Recently, a variety of growth factor-dependent subclones of the murine interleukin-3 (IL-3)-dependent cell line 32D have been isolated. These subclones include those dependent for growth on erythropoietin (Epo) (32D Epo), granulocyte-macrophage colony-stimulating factor (GM-CSF) (32D GM), or granulocyte colony-stimulating factor (G-CSF) (32D G). 32D Epo 1.1 is a revertant of 32D Epo and is capable of growing in IL-3. These cell lines express the differentiation program appropriate to the specific growth factor and depend on the growth factors not only for proliferation but also for survival. To determine how the signal for proliferation is triggered by various growth factors, we examined the DNA histograms and the expression of cell cycle-specific genes in the different cell lines. The cell cycle-specific genes analyzed were myc (early G1), myb (late G1), and the structural genes for the calcium-binding protein 2A9 (middle G1) and histone H3 (G1-S boundary). The DNA histogram analysis of cells in the logarithmic phase of growth showed that approximately 40% of 32D, 32D GM, 32D G, and 32D Epo 1.1 (growing in IL-3) were cells with a 2N DNA content (and therefore in G0/G1), and another 40% have a DNA content intermediate between 2N and 4N (in S phase). In contrast, 32D Epo and 32D Epo 1.1 (growing in Epo) had fewer cells in the G0/G1 phase of the cell cycle compared with the number of cells that were in the S phase (19% to 31% v 69% to 78%, respectively). Because all the cell lines have comparable doubling times (15 to 18 hours), the cell distribution among the phases of the cell cycle is proportional to the length of the phase. Therefore, cells growing in IL-3 (32D and 32D Epo 1.1) spend relatively less time in G0/G1, and corresponding more time in S. These data were confirmed by the analysis of the tritiated thymidine (H-TdR) suicide rate and of the expression of cell cycle-specific genes. The 32D and 32D Epo 1.1 cells growing in IL-3 had a suicide rate of ≤50%, whereas the suicide rate of 32D Epo and 32D Epo 1.1 growing in Epo was higher than 75%. All the cell lines expressed comparable levels of myc and myb, whereas 32D Epo expressed undetectable levels of 2A9 and increased levels of histone H3. These results indicate that cells may progress along the cell cycle according to different pathways depending on which growth factor triggers the process. The prolonged S phase induced by Epo and described here could provide a cellular mechanism for the high H-TdR suicide rate previously reported for normal murine and human Epo-dependent erythroid progenitors.

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study in the same genetic background proliferation and differen-
tiation induced by the different hematopoietic growth
factors. In this study we have used the cell lines to determine
how different growth factors signal proliferation and how the
cells respond.

The results indicate that although IL-3, GM-CSF, and G-
CSF trigger similar proliferation progression, Epo sustains a
“unique” progression characterized by a very short Go/G1
phase and, consequently, a longer S phase. These data are
consistent with the high 3H-TdR suicide rate previously ob-
erved for Epo-responsive hematopoietic cells6,7 and suggest
that untransformed cells may choose between alternative cell
cycle progressions, depending on the growth factor that trig-
gers the proliferation event.

MATERIALS AND METHODS

Cell lines. The 32D c3 cell line8 and its Epo-, GM-CSF-, or G-
CSF-dependent subclones9,10 were maintained by biweekly passage
in McCoy’s medium (GIBCO, Grand Island, NY) modified as de-
scribed, and supplemented with antibiotics, L-glutamine, and py-
ruvic acid (1% vol/vol; GIBCO), fetal bovine and horse serums (10%,
vol/vol of each; Hyclone Laboratories, Logan, UT), and the appro-
priate growth factor (described below). The 32D Epo1.1 is an IL-3-
dependent revertant of 32D Epo, which can respond to either Epo
or IL-3.12 All cell lines were periodically tested for the presence
of mycoplasma contamination with Mycoplasma T.C.11 (Genprobe, San
Diego, CA) and were found to be mycoplasma free.

Cell cycle analysis and measurement of 3H-TdR suicide rate.
DNA histograms of cells in the logarithmic phase of growth
were obtained by staining them with propidium iodide as de-
scribed.13 The 3H-TdR suicide rate was measured by incubating at
37°C for 20 minutes, 2 X 10^3 32D, 32D Epo1.1, or 32D Epo cells
(growing in the appropriate growth factors), either in medium alone
or in medium containing 5 to 10 µCi 3H-TdR (Amer sham, Arlington
Heights, IL), specific activity, 5 to 20 Ci/mmol) or 10 µCi of 3H-
Tdr plus 100 nmol of cold Tdr (500 X excess).17 At the end of the
incubation, the cells were extensively washed and the number of
cells able to form colonies evaluated in semisolid culture as described.18

Northern analysis. RNA was prepared from phenol-chloroform ex-
traction of acid guanidinium thiocyanate (lysatess).19 RNA, dissol
in MOPS buffer (20 mmol/L MOPS, 5 mmol/L EDTA, 5 mmol/L sodium
acetate, pH 7.0) containing 50% formamide and 1.9 mol/L sodium
formate, was heated at 65°C for 15 minutes, supplemented with
1 µg of ethidium bromide, and loaded onto a 1% agarose gel in
MOPS buffer containing 6.6 mol/L formaldehyde. The gel was run
at 30 V for 18 hours. After the gel was blotted to a nitrocellulose
membrane, hybridization was performed in 4 X SSPE, 50% forma-
mide, 0.47% Denhardt, 10% dextran sulphate, 0.1% sodium dodecyl
sulfate (SDS), 0.34% milk, and 180 µg/mL salmon sperm DNA at
42°C for 18 hours. After washing with 0.1 X SSC, 0.1% SDS at
50°C, the filters were hybridized with the probes described below that
had been labeled to a specific activity of 10^6 cpm/µg DNA with (a-32P)
deoxyctydine triphosphate using a random oligonucleotide priming
kit (Boehringer, Mannheim, Germany) as recommended by the
manufacturer.

Probes. To identify kinases that could be selectively associated
with the IL-3, GM-CSF, G-CSF, or Epo receptor, we analyzed by
Northern analysis the expression in these cell lines of two classes of
protein kinases: genes with homology with the src family of protein
kinases, including csk, hck, lyn, lyn, fgr, fes,13 and src28 (provided by
Dr Roger Perlmutter, University of Washington), and the serine-
threonine kinase, pem-121 (provided by Dr Anton Berns, Netherlands
Cancer Institute). We also analyzed the expression of the oncogenes
myc and myb (nuclear factors, provided by Dr Gianni Rovera, Wistar
Institute), histone H3 (H3),22 2A924 (a gene coding for a calcium-
binding protein), thymidine kinase (TK),25 and the β subunit of DNA
polymerase (PCNA)26 (provided by Dr B. Calabretta, Thomas Jef-
Ferson University, Philadelphia, PA). As controls of mRNA loading,
the gel was stained with ethidium bromide to determine the intensity
of the ribosomal RNA, and the membrane was hybridized with a
bovine β-actin probe.27

Growth factors. Conditioned medium of phytohemagglutinin
(PhA)-stimulated LBRM 33 cells was prepared under serum-deprived
conditions as described.28 The growth factors studied included pure
recombinant murine IL-3 and murine GM-CSF (provided by J.J.
Mermod, Glaxo, Basel, Switzerland), and pure recombinant human
Epo and human G-CSF (Amgen, Thousand Oaks, CA). Unless oth-
ewise stated, PHA-LBRM 33-conditioned medium was used at a
concentration of 3% (vol/vol). The growth factors were used at con-
centrations of 10 U/mL for IL-3, 1 ng/mL for GM-CSF, 10^4 U/mL
for G-CSF, and 1 U/mL for Epo.

RESULTS

Cell cycle progression of the subclones of 32D. The cell
cycle progression of the various cell lines induced by their
respective growth factors was analyzed by DNA histogram
of the cells in log growth phase. The results are presented in
Fig 1 and Table 1. As a marker of DNA content, we used
bone marrow cells from syngeneic mice. Usually, the peaks
of fluorescencce corresponding to a DNA content 2N (G0 +
G1) or 4N (G2 + M) were readablel detected (Fig 1, top
and bottom panels). In contrast, 32D Epo and 32D Epo1.1
growing in Epo (Fig 1 and Table 1), had fewer cells with a 2N
DNA content, whereas most of the cells had a fluorescence
intensity corresponding to a DNA content between 2N and
4N.

The cell distribution among the phases of the cycle is pro-
portional to the length of the phase. Because all the cell lines
have comparable doubling times (15 to 18 hours),19 we can
deduce the relative length of the phases of the cycle in the
different cell lines from the cellular distribution in the DNA
histogram. The cells growing in IL-3, GM-CSF, or G-CSF
showed a cell cycle phase distribution consistent with that
described for other IL-3-dependent cell lines15 as well as for
fibroblasts and T cells.12 In contrast, 32D Epo cells spend
relatively more time in S than in G0–G1.

In subsequent experiments, we determined the effect of
growth factor starvation and suboptimal IL-3 and Epo
concentrations on the proliferation of 32D, 32D Epo1.1, and
32D Epo cells. Within 3 hours, growth factor starvation
induced the appearance of dead (trypan blue-positive) cells in
the culture. The amount of mRNA per cell also decreased
(from 14 X 10^-6 µg/cell to 9.6 and 1.8 X 10^-6 µg/cell at 3
and 6 hours after growth factor had been withdrawn, respec-
tively). Twenty-four hours after the withdrawal of growth
factor, more than 50% of the cells in the culture were dead;
the cells that were still alive contained a very low amount of
mRNA (0.3 X 10^-6 µg/cell). After 24 hours of growth factor
starvation, a slight increase in the number of cells in the G0/
G1 (from 55% to 70%) phase of the cycle was observed for
32D cells. The cellular distribution of 32D Epo and Epo1.1
cells among the phases of the cell cycle did not change after
growth factor starvation.

Suboptimal concentrations of IL-3 or Epo did not affect
the length of the cycle or the distribution of the cells in the
cycle during the logarithmic phase of growth but did affect the maximal cell concentration (saturation density) the culture supported (results not shown).

The \( ^3 \text{H}-\text{TdR} \) suicide rate was also measured in 32D cells (cloned in IL-3), in 32D Epo1.1 (cloned in IL-3 or in Epo), and in 32D Epo (cloned in Epo). The results are presented in Table 2. Whereas 32D and 32D Epo1.1 (cloned in IL-3) had a \( ^3 \text{H}-\text{TdR} \) suicide rate of 46% to 57%, 32D Epo and 32D Epo1.1 (cloned in Epo) had a \( ^3 \text{H}-\text{TdR} \) suicide rate of 76% to 99%.

**Expression of cell cycle markers in the different clones of 32D.** To further compare the differences in cell cycle progression found with the DNA histogram, we performed Northern analysis to determine the expression of genes that are usually activated at specific stages of the cell cycle and that are considered cell cycle markers.

All the cell lines expressed myc and myb (Figs 2 and 3 and Table 3) at reasonably comparable levels. The 32D Epo (Fig 4) and 32D Epo1.1 growing in Epo (not shown) did not express detectable levels of 2A9 but did express high levels of H3. These data indicate that very few 32D Epo cells are in middle G1, in agreement with the DNA histogram analysis. No changes were observed in the levels of 2A9 and H3 mRNA in cells starved for 24 hours of growth factor or growing at suboptimal growth factor concentrations (results not shown). TK and PCNA, genes usually expressed at very low levels, were not detected (data not shown).

As shown in Fig 2, lane 3, and in Fig 4, lane 2, the expression of the \( \beta \)-actin gene was lower in the 32D Epo cell line. This is likely because \( \beta \)-actin is also relatively cell cycle-dependent being more abundantly expressed in G1.7

**Expression of protein kinases in the different subclones of 32D.** The IL-3, GM-CSF, G-CSF, and Epo receptors belong to the hematopoietic growth factor receptor superfamily. One of the peculiarities of this family of receptors is the lack of protein kinase domain(s) in the intracellular region. However, stimulation of responsive cell lines with these growth factors is followed by rapid (within 1 minute) phosphorylation of several different proteins.29-33 Therefore, it is postulated that protein kinases are closely associated in a receptor complex form with the receptors of this family.

In these experiments, the only tyrosine kinase detectable was the 3.0-kb transcript for lyn (Fig 5). All the cell lines expressed detectable levels of pim-1 (Fig 2). The level of expression of pim-1 varied greatly between the cell lines and was barely detectable in 32D (Fig 2, lane 1). The highest levels of expression were observed in 32D Ro (a GM-CSF-responsive version of 32D with a mast cell phenotype34) (Fig 2, lane 2) and 32D GM (Fig 2, lane 6). This indicates that the expression of the pim-1 gene could be upregulated together with activation of the GM-CSF receptor.

**DISCUSSION**

Epo-dependent cell lines, such as DA-13 and HCD-57,4 have been used to study the mechanisms by which Epo induces proliferation of erythroid cells. These cell lines require Epo to progress from the G1 to the S phase of the cell cycle, the relative lengths of the phases being very similar to those observed in the IL-3-dependent cell lines C-63' and FDCP-1.6 However, the HCD-57 and DA-1 cell lines are transformed and differentiate very poorly in vitro.

We have used several growth factor-dependent cell lines to study the cell cycle progression induced by IL-3, GM-CSF, G-CSF, and Epo. With the appropriate target, IL-3, GM-

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**Table 1. Cell Cycle Analysis of the Different Clones of 32D**

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Growth Factor</th>
<th>G1 (%)</th>
<th>G2+M (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>32D</td>
<td>IL-3</td>
<td>55 ± 14</td>
<td>36 ± 13</td>
</tr>
<tr>
<td>32D Epo</td>
<td>Epo</td>
<td>19 ± 6</td>
<td>76 ± 81</td>
</tr>
<tr>
<td>32D Epo1.1</td>
<td>IL-3</td>
<td>53 ± 15</td>
<td>32 ± 13</td>
</tr>
<tr>
<td>32D Epo1.1</td>
<td>Epo</td>
<td>18 ± 8</td>
<td>78 ± 81</td>
</tr>
<tr>
<td>32D GM</td>
<td>GM-CSF</td>
<td>33 ± 2</td>
<td>58 ± 4</td>
</tr>
<tr>
<td>32D G</td>
<td>G-CSF</td>
<td>42 ± 4</td>
<td>49 ± 9</td>
</tr>
</tbody>
</table>

The phase cycle values represent the percent of total cells analyzed. Mean ± SD of three separate experiments with the exception of 32D Epo in IL-3 and 32D G for which data obtained in a single experiment are presented. The data are significantly different (\( P < .025 \), \( P < .01 \)) from those obtained with 32D cells by a paired t-test analysis.
Table 2. \(^{3}H\)-TdR Suicide Rate in the 32D, 32D Epo1.1, and 32D Epo Cell Lines

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Growth Factor</th>
<th>(^{3}H)-Thymidine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>None</td>
<td>5 (\mu)Ci</td>
</tr>
<tr>
<td>32D</td>
<td>IL-3</td>
<td>2,180 ± 110</td>
</tr>
<tr>
<td>32D</td>
<td>Epo1.1</td>
<td>279 ± 2</td>
</tr>
<tr>
<td>32D</td>
<td>Epo</td>
<td>1,245 ± 200</td>
</tr>
<tr>
<td>32D</td>
<td>G</td>
<td>846 ± 34</td>
</tr>
</tbody>
</table>

Values are colonies per 10⁴ cells.

CSF, and G-CSF induced a cell cycle progression similar to that described for the C-63 and FDCP-1 cell lines, as analyzed by DNA histogram. In contrast, Epo induced a specific cell cycle progression characterized by a shorter G₁/G₀ phase of the cycle and by a correspondingly longer S phase. This Epo-induced cell cycle pattern is similar to the cell cycle progression observed for purified CFU-E in vitro and explains why CFU-E exhibit such a high \(^{3}H\)-TdR suicide index.

The DNA histogram analysis was confirmed by the analysis of the \(^{3}H\)-TdR suicide rate (Table 2) and of the expression of cell cycle-specific genes (Table 3). Some of the genes investigated (myc and myb) are also proto-oncogenes involved in the control of hematopoietic cell proliferation. Myc is expressed specifically in early G₁ and is required for cells to enter S phase. In fact, progression of cells in S phase is induced in fibroblasts by activating ectopic myc expression and is inhibited in T cells by exposing them to myc antisense oligomers. Myb seems to be important in the proliferation of adult progenitors of all types because myb antisense oligomers inhibit the in vitro growth and differentiation of both adult erythroid bursts and GM colonies, and transgenic mice lacking this gene have impaired fetal-adult but normal embryonic hematopoiesis. Downmodulation of myb expression could be a prerequisite for both erythroid and myeloid differentiation because its expression decreases during these processes and its ectopic expression blocks the Epo-induced differentiation in an erythroleukemic cell line.

All the cell lines studied here expressed reasonably comparable levels of myc and myb (Table 3). We did not find the expected downregulation of myb expression in the 32D Epo and G cell lines. Such a downmodulation could have been masked by the different relative ratios between proliferating and differentiated cells in the cell populations investigated.
Table 3. Expression of Cell Cycle Markers and Protein Kinase Genes in the Different Clones of 32D

<table>
<thead>
<tr>
<th>Cell Cycle Phase</th>
<th>Markers</th>
<th>32D</th>
<th>32D Epo</th>
<th>32D GM</th>
<th>32D G</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1 early</td>
<td>myc</td>
<td>45</td>
<td>59</td>
<td>39</td>
<td>30</td>
</tr>
<tr>
<td>G1 middle</td>
<td>2A9</td>
<td>216</td>
<td>160</td>
<td>240</td>
<td></td>
</tr>
<tr>
<td>G1 late</td>
<td>mmy</td>
<td>16</td>
<td>16</td>
<td>19</td>
<td>36</td>
</tr>
<tr>
<td>G1/S boundary</td>
<td>H3</td>
<td>65</td>
<td>190</td>
<td>99</td>
<td>70</td>
</tr>
<tr>
<td>G2</td>
<td>PCNA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S</td>
<td>TK</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Not related to cell cycle</td>
<td>pim-1</td>
<td>12</td>
<td>16</td>
<td>63</td>
<td>20</td>
</tr>
<tr>
<td></td>
<td>lyn</td>
<td>60</td>
<td>100</td>
<td>40</td>
<td>50</td>
</tr>
</tbody>
</table>

The levels of expression are given as a percent of the level of expression of β-actin. Because of the different exposure times and the different hybridization affinities of the probes, the results should be compared only within each probe. The exposure times were: actin, 2 hours; H3, 5 hours; mmy, 2 days; lyn and pim-1, 3 days; mmy, 5 days; 2A9, 10 days. Where numerical values are absent, the results were below detectable levels.

The pattern of progression through the cell cycle seems to be the result of stimulation with Epo and not an intrinsic property of the erythroid differentiation program. In fact, the IL-3-responsive revertant, 32D Epo 1.1, growing in IL-3 progressed along the cycle in a fashion similar to the parent 32D cell line despite the fact that the cells maintained their erythroid phenotype. Therefore, the control of cell cycle progression induced by Epo occurs through a signal transduction pathway at least partially uncoupled from that of the differentiation program. A similar dissociation between induction of differentiation and induction of proliferation has been described for M-CSF in macrophages and may represent a general feature of late-acting hematopoietic growth factors.

Cell proliferation does not occur until the cells reach a critical size. Such an increase in cell mass occurs usually in G1. Because the total length of the cycle in 32D Epo 1.1 did not change when the cells were stimulated with IL-3 or Epo, the functions that lead to increased cellular mass in these cells had to be completed in G1 when the cells were growing in IL-3 or in S when the cells were stimulated by Epo. This aspect, as well as other aspects of cell cycle regulation, could be investigated further by synchronizing the cells in some fashion. We have tried to synchronize the cells by growth factor starvation, growing the cells in suboptimal growth factor concentrations, and size fractionation by cell sorting. In the absence of IL-3 or Epo, the cells seem to die, independent of the phase of the cycle in which they are, and the presence of suboptimal growth factor concentrations affects the maximal saturation density but not the progression along the cell cycle. Furthermore, we have also failed to synchronize the cells by size fractionation because these cells not only proliferate but also differentiate in culture. Therefore, size differences also reflect different stages of differentiation.

To characterize partially the signal transduction pathways that could differentiate cells growing in Epo from cells growing in IL-3, GM-CSF, or G-CSF, we analyzed the expression in these cell lines of protein kinases believed to be involved in signal transduction in hematopoiesis. We found that all the cell lines expressed the tyrosine kinase lyn and the serine-threonine kinase pim-1. These data are consistent with the recent studies of Yi et al. and Stanley et al. who have shown that lyn is expressed in normal cells derived from murine BFU-E and GM-CFC and in the FDCP-1 cell line. Interestingly, the expression of pim-1 was increased in cells that had acquired the potential to respond to GM-CSF. This fact suggests that the regulation of the pim-1 gene is coupled with the regulation of the GM-CSF receptor.

Immortalized fibroblasts infected and therefore transformed by adenovirus have a cell cycle progression similar to that described here for 32D Epo growing in Epo. Until now, this pattern of cell cycle progression was interpreted to be a result of the transformation process and therefore of the altered proliferation control in these cells. Our data suggest that this cell cycle progression pattern represents an alternative proliferation pathway and that the cells switch the pathways.
used according to the growth factor that delivers the proliferation signal.

Until now, cell cycle progression has been mainly studied with cell cycle mutants in yeast. Such studies have confirmed that cell cycle progression requires the interaction between two classes of proteins, cdc2 and cyclins. Recently, eukaryotic cdc23 and cyclin32 genes specific for the hematopoietic system have been cloned. Of the three cyclin genes cloned from a macrophage library, one is particularly involved in the induction of proliferation induced by M-CSF. It is possible that different cyclins could mediate the response to different growth factors. For the present, we do not have any data on the expression of the cdc2 and cyclin genes in our cell lines. However, we propose that these cells could represent a model to study lineage-specific interactions between different cyclin and cdc2 genes important in hematopoiesis.

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REFERENCES

EPO-SPECIFIC CELL CYCLE PROGRESSION


47. Liu HT, Baserga R, Mercer WE: Adenovirus type 2 activates cell cycle-dependent genes that are a subset of those activated by serum. Mol Cell Biol 5:2936, 1985


Erythropoietin-specific cell cycle progression in erythroid subclones of the interleukin-3-dependent cell line 32D [published erratum appears in Blood 1993 Jun 1;81(11):3168]

Y Shimada, G Migliaccio, H Ralph, AR Migliaccio and H$[corrected to Ralph]