c-myc Down Regulation and Precommitment in HL-60 Cells Due to Bromodeoxyuridine

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ABSTRACT

HL-60 human nonlymphocytic leukemia cells undergo terminal differentiation along either the myeloid or monocytic pathway in a process previously shown to involve two sequential steps, early events leading to a precommitment state and late events leading to onset of terminal differentiation. The present report shows that bromodeoxyuridine induces the early events leading to precommitment. In this course bromodeoxyuridine causes the rapid down regulation of the c-myc protooncogene. The course is similar to other common inducers of HL-60 differentiation including retinoic acid, dimethyl sulfoxide, 1,25-dihydroxyvitamin D3, and sodium butyrate. HL-60 cells which were initially exponentially proliferating were exposed to 10 μM bromodeoxyuridine for 24 h, a period corresponding to one division cycle in these cells. When the cells were subsequently exposed to either retinoic acid or 1,25-dihydroxyvitamin D, onset of G8n specific growth arrest and display of the differentiated phenotype occurred within 24 h. This is in contrast to the 48-h exposure needed for onset of terminal differentiation if either inducer is used singly during continuous exposure, as has been reported previously. Thus bromodeoxyuridine consummated the early events, including the rapid down regulation of c-myc message levels, which occur during the first division cycle of the induced cellular metabolic cascade leading to onset of terminal differentiation. The ability of bromodeoxyuridine to drive events in the metabolic cascade leading to onset of terminal differentiation was specific for early events, inasmuch as it was relatively ineffective at driving late events. Down regulation of c-myc was not in itself sufficient to result in subsequent terminal differentiation, since pulse exposure to bromodeoxyuridine followed by culture in inducer free medium resulted in little G8n specific growth arrest or phenotypic differentiation. Continuous exposure to bromodeoxyuridine, in contrast, resulted in significant G8n specific growth arrest but little phenotypic differentiation, indicating that the regulation of cell cycle transit and differentiation are separable.

INTRODUCTION

Control of terminal cell differentiation can be studied using the human nonlymphocytic leukemia cell line, HL-60, as an in vitro model (1). These cells have become an archetype model for studying terminal differentiation in vitro. Originally isolated from the peripheral blood of a patient with acute promyelocytic leukemia, later retrospectively reevaluated to be French-American-British M2 versus the original French-American-British M3 classification (2), HL-60 cells terminally differentiate along either the myeloid or monocytic lineages in response to various chemically defined and biologically derived inducer agents (3-6). For example, retinoic acid induces myeloid differentiation, whereas 1,25-dihydroxyvitamin D3 induces monocytic differentiation. The cells are thus an uncommitted precursor cell (9). Although still largely unknown, the metabolic cascade leading from this proliferatively active cell to its terminally differentiated myeloid or monocytic descendants appears to have certain features. The induced metabolic cascade leading to onset of terminal differentiation occurs over a period corresponding to two division cycles, during which the inducer must be continuously present (7). This process occurs in two steps involving an intermediate regulatory state called the “precommitment state” (8-11). The essential “early events,” defined as those occurring over the first division cycle and leading to precommitment, appear to be the same whether the cell ultimately undergoes myeloid or monocytic differentiation. In contrast, “late events,” defined as those occurring over the second division cycle and leading from precommitment to onset of terminal differentiation, specify the explicit differentiation lineage as either myeloid or monocytic. Early events thus respond to an initial inducer signal to prime a cell to differentiate without regard to lineage specificity, while late events respond to the subsequent signal to determine which terminally differentiated phenotype is displayed.

Evidence has accumulated to indicate that the seminal cellular processes eliciting early events is S-phase specific. In the cases of retinoic acid and DMSO,2 both inducers of myeloid differentiation, a kinetic analysis of cellular response to inducers indicates an S phase specificity to the initial cellular response to inducer (7, 12). Consistent with this, various agents specifically affecting nucleotide metabolism and DNA synthesis have also been found to induce a precommitment like state or differentiation (13-19). Gene amplification has also been implicated to be involved in the early events (13). In this case, it was found that a subcytotoxic pulse and release treatment with hydroxyurea consummated early events resulting in precommitment. This same hydroxyurea pulse exposure and release had been shown previously to cause cellular resistance to methotrexate through amplifying the dihydrofolate reductase gene in rodent cells (20). Significantly, the c-myc protooncogene, which has been implicated with a role in regulating cell growth and differentiation, has been found to be down regulated early in response to various inducers of differentiation (21, 22). This down regulation may thus be essential to early events leading to precommitment. This picture of control of HL-60 cell differentiation has been pieced together from a variety of studies utilizing various different agents applied to HL-60 cells. If indeed early events (a) are elicited through S-phase specific cellular events, (b) involve gene amplification, (c) occur without specificity for the ultimate differentiation lineage elected, and (d) require down regulation of c-myc, then it might be expected that early events can be elicited by a single agent which has S phase specificity and the capability to induce gene amplification, primes cells to differentiate along either the myeloid or monocytic lineages, and causes early down regulation of c-myc. The existence of such an agent thus provides a test of this hypothesized model of control of terminal differentiation. This report shows that bromodeoxyuridine, an S-phase specific agent (23) previously implicated with inducing gene amplification (24), can elicit early events which prime cells for myeloid or monocytic differentiation and causes the early down regulation of c-myc.

1 Supported in part by grants from the USPHS (NIH), American Institute for Cancer Research, Council for Tobacco Research, Cornell Biotechnology Program, and Cornell BRSG.

2 The abbreviations used are: DMSO, dimethyl sulfoxide; poly(A), polyadenylate; oligo(dT), oligodeoxynucleotide.
MATERIALS AND METHODS

Cells and Culture Conditions. HL-60 human leukemia cells originally from the laboratory of Dr. R. Gallo were defrosted and initiated in culture within the past year. The cells were maintained as described previously in constant exponential growth using RPMI 16400 with 10% heat inactivated fetal calf serum (25). The capability of the cells to undergo terminal differentiation in response to retinoic acid, DMSO, 1,25-dihydroxyvitamin D3, sodium butyrate, 12-0-tetra-decanoylphorbol-13-acetate was verified.

For experimental cultures, exponentially proliferating cells were initiated in culture with new medium at an initial cell density of 0.2 x 10^6 cells/ml with 20 ml/75-cm² flask. For cultures treated with 10^-4 M retinoic acid, the retinoic acid was added from a 10^-3 M stock made in ethanol and stored protected from light at -20°C. For cultures treated with 10^-4 M, 1,25-dihydroxyvitamin D3, the 1,25-dihydroxyvitamin D3 was added from a 10^-3 M stock made in propylene glycol and stored protected from light at -20°C. 1,25-Dihydroxyvitamin D3 was a generous gift of Dr. Milan Uskokovic, Hoffman-LaRoche Inc., Nutley, NJ. For cells treated with 10^-6 M bromoodeoxyuridine, the bromodeoxyuridine was added from a 10 MM stock made in medium. As reported before (9), carrier blanks had no effect on cell proliferation or differentiation. For cultures treated initially with 1,25-dihydroxyvitamin D3 prior to another agent, the cells were grown in 75-cm² flasks which were inverted to obviate cell adherence (26). In experiments where culture conditions were shifted, the cells were washed twice in an equal volume of serum supplemented culture medium and then reintroduced in the new culture conditions preserving the same cell density at the time of the shift. The indicated agent was then added to the new cultures. For cultures subsequently treated with 1,25-dihydroxyvitamin D3, cells were cultured in 100-mm bacteriological Petri dishes to obviate cell adherence, using 5 ml/plate. In the case of cultures treated first with bromodeoxyuridine for 24 h and then maintained in culture medium only for a subsequent 24 h before treatment with retinoic acid or bromoodeoxyuridine, cultures were diluted to a cell density of 0.5 x 10^6 cells/ml at the time of addition of the final agent to avoid potential growth inhibitory effects due to eventual high cell density of the cultures. To generate cells used for isolation of poly(A) containing RNA, cell cultures were initiated at a density of 0.4 x 10^6 cells/ml with 20 ml/75-cm² flask. For cultures treated with 10^-6 M retinoic acid, onset of G1/O specific growth arrest and functional differentiation, measured by induction of refractory oxidative metabolism characteristic of differentiated cells, was less than 0.05. The mixture was then added to a column which had been equilibrated with 0.01 M Tris-HCl buffer. The binding buffer was 0.5 M NaCl-0.01 M Tris-HCl (pH 7.5). This was repeated 3-4 times until the supernatant was clear and its A_260 was less than 0.05. The mixture was then added to a column which was allowed to run dry. RNA was eluted from the column by adding 3 ml of elution buffer to the column. The 3 ml of eluate were collected, using a 15-ml polypropylene tube, to which 2 volumes (6 ml) of 100% ethanol and 0.1 volume (90 ml) sodium acetate were added. The RNA was allowed to precipitate over at least 24 h at -20°C. It was precipitated by centrifugation at 9500 rpm for 20 min at 0°C. The RNA was resuspended in double distilled water for quantitation. The RNA was then immobilized on nitrocellulose paper, probed with a 1.8-kilobase CiaI-EcoRI subclone of the human c-myc third exon (Oncor, Gaithersburg, MD) which was nick-translated (kit; Bethesda Research Laboratories), as described previously (31), using standard procedures (32). Autoradiographs of the blots were densitometrically scanned (Chromoscan; Joyce-Lobel, Ltd.) with computer driven background correction and normalization using autoradiograph exposures which were nonsaturated at the highest RNA concentrations, as described before (31). The computer implemented traces are shown.

RESULTS

Myeloid and Monocytic Differentiation. Retinoic acid is well known to induce the terminal myeloid differentiation of HL-60 cells. When initially exponentially proliferating cells were continuously exposed to 10^-6 M retinoic acid, onset of G1/O specific growth arrest and functional differentiation, measured by inducible oxidative metabolism, occurred at 48 h, a period corresponding to two division cycles in these cells (25). The percentage of G1/O growth arrested and functionally differentiated cells increased progressively thereafter. Fig. 1 (top) shows the population size, percentage of cells with G1 DNA, and percentage of cells capable of inducible oxidative metabolism, a hallmark of mature myeloid or monocytic cells, as a function of time during continuous exposure to retinoic acid. Population size is shown as multiples of the starting cell density. The percentage of cells with G1 DNA was determined from flow cytometrically derived DNA histograms of the cell population. Incidence of G1/O specific growth arrest would be evidenced as an enrichment in the percentage. If the cells were exposed to 1,25-dihydroxyvitamin D3, an inducer of monocytic terminal differentiation, instead of retinoic acid, then onset of G1/O specific growth arrest and functional differentiation likewise occurred at 48 h. Fig. 1 (bottom) shows the population size, percentage of cells with G1 DNA, and percentage of cells capable of oxidative metabolism for cells continuously exposed to 10^-4 M 1,25-dihydroxyvitamin D3. The induced appearance of cell surface differentiation specific markers characteristic of myeloid or monocytic differentiation generally paralleled the
Fig. 1. Top, HL-60 cells continuously exposed to 10^{-8} M retinoic acid. Upper left: N(t)/N(0), population size shown as multiples of the original population size (0.2 x 10^6 cells/ml); %GI, percentage of cells with G1 DNA content; %SO(+), percentage of cells capable of phorbol myristate acetate induced cellular superoxide production characteristic of functionally mature cells, all as a function of time during exposure to inducer. Upper right: Mo1(%), Mo2(%), My4(%), percentage of cells expressing the Mo1, Mo2, or My4 cell surface differentiation specific markers respectively as a function of time during exposure to inducer. Bottom, HL-60 cells continuously exposed to 10^{-8} M 1,25-dihydroxyvitamin D_3.

occurrence of growth arrest and functional differentiation. Fig. 1 also shows the percentage of cells expressing Mo1, Mo2, and My4 as a function of time during exposure to retinoic acid or 1,25-dihydroxyvitamin D_3. As has been shown previously (9), the myeloid and monocytic differentiation lineages can be distinguished by the expression pattern of such markers. In particular, among the cell surface markers, Mo1, Mo2, and My4, retinoic acid characteristically induced Mo1 expression during myeloid differentiation, while 1,25-dihydroxyvitamin D_3 induced Mo2 and My4 expression, as well as Mo1, during monocytic differentiation.

Bromodeoxyuridine Induced Precommitment. Bromodeoxyuridine induces the early events needed in the metabolic cascade leading over 48 h to the onset of terminal myeloid differentiation. Cells exposed first to 10 \textmu M bromodeoxyuridine for 24 h (one division cycle), washed, and then exposed to 10^{-8} M retinoic acid exhibited onset of terminal differentiation only 24 h after exposure to retinoic acid. Fig. 2 shows the proliferation and functional differentiation of these cells. Proliferation and differentiation were assayed as described above for the cases of retinoic acid or 1,25-dihydroxyvitamin D_3 shown in Fig. 1. Onset of G_{1/0} specific growth arrest occurred at 48 h, i.e., 24 h after exposure to retinoic acid. Likewise onset of functional differentiation, occurred at 48 h. The percentage of growth arrested and functionally differentiated cells progressively increased thereafter. Thus cells treated with bromodeoxyuridine and then retinoic acid had kinetics of growth arrest and differentiation (Fig. 2) similar to those of cells continuously treated
with retinoic acid only (Fig. 1). In the first 24 h of the metabolic cascade leading to terminal myeloid differentiation, bromodeoxyuridine and retinoic acid thus induced cellular events which were apparently equivalent. The bromodeoxyuridine induced early events are also those needed in the metabolic cascade leading to terminal monocytic differentiation. In experiments analogous to those above, cells exposed to bromodeoxyuridine for 24 h, washed, and then exposed to 1,25-dihydroxyvitamin D3 exhibited onset of terminal monocytic differentiation at 48 h which was 24 h after exposure to 1,25-dihydroxyvitamin D3. Fig. 3 shows the proliferation and differentiation of these cells as a function of time during treatment with bromodeoxyuridine and then 1,25-dihydroxyvitamin D3. While the retinoic acid treated cells described in Fig. 2 express only the Mo1 cell surface determinant, consistent with their myeloid lineage (Fig. 2), the 1,25-dihydroxyvitamin D3 treated cells express Mo2 and My4, as well as Mo1, consistent with their monocytic lineage (Fig. 3). Table 1 shows differential counts of the morphological assessment of the cells at 96 h for the bromodeoxyuridine/retinoic acid and bromodeoxyuridine/1,25-dihydroxyvitamin D3 treated cells, confirming the differentiation lineages indicated by the cell surface markers. Thus in the first 24 h of the metabolic cascade leading to terminal differentiation, bromodeoxyuridine induced early events which were apparently equivalent to those induced by 1,25-dihydroxyvitamin D3, as well as by retinoic acid. Bromodeoxyuridine could thus consummate the early events needed for either subsequent myeloid or monocytic differentiation. The
differentiation lineage was specified late in the metabolic cascade by the character of the second agent. Bromodeoxyuridine had thus induced the early events leading to a precommitment state.

Cells retain a memory of the bromodeoxyuridine induced early events which prime or "precommit" them to terminal differentiation. HL-60 cells were cultured in bromodeoxyuridine for 24 h as before, washed, and recultured in medium free of bromodeoxyuridine for 24 h, and then retinoic acid was added to the medium. Fig. 4 shows the proliferative cell cycle distribution and functional differentiation of the cells during this time. Onset of G1/0 specific growth arrest and functional differentiation occurred by 24 h after the addition of retinoic acid. This is in contrast to the 48 h usually required for onset of terminal differentiation when cells were continuously exposed to retinoic acid without a previous exposure to bromodeoxyuridine. A similar result occurred when 1,25-dihydroxyvitamin D3 was used in lieu of retinoic acid. Fig. 5 shows the results in this case. In the case of retinoic acid, the Mo1 marker was expressed; whereas in the case of 1,25-dihydroxyvitamin D3, the Mo2 and My4 markers were expressed as well as Mo1. Onset of the induced expression of the markers occurred 24 h after addition of the retinoic acid or 1,25-dihydroxyvitamin D3, consistent with the time of onset of functional differentiation measured by superoxide production. Thus cells retained a memory of the bromodeoxyuridine induced precommitment state such that they required only an abbreviated period of subsequent exposure to induce for onset of terminal differentiation.

The pulse exposure to bromodeoxyuridine by itself induced little subsequent G1/0 specific growth arrest or differentiation. Fig. 6 (left) shows the proliferation and functional differentiation of cells cultured for 24 h in bromodeoxyuridine as before, washed, and then recultured in bromodeoxyuridine free medium. Fig. 6 (right) shows the expression of Mo1, Mo2, and My4 cell surface markers. Most cells failed to show any G1/0 specific growth arrest or differentiation, although a few showed weak expression of Mo1. Thus the pulse exposure to bromodeoxyuridine used above was ineffective in itself for inducing terminal differentiation in these cells.

Continuous exposure to bromodeoxyuridine, however, did induce G1/0 specific growth arrest but with little differentiation. Fig. 7 (left) shows the proliferation and functional differentiation of cells continuously cultured in bromodeoxyuridine. Fig. 7 (right) shows the expression of the Mo1, Mo2, and My4 cell surface markers during this time. There was G1/0 specific growth arrest as evidenced by the enrichment in the relative number of G1 DNA cells but little differentiation as measured by incidence of cellular superoxide production or display of the differentiation specific cell surface markers. Thus in this case growth arrest had been uncoupled from expression of differentiation specific functional or cell surface markers. Morphological assessment of the bromodeoxyuridine pulse or continuously treated cells confirms that most cells failed to differentiate, although a few showed progress along the monocytic lineage (Table 2). However, the differentiation of these few cells was apparently incomplete since they failed to display the Mo2 and My4 markers found above and shown previously (9) to be characteristic of monocytic differentiation.

While bromodeoxyuridine is effective at inducing early events leading to precommitment, it is relatively ineffective at inducing late events leading from precommitment to onset of terminal differentiation. HL-60 cells were cultured in the presence of

| BrdUrd/RA | 53 | 31 | 2 | 37 | 10 | 20 | 33 |
| BrdUrd/D3 |   |   |  |   |   |   |   |

Fig. 4. HL-60 cells exposed to $10^4$ M bromodeoxyuridine (BrdU) for 24 h, subsequently released in culture medium (o) for 24 h, and thereafter exposed to $10^{-8}$ M retinoic acid (RA). %G1, %SO(+), Mo1(%), Mo2(%), My4(%) as defined in legend to Fig. 1.
Fig. 5. HL-60 cells exposed to $10 \times 10^{-6}$ M bromodeoxyuridine (BrdU) for 24 h, subsequently released in culture medium (o) for 24 h, and thereafter exposed to $10^{-6}$ M 1,25-dihydroxyvitamin D$_3$ (D$_3$). %G1, %SO(+), Mo1(%), Mo2(%), My4(%) as defined in legend to Fig. 1.

Fig. 6. HL-60 cells exposed to $10 \times 10^{-6}$ M bromodeoxyuridine (BrdU) for 24 h and subsequently released in culture medium (o). N(o)/N(O), %G1, %SO(+), Mo1(%), Mo2(%), My4(%) as defined in legend to Fig. 1.
either $10^{-6}$ M retinoic acid or 1,25-dihydroxyvitamin D$_3$ for 24 h, washed, and then recultured in the presence of bromodeoxyuridine. Table 3 shows the percentage of cells with G$_1$ DNA, capable of superoxide production, and expressing the Mo1, Mo2, and My4 cell surface markers at 72 and 96 h. By 96 h there was some G$_{1/0}$ specific growth arrest, as evidenced by an enrichment in the number of G$_1$ DNA cells, but little differentiation assessed by the functional and cell surface markers. This is consistent with the capability of continuous bromodeoxyuridine treatment to induce G$_{1/0}$ specific growth arrest observed above. Thus the capability of bromodeoxyuridine to induce elements of the metabolic cascade leading to expression of differentiation was specific for early events.

**Down Regulation of c-myc.** Bromodeoxyuridine causes a down regulation of c-myc transcription, the course of which over 12 h is similar to that caused by other inducers of differentiation. Cells were cultured in bromodeoxyuridine as before and harvested at 0, 6, 12, and 24 h. Poly(A) containing RNA was isolated using oligo(dT) affinity columns and subject to dot blot hybridization analysis, using a 1.8-kilobase ClaI EcoRI probe specific for the c-myc third exon. Autoradiographs of the dot blots were densitometrically scanned with a microprocessor based automatic scanner. Fig. 8 shows the resulting densitometric trace of the autoradiogram, indicating the relative c-myc specific message abundance. There was a progressive decline in relative c-myc specific message abundance over 12 h, which persisted at 24 h. This down regulation is similar to that seen in cells continuously treated with $10^{-6}$ M retinoic acid. In this case, cells were treated with $10^{-6}$ M retinoic acid, as before, and poly(A) containing RNA was likewise isolated. The densitometric scan of the autoradiogram resulting from the dot blot

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**Table 2** Morphological assessment (96 h) of cells treated with bromodeoxyuridine (BrdUrd) continuously or from 0 to 24 h

<table>
<thead>
<tr>
<th></th>
<th>Monocyte</th>
<th>Promonocyte</th>
<th>Monoblast</th>
<th>Promyelocyte</th>
<th>Myelocyte</th>
<th>Metamyelocyte</th>
<th>Band form</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>BrdUrd</strong></td>
<td>8</td>
<td>16</td>
<td></td>
<td>2</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>BrdUrd/G0</strong></td>
<td>4</td>
<td>8</td>
<td></td>
<td>8</td>
<td>88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*G0, culture medium (from 24 to 96 h after BrdU).*

**Table 3** Cells treated with retinoic acid from 0–24 h and bromodeoxyuridine (BrdUrd) thereafter

<table>
<thead>
<tr>
<th></th>
<th>% GI</th>
<th>% SO</th>
<th>% Mo1</th>
<th>% Mo2</th>
<th>% My4</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>RA/BrdUrd</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>58</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>96 h</td>
<td>73</td>
<td>13</td>
<td>17</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td><strong>D$_3$/BrdUrd</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>72 h</td>
<td>61</td>
<td>9</td>
<td>11</td>
<td>1</td>
<td>3</td>
</tr>
<tr>
<td>96 h</td>
<td>77</td>
<td>16</td>
<td>20</td>
<td>0</td>
<td>8</td>
</tr>
</tbody>
</table>

*For definitions of abbreviations, see legend to Fig. 1. D$_3$, 1,25-dihydroxy vitamin D$_3$.
hybridization analysis of this poly(A) containing RNA using the same c-myc specific probe as above is also shown in Fig. 8. A similar down regulation over the initial 12 h also occurred in cells treated with 10^{-6} M 1,25-dihydroxyvitamin D_{3}. In the case of 1,25-dihydroxyvitamin D_{3}, the down regulation of c-myc observed at 12 h did not persist at 24 h as it did for bromodeoxyuridine and retinoic acid. Nevertheless over the initial 12 h bromodeoxyuridine; the myeloid inducer, retinoic acid; and the monocytic inducer, 1,25-dihydroxyvitamin D_{3}; all induced a similar down regulation of c-myc. The down regulation of c-myc induced by bromodeoxyuridine is also similar to that caused by the myeloid inducer (1.25%) DMSO or the monocytic inducer (10^{-3} M) sodium butyrate. There was a similar down regulation of c-myc observed over the initial 12 h in all cases. Thus during the early events antecedent to precommitment, bromodeoxyuridine induces a similar pattern of c-myc regulation as two inducers of myeloid differentiation and two inducers of monocytic differentiation. c-myc down regulation may thus be an essential feature of early events in the metabolic cascade leading ultimately to terminal differentiation.

**DISCUSSION**

The present results show that bromodeoxyuridine induces the early events needed in the metabolic cascade leading to onset of either terminal myeloid or monocytic differentiation in HL-60 cells. Like other inducers of HL-60 myeloid or monocytic differentiation, bromodeoxyuridine causes the down regulation of c-myc message levels. While bromodeoxyuridine is able to consummate early events and induce the precommitment state, it is relatively ineffective at eliciting late events leading from precommitment to onset of terminal differentiation. The results support the hypothesis that early events in the metabolic cascade leading to precommitment do not have differentiation lineage specificity and involve a similar down regulation of c-myc for either differentiation lineage.

Previous studies on the induced metabolic cascade leading to the onset of terminal differentiation have shown that the process involves an intermediate regulatory state called precommitment (8, 14). The precommitment state thereby segregates the induced metabolic cascade into two steps: early events leading to precommitment and late events leading from precommitment to the onset of terminal differentiation. The character of the essential processes comprising the early events remains largely obscure. On the basis of cell kinetic analysis of cellular sensitivity to inducer, it has been hypothesized that the seminal cellular event initiating the cascade of early events is S-phase specific (7, 12, 25). The S phase specificity of bromodeoxyuridine supports this S-phase specific initiation. It has been shown before that bromodeoxyuridine continuously applied over a long period (7 days) will result in a modest level of HL-60 differentiation (33). In this case thymidine kinase negative mutants of HL-60 failed to respond to bromodeoxyuridine, indicating that S-phase specific incorporation was essential. The present findings extend those results and suggest that a partial explanation of the previous findings of weakly induced differentiation were due to the relative inefficiency of bromodeoxyuridine at eliciting late events compared to early events.

How bromodeoxyuridine effects the needed early events leading to precommitment is not clear. Earlier work has shown that a pulse and release treatment with a subcytotoxic dose of hydroxyurea could induce precommitment (14). Such cells were thus primed to undergo either myeloid or monocytic differentiation. It had been shown previously that this protocol of hydroxyurea treatment would induce gene amplification (20). In particular it had been found that the incidence of methotrexate drug resistance could be promoted by amplification of the gene for dihydrofolate reductase resulting from this hydroxyurea treatment. One hypothesis that has been advanced for the mechanisms of this is that the hydroxyurea pulse and release caused the overreplication of genes normally replicated in early S phase. Bromodeoxyuridine has also been found to be able to induce gene amplification in other cell systems (24). Of significant relevance, it has also been found that the capability of bromodeoxyuridine to modulate induced differentiation of erythroleukemia cells requires its incorporation into DNA sequences replicated during early S phase (23). The two agents thus appear to also have in common the ability to affect genes replicated in early S phase. The ability of two agents associated with inducing gene amplification to effect the needed early events leading to precommitment suggests that gene amplifi-
cation may be a component of these events.

One feature of the bromodeoxyuridine induced precommitment is that memory of this priming for differentiation persists for a further division cycle in the absence of bromodeoxyuridine. The uniliarly substituted of bromodeoxyuridine in all cells resulting from the original exposure for one division cycle will thus have been diluted to only one-half of the cells after the second division cycle in the absence of bromodeoxyuridine. Thus the persistence of precommitment memory appears not to be dependent upon the uniliarly substitution by bromodeoxyuridine, but rather on events resulting from it.

A priori one might not expect bromodeoxyuridine to modulate c-myc message levels. However, the finding that bromodeoxyuridine can consummate needed early events leading to onset of terminal differentiation. The capability of bromodeoxyuridine to down regulate c-myc is thus a test of the hypothesis that down regulation of c-myc is an essential element of early events. The observation that other inducers of either myeloid or monocytic differentiation cause an early down regulation similar to that induced by bromodeoxyuridine is consistent with this hypothesis.

It is noteworthy that the down regulation of c-myc by bromodeoxyuridine as well as by retinoic acid is sustained at 24 h in the HL-60 cells studied. A variety of HL-60 cell sublines have been reported on (34). Some of them can differ markedly in their response to the same inducing agent (35). The variety of HL-60 sublines currently outstanding allows one to compare the responses of different sublines to advantage. In particular if two sublines which both undergo differentiation differ in some feature of a molecular response, then the disposition of that response is not likely to be essential to deriving terminal differentiation. We have previously reported on another subline of HL-60 cells which underwent myeloid and monocytic differentiation with kinetics indistinguishable from the present subline. In those cells, there was an early down regulation of c-myc followed by a rebound up (31, 34). The elevated c-myc level which was apparent at 24 h after addition of inducer persisted before finally declining when the cells were largely G1o arrested and differentiated at 96 h. A similar rebound phenomenon for c-myc has been observed in Friend erythroleukemia cells induced to differentiate terminally (36), where there was likewise an initial down regulation of c-myc followed by a rebound up at approximately 24 h and a final decline when the cells were largely differentiated. In the present HL-60 subline reported on there was no rebound at 24 h. Since these cells also subsequently differentiated, it appears that the rebound is not an essential feature of the metabolic cascade leading to terminal differentiation. Among the HL-60 sublines studied and the erythroleukeemia cells, the consistently appearing feature of c-myc regulation is its initial down regulation. This feature is common to the bromodeoxyuridine, retinoic acid, DMSO, 1,25-dihydroxyvitamin D3, and sodium butyrate induced responses observed here. It also suggests that the apparent up regulation observed at 24 h in the case of 1,25-dihydroxyvitamin D3 is not of regulatory significance compared to the initial down regulation. Relevant to these considerations, it has been found using a panel of HL-60 sublines that the initial level of c-myc expression did not influence cellular capability to differentiate (37). This and the above suggest that it is not the absolute level of c-myc expression, but rather its change, i.e., reduction in this case, which is of regulatory significance as perceived by the cell. Finally, since the pulse exposure to bromodeoxyuridine caused c-myc down regulation but did not by itself elicit subsequent terminal differentiation, down regulation of c-myc is by itself not sufficient to induce differentiation. This is consistent with its hypothesized role as a component of early events within a basically two step metabolic cascade consisting of early and late events leading to onset of terminal differentiation.

ACKNOWLEDGMENTS

We are grateful to Mary Galles for technical assistance, Phyllis R. Dague and Amy Pellegrino for secretarial assistance in the preparation of the manuscript, and Ed Dougherty for assistance in preparing figures.

REFERENCES

20. Mariani, B. D., and Schimke, R. T. Gene amplification in a single cell cycle
c-myc Down Regulation and Precommitment in HL-60 Cells Due to Bromodeoxyuridine

Andrew Yen and Mary E. Forbes

Cancer Res 1990;50:1411-1420.

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