Regulation of Transferrin Receptor Expression in Myeloid Leukemia Cells

By Raymond Taetle, Steven Ralph, Shelley Smedsrud, and Ian Trowbridge

Surface transferrin (Tf) receptors are displayed by cultured human hematopoietic cells and provide Fe required for cell growth. Cell cycle status, cell density in culture, exposure to Fe, and differentiation alter Tf receptor display by myeloid leukemia cells. To investigate mechanisms controlling Tf receptor expression, rates of receptor synthesis and steady-state mRNA levels were measured in HL6O promyelocytic cells grown in serum and serum-free media or after differentiation in response to dimethylsulfoxide (DMSO). Although surface binding sites were unchanged during the first three days in culture with serum or in serum-free media containing Tf, by the third day, rates of receptor biosynthesis and steady-state mRNA levels declined, consistent with cell density-dependent receptor regulation. Cells grown with soluble Fe instead of Tf showed reduced Tf binding sites, rates of receptor synthesis, and Tf receptor mRNA. When cells grown with Fe were subcultured, Tf receptor mRNA levels increased within 15 minutes and peaked by 24 hours. This was followed by a decline in receptor and gene expression so that by three days cells grown in the presence of Fe expressed approximately four times fewer receptors and had half the rates of Tf receptor synthesis and mRNA levels of cells grown in serum or Tf. Cells treated with DMSO showed a rapid decline in surface receptors, receptor synthesis, and steady-state mRNA levels. Modulation of Tf receptor expression was not due to redistribution between the cell surface and an internal receptor pool. In each instance, concurrent assessment of N-ras transcripts showed that changes in Tf receptor mRNA levels were not due to generalized alterations in protein synthesis. Exposure of cells grown in Fe or treated with DMSO to cycloheximide did not alter Tf receptor mRNA levels, thereby suggesting that receptor expression was not regulated by posttranscriptional processes dependent on protein synthesis. Actinomycin D inhibition of Tf receptor mRNA was compatible with a transcript half-life of approximately 2.2 hours. Nuclear transcription studies showed reduced rates of Tf receptor transcription after culture with Fe or exposure to DMSO. The present studies show complex patterns of Tf receptor gene regulation in cultured myeloid leukemia cells and demonstrate that transcriptional regulation is a major mechanism controlling Tf receptor gene expression in response to Fe and differentiation.

© 1987 by Grune & Stratton, Inc.

From the Departments of Pathology and Medicine, Division of Laboratory Medicine and Hematology/Oncology, and Cancer Center, University of California, San Diego; and the Salk Institute for Biologic Studies, La Jolla, CA.


Supported by American Cancer Society Grant No. CH333 and Public Health Service Grants No. CA32094, CA37641, and CA23100.

Dr Ralph was an overseas training fellow of the Cancer Society of New Zealand, Inc, and a recipient of a J. Aron Charitable Foundation Fellowship.

Address reprint requests to Raymond Taetle, M.D., University of California Medical Center (H720), 225 Dickinson St, San Diego, CA 92103.

The publication costs of this article were defrayed in part by charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

© 1987 by Grune & Stratton, Inc.

0006-4971/87/7003-0042$3.00/0

METHODS

Cell cultures. HL60 cells were originally a gift from Dr. Stephen Collins, University of Washington, Seattle, and have been maintained in RPMI 1640 medium with 10% FBS (JMI International, Kansas City, CA). Serum-free cultures were established from these cells and maintained for more than three years in RPMI 1640 medium supplemented with ethanolamine, 5 μmol/L; insulin, 5 μg/mL; selenium, 2.5 nmol/L; and either transferrin, 25 μg/mL, or 5 μmol/L Fe nitrolacetate (NTA).
For growth assays, cells were seeded at 2 x 10^5/mL and viable cell counts performed daily using trypan blue dye exclusion. To induce granulocyte differentiation, cells grown with FBS were seeded at 3 x 10^5/mL with 1% DMSO and viable cell counts performed daily.

**Immunofluorescence staining.** Staining with monoclonal, anti-TF receptor antibody B3/25 was performed as previously described. A mixture of mouse antibodies served as a control. Positive cells were enumerated by using an Ortho 50H cytofluorograph (Ortho Diagnostics, Westwood, MA). DNA content was determined by propidium iodine staining and cell cycle status determined by a computer program as previously described.

The percentage of cells in S phase was confirmed by immunofluorescent staining of incorporated bromodeoxyuridine (BrdU). Radioligand binding. Surface TF binding was measured at 4°C and 37°C as described.

Briefly, Fe-saturated TF (Miles Pharmaceuticals, Naperville, IL) was radiolabeled in a 30-second incubation and 37°C as described. Cells grown under varying conditions were washed twice, incubated for two hours at 37°C in TF-free medium, and then incubated with 100 nmol/L labeled TF for two hours at 4°C. The cells were then washed and counted in a gamma counter. Nonspecific binding was determined by incubation with 200x unlabeled TF.

The total cell-associated TF was determined by incubation with labeled TF at 37°C. Cells were incubated in TF-free medium, labeled with TF at 4°C as described earlier, and then incubated for periods of five minutes to 24 hours at 37°C. All three lines reached a maximum binding within 30 minutes, and this time point was used for subsequent studies. Labeled cells were placed on ice and washed in phosphate-buffered saline (PBS) containing 100 mmol/L labeled TF for two hours at 4°C. The cells were then washed and counted in a gamma counter. Nonspecific binding was determined by incubation with 200x unlabeled TF.

**Immunoprecipitation of metabolically labeled TF receptors.** To determine rates of TF receptor synthesis, cells were metabolically labeled with 35S-methionine (Amersham Corp, Arlington Heights, IL) and TF receptors immunoprecipitated with B3/25. A total of 5 x 10^6 HL60 cells grown under varying conditions were washed, incubated for one hour in methionine-free medium, and resuspended in 1 mL of methionine-free medium containing 100 μCi 35S-methionine. After four hours at 37°C, medium containing excess unlabeled methionine was added and the cells incubated for another hour. Labeled cells were washed three times in cold PBS and resuspended in 200 μL PBS with 10 μL 0.01 mol/L phenylmethyl-sulfonylfluoride.

To study cells grown in FBS or serum-free media, aliquots containing equal trichloroacetic acid (TCA) precipitable counts (5 to 10 x 10^5 cpm) were removed and incubated at 4°C for two hours on a mechanical rocker with 100 μL 10% Panson suspension (Calbiochem-Behring, La Jolla, Ca) complexed to 28 μg rabbit antirabbi antimunoglobulin (Zymed Laboratories, S San Francisco) and 10 μg B3/25, monoclonal anti-TF receptor antibody, or an isotype-specific control. For studies of DMSO-treated cells, aliquots of cell lysates representing equal numbers of control and treated cells were analyzed. The immune complexes were pelleted in a microfuge, washed with PBS containing 0.3% NP-40, and layered over a 20% sucrose cushion. The tubes were spun for ten minutes in a microfuge and washed three times with buffer. The pellet was incubated for ten minutes with 0.5 mol/L LiCl and resuspended in buffer. The pellet was resuspended in E15 medium and assayed by freezing and thawing on acetone/dry ice. Complete lysis was confirmed byvisual examination.

**Determination of cellular ferritin.** Cellular ferritin was quantitated by radioimmunoassay according to the manufacturer’s instructions (Becton Dickinson, Orangeburg, NY).
Effects of cycloheximide and actinomycin D on transferrin receptor mRNA. To determine the effects of inhibiting protein or RNA synthesis on Tf receptor gene expression, HL60 cells were grown for 48 hours in FBS containing 1.0% DMSO or for 72 hours in ethanolamine, transferrin, insulin, and selenium (ETIS) or EIS-Fe. Cells were then incubated with media, 10 μg/mL cycloheximide (Sigma Chemical Co, St Louis) for 2.5 hours, or 5 μg/mL actinomycin D (Sigma) for two hours and washed and cellular RNA extracted.

Nuclear transcription assays. Transcription of nuclear mRNA was compared in cells cultured for 72 hours in FBS or EIS-Fe and in cells induced to differentiate with DMSO. The assays were performed as described by others. Briefly, 2 to 5 × 10⁷ nuclei were obtained by lysis in STKM buffer (30% sucrose, 40 mmol/L Tris, 37 mmol/L KCl, 12 mmol/L MgCl₂) with 0.5% NP-40. Extension of nuclear transcripts was carried out in a ten-minute incubation with 30 μg/mL DNase (BRL) in the presence of 1 mmol/L CaCl₂ with 0.8 mCi/mL ³²P-guanosinetriphosphate (3000 Ci/mmol/L), 0.5 mmol/L adenosine triphosphate, 0.25 mmol/L uridine triphosphate, and 0.25 mmol/L cytidine triphosphate, followed by a 30-minute treatment at 45°C. The RNA was purified free of DNA, protein, and unincorporated isotope and precipitated with 10% TCA (4°C for 30 minutes) and the labeled RNA recovered on type HA nitrocellulose filters (0.45-μm pore size). The filters were incubated with RNase I with 50 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, and 1 mmol/L CaCl₂ and the RNA eluted with 5 mmol/L EDTA/1% SDS at 68°C. The eluate was treated with protease K, extracted with phenol/chloroform, and precipitated at least twice in ethanol. Filters containing immobilized Tf receptor cDNA or pBR322 plasmid control DNA were prehybridized in 4x Denhardt's, 50% formamide, and 1 mg/mL phenol/chloroform-extracted tRNA (Boehringer-Mannheim, Indianapolis) at 42°C for three hours. Hybridization was carried out as described. Hybridization to control wells containing no DNA was also performed in each experiment and showed no specific binding. Equal radioactive counts derived from each experimental treatment, including DMSO exposure, were loaded into each well. After hybridization for three days at 42°C, the filters were washed twice in 300 mmol/L NaCl, 2 mmol/L EDTA, and 10 mmol/L Tris, followed by a wash in 10 mmol/L NaCl, 2 mmol/L EDTA, and 5 mmol/L Tris. The filters were then incubated with 10 μg/mL RNase A and 1 μg/mL RNase T1 and autoradiographed. Incubation of nuclei with 2 μg/mL α-amanitin completely abrogated new Tf receptor transcription.

RESULTS
In previous studies, we showed that log-phase cultures of HL60 cells grown in FBS- or TF-supplemented medium (ETIS) had similar numbers of Tf receptors, but whether measured by Tf binding or staining with monoclonal antibody B3/25, cells grown with soluble Fe (EIS-Fe) had substantially fewer receptors. As shown in Table 1, more extensive studies showed that this difference results from a marked temporal variation in Tf receptor expression on HL60 cells grown in EIS-Fe that was not observed under other culture conditions. During the first three days in culture, Tf receptor expression remained unchanged on cells grown with FBS or ETIS, whereas during the first day in culture, cells grown with EIS-Fe showed a transient five- to tenfold increase in Tf receptor expression. As a consequence, after one day in culture, cells grown with EIS-Fe expressed Tf receptor numbers similar to cells grown in FBS or ETIS but showed a rapid decrease in receptor expression during the ensuing 48 hours.

This modulation in Tf receptor expression by cells grown in EIS-Fe was not directly related to cell density or growth rate. As shown in Table 1, for the three-day culture period studied, HL60 cell growth was identical under all three culture conditions, and doubling times ranged from 22 to 24 hours. Further, cell cycle distributions were similar. As determined by propidium iodine staining, the proportion of cells in G₂ phase of the cell cycle ranged from 49% ± 4% to 53% ± 5% (mean ± SE, three to five determinations). In three independent experiments, the fraction of cells in S phase was determined by using BrdU incorporation. Consistent with data obtained by propidium iodine staining, cells grown in ETIS and EIS-Fe had similar relative proportions of cells synthesizing DNA (48% ± 5% and 44% ± 10% respectively).

To determine whether changes in Tf receptor expression on cells grown with EIS-Fe represented a redistribution between internal and cell surface pools, the total cell-associated Tf was measured at 37°C. As shown in Table 1, changes in the total receptor pool assessed by binding at 37°C closely paralleled changes in cell surface receptors, and relative distributions between surface and intracellular compartments remained constant. Thus, changes in surface Tf binding by cells grown with EIS-Fe were not the result of receptor mobilization from a preexisting, intracellular receptor pool.

To further characterize Tf receptor regulation in HL60 cells grown with FBS, ETIS, or EIS-Fe, steady-state Tf receptor mRNA and rates of receptor biosynthesis were investigated. Transferrin receptor mRNA levels were quanti-
titated by slot-blot analysis of total cellular mRNA. When hybridized to the human Tf receptor cDNA probe on Northern blots, RNA preparations used in these studies showed a single 4.9-kb band. The rate of Tf receptor biosynthesis was estimated by pulse labeling with $^{35}$S-methionine for four hours and analyzing immunoprecipitates by using SDS-PAGE under nonreducing conditions. As shown in Fig 1, the rates of receptor synthesis correlated well with steady-state levels of Tf receptor mRNA. After one day in culture, mRNA levels and receptor synthesis by cells grown in EIS-Fe were about one third that of cells grown in ETIS or FBS and declined even further after three days in culture. A twofold decrease was also observed in Tf receptor mRNA levels and rates of synthesis in cells grown in ETIS and FBS for three days. This suggests that, in cultures with FBS or Tf, cell density-dependent downregulation20'21 had been initiated through reduced Tf receptor biosynthesis, but because of the relatively long half-life of the Tf receptor glycoprotein,22 was not yet reflected by cell surface receptor expression.

Although these data were consistent with previous studies showing reduced Tf receptor expression by HL60 cells grown in EIS-Fe,4 they did not account for the transient elevation in Tf receptor levels detected in these cells after one day in culture. To address this point, Tf binding sites, Tf receptor synthesis, and mRNA levels at various times during the first 24 hours of culture in EIS-Fe were determined. A reproducible, rapid increase in Tf binding sites was observed during the first few hours, which increased to maximum levels within 24 hours (Fig 2). Although the precise time at which maximal mRNA and receptor synthesis occurred varied from experiment to experiment, Tf receptor synthesis and steady-state mRNA levels also consistently increased within 30 minutes after subculture and peaked by 24 hours (Fig 3A and B). In contrast, no change was observed in N-ras transcripts. Despite the fact that Tf receptor numbers on cells grown in ETIS remained relatively constant during the three-day culture period, an increase in Tf receptor mRNA levels was also observed in these cells during the first 24 hours after subculture (Fig 3B).

When HL60 cells are cultured in EIS-Fe, reduced Tf receptor levels are accompanied by a tenfold increase in intracellular ferritin.4'7 To determine whether the transient increase in Tf receptors during the first day in culture was accompanied by reciprocal changes in ferritin, ferritin levels were determined at various times during the first 24 hours of
culture with EIS-Fe. Values were similar to those previously reported4-7 (450 to 900 ng/10^7 cells) and showed no change during the first day in culture. HL60 cells were also resuspended for 24 hours in “spent” three-day EIS-Fe medium that had been dialyzed against fresh medium by using tubing with a 3,000-dalton exclusion limit. Increased Tf receptors were again noted. Thus, the transient increase in Tf receptor expression by HL60 cells grown in EIS-Fe was not accompanied by reduced cell ferritin, nor was it influenced by macromolecules in HL60 conditioned medium.

Downregulation of Tf receptors also occurs when HL60 cells grown in FBS are induced to differentiate into granulocytes with DMSO2 (Table 1). As shown in Fig 4, when HL60 cells were exposed to DMSO, within one day Tf receptor biosynthesis decreased by 90% and was accompanied by a similar decrease in steady-state levels of Tf receptor mRNA. In contrast, during the first two days, N-ras transcripts increased after DMSO treatment (Fig 4A). Thus, the early decrease in Tf receptor synthesis was specific and occurred before a generalized decline in protein synthesis seen on day

---

Fig 3. (A) Transferrin receptor mRNA levels increased rapidly in EIS-Fe–containing cells during the first 24 hours. At the times shown, total cellular mRNA was extracted and hybridized to a transferrin receptor or N-ras probe. Representative results from one of three studies are shown. (B) By scanning densitometry, transferrin receptor mRNA levels (E) and metabolically labeled receptors (X) increased during the first day in culture with EIS-Fe. A similar smaller increase occurred in Tf receptor transcripts in HL60 cells cultured in ETIS (.getD). In contrast, N-ras transcripts in EIS-Fe (O) were unchanged. Transferrin receptor transcripts and protein synthesis increased with varying kinetics from study to study.

---

Fig 4. (A) Relative levels of metabolically unlabeled transferrin receptors, transferrin mRNA, and N-ras mRNA in HL60 cells cultured with 1% DMSO. Labeled receptor protein, receptor mRNA, and N-ras mRNA were quantitated by scanning gels or slot blots. Representative results from one of three studies are shown. (B) Total cellular RNA extracted from HL60 cells cultured for one to three days with DMSO and analyzed by Northern blotting. The decrease in transferrin receptor mRNA after one day is apparent.
3 that was reflected by decreased incorporation of $^{35}$S-methionine into TCA-precipitable material and a reduction in $N$-ras transcripts.

Recent studies indicate that expression of some eukaryotic genes such as $c$-myc$^{15,16}$ is regulated by posttranscriptional mRNA stabilization. When cells are cultured with protein synthesis inhibitors such as cycloheximide, the rate of mRNA degradation decreases, and steady-state mRNA levels increase. To investigate whether similar mechanisms regulate Tf receptor expression, HL60 cells were incubated for two hours with cycloheximide and Tf receptor mRNA levels analyzed by Northern (not shown) and slot-blot hybridization. After cycloheximide treatment, cells cultured in EIS-Fe or ETIS for three days or incubated with DMSO for two days contained slightly decreased Tf receptor transcripts (Fig 5). In contrast, after incubation for two hours with cycloheximide and Tf receptor mRNA levels analyzed by Northern (not shown) and slot-blot hybridization. After cycloheximide treatment, cells cultured in EIS-Fe or ETIS for three days or incubated with DMSO for two days contained slightly decreased Tf receptor transcripts (Fig 5). In contrast, after incubation for two hours with cycloheximide and Tf receptor mRNA levels analyzed by Northern (not shown) and slot-blot hybridization. After cycloheximide treatment, cells cultured in EIS-Fe or ETIS for three days or incubated with DMSO for two days contained slightly decreased Tf receptor transcripts (Fig 5). In contrast, after incubation for two hours with cycloheximide and Tf receptor mRNA levels analyzed by Northern (not shown) and slot-blot hybridization. After cycloheximide treatment, cells cultured in EIS-Fe or ETIS for three days or incubated with DMSO for two days contained slightly decreased Tf receptor transcripts (Fig 5).

To confirm that Tf receptor gene regulation occurred at the level of gene transcription, nuclear transcription of Tf receptor mRNA was compared in cells cultured in FBS and Fe for 72 hours or induced to differentiate with DMSO. As shown in Fig 6A, cells grown with Fe showed rates of Tf receptor transcription approximately $1/2$ that of cells grown in FBS. Similarly, Tf receptor transcription was three- to tenfold less in cells treated with DMSO for 24 or 48 hours than in control cells (Fig 6B). Because equal numbers of radiolabeled mRNA counts from each experimental condition were hybridized to the Tf receptor probe, these results may underestimate the actual decrease in Tf receptor gene transcription. However, these results confirm that Tf receptor gene expression in response to Fe or DMSO is controlled at the level of gene transcription.

**DISCUSSION**

Tf receptor expression is closely coupled to important events in hematopoietic cell growth such as the transition of resting cells ($G_0$) to the DNA synthesis (S) phase of the cell cycle and induction of terminal myeloid leukemia cell differentiation. Receptor expression is required for progress through the cell cycle and, in leukemia cell differentiation, is suppressed before detection of other differentiation programs. Therefore, mechanisms controlling Tf receptor gene expression are intimately involved in regulation of hematopoietic cell growth and differentiation.

The use of stable, variant HL60 lines allowed us to examine steady-state Tf receptor mRNA in a variety of regulatory settings and to correlate transcript levels with receptor synthesis. To minimize effects of cell growth on Tf receptor display, we initially studied growing cells with identical proliferation rates and cell cycle distributions. Using these conditions and DMSO induction, we determined that altered surface Tf binding reflected changes in the total receptor pool.

In previous studies of HL60 and other hematopoietic cells, Tf receptor display declined with increased cell density in culture. In the present studies, cells were grown in FBS and ETIS for only three days, and no change was observed in Tf binding. However, Tf receptor protein synthesis and mRNA levels were reduced by three days, thus indicating the onset of decreased receptor synthesis. It is likely, therefore, that prolonged culture would lead to decreased levels of surface transferrin receptors as described by others. The basic pattern of the cell density-dependent regulation of transferrin receptor expression in cells cultured in EIS-Fe was similar to that of cells grown in FBS and ETIS. The
Thus, regulation of the If receptor gene does not involve posttranscriptional mechanisms requiring protein synthesis. Consistent with results recently reported by Rao et al.,

nuclear transcription assays confirmed that cells grown with Fe had lower rates of Tf receptor gene transcription. After DMSO treatment, Tf receptor gene transcription was also reduced. This was observed despite the fact that equal radioactive counts were hybridized to each probe. Thus, the observed changes were not attributed to a generalized reduction in nuclear RNA synthesis.

Although cycloheximide did not alter Tf receptor gene expression, RNA synthesis was inhibited by actinomycin D. A two-hour pulse decreased Tf receptor transcript levels by slightly less than 50%, consistent with a transcript half-life of 2.2 hours. This figure is in close agreement with recent studies examining Fe effects on K562 Tf receptor gene transcription.

In vitro, Tf receptor gene expression is rapidly modulated and results obtained when using cells grown with Fe indicate that multiple regulatory mechanisms interact. Regulation of this gene is remarkable for both its rapidity and multiplicity of control mechanisms. The present and other recent studies indicate that Tf receptors are controlled at the level of gene transcription. Recently, the S' noncoding regulatory regions of the Tf receptor gene have been cloned and sequenced and appear to control serum-stimulated Tf receptor gene transcription. Specific binding of proteins to this region has been demonstrated, and in the future, it may be possible to determine whether the same or different proteins mediate Tf receptor gene expression in these diverse settings. In mouse cells transfected with mutant human Tf receptor constructs, the S' noncoding regions also appear to regulate Tf receptor expression in response to iron. Further studies will determine how these regions of the Tf receptor gene interact during regulation of Tf receptor gene expression.

ACKNOWLEDGMENT
We wish to express our appreciation to Drs Roland Newman and Miles Wilkinson for their many helpful discussions during this project.

REFERENCES

32. Owen D, Kuhn LC: Noncoding 3′ sequences of the transferrin receptor gene are required for mRNA regulation by iron. EMBO J 6:1287, 1987
Regulation of transferrin receptor expression in myeloid leukemia cells

R Taetle, S Ralph, S Smedsrud and I Trowbridge