A GM-colony-stimulating factor (CSF) Activated Ribonuclease System Transregulates M-CSF Receptor Expression in the Murine FDC-P1/MAC Myeloid Cell Line

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The murine myeloid precursor cell line FDC-P1/MAC simultaneously expresses receptors for multi-colony-stimulating factor (CSF), granulocyte-macrophage (GM)-CSF, and macrophage (M)-CSF. Growth of FDC-P1/MAC cells in either multi-CSF or GM-CSF results in the posttranscriptional suppression of M-CSF receptor (*c-fms* proto-oncogene) expression. We use the term transregulation to describe this control of receptor expression and have further characterized this regulatory process. The removal of FDC-P1/MAC cells from GM-CSF stimulation resulted in the re-expression of *c-fms* mRNA independent of M-CSF stimulation and new protein synthesis. Switching FDC-P1/MAC cells from growth in M-CSF to GM-CSF caused the selective degradation of *c-fms* mRNA within 6 h after factor switching. Blocking protein synthesis or gene transcription with metabolic inhibitors effectively prevented GM-CSF stimulated degradation of *c-fms* mRNA. These results suggest that the transregulation of *c-fms* transcripts by GM-CSF requires the transcriptional activation of a selective mRNA degradation factor. In vitro analysis, the use of cytoplasmic cell extracts, provided evidence that a ribonuclease is preferentially active in GM-CSF stimulated cells, although the specificity for mRNA degradation in vitro is broader than seen in vivo. Together, these data suggest that GM-CSF can dominantly transregulate the level of *c-fms* transcript through the transcriptional activation of a ribonuclease degradation system.

INTRODUCTION

Circulating red and white blood cells have finite life spans and must constantly be replenished by a process known as hematopoiesis. These cells are derived from self-replicating stem cell populations that give rise to committed precursor cells that ultimately differentiate along a specific lineage to generate the various mature blood cells. Development along a specific lineage is regulated by a complex set of humoral and microenvironmental factors (reviewed Ogawa *et al.*, 1983; Metcalf, 1989a). However, studies on cultured myeloid and lymphoid cells have demonstrated that much of this process can be controlled by a diverse family of hematopoietic growth factors comprised of the colony-stimulating factors (CSFs) and interleukins (Metcalf,

1984; Clark and Kamen, 1987; Metcalf, 1989b; Nicola, 1989). During myeloid cell development, CSFs play a major role in guiding the differentiation of cells in the monocyte/macrophage and granulocyte lineages. In general, multi-CSF (also called IL-3) and granulocyte-macrophage CSF (GM-CSF) can support the proliferation and maturation of pluripotent myeloid progenitor cells, whereas macrophage CSF (M-CSF or CSF-1) and granulocyte CSF (G-CSF) stimulate primarily lineage specific differentiation and survival.

The receptors for multi-CSF and GM-CSF are found on a wide range of myeloid cells with different proliferative and developmental capacities (Nicola, 1987; Nicola, 1989). In contrast, during hematopoiesis the expression of G-CSF and M-CSF receptors become restricted to cells of the neutrophilic granulocyte lineage

and the monocyte/macrophage lineage, respectively (Nicola *et al.*, 1983; Nicola, 1989). Isolation of enriched granulocyte/macrophage progenitor cells have demonstrated that multiple CSF receptors are expressed on the surface of these cells (Cook *et al.*, 1989). Therefore, it appears that differentiation along a specific lineage requires the regulated expression of CSF receptors that are appropriate for that lineage.

How CSF receptor expression becomes restricted to a specific lineage remains poorly understood at the molecular level. One proposed model of receptor expression regulation suggests that a hierarchy of growth factor action exists where multi-CSF and GM-CSF can act dominantly to regulate the expression of the G-CSF and M-CSF receptors (Walker *et al.*, 1985; Nicola, 1987). Another model has been proposed based on a recently isolated murine myeloid precursor cell line, designated FDC-P1/MAC, that simultaneously expresses receptors for multi-CSF, GM-CSF, and M-CSF (Gliniak and Rohrschneider, 1990). Using this cell line, we have demonstrated that the expression of the M-CSF receptor (*c-fms* proto-oncogene) is regulated at a posttranscriptional level by multi-CSF or GM-CSF. Moreover, both multi-CSF and GM-CSF act dominantly and in a factor concentration-dependent manner to suppress *c-fms* expression. Consistent with these findings, bone marrow soft agar colony assays reveal that high concentrations of GM-CSF can suppress macrophage colony development even in the presence of high concentrations of M-CSF. Thus it appears that the concentration of multi-CSF or GM-CSF can dramatically influence the developmental outcome of bipotential myeloid progenitor cells by suppressing the expression of specific CSF receptors. We have termed the regulation of *c-fms* mRNA expression by multi-CSF or GM-CSF as transregulation. This effect is distinct from that reported by Walker *et al.*, who proposed that the downregulation of a receptor by a heterologous growth factor results in the activation of that receptor's signal transduction pathway.

The posttranscriptional transregulation of *c-fms* suggests a potentially complex level of cellular regulation controlling specific CSF receptor expression. Conceivably, this could involve the activation/inactivation of specific stabilizing and/or destabilizing factors. Utilizing the FDC-P1/MAC cell line, we have begun to further define the level of posttranscriptional regulation of *c-fms* and the transcriptional activation and protein synthesis requirements necessary for GM-CSF stimulated transregulation.

MATERIALS AND METHODS

Cells and Culture Conditions

FDC-P1/MAC cl. 11 cells were isolated as previously described (Gliniak and Rohrschneider, 1990). FDC-P1/MAC cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) with 2.5% pokeweed mitogen-stimulated spleen-

cell conditioned medium as a source of GM-CSF and multi-CSF. Before use in specific experiments, cells were switched to DMEM with 10% FBS plus 200 U/ml purified CSF for 48 h. Recombinant murine GM-CSF was obtained from Immunex (Seattle, WA) and recombinant murine M-CSF was expressed in yeast and purified over a DEAE-cellulose column (Stanley and Heard, 1977). Actinomycin D (Calbiochem, San Diego, CA; 2.5 µg/ml) and cycloheximide (Sigma, St. Louis, MO; 10 µg/ml) were used as indicated in specific figure legends.

RNA Extraction and Northern Blot Analysis

Total cellular RNA was extracted by the guanidine thiocyanate-CsCl method (Chirgwin *et al.*, 1982). Poly(A)⁺ RNA was isolated with oligo(dT)-cellulose (Collaborative Research, Bedford, MA) and quantified by measuring the O.D.260 (Maniatis *et al.*, 1982). Glyoxal denatured poly A⁺ RNA was analyzed by electrophoresis through 1.2% agarose gels followed by Northern blot transfer to nitrocellulose (Thomas, 1983). Blots were probed with nick-translated plasmids to *c-fms*, p755 (Rothwell and Rohrschneider, 1987), and actin, pBam-βA2 (Tokunaga *et al.*, 1986), to specific radioactivities of 1×10^6 cpm/µg DNA (Maniatis *et al.*, 1982). Filters were prehybridized at 42°C for 6 h in a solution consisting of 50% formamide, 5× SSPE, 5× Denhardt's, 0.1% SDS, and 200 µg/ml salmon sperm DNA. Hybridization was carried out for 20 h at 42°C with 10^6 cpm nick-translated probe/ml hybridization buffer (same as prehybridization buffer except with 1× Denhardt's and 100 µg/ml salmon sperm DNA). Blots were washed twice in 1× SSC, 0.1% SDS at 65°C for 30 min and exposed to X-ray film with an intensifying screen at -70°C.

Nuclear Run-Off Assays

Approximately 10^8 cells were used per assay after growth for 48 h in the indicated colony-stimulating factor or in no factor for 12 h. Cells were washed once with ice-cold phosphate-buffered saline and then lysed in 5 ml of lysis buffer (10 mM tris(hydroxymethyl)aminomethane [Tris]-HCl [pH 7.4], 10 mM NaCl, 3 mM MgCl₂, 0.5% Nonidet P-40). Nuclei were pelleted for 5 min at $500 \times g$ and lysed an additional time. Nuclei were collected and resuspended in 200 µl nuclei-freezing buffer (50 mM Tris-HCl [pH 8.0], 5 mM MgCl₂, 40% glycerol, 0.5 mM dithiothreitol) and stored at -70°C until use. Nuclear run-off assays were performed exactly as described by Bentley and Groudine (1986) with α-³²P-UTP >3000 Ci/mM (ICN Radiochemicals, Irvine, CA). Run-off transcription products were purified through G-50 spin columns and hybridized to 5 µg denatured plasmids of pGEM2, *c-fms* (p755), and actin (pBam-βA2'). Plasmids were denatured in 0.3 M NaOH for 1 h at 65°C, neutralized with 2 M ammonium acetate, and then slot blotted onto nitrocellulose. Filters were hybridized for 48 h and washed twice with 0.1× SSC, 0.1% sodium dodecyl sulfate at room temperature for 5 min and then once in 2× SSC with 2 µg/ml RNase A for 30 min at room temperature.

GM-CSF Binding Analysis

Recombinant murine GM-CSF was radiolabeled with enzymobead radioiodination reagent (Bio-Rad Laboratories, Richmond, CA) as previously described (Park *et al.*, 1986). Cells (2×10^6) were incubated with various concentrations of ¹²⁵I-GM-CSF in 150-µl binding medium (Park *et al.*, 1986) for 1 h at 37°C and assayed for binding as described in detail elsewhere (Park *et al.*, 1986, 1989). Nonspecific binding was measured with the use of a 1000-fold molar excess of unlabeled GM-CSF. Specific binding was plotted in the Scatchard coordinate system (Scatchard, 1949) with the use of RS/1 (Bolt, Beranek, and Newman, Boston, MA), a commercially available data processing package running on a Microvax II under the VMS operating system.

In vitro Analysis of RNA Stability

In vitro analysis of *c-fms* RNA stability was carried out as described previously by Wager and Assoian (1990). Briefly, FDC-P1/MAC cells

Figure 1. Analysis of *c-fms* expression after the removal of GM-CSF. FDC-P1/MAC cells were grown in GM-CSF (200 U/ml) and then switched to no factor (DMEM plus 10% FBS) for the indicated time. (A) Nuclear run-off assays performed on nuclei isolated from FDC-P1/MAC cells grown in GM-CSF or no factor for the indicated times. 32 P-labeled nuclear RNA was hybridized to the indicated plasmid DNAs. (B) Northern blot analysis of *c-fms* and actin mRNA levels. Approximately 5 μ g of poly(A)⁺ RNA was analyzed for each sample and hybridized to 32 P-labeled *c-fms* or actin probes. (C) Protein synthesis inhibition effect on the re-expression of *c-fms*. FDC-P1/MAC cells were grown for 72 h in 200 U/ml GM-CSF (lanes 1 and 4) or preincubated in cycloheximide plus GM-CSF for 1 h (lane 5). Cells were then switched to no factor (DMEM plus 10% FBS) for 6 and 12 h in the absence of a protein synthesis inhibitor (lanes 2 and 3, respectively) or in the presence of cycloheximide (10 μ g/ml) (lanes 6 and 7, respectively). Approximately 5 μ g of poly(A)⁺ RNA was analyzed for each sample and hybridized to 32 P-labeled probes for *c-fms* and actin.

were grown for 48 h in either M-CSF (200 U/ml) or GM-CSF (200 U/ml). Approximately 10^6 cells grown in each factor were lysed in 250 μ L of 10 mM Tris-HCl (pH 7.5), 10 mM NaCl, and 0.5% Nonidet P-40. Nuclei were removed by pelleting for 15 min at $500 \times g$. One hundred micrograms of guanidine thiocyanate-CsCl purified total RNA isolated from FDC-P1/MAC cells grown in M-CSF was added to the nucleus-cleared cytoplasmic lysates for the indicated times. The incubations were terminated by adding an equal volume of chloroform:phenol. After a second chloroform:phenol extraction, the RNA was isolated by ethanol precipitation and analyzed by Northern blotting.

RESULTS

Posttranscriptional Expression of *c-fms* Occurs Independently of M-CSF and Protein Synthesis

The murine promyelocytic FDC-P1/MAC cell line is dependent on either multi-CSF, GM-CSF, or M-CSF for its proliferation and survival (Gliniak and Rohrschneider, 1990). Growth of these cells in multi-CSF or GM-CSF maintains them in a relatively undifferentiated state where they undergo rapid proliferation. In contrast, growth in M-CSF results in partial differentiation along the monocyte/macrophage lineage, but the differentiation is not terminal, and the cells can be switched from one factor to another with minimal loss of viability.

Growth of FDC-P1/MAC cells in multi-CSF or GM-CSF has been shown to regulate the expression of the M-CSF receptor (*c-fms* proto-oncogene) at the posttran-

scriptional level (Gliniak and Rohrschneider, 1990). Consistent with our previous study, the level of both *c-fms* and actin transcription is high in FDC-P1/MAC cells stimulated with 200 U/ml GM-CSF (Figure 1A). On removal of CSF, the level of both *c-fms* and actin transcription steadily decreased throughout the 24-h period. In contrast, there was little detectable *c-fms* mRNA in the presence of GM-CSF, whereas switching the cells from growth in GM-CSF to no CSF resulted in the strong re-expression of *c-fms* transcript occurring by 6 h and continuing through 24 h (Figure 1B). These results are consistent with the re-expression kinetics of the *c-fms* glycoprotein previously reported (Gliniak and Rohrschneider, 1990). Therefore, the dramatic accumulation of *c-fms* mRNA that is observed on the removal of GM-CSF occurs without a similar increase in *c-fms* transcription. These results suggest that the accumulation of *c-fms* mRNA can be achieved exclusively at a posttranscriptional level and does not require M-CSF stimulation. In contrast to *c-fms*, the removal of CSF resulted in decreased actin transcription along with the corresponding decrease of cytoplasmic actin mRNA and suggests that actin expression is regulated at a transcriptional level in FDC-P1/MAC cells.

The need for continual protein synthesis in the re-expression of *c-fms* was analyzed by including a protein

synthesis inhibitor in the medium after the removal of GM-CSF (Figure 1C). Switching FDC-P1/MAC cells from growth in GM-CSF to no CSF resulted in the expected accumulation of *c-fms* transcript (lanes 1–3). Preincubation of cells with cycloheximide for 1 h in the presence of GM-CSF had no detectable effect on the level of *c-fms* transcript (lanes 4 vs. 5). Switching the FDC-P1/MAC cells to no CSF for up to 12 h in the continuous presence of cycloheximide did not prevent the accumulation of *c-fms* transcript (lanes 6 and 7). Moreover, the accumulation of *c-fms* mRNA 6 h after the removal of GM-CSF was greater in the presence of cycloheximide (lane 6) than in its absence (lane 2). However, by 12 h after the removal of GM-CSF in the presence of cycloheximide (lane 7), there was less accumulation of *c-fms* than without cycloheximide (lane 3). The decrease of *c-fms* transcript does not appear to result from a general cellular toxicity to cycloheximide as judged by the consistently high levels of actin transcript (Figure 1C, bottom).

These findings suggest that the synthesis of a protein is not necessary for the posttranscriptional accumulation of *c-fms*. However, synthesis of potential stabilizing factors for long-term *c-fms* expression can not be ruled out. Elevated *c-fms* mRNA levels occurred within 6 h, but longer exposure of the cells to cycloheximide resulted in a lower level of transcript than was accumulated in the absence of cycloheximide (Figure 1C, lanes 3 vs. 7). This is probably not a general effect of cycloheximide on *c-fms* transcription levels because the removal of factor naturally caused a decrease in *c-fms* transcription (Figure 1A). Also, the level of actin transcript did not change significantly throughout the cycloheximide treatment. Instead, the lower level of *c-fms* transcript may represent the inhibition of a protein factor necessary for long-term transcript stabilization.

A labile stabilizing factor has been recently described for the *c-fms* transcript during the differentiation of HL-60 cells (Weber *et al.*, 1989). To further analyze the role of protein stabilizing factors in the expression of *c-fms*, FDC-P1/MAC cells continuously growing in M-CSF were treated with the protein synthesis inhibitor cycloheximide (Figure 2). The addition of cycloheximide caused a super-induction of *c-fms* transcript between 2 and 4 h after addition. A superinduction of the actin transcript was also seen with similar kinetics. Similar results have been observed with the protein synthesis inhibitor anisomycin. Super-inductions of mRNA transcripts are often observed after cycloheximide blocks and presumably represent the inhibition of ribonucleases responsible for steady-state expression. However, the high level of superinduced transcript was not maintained and decreased to near normal levels after 10 h in the presence of cycloheximide. This decrease in the level of *c-fms* mRNA may be caused by a loss of a transcript stabilizing protein. Alternatively, culturing the cells for 10 h in cycloheximide may result in decreased

Figure 2. Effect of protein synthesis inhibition on *c-fms* expression in M-CSF growing FDC-P1/MAC cells. FDC-P1/MAC cells were grown in M-CSF (200 U/ml) and then treated with cycloheximide for the indicated times. Approximately 5 μ g of poly(A)⁺ RNA was analyzed for each sample and hybridized to ³²P-labeled *c-fms* probe. The blot was then stripped and rehybridized with a ³²P-labeled actin probe.

c-fms transcription through the loss of an essential transcription factor. Therefore, if transcript stabilizing proteins are necessary for long-term expression of *c-fms*, they do not appear to be more labile than the ribonucleases that regulate the level of the transcript.

Trans-Regulation of c-fms Occurs Through Selective Transcript Degradation.

The fully processed, polyadenylated murine myeloid *c-fms* transcript is ~4.0 kb and is believed to be derived from a relatively large region (>30 kb) of genomic DNA containing the coding sequences and multiple introns (Roberts *et al.*, 1988; Hampe *et al.*, 1989). The posttranscriptional regulation of *c-fms* could conceivably occur at a number of potential levels; for example, the amount of transcript could be regulated at the processing stage, transport out of the nucleus, or at the level of cytoplasmic mRNA stability. If the regulated step involves transcript processing or transport to the cytoplasm, an accumulation of RNA would be expected to develop at the blocked step. Factor-switching experiments were performed and total RNA analyzed to help identify the point of posttranscriptional regulation.

When the FDC-P1/MAC cells were initially grown in M-CSF and then switched to no CSF (Figure 3A), the level of *c-fms* transcript remained high and constant throughout the 10-h analysis. The actin transcript gradually decreased when the M-CSF was removed from the cells as would be expected for a gene transcriptionally activated by CSFs (Figure 3A, bottom). In contrast, switching parallel cultures from M-CSF to GM-CSF resulted in the disappearance of the *c-fms* transcript occurring by 6 h in GM-CSF (Figure 3B). However, this

Figure 3. Factor switching effects on the expression of *c-fms*. FDC-P1/MAC cells growing in M-CSF (200 U/ml) were spun down, washed thoroughly with DMEM plus 10% FBS, and then switched to no factor (DMEM plus 10% FBS) (A) or 200 U/ml GM-CSF (B) for the indicated time. Approximately 25 μ g of total cellular RNA was analyzed for each sample and hybridized to 32 P-labeled probe for *c-fms*. The blots were then stripped and reprobed with actin.

CSF switch had little effect on the level of actin transcript (Figure 3B, bottom) and demonstrated the selectivity of *c-fms* transcript destabilization in GM-CSF-stimulated cells. Because no larger *c-fms* RNA processing intermediates were observed and all of the mature transcript was degraded, it appears that *c-fms* RNA is posttranscriptionally regulated by the selective destabilization of cytoplasmic transcript.

The posttranscriptional transregulation of *c-fms* must require the interaction of some factor with the *c-fms* transcript to regulate its level in vivo. This regulation could occur through the activation of a destabilization factor(s) and/or the inactivation of a *c-fms* transcript-stabilizing factor. The data presented thus far demonstrate that the regulation of a destabilizing factor is the dominant regulatory mechanism for controlling *c-fms* transcript levels. Removal of GM-CSF inactivates the degradation pathway, whereas the addition of GM-CSF to FDC-P1/MAC cells stimulates the selective degradation of *c-fms* mRNA. Therefore, an important biochemical consequence of the GM-CSF signal transduction pathway is the activation of a mRNA degradation system.

Synthesis of a Trans-Regulation Factor is Required for GM-CSF Stimulated Degradation of c-fms

The regulation of the *c-fms* mRNA degradation factor could occur through the posttranslational modification of a pre-existing cellular pool of factor, possibly by phosphorylation of the factor. Alternatively, second messengers stimulated by GM-CSF could transcriptionally activate the gene for the factor. To distinguish between these possibilities, the ability of GM-CSF to transregulate *c-fms* expression in the presence of protein synthesis and transcriptional inhibitors was determined.

Switching FDC-P1/MAC cells from growth in M-CSF to no CSF and cycloheximide resulted in the gradual increase of *c-fms* transcript over 10 h (Figure 4A). This observation would be consistent with the inhibition of ribonuclease synthesis and the elimination of the *c-fms* degradation pathways. Likewise, switching cells from M-CSF to GM-CSF and cycloheximide also resulted in the stable expression of *c-fms* transcript throughout 10 h (Figure 4B), although the transcript level did not increase as it did in the switch to no CSF and cycloheximide. This may be due to a less than complete block of translation by cycloheximide and the synthesis of small amounts of transregulating factor. Therefore, protein synthesis is required for GM-CSF-stimulated transregulation and suggests that GM-CSF activates a transregulating factor through the induction of its synthesis.

A general inhibition of protein synthesis by cycloheximide may result in the stabilization of *c-fms* by some means other than the inhibition of a protein destabilizing factor; for example, translation of the *c-fms* transcript on the polysome complex may be required for its turnover as has been described for tubulin (Yen *et al.*, 1988). To analyze the need for the transcriptional activation of a transregulating factor, CSF switching experiments were performed after a transcriptional block with the use of actinomycin D (Figure 5). Switching cells from the growth in M-CSF to M-CSF and actinomycin D resulted in the stable expression of *c-fms* throughout the first 4 h and then was followed by a gradual decrease to barely detectable levels by 8 h (Figure 5A). Switching FDC-P1/MAC cells from M-CSF to GM-CSF and actinomycin D resulted in a decrease of *c-fms* and actin transcripts with kinetics similar to those of M-CSF and

Figure 4. Inhibition of GM-CSF stimulated transregulation by blocking protein synthesis. FDC-P1/MAC cells growing in M-CSF (200 U/ml) were switched to no factor (DMEM plus 10% FBS) (A) or 200 U/ml GM-CSF (B) in the presence of cycloheximide (10 μ g/ml) as described in the legend of Figure 4. Approximately 25 μ g of total cellular RNA was analyzed with 32 P-labeled *c-fms* and actin probes. The decrease in actin intensity in B at 6 and 8 h is not experimentally significant and is due to technical problems during RNA transfer to nitrocellulose.

Figure 5. Effect of actinomycin D on *c-fms* mRNA levels after factor switching. FDC-P1/MAC cells growing in M-CSF (200 U/ml) were spun down, washed thoroughly, and resuspended in 200 U/ml M-CSF plus 2.5 µg actinomycin D (A) or 200 U/ml GM-CSF plus 2.5 µg actinomycin D (B) for the indicated times. Approximately 25 µg of total cellular RNA was analyzed with ³²P-labeled *c-fms* and actin probes.

actinomycin D (Figure 5B). However, the stability of *c-fms* transcripts on switching to GM-CSF and actinomycin D was significantly greater than the switch to GM-CSF alone where the transcript was degraded within 4–6 h (Figure 3B). Thus active transcription must be required for the accelerated degradation of the *c-fms* transcript in response to GM-CSF stimulation. These results are consistent with the cycloheximide data and suggest that GM-CSF stimulates the transcriptional activation of a degrading factor for the transregulation of *c-fms*.

Prolonged cellular exposure to the metabolic inhibitors cycloheximide and actinomycin D is known to be eventually cytotoxic. Although there was no detectable loss of cell viability under these experimental conditions, the rapid decrease in *c-fms* intensity after 4 h of actinomycin D treatment could be interpreted as a cytotoxic effect of the agent. To address this possibility, FDC-P1/MAC cells growing in M-CSF were grown for 10 h in the presence of both cycloheximide and actinomycin D (Figure 6). Under these experimental conditions, the fate of the pre-existing transcripts can be followed over time as both new transcription and RNase synthesis is simultaneously inhibited.

The level of both *c-fms* and actin transcripts remained relatively the same and were still detectable after 10 h (Figure 6). These results demonstrate that any potential cytotoxicity of cycloheximide and actinomycin D does not result in the decline of *c-fms* or actin transcripts. Therefore, the decrease in *c-fms* transcript observed after actinomycin D addition must represent the experimental half-life of the mRNA and not a general toxic effect of these agents on the cells.

The broad inhibitory effects of both cycloheximide and actinomycin D must also eventually result in the reduction of cell surface GM-CSF binding sites. If the

Figure 6. Effect of cycloheximide and actinomycin D on *c-fms* mRNA levels. Cycloheximide (10 µg/ml) and actinomycin D (2.5 µg/ml) were simultaneously added to FDC-P1/MAC cells growing in M-CSF (200 U/ml). Approximately 25 µg of total cellular RNA was analyzed after the indicated times of incubation. Transcripts were detected with ³²P-labeled *c-fms* and actin probes.

GM-CSF receptor was particularly labile and quickly lost from the cell surface, then the observed effects of the inhibitors could be explained by the inability of GM-CSF to initiate a biological signal through binding to cell surface receptors rather than the inhibition of a specific gene product stimulated by GM-CSF. To address this possibility, the availability of GM-CSF binding sites were determined after treatment of FDC-P1/MAC cells with actinomycin D (Table 1). The FDC-P1/MAC cells expressed ~780 high-affinity GM-CSF binding sites when the cells were grown in M-CSF. Transfer of these cells to M-CSF and actinomycin D resulted in a loss of <50% of the GM-CSF binding sites within the first 3 h of incubation with inhibitor. Moreover, the affinity of

Table 1. High-affinity GM-CSF receptor expression after actinomycin D treatment

Incubation time (h)	GM-CSF bound	
	Molecules/cell	Affinity (K_a , M^{-1})
0	780	1.0×10^{10}
1	660	1.2×10^{10}
3	480	1.7×10^{10}
6	110	1.8×10^{10}

FDC-P1/MAC cells grown in M-CSF (200 U/ml) were resuspended at 2×10^6 cells/ml in M-CSF (200 U/ml) plus actinomycin D (2.5 µg/ml) and maintained at 37°C. At the indicated time points, cells were analyzed for GM-CSF receptor expression by Scatchard analysis of complete sets of binding data as described in MATERIALS AND METHODS.

GM-CSF binding did not change significantly during the entire incubation with actinomycin D. By 6 h, the effect of actinomycin D became more significant and caused a sevenfold decrease in GM-CSF binding sites. These results demonstrate that the majority of high-affinity GM-CSF binding sites remain on FDC-P1/MAC cells within 3 h after the inhibition of cellular transcription. Therefore, the GM-CSF receptor is not rapidly cleared from the cell surface and the action of actinomycin D does not appear to be mediated at this level.

In Vitro Demonstration of an Active Ribonuclease in GM-CSF-Stimulated Cell Extracts

The metabolic inhibitor studies demonstrate that a ribonuclease system is activated by GM-CSF stimulation. To further define the activity of this ribonuclease, a simple in vitro system to analyze RNA degradation was utilized. A similar in vitro system was used successfully to define a phorbol ester-regulated ribonuclease system that controls the expression of transforming growth factor β 1 in hematopoietic cells (Wager and Assoian, 1990). In this assay, total deproteinated RNA isolated from FDC-P1/MAC cells grown in M-CSF (therefore containing *c-fms* transcripts) was added to nucleus-cleared cytoplasmic extracts from FDC-P1/MAC cells stimulated with M-CSF or GM-CSF (see MATERIAL AND METHODS). Cytoplasmic extracts from FDC-P1/MAC cells stimulated with M-CSF contained no detectable *c-fms* or actin transcripts in the absence of exogenously added RNA (Figure 7, A and B, lane 1). When exogenous RNA containing the *c-fms* transcript was added to cytoplasmic extracts of M-CSF stimulated cells, both the *c-fms* and actin transcripts could be detected after 30- and 60-min incubations (Figure 7, A and B,

lanes 2 and 3, respectively). In comparison, addition of the equivalent RNA to GM-CSF-stimulated extracts resulted in the complete degradation of *c-fms* and nearly complete degradation of actin (Figure 7, A and B, lanes 4 and 5, respectively).

The degradation of RNA transcripts in GM-CSF-stimulated extracts demonstrates the activity of a cytoplasmic ribonuclease that does not appear to function in M-CSF-stimulated cells. However, this in vitro system does not perfectly reproduce the transregulation seen in whole cells, as demonstrated by the extensive degradation of the actin transcript in GM-CSF-stimulated extracts. Repeated attempts using different incubation times and/or in vitro transcribed RNA substrates consistently demonstrated a more active RNase system in GM-CSF-stimulated extracts, but both the *c-fms* and actin transcripts were degraded with similar kinetics. Thus the selectivity of this RNase is not maintained in vitro and suggests that the in vitro system has lost some factor or complex necessary for the specificity of mRNA depletion seen in vivo.

DISCUSSION

The simultaneous expression of the multi-CSF, GM-CSF, and M-CSF receptors in the FDC-P1/MAC cell line makes it possible to study the intracellular communication between these receptors. We have previously shown that multi-CSF or GM-CSF can suppress the expression of the M-CSF receptor (*c-fms* proto-oncogene) at a posttranscriptional level (Gliniak and Rohrschneider, 1990). Our previous results and the additional data presented in this report demonstrate that this regulation is controlled by the dominant actions of GM-CSF (or multi-CSF) and that these factors modulate

Figure 7. Preferential RNA degradation in GM-CSF stimulated cell extracts. Cytoplasmic lysates prepared from FDC-P1/MAC cells growing in M-CSF or GM-CSF were prepared as described in MATERIALS AND METHODS. The RNA contribution from a M-CSF stimulated cell lysate alone is shown in lane 1. Approximately 100 μ g of total cellular RNA isolated from M-CSF growing FDC-P1/MAC cells was added for 30 or 60 min to an M-CSF stimulated cell lysate (lanes 2 and 3, respectively) or a GM-CSF stimulated cell lysate (lanes 4 and 5, respectively). RNA was extracted and analyzed by Northern blot with the use of 32 P-labeled *c-fms* (A) and actin (B) probes.

c-fms expression by controlling the stability of *c-fms* mRNA.

Trans-Regulation of c-fms Requires the Activation of a Selective Ribonuclease System

The mechanism of GM-CSF regulation could conceivably occur through the transcriptional activation of a transregulating factor or by the posttranslational modification of pre-existing factors, such as downstream phosphorylation events. However, both the re-expression and suppression of *c-fms* require between 6 and 12 h for the transcript level to be affected. These slow kinetics are more consistent with the regulation of a transregulating factor at the transcript and/or protein synthesis level and not by the posttranslational modification of a pre-existing population of inactive factors. More direct evidence for the transcriptional activation of a transregulating factor was obtained by the use of metabolic inhibitors in CSF switching experiments. Adding GM-CSF to *c-fms* expressing FDC-P1/MAC cells effectively suppressed the transcript, and this suppression could be blocked with the protein synthesis inhibitor cycloheximide or with the transcription inhibitor actinomycin D. Therefore, it appears that GM-CSF directly stimulates the activation of a transregulating factor through the induction of its synthesis.

The transregulation of *c-fms* expression requires the functional activation of a selective ribonuclease degradation system. The induction of this ribonuclease system could be accomplished through the direct synthesis of a specific RNase in GM-CSF-stimulated cells. Alternatively, a co-factor could be synthesized that specifically regulates the activity of an RNase. This co-factor could directly activate an inactive RNase or serve as a RNA binding protein to direct an RNase to its substrate. The results of the *in vitro* mRNA degradation analysis suggest that a broad acting RNase is active in GM-CSF stimulated lysates, but a co-factor that controls mRNA specificity *in vivo* may be lost. Recent studies have identified several mRNA binding proteins (Bohjanen *et al.*, 1991; Brewer, 1991; Vakalopoulou *et al.*, 1991), of which one has associated ribonuclease activity (Brewer, 1991). Currently, it remains unknown how these factors regulate mRNA levels and how the factors themselves are regulated. Our results demonstrate that the synthesis of some critical factor is required to transregulate *c-fms*, but the identity of this factor will require its isolation and characterization.

The involvement of transcript stabilizing factors, or other mRNA binding proteins other than RNases, can not be ruled out as being necessary for *c-fms* expression. Recent work has demonstrated that a variety of protein factors can bind to RNA transcripts. These include poly A binding proteins (Blobel, 1973), AUUUA sequence binding factors (Malter, 1989), and stabilizing factors that bind to mRNA stem-loop structures (Mullner *et al.*,

1989). However, unless mRNA stabilizing proteins are extremely labile, general protein synthesis and transcriptional blockers would be expected to have the most noticeable effect on ribonucleases resulting in more stable transcripts.

An extremely labile stabilizing factor was described for *c-fms* when HL-60 cells were induced to differentiate along the monocyte/macrophage pathway with TPA (Weber *et al.*, 1989). Treatment of *c-fms* expressing FDC-P1/MAC cells with cycloheximide did not result in a rapid decrease of *c-fms* transcript as was found in HL-60 cells. Instead, cycloheximide caused a super-induction of the *c-fms* transcript, and we have no evidence of a labile stabilizing factor in FDC-P1/MAC cells. However, indirect evidence for a potential stabilizing factor needed to maintain *c-fms* expression is seen by the decreased level of *c-fms* transcript after removal of GM-CSF in the presence of cycloheximide and by long-term cycloheximide treatment of cells growing in M-CSF. If stabilizing proteins are present in FDC-P1/MAC cells, they are not readily apparent and the dominant regulatory mechanism stimulated by GM-CSF is the transcript degradation pathway.

Posttranscriptional regulation of gene expression is emerging as a major form of gene regulation in hematopoietic cells. Examples of other genes regulated at the posttranscriptional level include transforming growth factor $\beta 1$ (Wager and Assoian, 1990), nuclear proto-oncogenes *myc* and *fos* (Schuler and Cole, 1988), and a variety of hematopoietic cytokines (Thorens *et al.*, 1987; Ernst *et al.*, 1989; Wodnar-Filipowicz and Moroni, 1990). The 3' untranslated region of several of the posttranscriptionally regulated transcripts has been shown to play a significant role in governing their stability. A common feature of this region is a stretch of AU-rich sequences and the presence of multiple AUUUA motifs (reviewed Brawerman, 1989). How these sequences may regulate the expression of a transcript remains unknown, but recent work has shown that this region can mediate both the stability and instability of the same transcript, depending on the cell type in which it is expressed (Schuler and Cole, 1988). The murine *c-fms* transcript contains two AUUUA motifs in its 3' untranslated region but no stretch of AU-rich sequences (Rothwell and Rohrschneider, 1987). However, the AUUUA motifs are not conserved in the human *c-fms* transcript sequence (Coussens *et al.*, 1986). Experiments are currently in progress to identify a potential transregulating signal in the *c-fms* transcript and determine what role, if any, the AUUUA motifs play in transregulation.

Implications for CSF Receptor Regulation and Myeloid Lineage Restriction

The regulated expression of other myeloid lineage specific receptors remains poorly understood. Studies on the murine myeloid line 32D, which is dependent on

multi-CSF or G-CSF for growth, suggest that multi-CSF can transregulate the G-CSF receptor in a factor concentration-dependent manner (Valtieri, 1987). In addition, multi-CSF has been shown to be able to suppress high-affinity erythropoietin binding on murine erythroblasts (Fraser *et al.*, 1988). A potentially more interesting example of receptor regulation is provided by *c-kit*, which is the receptor for the recently described stem cell factor (reviewed Witte, 1990). Recent studies suggest that *c-kit* is expressed in relatively early progenitor cells and can synergize with a variety of CSFs to produce more and larger colonies in vitro (Zsebo *et al.*, 1990) Broxmeyer *et al.*, 1991). However, with further hematopoietic differentiation *c-kit* becomes restricted to primarily the erythroid and mast cell lineages (Nocka *et al.*, 1989). A recent report using the FDC-P1 cell line suggested that *c-kit* mRNA levels, like *c-fms*, can be regulated by GM-CSF or multi-CSF (Welham and Schrader, 1991). However, the regulatory mechanisms used in these other cell systems has yet to be elucidated at the molecular level. The transregulation of *c-fms* begins to provide a molecular description of how this regulation may occur at the cellular level and may be one mechanism used to restrict a developing hematopoietic cell to a specific lineage.

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