commitment assays were performed after 60 hr of culture. Results are % benzidine-reactive colonies.

‡ $\alpha$-Globin mRNA was assayed in cells after 65 hr of culture plus 2 hr of culture with [$^3$H]uridine (50 $\mu$Ci/ $2 \times 10^6$ cells per ml). Results are % of total cytoplasmic [$^3$H]RNA.

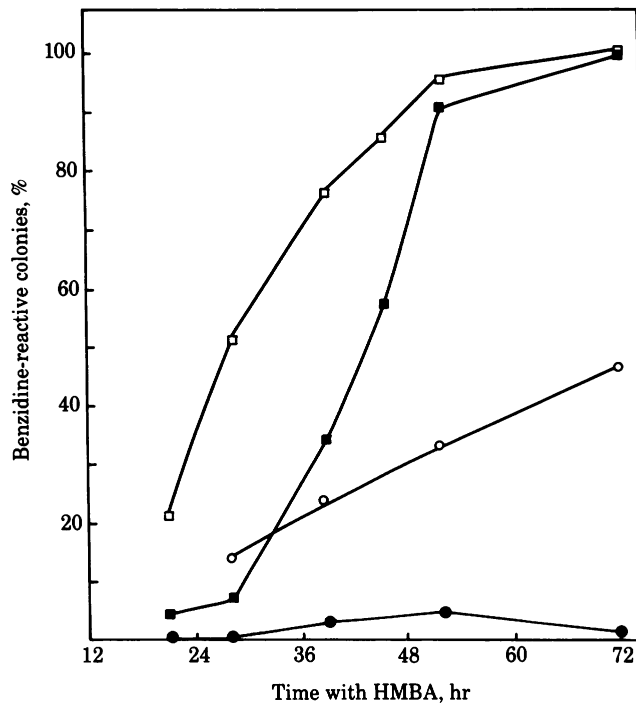


FIG. 1. Effect of dexamethasone on HMBA-mediated commitment of MELC to terminal erythroid differentiation. MELC were cultured in liquid suspension medium with 4 mM HMBA and, at the times indicated, aliquots were removed and transferred to 1.8% methylcellulose cloning medium without (□) or with (■) 4 $\mu$M dexamethasone. In parallel experiments, MELC were cultured in liquid suspension medium with 4 mM HMBA and 1 $\mu$M dexamethasone and, at the times indicated, aliquots were removed and transferred to 1.8% methylcellulose cloning medium without (○) or with (●) 4 $\mu$M dexamethasone. Commitment is expressed as % benzidine-reactive colonies.

committed cells paralleled but lagged behind that of cells cultured continuously with HMBA.

These findings are consistent with the interpretation that: (*i*) there are initial inducer-mediated changes leading to commitment which can persist for more than one cell cycle without expression of the manifestations of commitment, such as terminal cell division or hemoglobin accumulation; (*ii*) later inducer-mediated effects, involving expression of terminal cell division and hemoglobin accumulation, are reversibly suppressed by dexamethasone; and (*iii*) an early step in the commitment process appears to be rate limiting.

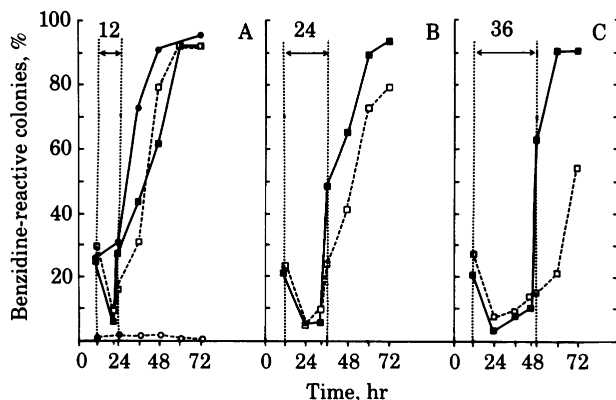


FIG. 2. Effect of interrupted exposure of MELC to HMBA on commitment. MELC were cultured with 5 mM HMBA for 12 hr, and then the culture was interrupted by a period of 12 hr (*A*), 24 hr (*B*), or 36 hr (*C*) in which culture was with either no HMBA (□) or 5 mM HMBA and 1 $\mu$M dexamethasone (■). After the period of interruption, in each case the cells were returned to fresh medium containing HMBA alone. Commitment assays were performed at each time point indicated. As controls, cells were cultured continuously without interruption, either without (○) or with HMBA (●) and assayed for commitment.

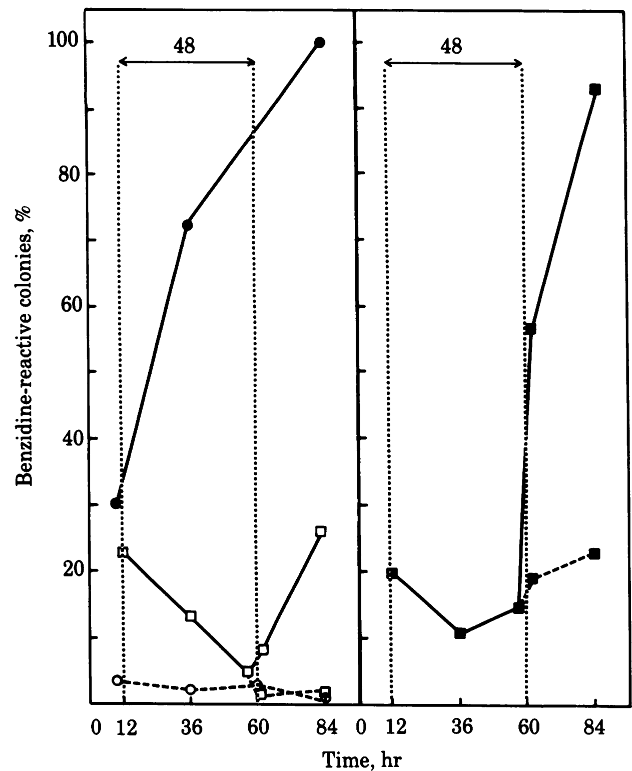


FIG. 3. Effect of interrupted exposure of MELC to HMBA on commitment. MELC were cultured with 5 mM HMBA for 12 hr and then the culture was interrupted for 48 hr by transfer to fresh medium without inducer (□; *Left*) or with HMBA and dexamethasone (■; *Right*) for 48 hr. The cells were then transferred to fresh medium either with (——) or without HMBA (----). As controls, MELC were cultured continuously with HMBA (●——●) or without HMBA (○---○). As additional controls, (data not shown), cells were cultured continuously with or without HMBA and were subjected to washing and resuspension at 12 hr and at 60 hr; the kinetics of commitment were not affected by these manipulations.

**Effect of Hemin on Dexamethasone-Suppressed Globin mRNA Accumulation and Terminal Cell Division.** Addition of hemin to MELC cultures induces an increased rate of accumulation of newly synthesized $\alpha$- and $\beta$-globin mRNA but does not initiate terminal cell division (17, 31). In the present experiments, MELC were cultured with HMBA, with HMBA and dexamethasone, with HMBA, dexamethasone, and hemin, and with hemin alone. Addition of hemin to cultures with inducer and steroid was associated with accumulation of newly synthesized $\alpha$-globin mRNA to about the same level as observed in cells cultured with HMBA alone (Table 2). Addition of hemin did not affect dexamethasone-mediated inhibition of HMBA-induced commitment to terminal cell division.

**Changes in Chromatin Protein Associated with HMBA-Mediated MELC Commitment.** Eisen and coworkers (29) reported a chromatin-associated protein, $IP_{25}$, which appears early during induced differentiation *in vitro*. They suggested that this protein plays a role in the control of proliferation and differentiation of MELC, as well as other cell types (29, 32). $IP_{25}$ appears to be identical to the chromatin protein, $H1_0$, described earlier by Panyim and Chalkley (33). To examine the relationship between the accumulation of $IP_{25}$ and inducer-mediated

Table 2. Effect of hemin on cultures in which dexamethasone has suppressed HMBA-mediated MELC differentiation

| Addition | Cell density,* no. × $10^{-6}$/ml | Commitment,† % | α-Globin [$^3$H]mRNA,‡ % |
|---|---|---|---|
| None | 1.82 | 1 | 0.007 |
| HMBA (5 mM) | 2.12 | 94 | 0.038 |
| HMBA (5 mM) and dexamethasone (1 μM) | 2.04 | 21 | 0.016 |
| HMBA and dexamethasone and hemin (0.1 M) | 1.78 | 12 | 0.034 |
| Hemin (0.1 M) | 2.00 | 3 | 0.036 |

* Assayed at 120 hr after initiation of culture.

† Assayed at 72 hr after initiation of culture.

‡ Assayed after 25 hr of incubation and then 2 hr of culture with [$^3$H]uridine (50 μCi/2 × $10^6$ cells per ml). Results are shown as % of total cytoplasmic [$^3$H]RNA.

commitment of MELC, the following experiments were performed.

MELC were cultured with 5 mM HMBA, with 5 mM HMBA and 1 μM dexamethasone, with 5 mM HMBA, 1 μM dexamethasone, and 0.1 mM hemin, or with 0.1 M hemin as the only addition. Consistent with the findings by Eisen and coworkers (29), $IP_{25}$ accumulated in cells induced with HMBA (Fig. 4). Dexamethasone did not inhibit the accumulation of $IP_{25}$, although it suppressed the expression of commitment to terminal cell division. Hemin, which did not induce terminal cell division but did induce an increased rate of accumulation of newly synthesized globin mRNA, did not induce accumulation of $IP_{25}$. In cultures with HMBA, dexamethasone, and hemin, $IP_{25}$ accumulated although the cells were not committed to terminal cell division. These studies suggest that $IP_{25}$ may reflect an inducer-mediated change in chromatin protein, but the accumulation of $IP_{25}$ is not, in itself, sufficient for expression of commitment to terminal erythroid cell differentiation.

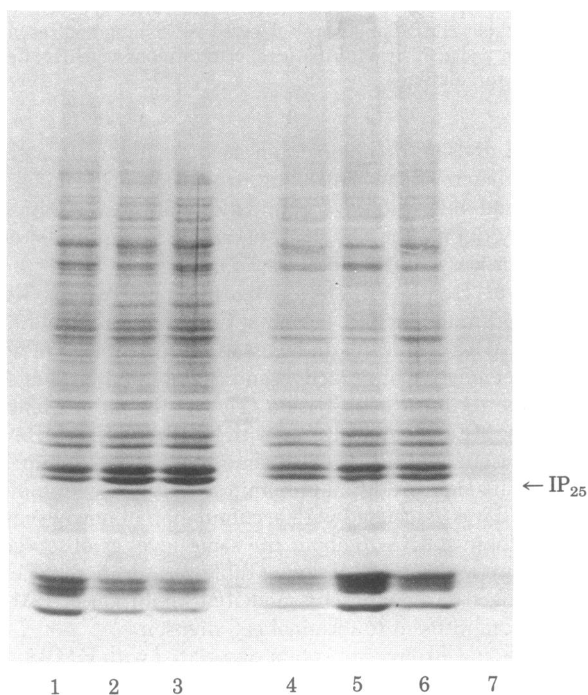


FIG. 4. Chromatin proteins, prepared from MELC cultured under various conditions, were separated by electrophoresis on a sodium dodecyl sulfate/polyacrylamide gradient gel. Lanes: 1, without addition; 2, 5 mM HMBA; 3, 5 mM HMBA and 1 μM dexamethasone; 4, 5 mM HMBA, 1 μM dexamethasone, and 0.1 M hemin; 5, 0.1 M hemin; 6, 0.1 M hemin and 5 mM HMBA; and 7, 0.1 M hemin and 1 μM dexamethasone. Gels were stained with Coomassie brilliant blue.

## DISCUSSION

These studies provide evidence that HMBA-mediated MELC commitment to terminal erythroid cell differentiation is a multistep process. The evidence includes: (*i*) the observation that some inducer-mediated changes early in the process of commitment are not suppressible by dexamethasone, whereas later steps, involving initiation of terminal cell division and globin mRNA synthesis, are sensitive to this steroid; (*ii*) the rate-limiting step in commitment appears to be in the early stage of the process; (*iii*) later steps in the expression of commitment occur relatively rapidly; and (*iv*) MELC can retain a memory for prior exposure to HMBA which can persist, without expression of terminal cell division or of the globin genes, for up to 24 hr. The molecular basis of this memory is unknown, but this inducer-mediated change is associated with an early stage of the commitment process and can persist through more than one cell cycle, because the generation time for MELC is 11–12 hr (17).

In earlier studies, the tumor promotor TPA, another potent inhibitor of inducer-mediated MELC differentiation (18), was also found to suppress terminal cell division and accumulation of newly synthesized globin mRNA and hemoglobin formation but not the memory for inducer-mediated changes that could lead to commitment. It would appear that a late step in the commitment process is the target for the suppression of terminal differentiation observed with both the tumor promoters, such as TPA, and the steroid dexamethasone. These observations do not necessarily mean that these agents act at the same molecular site.

Studies of inducer-mediated MELC differentiation (34–39) and a number of other differentiating cell systems (40–44), including normal erythropoiesis (45–47), suggest that DNA synthesis is required for the transition to expression of differentiated characteristics. For example, we found that inducer-mediated events during early S phase appear to be required for the expression of α- and β-globin genes in MELC cultured with HMBA (37, 38). Conkie *et al.* (39), working with a temperature-sensitive variant of MELC, found that, under conditions nonpermissive for cell division, MELC could not be induced to erythroid differentiation. On the other hand, it has been suggested that inducer-mediated MELC commitment is unaffected by inhibition of DNA synthesis (48, 49). These studies are difficult to interpret, owing to the fact that cells were either cultured in the presence of inducer for a protracted period prior to addition of inhibitor or that the inhibitor used, such as hydroxyurea, does not completely block DNA synthesis and does itself induce MELC differentiation (2).

The evidence for a requirement for DNA synthesis for inducer-mediated commitment of MELC may reflect the need to restructure chromatin during commitment. The present study confirms (29, 31, 32) that inducer-mediated MELC commitment is associated with a change in the accumulation of $IP_{25}$. The accumulation of this chromatin protein, however, is not sufficient for inducer-mediated expression of terminal erythroid cell differentiation. The possibility that inducer-mediated restructuring of chromatin configuration may be one step in the commitment process requires further investigation.

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