Translational regulation plays an important role in development. In terminally differentiating cells a decrease in translation rate is common, although the regulatory mechanisms are unknown. We utilized 32Dcl3 myeloblast cells to investigate translational regulation during granulocyte colony-stimulating factor (G-CSF)-induced differentiation. G-CSF causes a significant decrease in translation rate compared with interleukin-3, which is a mitogen for these cells. Although these two cytokines exhibit modest differences in their effect on translation factor phosphorylation, they exhibit dramatic differences in their effect on ribosomal abundance and ribosomal DNA transcription. However, because both cytokines stimulate cell cycling, G-CSF induces a dissociation of ribosomal biogenesis from cell cycle progression. This uncoupling of ribosomal biogenesis from cell cycle progression appears to be closely related to the transmission of a differentiation signal, because it is not observed in cells expressing a carboxyl-terminally truncated G-CSF receptor, which supports proliferation but not differentiation of these cells. Because a similar event occurs early in differentiation of murine erythroleukemic cells, this suggests that ribosomal content is a common target of differentiating agents.

Translational regulation of gene expression plays an important role in development and is the dominant means of regulating gene expression early in development (1–3). In terminally differentiating cells a decrease in translation rate is also common, occurring in species ranging from Dictyostelium (4) to humans (5). In murine erythroleukemic (MEL) cells a decrease in translation is an effect of virtually every differentiation-inducing agent (6, 7), and this precedes the earliest evidence of differentiation (8), suggesting that this change may play a role in the alterations in gene expression that occur during differentiation. Consistent with this possibility, in both MEL and HL-60 cells this translational change has selective effects upon individual mRNAs (5, 8). However, the mechanisms that regulate translation during terminal cell differentiation are largely unknown.

In cells exposed to mitogens, translation is primarily regulated by phosphorylation of translation factors (9). Regulation of the m7GTP "cap" recognition is mediated by phosphorylation of the cap-binding protein, eIF4E, as well as the eIF4E-binding protein, 4E-BP or PHAS (10). Cap-dependent translation has been proposed to be important for the expression of mRNAs with stable secondary structure in their 5′-untranslated regions (10), and a number of mRNAs whose translation have been demonstrated to be affected by eIF4E are involved in cell cycle progression (11–13). Consistent with this, microinjection of eIF4E into quiescent cells stimulates their entry into S phase, and overexpression of this protein is transforming (14–17). Phosphorylation of ribosomal protein S6 is also common following mitogenic exposure (18). This has been proposed to enhance translation of mRNAs that contain a 5′-terminal oligopyrimidine tract (19, 20), of which ribosomal protein mRNAs are the primary example (21). Microinjection of antibodies to the S6 kinase, p70(60k), inhibits entry of cells into S phase (22), suggesting that S6 phosphorylation also plays a role in cell cycle progression. More recent data suggest that this kinase may also play a role in regulating cell size (23).

In previous studies, we have provided evidence that translation in differentiating MEL cells utilizes distinct regulatory mechanisms from those employed during mitogenic signaling (8). In these cells, inducers of differentiation cause a 50–70% decrease in translation rate in the face of constitutive phosphorylations of PHAS I and eIF4E. Inducers also do not affect S6 phosphorylation, which is undetectable in MEL cells under normal growth conditions. We have recently provided evidence that S6 phosphorylation is suppressed in a lineage-specific manner in hematopoietic cells by an S6 phosphatase. Although these results do not identify the specific mechanism responsible for translational repression in differentiating cells, they do provide evidence that this is not mediated by the signaling pathways that regulate phosphorylation of PHAS I (phosphoinositol 3-kinase and mTOR (mammalian target of rapamycin)), eIF4E (the ERK and p38 kinases), or S6 (phosphoinositol 3-kinase, mTOR, and ERKs) for review of this regulation see Refs. 18 and 24).

The myeloid cell line, 32Dcl3, provides a useful system for
analyzing the signaling pathways required for differentiation. These cells grow continuously in IL-3 but differentiate into granulocytes over a time course of 1–2 weeks when grown in G-CSF (25). The mechanism(s) responsible for the differential effect of these two cytokines is unknown. Several lines of evidence suggest that the carboxyl terminus of the G-CSF receptor is necessary for transducing a differentiation signal (26). Naturally occurring mutations that delete this region cause severe congenital neutropenia with a high frequency of conversion to acute myeloid leukemia (27). When similar receptor mutants are expressed in hematopoietic cell lines, sustained activation occurs in response to G-CSF, presumably because of defective receptor internalization (28, 29). In addition, 32D cells expressing this truncated receptor do not differentiate but proliferate indefinitely in response to G-CSF (30). Carboxyl-terminal truncation of the G-CSF receptor also results in defective activation of STAT3 (31), a transcription factor that appears to play a role in G-CSF-induced myeloid differentiation (32). However, the functional significance of these changes for differentiation is unclear.

The experiments described in this manuscript have investigated the mechanisms of translational regulation in differentiating 32D cells. These results demonstrate that G-CSF and IL-3 have dramatically different effects on overall translation rate, although these agents exhibit only modest differences in their effect on phosphorylation of eIF4E, PHAS I, or rpS6. However, despite the fact that both IL-3 and G-CSF stimulate cell cycle progression, they exhibit substantial differences in their ability to stimulate ribosomal RNA synthesis, which is inhibited in G-CSF-exposed cells. This uncoupling of ribosomal biogenesis from cell cycling results in a decrease in cellular ribosomal content, which is evident as early as the first cell cycle of growth in G-CSF. Cells expressing a carboxyl-terminally truncated G-CSF receptor that supports 32D cell proliferation but not differentiation do not demonstrate this dissociation of ribosomal content from cell cycling in response to G-CSF. These data demonstrate that one function of the carboxyl-terminal domain of the G-CSF receptor is to suppress ribosomal biogenesis, and this appears closely related to cell maturation.

**EXPERIMENTAL PROCEDURES**

**Materials**—Tissue culture supplies were from Life Sciences, Inc. except for fetal bovine serum, which was obtained from Intergen (Purchase, NY). Human G-CSF was from Amgen (Thousand Oaks, CA), and murine IL-3 was from R & D Systems (Minneapolis, MN). Cycloheximide was obtained from Calbiochem (San Diego, CA). Biochemical reagents were obtained from Sigma or Fisher except for polyacrylamide and nuclease membranes and transferred to nitrocellulose membranes by capillary blotting. Blotted RNA was hybridized at 65 °C in 5× SSPE (0.9 mM NaCl, 50 mM sodium phosphate, 5 mM EDTA, pH 7.7) with a cDNA encoding myeloperoxidase (34) that had been 32P-labeled by the method of Feinberg and Vogelstein (35). The blots were washed to a final stringency of 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) with 0.1% sodium dodecyl sulfate at 60 °C, developed by autoradiography, and quantified by PhosphorImager. For analysis of celluar 18 S rRNA content, the cells were counted in a Coulter counter, and equal numbers of cells were resuspended in equal volumes of lysis buffer and RNA extracted and recovered as described above. During extraction care was taken to ensure recovery of equal volumes throughout all steps. The RNA pellets were resuspended in equal volumes of running buffer, and equal volumes were loaded onto gels for Northern blotting. Because of the abundance of RNA species loads on these gels were reduced to 1/10 of that loaded on standard Northern blots to avoid local saturation of the membrane. Blots were hybridized with a 32P-labeled fragment of 18 S rDNA (36), washed, visualized, and quantified as described above.

**Determination of Translation Factor Phosphorylation**—PHAS I phosphorylation was assessed by gel shift and immunoblotting, as previously described (37) using antisera obtained from John Lawrence (University of Virginia). Cell extracts were prepared by lysis in Laemmli sample buffer, and proteins were separated in 12.5% polyacrylamide Laemmli gels. Phosphorylation of eIF4E was assessed by immunoblotting, using Ser203-phospho-specific antibodies obtained from Simon Morley (University of Sussex, Brighton, UK). Total eIF4E was determined in Church buffer (1% bovine serum albumin, 1 mM EDTA, 0.5 mM sodium pyrophosphate, 0.5% Nonidet P-40, on ice). RNA was extracted using RNeasy Mini Kit (Qiagen), and the entire of the recovered RNA was hybridized to DNA immobilized on a 0.4-μm Nytran membrane. Hybridization was performed in Church buffer (1× SSC, 0.5% sodium phosphate, pH 7.5, 7.5% sodium dodecyl sulfate) at 65 °C for 48 h. Blots were washed to a final stringency of 0.1× SSC (15 mM NaCl, 1.5 mM sodium citrate, pH 7.0) with 0.1% sodium dodecyl sulfate at 60 °C, developed by autoradiography, and quantified by PhosphorImager. The experiments were performed in triplicate.

**Determination of Amino Acid Incorporation Rate**—Two different protocols were used to determine the leucine incorporation rate in 32Dcl3 cells, as indicated in the text. Cells in the logarithmic phase of growth were pelleted, washed, and resuspended in prewarmed medium containing either G-CSF or IL-3. At the times indicated, cell counts were obtained, [3H]leucine (100 μCi/ml) was added to the cultures, and incubations continued for an additional 15 min. Amino acid incorporation was terminated by three washes in ice-cold phosphate-buffered saline, and the cells lysed in 10% trichloroacetic acid. tRNAs were decayslated by 15 min of incubation at 90 °C, and following an additional incubation of ice on collection and centrifugation, the tRNA pellet was washed by vacuum filtration onto Whatman G/F glass fiber filters. Retained radioactivity was determined by scintillation counting in a Beckman LS 7800 liquid scintillation counter. Experiments were performed in triplicate, and the results were standardized for cell number. Because in the previous experiments pelleting and washing the cells was noted to cause patient slowing of the translation rate, to examine early effects G-CSF and IL-3 on translation rate, cells were deprived of cytokines for 12 h and then added at the concentrations indicated above. At 0, 2, 4, and 8 h of exposure [3H]leucine incorporation was determined as described above. The effect of cycloheximide on translation rate was determined 24 h after its addition to normal growth medium (containing IL-3). A concentration of 50 ng/ml was determined to decrease translation rate by 54% (19,831 ± 1,269 cpm [3H]leucine incorporation in cells exposed to IL-3 versus 9,154 ± 717 cpm [3H]leucine incorporation in cells exposed to IL-3 plus cycloheximide).
G-CSF Receptor Suppression of Ribosomal Biogenesis

Primer extension analysis of rDNA transcription rate was performed as described by Aluzueri and White (40). The cells were synchronized and treated with IL-3 or G-CSF, equal numbers of cells were collected, and total RNA was extracted using the guanidium-acid phenol method. Equal volumes of RNA were hybridized to 500,000 cpm of a [γ-32P]ATP-labeled primer complementary to the 5′ end of the 45S rDNA precursor RNA (primer 5′-CAGGACCCCGGACAGCCGACG-3′, complementary to +100 to +122 of the mouse rDNA gene). Following hybridization for 24 h (40 μCi PIPES, 0.4 μM NaCl, 1 mM EDTA, 50% formamide) at 42 °C, the RNA and hybridized primer were precipitated with two volumes of ethanol and resuspended in buffer for reverse transcription (Life Technologies, Inc.). Reverse transcriptions were performed using SuperScript II RNase H− reverse transcriptase (Life Technologies, Inc.). The extended products were precipitated in ethanol, resuspended in sample buffer containing 90% formamide, and separated on 8% polyacrylamide, 8 μL urea gels. The extended products were visualized by autoradiography and quantified by PhosphorImager.

Gradient Sedimentation of Polysomes—Cytosolic extracts and gradients were prepared as previously described (8). The extracts were layered onto 10−50% sucrose gradients and centrifuged for 4 h at 27,000 rpm in a Beckman SW28.1 rotor. Fractions were collected with an ISCO 640 gradient fractionator with continuous monitoring at 254 nm by an ISCO UA6 absorbance detector at a sensitivity of 0.5 absorption units full-scale using a 5-mm path length flow cell.

Cell Cycle Analysis—The cells were incubated for 12 h without cytokines and separated into two equal aliquots, and either IL-3 or G-CSF was added at the concentration indicated above. At the times indicated, the cells were prepared for flow cytometric analysis of DNA content by fluorescence intensity of propidium as described by Schimienti and Jacobberger (41). Flow cytometry was performed on a Coulter Elite ESP. Excitation was with a 488-nm air-cooled argon ion laser at 15 mW, and propidium signal was collected using a 640-nm long pass optical filter. The percentage of distribution of cells in the cycle phases was determined using the ModFit DNA analysis program.

CDK2 Immunoprecipitation and Kinase Assay—32D cells were synchronized by growth in the absence of cytokines for 12 h. Equal cell aliquots were then treated with either IL-3 or G-CSF for 24 h. 1.5 × 107 cells were washed twice in phosphate-buffered saline and lysed in 0.5 mL of RIPA buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% sodium dodecyl sulfate, 10 mM p-nitrophenyl phosphatase, 5 mM β-glycerophosphate, 10 mM NaF, 2 μg/mL aprotinin, 10 μg/mL leupeptin, 10 μg/mL pepstatin, 0.25 mM phenylmethylsulfonyl fluoride) by incubating for 15 min on ice. CDK2/cyclin A complexes were immunoprecipitated with anti-cyclin A antiserum (Santa Cruz, 1:200 dilution) or with preimmune rabbit serum for control. Immunoprecipitations were performed for 3 h at 4 °C with protein A-coupled agarose beads added for the final 2 h of incubation. Immunoprecipitates were washed four times in RIPA buffer, suspended in 20 μL of kinase buffer (50 mM Tris-HCl, pH 7.4, 1 mM dithiothreitol, 10 mM MgCl2, 10 μM cold ATP, 10 μCi of [γ-32P]ATP, 0.5 μg of histone H1) and incubated 30 min at room temperature. The reactions were terminated by heating in Laemmli sample buffer (95 °C for 10 min). The recovered proteins were separated by electrophoresis in a 15% polyacrylamide Laemmli gel. The gel was dried, and the labeled proteins were visualized by autoradiography. The band intensities were quantified using a PhosphorImager system.

Transfections and Luciferase Assay—1 × 107 32D cells were suspended in 1 mL of normal growth medium and the pGL-3 bicistronic luciferase plasmid (15 μg) and introduced by electroporation (1600 microfarad capacitance and 250 V). The pGL-3 vector expresses both Renilla luciferase, translated in a cap-dependent manner, and firefly luciferase translated from a cap-independent internal ribosomal entry sequence located in the intercistronic space. The electroporated cells were allowed to recover overnight in normal growth medium. At time 0, the cells were washed and transferred into medium containing either IL-3 or G-CSF. After the indicated times cells were collected and analyzed for both firefly and Renilla luciferase activities according to a dual luciferase reporter assay system protocol (Promega).

RESULTS

G-CSF and IL-3 Differ in Their Effect on Translation Rate—Inducers of MEL cell differentiation cause a rapid decrease in translation rate that precedes differentiation (6−8). The timing of this effect suggests that translational regulation might play a role in the changes in gene expression that are necessary for differentiation. To determine whether a similar effect occurred during G-CSF induced myeloid differentiation, as well as to facilitate investigation of the signaling pathways affected, growth factor-dependent 32D cells were utilized. These cells grow indefinately as undifferentiated myeloblasts in IL-3 but in G-CSF mature into granulocytes over a time course of 7−14 days (25). To determine whether G-CSF and IL-3 had differing effects on translation rate, cells that had been maintained in IL-3 were washed, divided into two equal aliquots, and grown in the presence of either G-CSF or IL-3 for the times indicated. As demonstrated in Fig. 1A, a difference in rate of leucine incorporation, which ranged from 1.6- to 2.6-fold, was observed at all times examined. The decreased translation rate in the cells grown in G-CSF was not due to a lower effective concentration of G-CSF, because this dose induced granulocytic differentiation with normal kinetics, as assayed by expression of myeloperoxidase mRNA (Fig. 1B).

The preceding results demonstrate that translation rate decreases when 32D cells are switched from medium containing IL-3 to G-CSF. However, both the IL-3 and G-CSF receptors activate the phosphoinositot 3-kinase and ERK signaling pathways (42, 43), and these pathways generally increase translation initiation rate (44). In addition, it was difficult to assess
resulted in a slight decrease in abundance of eIF4E, although A exposure to G-CSF immunoblotting, using both pan-specific and phospho-specific eIF4E. Extracts were prepared from cells that had been shifted.

rylation of eIF2 translation factors, eIF4E, PHAS I, and eIF2/H9251 phosphorylation as described under density in IL-3 and normalized to histone H3 (D), which was bottom panel, translation rate was stimulated 2.2-fold by4hi ncells C Fig. 1 in IL-3 and G-CSF, the cells were grown overnight in medium containing G-CSF for the indicated times. The proteins were extracted, and eIF4E and phosphorylated eIF4E (A), PHAS I (B), eIF2α and phosphorylated eIF2α (C), and histone H3 (D) were identified by Western blotting as described under “Experimental Procedures.” Band intensities, determined using NIH Image, were expressed as a function of density in IL-3 and normalized to histone H3 (bottom panel), which was used as a control for loading.

the time of onset of this translational effect in the previous experiment because pelleting and washing the cells caused a transient reduction in translation rate. Therefore, to determine whether G-CSF stimulated translation, as well as to determine the earliest time where translation rate diverged in cells grown in IL-3 and G-CSF, the cells were grown overnight in medium containing cytokines, and the translation rate was assessed following re-exposure to either IL-3 or G-CSF. As demonstrated in Fig. 1C, translation rate was stimulated 2.2-fold by 4 h in cells exposed to both G-CSF and IL-3, demonstrating that both cytokines activate translation in 32D cells. However, by 8 h the translation rate of cells in these two cytokines diverged and was 2-fold less in cells grown in G-CSF as compared to IL-3.

To determine how the differential effect of these two cytokines on the translational apparatus was regulated, the phosphorylation of translational proteins was examined. Growth factors commonly regulate translation via phosphorylations of eIF4E, PHAS I, and ribosomal protein S6 (rpS6) (10, 18). Because we have previously demonstrated that rpS6 phosphorylation is suppressed in primary myeloid cells, as well as in 32D cells,2 these studies focused on phosphorylation of PHAS I and eIF4E. Extracts were prepared from cells that had been shifted from IL-3 to G-CSF. Phosphorylation of eIF4E was assessed by immunoblotting, using both pan-specific and phospho-specific eIF4E antibodies. As indicated in Fig. 2A, exposure to G-CSF resulted in a slight decrease in abundance of eIF4E, although this was not apparent until 24 h of growth in G-CSF. Phosphorylation of eIF4E did not change significantly during the initial 8 h of G-CSF exposure, but at 24 h decreased slightly. Because this paralleled the decrease in eIF4E abundance, it appears that G-CSF affects eIF4E abundance but not phosphorylation. However, this decrease in abundance is delayed relative to the translational effect as determined by leucine incorporation.

PHAS I phosphorylation was assessed over a similar time course by gel shift. PHAS I migrates as three discrete bands during electrophoresis in SDS-containing gels, and only the most slowly migrating (γ) isofrom does not bind eIF4E (35). As demonstrated in Fig. 2B, this protein was transiently dephosphorylated when the cells were shifted from IL-3 into G-CSF. This is evident as a decrease in the percentage of protein in the most slowly migrating γ-isoform and an increase in the most rapidly migrating α-isoform (Fig. 2B). However, by 24 h the greatest abundance of the protein was in the γ-isoform. Neither of the above-noted changes directly correlated with the overall change in translational rate as determined by leucine incorporation. However, it is possible that the combined effect of the transient decrease in PHAS I phosphorylation and the decrease in abundance of eIF4E might account for the overall effect on translation rate. To assess the significance of these changes, a reporter construct was used to determine the changes in cap-dependent translation initiation, which is the primary target of eIF4E. A plasmid DNA that expressed two different luciferase proteins (firefly and Renilla) from a single, bicistronic mRNA was introduced into the cells. The 3′-most luciferase (firefly) in this mRNA is translated via internal (cap-independent) initiation utilizing the encephalomyocarditis virus internal ribosomal entry sequence located in the intercistronic space. As demonstrated in Table I, the cap-dependent/cap-independent translation ratio showed only a modest decrease in G-CSF-treated cells (decreased by 13 and 6% at 8 and 24 h of exposure, respectively). This appears insufficient to account for the 39% reduction in translation rate at the times demonstrated in Fig. 1.

In contrast to mitogens, which primarily affect cap-dependent translation, growth inhibitory stimuli (45, 46) commonly cause phosphorylation of the α-subunit of eIF2. This causes sequestration of this protein by its GDP/GTP exchange factor, eIF2B, thus inhibiting translation initiation (47). Because G-CSF inhibits translation relative to IL-3 we determined whether this was associated with an increase in eIF2α phosphorylation. As demonstrated in Fig. 2C, using a pan-specific antibody, eIF2α abundance decreased slightly following exposure to G-CSF, similar to that observed for eIF4E. Using an antibody specific for the Ser51 phosphorylated form of eIF2α, this phosphorylation was seen to decrease following shift into medium containing G-CSF. Because this decrease exceeded that observed for eIF2 protein, it can be concluded that G-CSF decreases eIF2 phosphorylation. However, because this phosphorylation inhibits translation, the decreased translation rate in G-CSF-exposed cells cannot be attributed to this change.

**G-CSF Causes a Reduction in Cellular Ribosomal Content**—Because the preceding observations did not provide a definitive explanation for the persistent decrease in translation rate of cells grown in G-CSF, sucrose gradients were utilized to assess the effect of IL-3 and G-CSF on translation. With this approach, a decrease in initiation rate is shown as a decrease in the percentage of ribosomes that are in polysomes. In contrast, if the elongation rate is decreased, the amount of polysomal ribosomes should increase (48). As demonstrated in Fig. 3A, in cells that were not exposed to either cytokine only a small percentage of ribosomes were present in translationally active polysomes. In contrast, for cells grown in either G-CSF or IL-3,
the percentage of polysomal ribosomes was increased. How-

ever, it was not evident from these studies that the distribution of ribosomes in polysomal and subpolysomal (primarily 80 S) complexes was different in IL-3 and G-CSF-exposed cells. Rather, the amount of ribosomes recovered from the cells grown in G-CSF appeared dramatically reduced. This suggested that the decrease in translation rate might be due to a general reduction in the components of the translational apparatus. To quantify the effect of G-CSF on ribosomal content, cells were shifted from IL-3 to G-CSF and rRNA content determined at timed intervals by blot hybridization using a probe for 18 S rRNA. Because ribosomes account for ~80–90% of total cellular RNA, gel loads were standardized for cell number rather than amount of RNA. As demonstrated in Fig. 3, ribosomal content decreased 36% during the first 24 h of growth in G-CSF and by 72 h had decreased by 60%.

Ribosomal synthesis is coregulated with changes in protein synthesis (49), although the basis of this regulation is not understood. It is possible that the decrease in ribosomal content was simply the result of the decrease in protein synthesis rate. Therefore, we determined whether a similar decrease in translation rate induced by cycloheximide resulted in a similar decrease in ribosomal content. Cells were grown in IL-3 in the presence or absence of 50 ng/ml cycloheximide, which reduced the protein synthesis rate by 54%, as determined by leucine incorporation. RNA was extracted from equal numbers of cells at 24 and 48 h, and ribosomal content was determined by blot hybridization with an 18 S rRNA probe, as above. Cells grown in the presence of cycloheximide did not demonstrate a reduction in ribosomal content (18 S rRNA content of 99 and 114% of control values at 24 and 48 h) (Fig. 3B). Thus, the reduction in cellular ribosomal content does not simply result from the decrease in protein synthesis rate.

**Inducers Dissociate Ribosome Accumulation from Cell Cycle Progression—**Ribosomal content normally increases as cells progress through the cycle (49). Because cell growth continues at least transiently in G-CSF, the decrease in ribosomal content suggested that cell cycle progression and ribosomal biogenesis might be uncoupled during growth in G-CSF. To examine this possibility more closely, cells were partially synchronized by cytokine withdrawal, then split into two aliquots, and exposed to either G-CSF or IL-3. Following 6 h of exposure to either cytokine, a decrease in ribosomal content occurred (Fig. 4A). This is likely due to the increase in the percentage of G1 cells at this time (data not shown), because cytokine deprivation caused arrest in both G2/M and G1, as demonstrated below (Fig. 5A). Following this time, differences in 18 S rRNA accumulation became immediately evident. In IL-3-treated cells ribosomal content increased to a maximum of 223% of that present in quiescent cells. In contrast, in cells exposed to G-CSF, 18 S rRNA content reached a maximum of 121% of that present in quiescent cells, and by 42 h, 18 S rRNA content had decreased relative to that in quiescent cells (Fig. 4B).

To determine whether the differences in ribosome accumulation were attributable to differences in cell cycle progression in these two cytokines, flow cytometry was performed. In the absence of cytokines, the cells arrested in both the G1 and G2/M

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**TABLE I**

|          | RLU/100 μg | Mean ± S.E. | Ratio renilla/ | IL-3 0 h | 18S rRNA | 18S rRNA |
|----------|------------|-------------|---------------|----------|----------|----------|
|          | Replia     | Firefly     |               |          |          |          |
| IL-3 0 h | 22,698     | 5,458       | 22,274 ± 424  | 15,320 ± 856 | 3,312.5 ± 93.5 | 4.6 |
| IL-3 8 h | 21,850     | 5,572       | 12,170.5 ± 97.5 | 3,019 ± 10 | 4 |
| G-CSF 8 h| 14,464     | 3,219       | 12,451 ± 856  | 674.5 ± 5.5 | 6.3 |
| IL-3 24 h| 16,176     | 3,406       | 4,251 ± 34    | 710.5 ± 133.5 | 5.9 |
| G-CSF 24 h| 12,073    | 3,009       | 4,319 ± 680   | 4,178 ± 132.5 | 5.9 |
phases of the cell cycle (Fig. 5A). Exposure to either cytokine increased the percentage of S phase cells at 24 h, and this was followed by a modest decrease at 48 h. This demonstrates that cytokine deprivation did result in a modest degree of cell cycle synchronization. At the latter time the percentage of cells in S phase was slightly less for those grown in G-CSF, consistent with the decreased growth rate in this cytokine (Fig. 5B). However, by this latter time cells grown in either cytokine had doubled in number (Fig. 5B). These data demonstrate that, in contrast to IL-3, cell doubling in G-CSF occurs in the absence of a concurrent doubling of ribosomal content.

Although cell growth rate and S phase percentage were slightly reduced in the G-CSF-treated cells, this appeared insufficient to account for the differences in ribosome accumulation demonstrated above. However, because cyclin-dependent kinase activity has been proposed to regulate Pol I transcriptional activation, we determined whether G-CSF and IL-3 differed in their ability to activate CDK2, as assessed by phosphorylation of histone H1. As demonstrated in Fig. 5C, exposure of the 32D cells to either IL-3 or G-CSF resulted in a comparable increase in CDK2 activity at 24 h of exposure, as determined by H1 phosphorylation in an in vitro kinase assay. Thus, these two cytokines did not differ significantly in their ability to stimulate cell cycle progression, as judged by CDK2 activation. Rather, these data provide evidence that G-CSF dissociates ribosome accumulation from cell cycle progression.

**IL-3 and G-CSF Have Differential Effects on Pol I Transcriptional Activation**—Mitogens stimulate rDNA transcription (49). To determine whether the decreased number of ribosomes in G-CSF-treated 32D cells was due to a failure to activate rDNA transcription, nuclear run-offs were performed. In the preceding experiments ribosomal content began to diverge between 6 and 12 h of cytokine exposure. Therefore, we examined the differential effect of IL-3 and G-CSF on Pol I transcription at 8 and 16 h of cytokine exposure. Because rDNA transcription...
accounts for ~45% of total transcription (50), these experiments were standardized for the number of nuclei rather than the amount of radioactivity added to the blots. The recovered, radiolabeled transcripts were hybridized with slot-blotted probes for Pol I (28 and 18 S rRNA), Pol II (U1 and U2 small nuclear ribonucleoproteins), and Pol III (5 S rRNA) transcripts, As demonstrated in Fig. 6A, at 8 h of cytokine exposure, Pol I transcription was reduced 2–4-fold in cells grown in G-CSF compared with those grown in IL-3. A 2-fold reduction in transcription was also observed for 5 S rRNA, a Pol III transcript. In contrast, Pol II transcriptional activation did not appear to differ significantly in G-CSF- and IL-3-treated cells, because U1 and U2 run-off transcription rates were similar. Similar results were obtained when cytokine exposures were prolonged to 16 h (Fig. 6B).

As an independent means of confirming these results and to determine the time of onset of transcriptional activation, nascent rRNA transcripts were quantified by primer extension using a probe that hybridized with the 5’ end of the nascent transcript. This sequence is rapidly removed and degraded during rRNA processing, and thus its abundance provides a good measure of transcription rate (40). Following exposure to either G-CSF or IL-3, total cellular rRNA was obtained from equal numbers of cells and nascent rRNA transcripts identified. As demonstrated in Fig. 6C, there was no significant change in transcript abundance for the first 4 h of exposure. However by 8 h, the transcription rate increased in response to both cytokines. Consistent with the data from the nuclear run-offs, this increase is 2.5-fold greater in the IL-3-treated cells, as determined by PhosphorImager. Thus, IL-3 and G-CSF differ in their ability to activate Pol I transcription.

**The Carboxyl-terminal Domain of the G-CSF Receptor Is Required for Suppression of Ribosomal Biogenesis**—The previous data demonstrated that G-CSF dissociates ribosomal biogenesis from cell cycle progression. To determine whether this effect was closely related to the ability of the G-CSF receptor to transmit a maturation signal, a 32D cell line was employed that did not express endogenous G-CSF receptor but expressed either a recombinant wild-type or carboxyl-terminally truncated receptor. As previously demonstrated, cells expressing the truncated receptor proliferate but do not differentiate in response to G-CSF (30). Consistent with the data shown in Fig. 4, cells that expressed wild-type receptor accumulated 18 S rRNA when exposed to IL-3 but not to G-CSF (Fig. 7A). In contrast, in cells expressing the carboxyl-terminally truncated receptor, 18 S rRNA accumulation occurred in response to either G-CSF or IL-3 (although with slightly different kinetics). These results provide evidence that the carboxyl-terminal domain of the G-CSF receptor is required to suppress ribosomal biogenesis. Further, because cells that express the truncated receptor do not differentiate in response to G-CSF, this suppression appears closely associated with the transmission of a maturation signal.
DISCUSSION

The experiments described in this manuscript have investigated the basis of the differential effect of IL-3 and G-CSF on translation rate in 32D cells. These data provide evidence that this is due at least in part to the failure of G-CSF to stimulate rDNA transcription. Because both G-CSF and IL-3 stimulate cell cycling, the failure of G-CSF to activate rDNA transcription dissociates ribosomal biogenesis from cell cycling and decreases cellular content of ribosomes. This effect of G-CSF is dependent upon the carboxy-terminal domain of its receptor, because in cells that express a carboxy-terminally truncated receptor, ribosomal accumulation is similar in response to either G-CSF or IL-3. This provides evidence that cellular ribosomal content is a target for regulation early in cell maturation. A decrease in ribosomal content may be important for the changes in gene expression that accompany terminal differentiation.

Cell growth and proliferation are distinct but coupled processes; doubling of cell mass must occur if cell division is to result in the generation of two identical daughter cells (for review, see Refs. 51–53). Because many of the components that regulate G1 cyclin-dependent kinase activity are regulated translationally (12, 54–58), it has been proposed that coupling of cell growth and proliferation may be due to this translational dependence (51). However, as demonstrated here, cellular translation rate in G-CSF is nearly 50% less than in IL-3, yet cycling continues (at least transiently) in the presence of G-CSF. In contrast, in 3T3 cells a cycloheximide-induced reduction in protein synthesis of less than 30% is sufficient to completely block entry into S phase (59). In this regard, it may be speculated that the failure of cycloheximide to decrease ribosomal content in 32D cells is due to the growth arrest induced by this translational inhibitor. Because uncoupling of the translation rate from cell cycling also occurs in MEL cells exposed to chemical inducers (6), this may be a common event during maturation of hematopoietic cells.

Differences in transcriptional activation of rDNA are responsible at least in part for the differential effect of IL-3 and G-CSF on ribosomal abundance. Although the signaling mechanisms that regulate transcriptional activation of rDNA in response to mitogenic receptor activation are still largely unknown, recent evidence implicates cyclin-dependent kinases in this regulation. CDK2 and CDK4 have been demonstrated to phosphorylate UBF (upstream binding factor), a transcription factor that has an essential role in Pol I transcriptional activation (60). However, as demonstrated here, G-CSF and IL-3 have similar effects upon CDK2 activation. Interestingly, differentiation of 32D and MEL cells has been associated with altered activity of G1 cyclin-dependent kinases (61–65). It is possible that these alterations could play a role in dissociating ribosomal synthesis from cell cycling. Alternatively, the carboxy-terminal domain of the G-CSF receptor may provide a signal that actively represses transcription by Pol I, which is independent of mitogenic signaling and CDK activation.

Although it is likely that the decrease in ribosomal content contributes to the differential effect of G-CSF and IL-3 on translation rate, the significance of this for the regulation of gene expression remains to be established. Although modification of eIF4E, eIF2α, and rpS6 has been proposed to facilitate translation of specific subsets of mRNAs (for reviews see Refs. 10, 21, and 66), ribosomal availability as a specific translational determinant has not been extensively investigated. However, genetic evidence from yeast suggests that ribosomal abundance may be of particular importance for translation of nonadenylated mRNAs (67). It is noteworthy that the accumulation of globin mRNAs in differentiating erythrocytes is dependent upon a stability sequence in their 3′-untranslated regions, which has been proposed to inhibit deadenylation (68–70). This sequence has been detected in other mRNAs that accumulate to high levels in differentiated cells (71). Thus, limiting ribosomal availability may provide a mechanism to destabilize mRNAs that lack such a sequence and thereby enhance the expression of the mRNAs that possess such a sequence.

Alternatively, a decrease in ribosomal biogenesis may be necessary to allow cells to redirect gene expression away from proteins involved in growth and toward those required for differentiation. As previously noted, rDNA transcription in growing cells represents ~50% of total transcription, and all RNA polymerases share a requirement for TBP (TATA-binding protein), which is present in limiting abundance (72). Thus, a decrease in Pol I transcription could increase TBP availability. Experiments analyzing the effects of Myc proteins on gene expression suggest that cell growth (as exemplified by ribosomal accumulation) and differentiation are incompatible; Myc overexpression blocks differentiation of myeloid (and erythroid) cells (73–76), whereas the primary targets of Myc proteins appear to be gene products involved in ribosomal biogenesis (77, 78). Regardless of the exact effect on gene expression, the timing of the decrease in ribosomal content in differentiating 32D cells suggests that this is a contributing cause rather than an effect of the changes in gene expression that accompany differentiation.

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Sandra L. Kroll, Diane Barth-Baus and Jack O. Hensold

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