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Murine erythroleukemia cell differentiation: Relationship of globin gene expression and of prolongation of G_1 to inducer effects during G_1/early S

(erythroid cells/hexamethylenebisacetamide/cell cycle)

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ABSTRACT **Murine erythroleukemia cells (MELC) are induced to erythroid differentiation by culture with 4 mM hexamethylenebisacetamide. The relationship between onset of accumulation of globin mRNA and cell cycle phase has been examined in MELC fractionated with respect to cell cycle (G_1, early S, mid S, and late S/G_2) by the technique of centrifugal elutriation. This technique provides synchronized populations of cells without use of chemicals that block cell function (e.g., DNA synthesis). It is shown that the initial onset of accumulation of globin mRNA occurs during the first G_1 phase after a complete S traversed in culture with inducer. Once globin mRNA synthesis is initiated, it continues throughout the cell cycle. Studies of synchronized MELC populations exposed to hexamethylenebisacetamide starting in G_1, mid S, or late S/G_2, provide evidence that an effect of the inducer during G_1 or early S is critical to initiation of accumulation of newly synthesized globin mRNA and to the prolongation of G_1, both of which are associated with terminal erythroid differentiation.**

Murine erythroleukemia cells (MELC) are induced to express a program of erythroid differentiation, including various biochemical functions characteristic of normal erythropoiesis, by exposure *in vitro* to hexamethylenebisacetamide (HMBA) and other chemical agents (1). The cellular and molecular target(s) of inducer effect on MELC have yet to be elucidated although data have been adduced to support putative targets at the plasma membrane (2, 3) as well as at nuclear (chromatin) sites (4–6). A number of studies have suggested that the action of inducers of MELC differentiation may be a cell cycle-related phenomenon. Levy *et al.* (7) and McClintock and Papaconstantinou (8) demonstrated a requirement for exposure to inducer during at least one S phase in order to achieve induction of differentiation; Harrison (9) has demonstrated that MELC arrested as single cells in the presence of inducer fail to differentiate. More recently, Geller *et al.* (10) have shown that commitment to differentiate, the ability to express differentiation despite removal of the inducing agent (11, 12), is more rapidly achieved in cultures of MELC initiated with cells synchronized in the G_2 or G_1 phase of the cell cycle than in mid or late S. Previous studies in this laboratory revealed an inducer-mediated prolongation of the G_1 phase of MELC undergoing differentiation induced by HMBA or dimethyl sulfoxide (13). We have also found (14) that the initial detectable HMBA-induced increase in the rate of accumulation of newly synthesized globin mRNA coincides with the first (prolonged) G_1 phase after MELC synchronized at the G_1/S interface by the method of sequential treatment with thymidine and hydroxyurea (15) proceed through S, G_2, and M.

The present investigation was designed to evaluate the relationship between the cell cycle-related effects of inducers and the initiation of accelerated globin mRNA synthesis and of alteration in cell cycle transit, without the use of agents such as thymidine or hydroxyurea that may in themselves perturb the processes of cell differentiation (9). Geller *et al.* (10) have demonstrated that MELC may be fractionated according to their position in the cell cycle by unit gravity sedimentation, which sorts the cells according to their volume, and is directly proportional to their position between G_1 and G_2 (16). In the present studies, centrifugal elutriation has been used to achieve fractionation of MELC according to their size and cell cycle position. We have found that the initial increment in induced globin mRNA synthesis is confined to the first G_1 phase after a complete S phase traversed in the presence of HMBA. Once an accelerated rate of globin mRNA synthesis is initiated, synthesis continues through the S and G_1 phases of subsequent cell cycles. The data suggest that there is an effect of HMBA that occurs during early S phase and is critical for the initiation of globin mRNA synthesis and for the prolongation of the G_1 phase of the cell cycle.

MATERIALS AND METHODS

Cell Culture. MELC strain 745A was provided by Charlotte Friend. Our subclone, designated DS19, has been maintained in suspension culture (17) by diluting the cells twice a week at 10^5/ml in fresh medium. For experiments, cultures were inoculated at 2×10^5 cells/ml from 2-day logarithmic-phase cultures. HMBA was prepared as described (18). The proportion of hemoglobin-containing cells was determined by the benzidine reaction. Cells were deposited on a glass slide (cytocentrifuge; Shandon Southern Instruments Inc., Sewickley, PA), fixed in methanol, and stained by the alkaline benzidine/Wright/Giemsa reaction (18), and the proportion of orange-stained cells was scored. Cell number was determined with a model ZF Coulter Counter. Details of RNA labeling have been described (14). RNA was purified from cytoplasmic and nuclear fractions according to the method of Bastos and Aviv (19).

Preparation of Globin mRNA, Synthesis of [^{3}H]cDNA and cDNA-Cellulose, and Conditions for Hybridization. 9S globin mRNA was purified from DBA/2 mouse reticulocyte RNA by oligo(dT)-cellulose chromatography and sucrose gradient centrifugation as described (14, 19). Mouse globin [^{3}H]cDNA was prepared according to the procedures described by Nudel *et al.* (20). [^{3}H]dCTP [23–25 Ci/mmol (1 Ci = 3.7×10^{10} becquerels)] was used in the reverse transcriptase reaction to label the cDNA (approximately 12,000 cpm/ng). Newly synthesized globin mRNA was assayed by oligo(dT)-globin-cDNA-cellulose chromatography. Details of conditions for synthesis of the mouse globin-cDNA-cellulose and for hybridization between [^{3}H]RNA and the oligo(dT)-globin-cDNA-cellulose have been described (14).

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Abbreviations: MELC, murine erythroleukemia cells; HMBA, hexamethylenebisacetamide.

Cell Cycle Fractionation and Synchronization. The Elutriator Rotor (Beckman) was used to fractionate MELC, by countercurrent centrifugation, according to cell volume which is a measure of position in the cell division cycle, as reported (16). A maximum of 5×10^8 logarithmic phase MELC in Hanks' balanced salt solution without protein were introduced into the spinning rotor, and elutriation was accomplished at 5°C at a constant rotor speed of 2000 rpm by stepwise (2.5 ml/min) increments in flow rate from 10 to 35 ml/min. Fractions (250 ml) were collected at each flow rate, cells were recovered by brief centrifugation, and an aliquot (10^6 cells) was stained with propidium iodide and analyzed for DNA content per cell by flow microfluorometry (TPS-1 cell sorter, Coulter) (13). Fractions containing cells with 2C DNA content, determined by comparison with the DNA content distribution (flow microfluorometry pattern) of unfractionated logarithmic phase MELC, were designated G_1; cells with twice this DNA content (4C) were designated G_2; intermediate DNA content defined S phase cells. These cell cycle assignments have been confirmed by pulse labeling with [^{2}H]thymidine and radioautography. Cells designated G_1 by these criteria display a labeling index of <10%; mid-S phase cells display a labeling index of >95%. Cell fractions synchronized with respect to their position in the cell division cycle by this procedure are capable of sustained synchronized cell growth in culture for up to two cell cycles, as reported for MELC and other cell types (15, 21, 22).

RESULTS

Accumulation of Globin mRNA during HMBA-Induced Differentiation. As reported from this (20) and other laboratories (23, 24), there is a roughly 10-fold increase in globin mRNA concentration in MELC exposed for 2 days to differentiation-inducing agents such as HMBA, compared to cells cultured without inducer (Fig. 1). In order to examine the onset and kinetics of accumulation of newly synthesized globin mRNA, MELC were incubated with [^{3}H]uridine after 0, 8, 23, 28, 48, and 96 hr of culture with HMBA (Fig. 2). The rate of accumulation of newly synthesized globin mRNA (^{3}H-labeled globin mRNA per cell per 2-hr pulse of [^{3}H]uridine) increased strikingly between 8 and 23 hr of culture with HMBA, remained relatively constant from 23 to 48 hr, and then declined gradually, coincident with the progressive maturation of the differentiated MELC and the decline in total RNA synthesis that characterizes that process (1). The rate of accumulation of newly synthesized globin mRNA relative to newly synthesized total RNA (largely ribosomal RNA), in fact, increased steadily from 8 to 96 hr of culture with inducer (data not shown), suggesting that during the later stages of induced MELC differentiation the rate of ribosomal RNA synthesis declined at a faster rate than did globin mRNA synthesis.

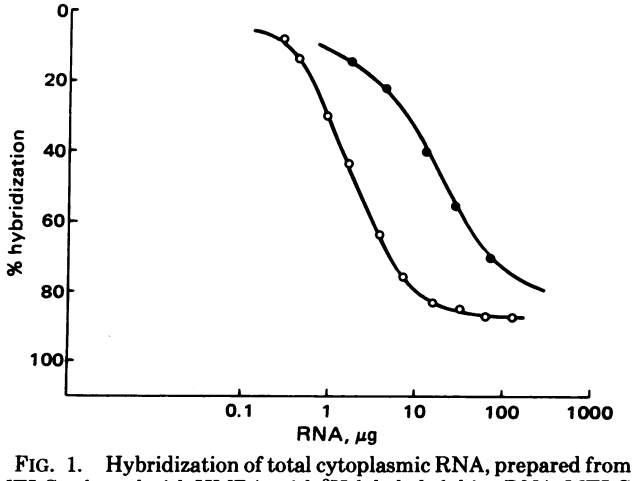

FIG. 1. Hybridization of total cytoplasmic RNA, prepared from MELC cultured with HMBA, with ^{3}H-labeled globin cDNA. MELC were cultured for 42 hr with 4 mM HMBA (○) or without HMBA (●). The relative concentration of cytoplasmic globin mRNA was determined by comparing the amounts of cytoplasmic RNA required to protect 50% of the labeled cDNA from micrococcal nuclease digestion.

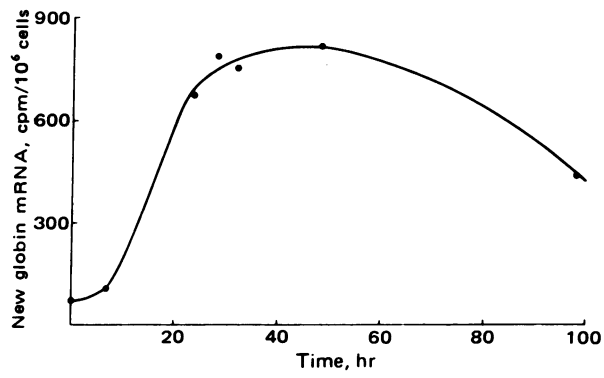

FIG. 2. Rate of accumulation of newly synthesized globin mRNA in MELC cultured with 4 mM HMBA. MELC were cultured for the times indicated with inducer, and aliquots of the culture were removed and labeled for 2 hr with [^{3}H]uridine (2×10^6 cells/ml; 50 μCi/ml; 30–40 Ci/mmol), extracted with phenol, and hybridized to oligo(dT)-globin-cDNA-cellulose. Accumulation of newly synthesized globin mRNA is expressed as cpm per 10^6 MELC retained on oligo(dT)-globin-cDNA-cellulose columns.

Onset of Accumulation of Newly Synthesized Globin mRNA in Relation to the Cell Cycle. These studies were designed to examine the time of onset of globin mRNA synthesis in HMBA-treated MELC in relation to the cell cycle. Cells were cultured in 4 mM HMBA for 12, 24, or 36 hr; at each time point, an aliquot of the culture was pulse labeled (1 hr) with [^{3}H]uridine and the labeled cells were separated, by centrifugal elutriation, into fractions corresponding to the G_1, early S, mid S, late S, and late S/G_2 phases of the cell cycle (Fig. 3). After 12 hr in culture with HMBA, an increased rate of synthesis of globin mRNA was detected in the G_1 and early S stages of the cell cycle. At later times (24 and 36 hr), the accumulation of newly synthesized globin mRNA in the cytoplasm occurred throughout the cell cycle (Table 1).

Because all cells in each of these cultures were exposed to HMBA for the same duration and each culture contained cells at all stages of the cell cycle, preferential initiation of globin mRNA synthesis in the G_1 or early S stages suggests that there may be a cell cycle-specific event critical to the process of induced differentiation and that this event may occur during a cell cycle stage before onset of globin mRNA synthesis.

Further experiments were designed to determine whether in induced differentiation there is such a critical step during a portion of the cell cycle preceding the G_1 stage. The total cell cycle time of these MELC is about 11–12 hr (10, 15, 16). Cells that were in G_1 after 12 hr of culture were largely in G_1 at the time of onset of culture with HMBA and proceeded through S, G_2, and M and then back into the G_1 during which accelerated globin mRNA synthesis was initiated. This suggests that exposure to inducer during S phase may be critical to induced differentiation.

Accumulation of Newly Synthesized Globin mRNA in Cell Cycle-Synchronized MELC. Three cell cycle fractions, corresponding to G_1, mid S, and late S/G_2, were prepared from logarithmic phase MELC by centrifugal elutriation. Each of these fractions was placed into culture with 4 mM HMBA and followed for 20 hr as each cohort of synchronized cells traversed the ensuing stages of the cell cycle (Fig. 4). Cells starting in G_1

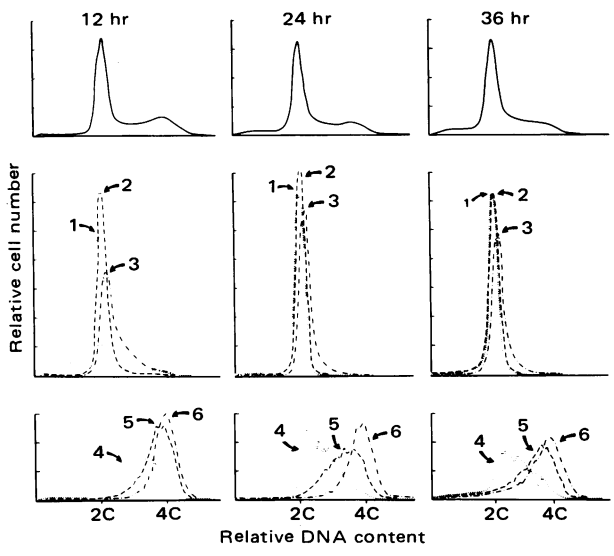

FIG. 3. Pattern of DNA content of MELC fractionated according to size, by centrifugal elutriation, after 12, 24, or 36 hr in culture with 4 mM HMBA. Relative DNA content (abscissa) was determined as relative fluorescence intensity of cells stained with propidium iodide. G_1 cells are characterized by a nominal 2C DNA content; G_2 cells have twice this DNA content (designated 4C). For each fraction, 10^5 cells were measured and the relative cell number at each level of fluorescence was plotted on the ordinate. The distribution of unfractionated MELC at each time point, according to DNA content, is displayed in the three top panels. Six fractions of increasing cell volume are shown for each time point: fraction 1, G_1 cells; fractions 2 and 3, mixed G_1 and early S; fraction 4, mid S; fractions 5 and 6, late S/G_2.

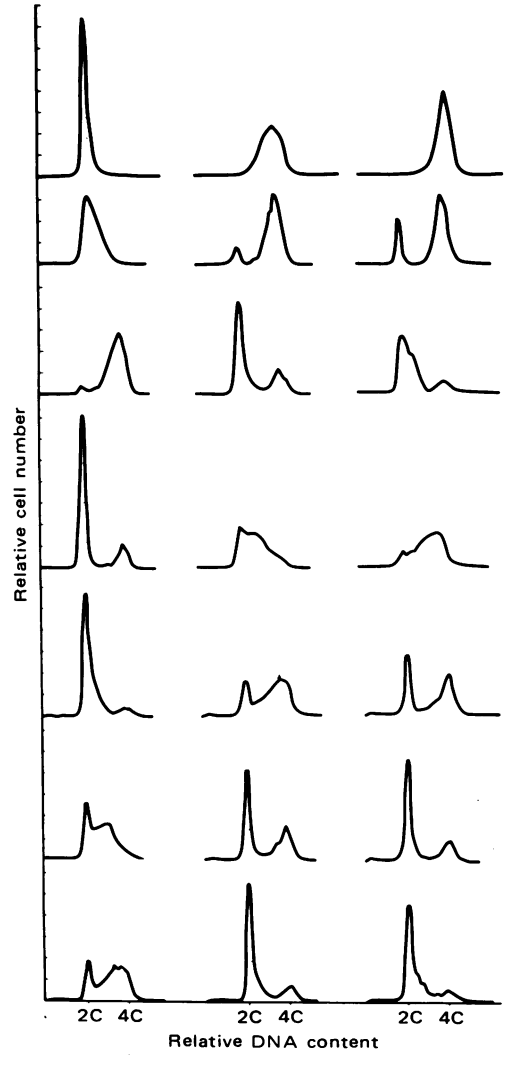

FIG. 4. Patterns of distribution of cellular DNA content in three cohorts of MELC synchronized by centrifugal elutriation and placed into culture, at 0 hr (top curve), with 4 mM HMBA. The first column represents MELC synchronized at 0 hr in G_1 phase; the second column is MELC synchronized in mid S phase; the third column is MELC synchronized in late S/G_2 phase. After 3, 7, 11, 14, 17, and 20 hr in culture with 4 mM HMBA (second through sixth curve), cells were removed for analysis of DNA content per cell as index of progression through cell cycle. The abscissa represents relative DNA content; 2C and 4C values, reflecting G_1 and G_2 cell populations, respectively, are indicated.

entered S phase by 3 hr and returned to G_1 by 11 hr (roughly one cell cycle); late S/G_2 cells entered their first G_1 by 3 hr and returned to G_1 between 14 and 17 hr; mid S cells were largely in G_1 by 7 hr and returned to G_1 at about 17 hr. The rate of accumulation of newly synthesized globin mRNA was determined for each population at sequential time points (3, 7, 11, 14, 17, and 20 hr) by incubating an aliquot of cells with [^{3}H]-uridine (50 μCi/ml) for 1 hr and analyzing extracted cytoplasmic RNA for [^{3}H]globin mRNA content. The "G_1/early S cohort" initiated globin mRNA synthesis at about 11 hr (Fig. 5). These cells had largely completed one full cell cycle and reentered G_1 (Fig. 3). The "mid-S cohort," which includes a portion of cells starting in early S as well as the mid S population (Fig. 3), displayed an increment in globin synthesis between 7 and 11 hr (the first G_1) and a second increase between 17 and 20 hr, a time when the cells again were largely in G_1. The "late S/G_2 cohort" displayed no increase in globin mRNA synthesis in their first passage through G_1 (3–7 hr); globin mRNA synthesis increased thereafter as cells reentered G_1. These observations suggest that cells must be exposed to inducer during early S phase if they are to initiate accumulation of newly synthesized globin in the subsequent G_1; after initiation, mRNA synthesis continued through both G_1 and S stages.

Table 1. Accumulation of newly synthesized globin mRNA, in relation to the cell cycle

Cell fraction*	Cell cycle stage	Globin mRNA synthesis,† cpm/10^7 MELC 12 hr	24 hr	36 hr
Unfractionated	All stages	114	210	520
1	G_1	121	100	582
2	G_1/early S	127	240	404
3	Early S	100	178	384
4	Mid S	78	199	894
5	Late S/G_2	58	305	768
6	Late S/G_2	56	250	956

* These fractions correspond to the fractions analyzed for cell cycle stage, according to DNA content, as illustrated in Fig. 3.

† Shown by duration of culture with 4 mM HMBA. Cells cultured without HMBA for up to 72 hr accumulated only 38 cpm/10^7 cells as globin mRNA.

Rates of Accumulation of Newly Synthesized Globin mRNA in Nucleus and Cytoplasm. Once accumulation of newly synthesized globin mRNA is initiated, it continues throughout the cell cycle. The following experiments were designed to determine whether the rate of accumulation of newly synthesized cytoplasmic globin mRNA during the cell cycle reflects the rate of accumulation of newly transcribed sequences in the nucleus or reflects the rate of processing of nuclear mRNA precursors. MELC were cultured for 42 hr in 4 mM HMBA, pulse labeled with [^{3}H]uridine (for 1 hr), and separated, by centrifugal elutriation, into four fractions: G_1,

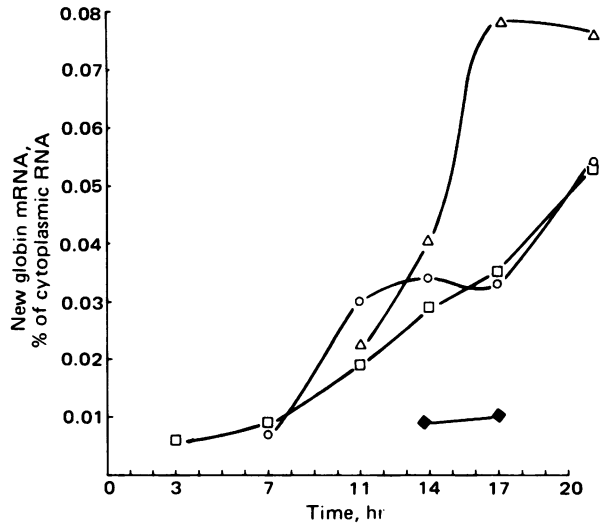

FIG. 5. Accumulation of globin mRNA in synchronized cells cultured with 4 mM HMBA; these cells were synchronized in G_1 phase (△), mid S phase (○), and late S/G_2 phase (□) as shown in Fig. 4. Also, cells were cultured without inducer synchronized in G_1 phase (◆). After 3, 7, 11, 14, 17, or 20 hr in culture with HMBA, 1.5×10^6 cells were removed and incubated in 5 ml of medium containing 4 mM HMBA and 0.3 ml of [^{3}H]uridine (30–40 Ci/mmol) for 1 hr, and the ^{3}H-labeled cytoplasmic RNA was extracted and hybridized to oligo(dT)-globin-cDNA-cellulose. Results are expressed as percentage of [^{3}H]RNA retained by the column.

early S, mid S, and late S/G_2. The ratio of newly synthesized globin mRNA to newly synthesized total RNA was relatively constant in both nuclear and cytoplasmic RNA taken from cells at each of the four stages of the cell cycle (Fig. 6). The rates of accumulation of nuclear and cytoplasmic globin mRNA sequences suggest that there is no cycle-related delay in transfer of these sequences from nucleus to cytoplasm (Table 2).

Relationship of G_1/Early S Phase of the Cell Cycle to HMBA-Mediated Prolongation of G_1. These experiments were designed to determine whether exposure to HMBA during a specific phase of the cell cycle is required for prolongation of

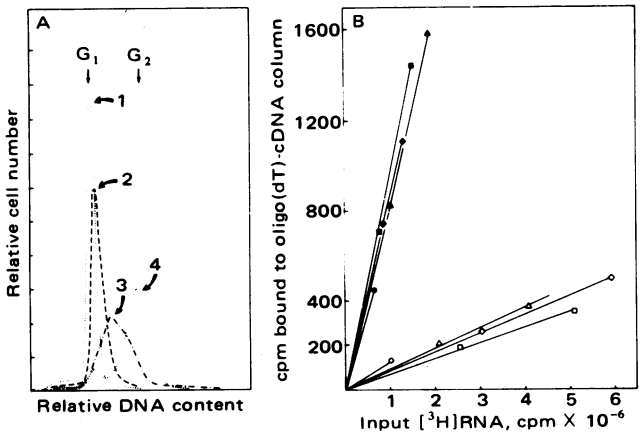

FIG. 6. (*A*) Fractionation, with respect to cell cycle, of MELC cultured for 42 hr with HMBA and then labeled for 1 hr with [^{3}H]-uridine. Curves: 1, cells in G_1 phase; 2, early S; 3, mid S; and 4, late S/G_2. (*B*) Hybridization, to oligo(dT)-globin-cDNA-cellulose, of increasing amounts of nuclear (open symbols) and cytoplasmic [^{3}H]RNA (solid symbols) extracted from MELC cultured for 42 hr with 4 mM HMBA and then labeled for 1 hr with [^{3}H]uridine (50 μCi/ml; 30–40 Ci/mmol). ○, ●, G_1 phase; □, ■, early S; ◇, ◆, mid S; △, ▲, late S/G_2.

Table 2. Accumulation of newly synthesized globin mRNA in nucleus and cytoplasm in relation to cell cycle stage of MELC cultured with 4 mM HMBA

Cell fraction*	Cell cycle stage	Globin mRNA synthesis, cpm/10^6 cells	
		Nucleus	Cytoplasm
1	G_1/early S	27.9	110
2	Early S	14.25	123
3	Mid S	31.24	344
4	Late S/G_2	35.5	221

* These cell fractions correspond to the fractions analyzed for cell cycle stage according to DNA content illustrated in Fig. 6.

the G_1 period that is associated with induced MELC differentiation by various chemical agents (10, 13, 15, 16). Cells were synchronized in G_1 and in late S/G_2 by centrifugal elutriation and then cultured without and with 4 mM HMBA, and their progress through the cell cycle was monitored by flow microfluorometry (Fig. 7). In the case of cells synchronized in G_1, the first G_1 phase after a single cell cycle through S and G_2 was prolonged. In the case of cells synchronized in late S/G_2, the first G_1 was not prolonged; (HMBA-treated and control cells move into S together from this first G_1); the first evidence of a prolonged G_1 was detected between 15 and 20 hr when the cells were in their second G_1. This suggests that the events in G_1 or early S required to achieve acceleration of globin mRNA synthesis may also be required for the expression of another characteristic of induced MELC differentiation—namely, prolongation of G_1.

DISCUSSION

These studies provide evidence that in HMBA-induced MELC differentiation an effect of the inducer during late G_1 or early S phase of the cell cycle is required for the initiation of accumulation of newly synthesized globin mRNA and for the change in cell cycle kinetics (prolonged G_1). MELC were synchronized with respect to the cell cycle by fractionation based on cell volume, thus avoiding the use of chemical inhibitors of DNA synthesis (e.g., thymidine or hydroxyurea). The present results also indicate that, once globin mRNA synthesis has commenced, it proceeds in all stages of the cell division cell (that is, both G_1 and S; the methods at present are not adequate to resolve a unique population of G_2 and M cells). No difference in the relative rates of accumulation of newly synthesized globin mRNA in nucleus and in cytoplasm among the several phases of the cell cycle was detectable; there is no evidence for cell cycle-related selective nuclear accumulation of mRNA precursors once globin mRNA synthesis is established. Newly synthesized globin mRNA formed during early stages of induced MELC has a half-life of >48 hr—i.e., stable for approximately three cell cycles (25, 26).

Both the prolongation of G_1 (13) and the onset of accelerated globin mRNA synthesis during that prolonged G_1 (14) in HMBA-treated MELC appear to be the consequence of a critical, inducer-mediated event that occurs during the preceding late G_1 or early S phase. Cells synchronized in late S/G_2 and exposed to HMBA display a delay in the onset of both these features of induced differentiation, suggesting that they are not induced to differentiate until they have traversed a full cell cycle, including the critical G_1/early S, in the presence of inducer. This suggests that both the expression of erythroid cell-specific genes (e.g., globin genes) and functions that govern the terminal cell divisions in terminally differentiating cell are regulated by events that affect G_1 or the early part of S phase of the cell cycle. The present data do not permit more precise localization of these effects in terms of cell cycle events.

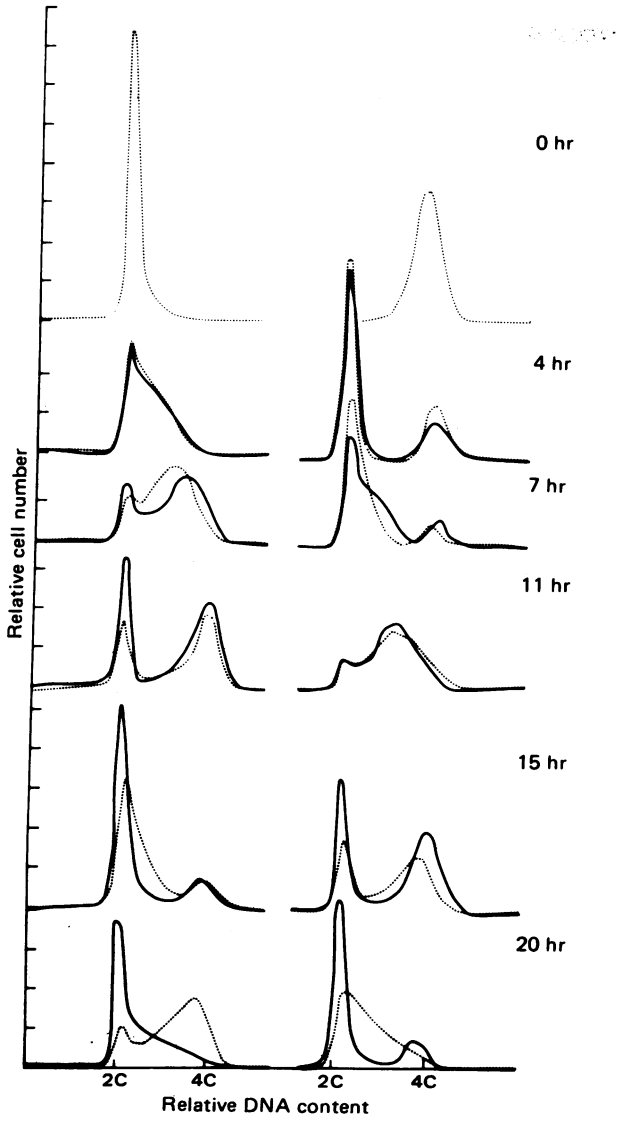

FIG. 7. Patterns of distribution of cellular DNA content in two cohorts of MELC synchronized and placed into culture at 0 hr without (dotted line) and with (solid line) 4 mM HMBA. The first column represents MELC synchronized in G_1 phase; the second column, MELC synchronized in late S/G_2. Progression of the cells through the cell cycle was monitored by flow microfluorometry at the times indicated (see legend to Fig. 3).

These studies are consistent with previous observations that implicate cell cycle-related events in the process of induced differentiation and of alteration in growth rate of cells induced to terminal differentiation (7–10). Whether inducers exercise a direct effect on a structure or function related to early replicating portions of the MELC genome or exert their primary influence by means of changes in cytoplasmic functions, such as cyclic nucleotide metabolism, cannot be distinguished at this time (1).

Several previous reports also point to the possibility that a portion of the genome that replicates during early S plays a critical role in regulation of growth rate and expression of differentiated characteristics. Brown and Schildkraut (15) have recently observed that unifilar substitution of bromodeoxyuridine for thymidine in DNA during early S phase inhibits the growth and differentiation of MELC subsequently exposed to dimethyl sulfoxide. It has been demonstrated that incorporation of bromodeoxyuridine into early, but not late, replicating DNA inhibits growth of HeLa cell (27) and hamster cells (28) in culture. Fusion of myoblasts (29) and the synthesis of certain enzymes in mouse L cells (30) are inhibited under conditions of bromodeoxyuridine substitution into early, but not late, replicating DNA. Taken together, these studies collectively focus attention on events occuring during the late G_1 or early S phase of the cell division cycle as a basis for further study of the mechanism of action of differentiation inducing agents.

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