THE JOURNAL OF BIOLOGICAL CHEMISTRY
© 1989 by The American Society for Biochemistry and Molecular Biology, Inc.

Vol. 264, No. 22, Issue of August 5, pp. 13181-13187, 1989
Printed in U.S.A.

Iron Up-modulates the Expression of Transferrin Receptors during Monocyte-Macrophage Maturation*

(Received for publication, December 30, 1988)

Ugo Testa‡, Marina Petrini‡, Maria Teresa Quaranta‡, Elvira Pelosi-Testa‡, Gianfranco Mastroberardino§, Antonio Camagna¶, Giovanni Boccoli‡, Massimo Sargiacomo‡, Giancarlo Isacchi‖, Anna Cozzi, Paolo Arosio**, and Cesare Peschle‡ ‡‡**

*From the ‡Department of Hematology, Istituto Superiore di Sanità, Viale Regina Elena 299 and the ‖Department of Hematology, Blood Transfusion Center, §Istituto Patologia Medica (VI), ¶II Clinica Medica, University "La Sapienza," Rome 00161, Italy and the **Department of Biomedical Sciences and Technology, University of Milan, Milan 20132, Italy*

We have investigated the effect of iron on the expression of transferrin receptors (TrfRs) and ferritin chains in cultures of human peripheral blood monocytes maturing to macrophages.

Monocyte-macrophage maturation is associated with a gradual rise of Trf-binding capacity in the absence of cell proliferation. At all culture times, treatment with ferric ammonium citrate induces a dose-dependent rise of the Trf-binding level as compared with nontreated cells. Scatchard analysis revealed that this phenomenon is due to an increase in receptor number rather than an alteration in ligand-receptor affinity. Biosynthesis experiments indicated that the rise in number of TrfRs is due to an increase of receptor synthesis, which is associated with a sustained elevation of the TrfR RNA level. The up-regulation of TrfR synthesis is specific in that expression of other macrophage membrane proteins is not affected by iron addition. Conversely, addition of an iron chelator induced a slight decrease of TrfR synthesis.

The expression of heavy and light ferritin chains at RNA and protein levels was markedly more elevated in cultured macrophages than in fresh monocytes, thus suggesting modulation of ferritin genes at transcriptional or post-transcriptional levels. Addition of iron salts to monocyte-macrophage cultures sharply stimulated ferritin synthesis but only slightly enhanced the level of ferritin RNA, thus indicating a modulation at the translational level.

These results suggest that in cultured human monocytes-macrophages, iron up-regulates TrfR expression, thus in sharp contrast to the negative feedback reported in a variety of other cell types. These observations may shed light on the mechanism(s) of iron storage in tissue macrophages under normal conditions and possibly on the pathogenesis of diseases characterized by abnormal iron storage.

Peripheral blood monocytes, originating from bone marrow progenitor cells, mature to different types of histiocytes and macrophages when they migrate from the bloodstream into various tissues (1). Different populations of macrophages can be distinguished on the basis of morphological and functional criteria (2, 3).

Monocytes-macrophages play an important role in host defense, particularly in the inflammatory process of acute and chronic disease (4). A number of these phenomena are iron-dependent, including the generation of oxidants for bacterial killing (5) and the production of arachidonic acid metabolites (6). Furthermore, monocyte-macrophages, together with other cells of the reticuloendothelial system, play a key role in the storage of iron, which is mostly derived from the breakdown of senescent red blood cells. Under normal conditions, the iron reserve is largely confined to liver, bone marrow, and skeletal muscle reticuloendothelial cells. The capacity of these elements to catabolize and store the iron of damaged red blood cells is enormous, amounting to as much as 20% of the red blood cell mass per day (7). In both humans (8) and animals (9), nearly equal amounts of processed iron are either returned to circulating transferrin (Trf)[1] or stored as ferritin and hemosiderin; the ratio is nearly constant over a 10-fold increase in reticuloendothelial iron load, but a further increase leads to enhanced iron storage.

When blood monocytes are grown in medium containing either human or bovine serum (5–20%), they adhere to plastic surfaces and undergo maturation to macrophages, as indicated by a number of morphological and biochemical parameters (2, 3, 10, 11). Thus, the intracellular level of lysosomal enzymes increases, the number and function of complement receptors are markedly modulated, the phagocytic capacity is enhanced, and the expression of various membrane antigens of known and unknown functions is up-regulated (10, 11). This is paralleled by an increase in cell size and granularity.

A majority of macrophages generated *in vitro* from monocytes reacts with anti-TrfR antibody, whereas circulating monocytes do not (12). Furthermore, 90–95% of macrophages from bronchoalveolar lavage fluids, as well as a minority of those from peritoneal and pleural cavities, possesses TrfRs (12). Presence of high affinity TrfRs on both murine and human macrophages has recently been confirmed (13). Consistent with these observations, TrfR mRNA was demonstrated in both alveolar and *in vitro* matured macrophages but not in blood monocytes (13). TrfR expression may reflect macrophage immune activation. Indeed, recent studies suggest that macrophage activation may affect macrophage iron release (14, 15). Furthermore, monocyte activators, *e.g.* inter-

* This work was supported by grants from the Italian-United States program on the therapy of tumors, "Associazione Italiana per la Ricerca sul Cancro" (AIRC), "Ministero della Pubblica Istruzione" (60%), and "Consiglio Nazionale delle Ricerche," Rome, Progetto Finalizzaro "Oncologia" (No. 87.01568.44). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡‡ To whom correspondence should be sent.

[1] The abbreviations used are: Trf, transferrin; TrfR(s), transferrin receptor(s); H, heavy; L, light; SDS, sodium dodecyl sulfate; BSA, bovine serum albumin; mAb(s), monoclonal antibody(ies); kb, kilobase.

This is an Open Access article under the CC BY license.

feron-γ, stimulate the expression of TrfR in human monocytes-macrophages (16–18).

Previous studies in other cell systems, *i.e.* fibroblasts (19), leukemic lines (20), mitogen-activated T lymphocytes (21), and hepatoma cells,[2] indicated that TrfRs are specifically modulated by the intracellular iron concentration via a negative feedback. Thus, iron treatment induces a decline in TrfR synthesis, whereas addition of an iron chelator enhances the receptor synthesis (*cf.* Ref. 22).

It seemed of interest to investigate the regulation of TrfR expression in human monocytes maturing *in vitro* to macrophages. In sharp contrast to the negative feedback observed in other cell types, iron induces an up-regulation of TrfR expression in macrophages via modulation at the RNA level. Heavy (H) and light (L) ferritin chain synthesis is strongly enhanced during monocyte-macrophage maturation. The expression is further up-regulated by iron treatment at the protein but not the RNA level, thus indicating a modulation via translational mechanisms.

EXPERIMENTAL PROCEDURES

Materials—Human Trf (Sigma) was purified by gel filtration chromatography to an electrophoretically homogenous preparation as estimated by SDS-polyacrylamide gel electrophoresis. Ferric ammonium citrate, bovine serum albumin (BSA, fraction V), and picolinic acid were purchased from Sigma. ^{125}I-Trf and ^{125}I-F(ab′)$_2$ sheep anti-mouse IgGs were obtained from Du Pont-New England Nuclear. Desferoxamine B (Desferal) was a gift from Ciba-Geigy.

Isolation and in Vitro Culture of Monocytes and Macrophages—Peripheral blood mononuclear cells were obtained from 18–40-year-old healthy male and female donors. Venous blood mixed with 12 IU/ml sodium heparinate was separated on Ficoll-Paque solution (Pharmacia LKB Biotechnology Inc.) at 450 × *g*/20 min. Peripheral blood mononuclear cells were washed three times with RPMI 1640 medium (Flow Laboratories, Inc.) and then suspended (4 × 10^6 cells/ml) in the same medium containing 20% fetal calf serum (Flow Laboratories). Monocytes were separated from lymphocytes by adherence (1 h at 37 °C) in plastic dishes. After removal of the nonadherent population, cells were washed three times with RPMI 1640 medium to remove residual nonadherent elements; cytochemical and surface marker analysis revealed that the adherent population consisted of >95% monocytes. Thereafter, cells were cultured in RPMI 1640 medium containing 15% fetal calf serum in 5% CO_2 in air at 37 °C. Mo-cell line conditioned medium (1%) (23) was added to the culture on different days to improve cell viability and monocyte to macrophage maturation. At various culture days, monocytes-macrophages were harvested by vigorous pipetting after a 20–30-min incubation at 4 °C. Cell viability ranged from 85 to 100% as determined by the trypan blue exclusion test. After 2–5 days of culture, monocytes were partially detached from the plastic surface and grew in suspension. However, both adherent and nonadherent cells terminally matured to macrophages.

Assay of Ferritin Concentration—Intracellular ferritin concentration was evaluated by radioimmunoassay (Behring Institute, Marburg, Federal Republic of Germany). Samples were lysed in distilled water, freeze-thawed three times, and centrifuged at 20,000 × *g*/30 min at 4 °C. The supernatant was then utilized for radioimmunoassay. Protein concentration was determined by the dye-binding method (Bio-Rad).

Trf Receptor (TrfR) Assay—Purified human Trf was conjugated with ^{125}I by the solid phase lactoperoxidase method (Du Pont-New England Nuclear radioiodination system) as described (16). The binding reaction was performed in polypropylene tubes (12 × 75 mm) in Hanks' saline medium containing 0.1% BSA (5 × 10^6 cells/ml, labeled and unlabeled Trf, 500 ng and 1 mg/ml, respectively). Unbound ligand was removed by passaging cells through a density cushion (21, 24).

200-microliter aliquots of the cell suspension were incubated and layered on 150 µl of a dibutyl- and dinoyl-phtalate mixture (Merck) up to a final density of 1.205 in 400-µl plastic tubes and then centrifuged in an Eppendorf microcentrifuge (13,000 × *g*/2 min). The resulting supernatant and a major part of the phtalate cushion were then aspirated. The tips of the vials, containing cell pellets, were severed with a scalpel and transferred to plastic vials, and radioactivity was measured in a γ-counter. "Total" binding corresponded to the radioactivity in the cell pellet. "Nonspecific" binding was represented by the radioactivity detected in the presence of a large excess of unlabeled Trf. "Specific" binding data were the differences between total and nonspecific values.

Before binding, cells were washed three times in 40 ml of Hanks' saline solution. The number of washes did not modify the Trf-binding capacity since cells were grown in fetal calf serum and bovine Trf, the latter having a low affinity for human TrfR.

Trf Receptor Biosynthesis—10^7 cells, washed three times in RPMI 1640 methionine-free medium, were incubated for 60 min at 37 °C in the same medium containing 4 mg/ml BSA and 100 µCi of [^{35}S] methionine (Du Pont-New England Nuclear). Cells were then washed three times in Hanks' saline solution and dissolved in phosphate-buffered saline containing 1% Triton X-100 and 2 mM phenylmethylsulfonyl fluoride (Sigma). After 30 min at 4 °C, cells were centrifuged for 30 min at 20,000 × *g* and the supernatant added to 50 µl of Sepharose-Trf, obtained by coupling purified human Trf to CNBr-activated Sepharose 6B (Pharmacia). The supernatant and resin were incubated for 30 min at 20 °C and then 90 min at 4 °C. The resin was first washed three times with Tris-HCl containing 1 mM phenylmethylsulfonyl fluoride, 0.5% Triton X-100, 0.2 M NaCl and then washed three times with the same buffer containing 0.5 M NaCl. 100 micro-liters of Tris-HCl (20 mM, pH 7.20) containing 2% sodium dodecyl sulfate (SDS) and 5% β-mercaptoethanol were added to the resin and boiled 5 min at 100 °C. After electrophoresis, the gel was stained with Coomassie Blue and treated for autoradiography as reported (25). The intensity of the radioactive bands was evaluated by densitometry.

It is emphasized that the amount of Sepharose-Trf (50 µl) utilized to purify TrfR fixes several µg of TrfR, *i.e.* was excessive as compared with the Trf-binding capacity of 10^7 cells. In control experiments, there was the recovery of Trf-binding capacity since no Trf-binding capacity was detected in the cell supernatants after reaction with 50 µl of Trf-Sepharose.

Immunofluorescence Studies—0.5 × 10^6 monocytes-macrophages were first washed three times in Hanks' saline solution and then incubated for 60 min at 4 °C with 200 µl of a 1:100 dilution (in Hanks' saline solution containing 1 mg/ml BSA) of the monoclonal antibody (mAb). Two antibodies were used: B3/25 to human TrfR (a generous gift of Dr. I. S. Trowbridge, Salk Institute, San Diego, CA) and anti-HLA-DR or anti-human monocyte mAbs (Leu-M3, Leu-M5) (Becton-Dickinson).

Cells washed at 4 °C in Hanks' saline solution were incubated for 60 min at 4 °C with fluorescein isothiocyanate-labeled F(ab′)$_2$ fragments of immunoadsorbent-purified sheep antibodies against mouse IgGs. After three additional washes, cells were mounted on slides in 50% glycerol in phosphate-buffered saline. The proportion of stained cells was scored in incident light with a Leitz standard universal fluorescent microscope, equipped with a set of filters for narrow fluorescence.

RNA Analysis—Total RNA was extracted from 2–3 × 10^7 macrophages by the guanidine isothiocyanate phenol-chloroform technique (26). Integrity of RNA was assessed by evaluation of the 28 S/18 S RNA ratio on 1% agarose Minigels stained with ethidium bromide. The 9.5–0.3-kb RNA ladder was used as molecular size marker. The levels of TrfR and ferritin RNA were evaluated by dot and Northern blot analysis. For dot blot analysis, decreasing amounts of total RNA (4–0.5 µg) were dotted onto nitrocellulose paper (BA-85, Schleicher & Scheull) through a 96-well Minifold apparatus, fixed by baking for 2 h at 80 °C in a vacuum, and hybridized to 10^7 cpm of nick-translated probe (specific activity, 3–6 × 10^8 dpm/µg) as described (27). Filters were washed under stringent conditions (15 mM NaCl, 1.5 mM sodium citrate, 0.1% SDS, pH 7.0, at 65 °C) and exposed at −70 °C to Kodak SO-282 x-ray films using X-Omatic intensifying screens. For Northern analysis, 5 µg of total RNA was run on 1% formaldehyde gels, transferred onto nitrocellulose filter by capillary blot (27), hybridized, washed, and exposed as for dot blots. Rehybridization was carried out after removal of probe by washing with 5 mM Tris-HCl, pH 8.0, 2 mM EDTA, 0.002% Ficoll (Pharmacia), 0.002% polyvinylpyrrolidone (Sigma), and 0.002% BSA (Sigma) for 1 h at 65 °C. Appropriately exposed autoradiograms were analyzed by densitometric scanning. Expression of H- and L-ferritin genes was evaluated by densitometric comparison with standard dots of 4 µg of human rRNA containing various amounts (usually 1–250 pg) of the human H- and L-ferritin cDNA probes. Appropriate conversion factors were formulated for

[2] U. Testar and C. Peschle, unpublished observations.

each probe, thus accounting for their size as compared with full length mRNA. The final data were expressed as pg of mRNA/μg of total RNA dotted onto the filters.

The probes used were the 4.9-kb full length cDNA of human TrfR (pcD-TR1) (28), the 0.86- and 0.70-kb cDNA of human H- and L-ferritin chains (FR 33 and FR 36) (29), and the 11-kb genomic probe of human 28 S ribosomal RNA (pHR 28A).

Phagocytic Activity—10^6 cells were incubated for 16 h in 1 ml of culture medium (RPMI 1640) supplemented with 10% fetal calf serum in 17 × 100-mm silicone-coated glass tubes containing a 0.02% suspension of latex particles (0.8-μm bead diameter, Sigma). Cells containing more than five latex particles were scored as phagocytic cells. Experiments described in Table II were performed four times with similar results.

H- and L-Ferritin Content—H- and L-ferritin was evaluated by a sensitive immunoenzymatic method using specific mAbs (see Ref. 30).

RESULTS

Iron Salts Up-regulate the TrfR-binding Capacity in Cultured Monocytes-Macrophages—To evaluate the effect of iron salts on the expression of TrfR in maturing monocytes-macrophages, we added ferric ammonium citrate (50 μg/ml) to the culture and measured TrfR expression at different times thereafter.

Throughout the observation period (*i.e.* from day 1 to 15) the [^{3}H]thymidine incorporation level was not modified, thus suggesting that under these experimental conditions monocytes are unable to proliferate (data not shown). TrfRs were detected on monocytes from day 3 onward. Peak expression was observed at days 10–12 when a large majority of cells was morphologically identified as macrophages (Fig. 1).

At all culture times, iron-treated cells showed a higher Trf-binding capacity than controls (Fig. 1), *e.g.* at day 12, macrophages grown in the presence of iron exhibited a mean Trf-binding capacity (4.1×10^4 cpm/10^6 cells) significantly higher than controls (2.55×10^4). This difference was consistently observed in monocyte-macrophage cultures from six different donors (Table I). The increase in Trf-binding capacity was concentration-dependent and peaked at 100–200 μg/ml of iron salts (Fig. 2). Scatchard analysis (31) revealed that it was due to an increase in receptor number rather than an alteration in ligand-receptor affinity (Fig. 3), *e.g.* on day 12, control macrophages possessed 1.46×10^5 TrfRs/cell as compared with 3.3×10^5 in iron-treated cultures.

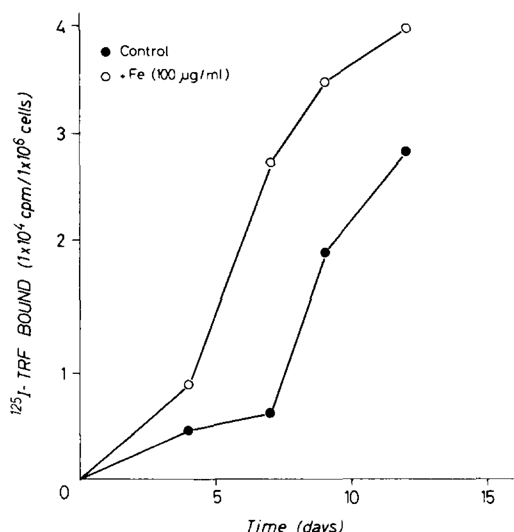

FIG. 1. **Time course of the expression of TrfR on *in vitro* cultured blood monocytes grown in the absence (control) or presence of ferric ammonium citrate (100 μg/ml).** At different days of culture, differentiating monocytes were harvested and analyzed for their capacity to bind ^{125}I-Trf. The results of a single representative experiment are reported.

TABLE I

^{125}I-Trf-binding capacity of human monocytes grown in vitro in either the absence (control) or presence of iron salts

The data represent the mean level of Trf binding from six separate experiments. Statistical analysis showed that the increase of binding values at days 2–12 in iron-supplemented *versus* control cultures is significant (t, 3.76; $p < 0.05 > 0.01$).

Day of culture	Control	Ferric ammonium citrate (100 μg/ml)
	10^4 cpm/10^6 cells	
0	0.012	0.012
2	0.13	0.21
5	0.5	1.033
7	1.05	2.22
9	1.65	3.35
12	2.55	4.1

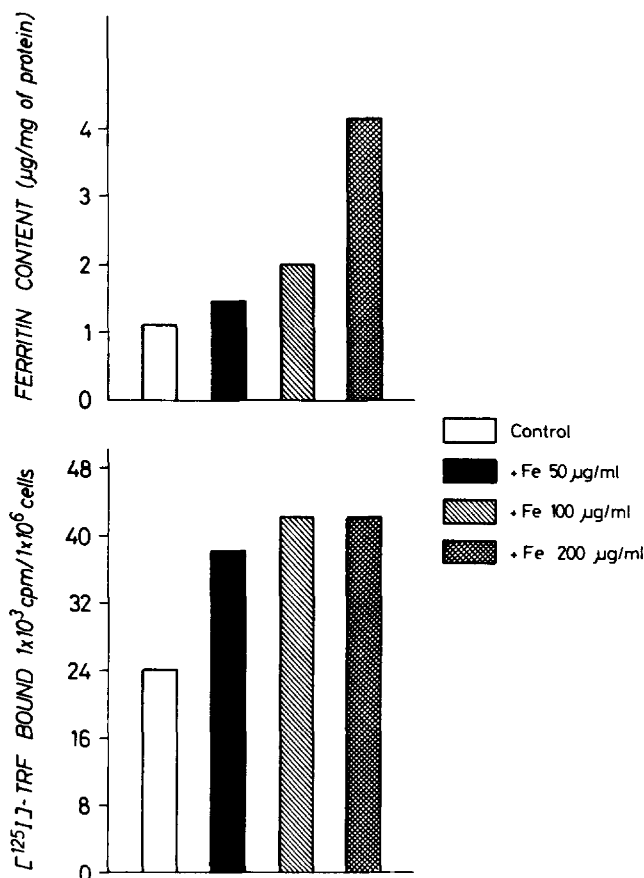

FIG. 2. **Effect of increasing concentrations of iron on the ferritin content and ^{125}I-Trf binding of human monocytes grown *in vitro*.** Human monocytes were grown *in vitro* in the absence (control) or presence of increasing concentrations of ferric ammonium citrate (50, 100, and 200 μg/ml). At day 12 of culture, the cells were harvested and processed for evaluation of their ferritin content and Trf-binding capacity. The data represent the mean level from three separate experiments.

Iron Positively Regulates the Synthesis of TrfR Protein and RNA in Cultured Monocytes-Macrophages—To verify whether iron modulates the rate of TrfR synthesis, macrophages were incubated in the presence of [^{35}S]methionine at day 10 of culture, and TrfRs were then purified by affinity chromatography on Sepharose-Trf. Synthetic values were lower in control cells than in macrophages grown in the presence of ferric ammonium citrate, *i.e.* densitometric analysis of the 95-kDa

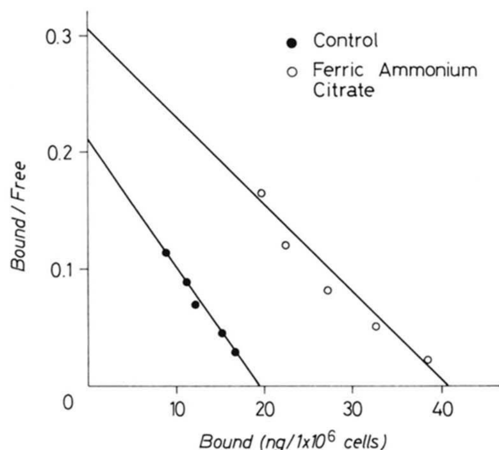

FIG. 3. **Scatchard analysis (36) of ^{125}I-Trf binding to differentiating human monocytes grown in culture for 12 days in the absence (control) or presence of ferric ammonium citrate (100 μg/ml).** The cells were washed and then incubated for 30 min at 20 °C with a fixed nonsaturating concentration of ^{125}I-Trf (500 ng/ml) and increasing concentrations of cold Trf, ranging from 500 to 5 mg/ml. The data are mean values from three separate experiments. Coefficients of variation are <5%.

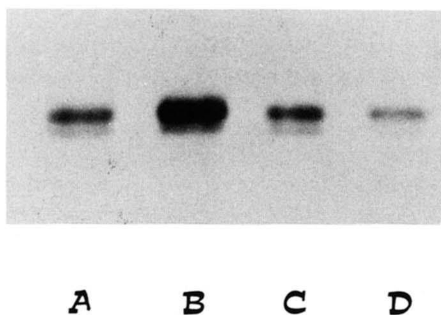

FIG. 4. **SDS-polyacrylamide gel electrophoresis of affinity chromatography-purified [^{35}S]methionine-labeled TrfR.** Human monocytes were grown for 12 days in the absence (*A*, control) or presence of ferric ammonium citrate (100 μg/ml) (*B*) and then labeled for 1 h with [^{35}S]methionine. Control experiments were performed on U937 promonocytic human cells grown in the absence (*C*, control) or presence of ferric ammonium citrate (5 μg/ml) (*D*). The cells were lysed with 1% Triton X-100-TrfR, purified by affinity chromatography, and then analyzed by SDS-polyacrylamide gel electrophoresis.

TrfR band showed that the level of synthesis in controls was 60% of that in iron-treated cells (Fig. 4). Conversely, iron addition induced a marked decrease of TrfR biosynthesis in a promonocytic line (U937) (Fig. 4) and an erythroleukemic one (K562) (not shown) thus in agreement with previous observations in a variety of other cell types (10, 11).

These results clearly show that the level of intracellular iron in cultured macrophages is directly related to the rate of TrfR synthesis.

We have investigated TrfR synthesis in human macrophages cultured for 12 days and then subjected to a 4-h treatment with an iron chelator. The chelator induced a significant decrease of TrfR synthesis (Fig. 5) again in sharp contrast to the rise observed in other cell types (22). These results strengthen the hypothesis that iron positively regulates the synthesis of TrfRs in maturing macrophages.

Further experiments were carried out to analyze the effect of iron on the level of TrfR RNA. Total RNA was extracted from macrophages at different times of culture (from day 1 to 8) in the presence or absence of ferric ammonium citrate (50 μg/ml). No TrfR RNA was detected in control fresh blood

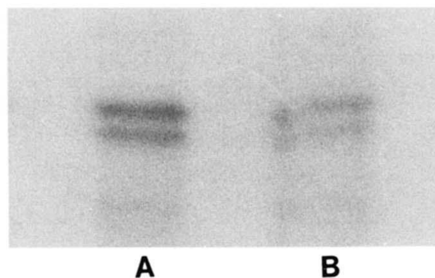

FIG. 5. **Effect of an iron chelator on the biosynthesis of TrfR in cultured human monocytes-macrophages.** Monocytes, grown *in vitro* for 10 days under standard conditions (*A*, control), were incubated for 4 h at 37 °C in the presence of an iron chelator (0.5 × 10^{-3} M desferrioxamine) (*B*) and then labeled with [^{35}S]methionine. The cells were then processed as described in the legend of Fig. 4.

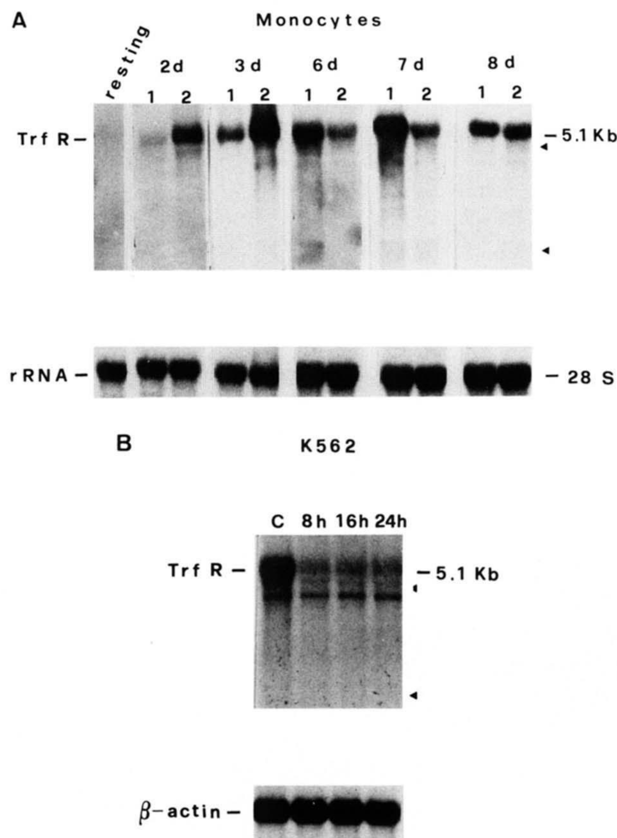

FIG. 6. *A*, Northern blot analysis of TrfR RNA expression in human monocytes grown for different times (0–8 days) in the absence (control, *lane 1*) or presence of ferric ammonium citrate (100 μg/ml, *lane 2*); a representative experiment is shown here. Five μg of total RNA was run on 1% formaldehyde/agarose gels, transferred onto nitrocellulose paper, and hybridized to the human TrfR cDNA probe (pcD-TR1). Hybridization to a human 28 S rRNA genomic probe is also shown. *B*, Northern blot analysis of TrfR RNA expression in K562 cells at different times (8, 16, and 24 h) after ferric ammonium citrate (5–10 μg/ml) addition. Hybridization with the β-actin probe is shown for normalization of RNA level.

monocytes. However, starting from day 1 of culture, the monocytes showed a gradual rise of the level of the 5.1-kb TrfR RNA transcript up to peak expression on days 6–8 (Fig. 6). During the initial culture days, cells treated with ferric ammonium citrate exhibited significantly higher levels of TrfR RNA as compared with controls (Fig. 6*A*). However, after day 6, the TrfR RNA level was slightly lower in iron-supplemented cultures than in controls (Fig. 6*A*).

TrfR RNA iron modulation was also tested in K562 cells;

addition of as little as 5–10 μg/ml ferric ammonium citrate dramatically decreased the TrfR RNA level starting from as early as 8 h after iron loading (Fig. 6B).

Specificity of TrfR Modulation by Iron in Cultured Monocytes-Macrophages—It is well known that maturation of monocytes to macrophages is associated with a marked increase of cell size and protein content. The size of fresh monocytes ranged between 8 and 12 μm, and their cytoplasm nucleus ratio was 1:1. In both control and iron-treated cultures, these parameters showed a significant increase: after

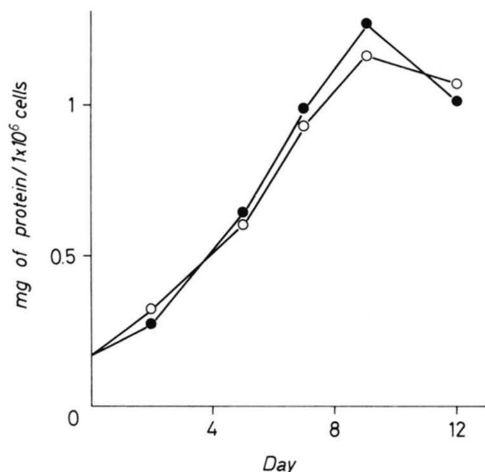

FIG. 7. **Protein content in human monocytes grown *in vitro* in the absence (control) (●) or presence (○) of ferric ammonium citrate (100 μg/ml).** The cells were harvested at different days of culture, lysed, and their protein content was assessed by the dye-binding method. The results represent the mean value obtained from three separate experiments.

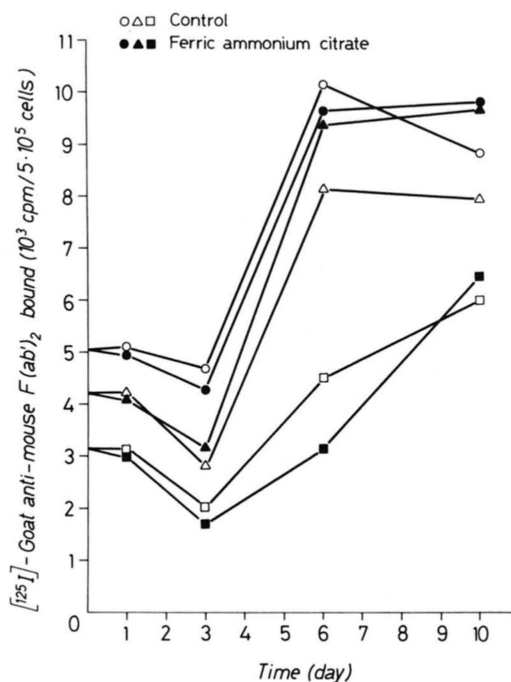

FIG. 8. **Expression of membrane antigens recognized by mAbs Leu-M1, Leu-M3, and Tec-M1 in human monocytes grown in the absence (control) or presence of ferric ammonium citrate (100 μg/ml).** The cells were harvested at different days of culture, incubated with mAbs, washed, and then incubated with ^{125}I-F(ab′)$_2$ anti-mouse Igs. The results represent the mean value obtained from two separate experiments. ○ and ●, Leu-M3; △ and ▲, Tec-M1; □ and ■, Leu-M1.

TABLE II

Phagocytic activity of monocytes grown in either the absence (control) or presence of ferric ammonium citrate

Monocytes were cultured for 10 days in either the absence (control) or presence of ferric ammonium citrate (50 μg/ml), and the percentage of latex phagocytic cells was then evaluated.

Experiment no.	Control	Ferric ammonium citrate (50 μg/ml)
	% phagocytic cells	
1	81	84
2	85	91
3	79	76

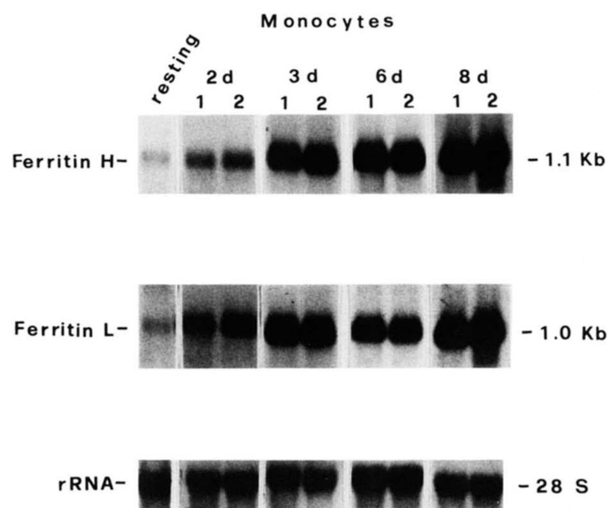

FIG. 9. **Northern blot analysis of H- and L-ferritin RNA expression in human monocytes grown for different times (0–8 days) in the absence (control, *lane 1*) or presence of ferric ammonium citrate (50 μg/ml, *lane 2*).** For other details, see legend of Fig. 6A.

12 days of culture, the size was 18–35 μm, and the cytoplasm/nucleus ratio was 3:1. In both conditions, the mean cell protein content progressively increased from day 1 to days 9–12 of incubation (Fig. 7).

The expression of several membrane monocytic markers (recognized by mAbs Leu-M3, Leu-M5, Tec-M1) was evaluated at different days in both control and iron-treated cultures (Fig. 8). Iron salts did not modify the expression level of these membrane antigens. Conversely, the number of TrfRs was significantly increased in iron-treated cultures as estimated by mAb to human TrfR.

Finally, the phagocytic activity of human monocytes, as evaluated by their capacity to phagocytize latex particles, was comparable in control and unsupplemented cultures (Table II).

Altogether, these results strongly suggest a specific effect of iron salts on TrfR expression in cultured monocytes-macrophages.

Expression of Ferritin Genes in Cultured Monocytes-Macrophages—Human monocytes-macrophages were grown *in vitro* in the absence or presence of ferric ammonium citrate (50 μg/ml). RNA from these cells was extracted at different days of culture and hybridized to cDNA probes specific for H- and L-ferritin genes. The sizes of transcripts are 1.1 and 1.0 kb for H- and L-ferritin, respectively. In fresh monocytes, low levels of both H- and L-ferritin RNAs were detected by dot or Northern blot. However, the cultured monocytes showed a progressive and marked increase of H- and L-ferritin RNA up to peak levels on days 6–8 (Fig. 9). Densitometric analysis of dot blot results indicated that fresh monocytes contained

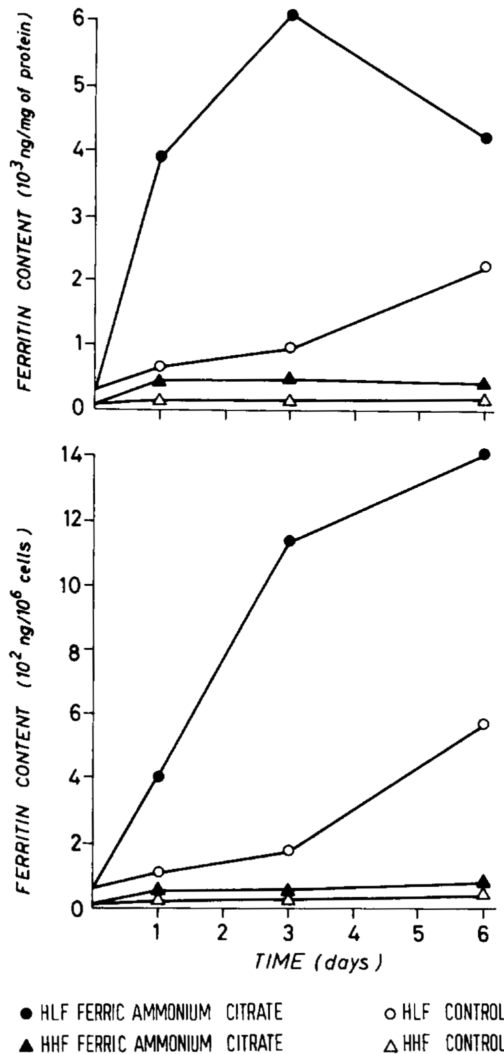

FIG. 10. **Basic human liver ferritin (*HLF*) and acidic human heart ferritin (*HHF*) concentrations in human monocytes grown *in vitro* in the absence (control) or presence of ferric ammonium citrate (100 μg/ml).** At different days of culture, the cells were harvested and then processed for evaluation of H- or L-ferritin content. The data are expressed as the ferritin concentration per 10^6 cell or per mg of protein. For further details see "Results."

19 or 30 pg of H- or L-ferritin RNA/μg total RNA, respectively. At day 3 of culture, the corresponding levels reached 500 and 1000 pg/μg, respectively. In iron-loaded cultures, the abundance of ferritin RNA was comparable or slightly lower than in controls (Fig. 9). In all cases, a higher level of L- and H-ferritin RNA was observed (Fig. 9).

We have evaluated the ferritin concentration in maturing monocytes in the presence or absence of iron supplementation. Control monocytes contained 0.2–0.4 μg of ferritin/mg of protein, a higher percentage represented by L- than H-ferritin (Fig. 10).

This base-line level progressively increased to 2–2.5 μg/mg of protein at day 6 of culture, when the majority of monocytes had differentiated (Fig. 10). Significantly higher levels of ferritin (*e.g.* up to 6 μg/mg of macrophage protein on day 6) were observed in macrophages grown in the presence of ferric ammonium citrate (Fig. 10). In all cases, the basic ferritin (human liver ferritin) content was much higher than that of acidic ferritin (human heart ferritin) (Fig. 10); a similar difference is observed when results are expressed in terms of ferritin concentration/10^6 cells (Fig. 10).

These results clearly show that maturing macrophages actively incorporate iron from the medium, which in turn markedly stimulates ferritin synthesis. This stimulation seems largely mediated by a translational mechanism since iron does not modify ferritin RNA levels but markedly stimulates its synthesis.

DISCUSSION

The expression of TrfR is regulated by complex mechanisms. A variety of factors, *e.g.* hemin, protoporphyrin, and growth factors, modulates the synthesis of the receptor (see Ref. 31). It is generally conceded that the expression of TrfR is directly correlated with the rate of cell growth (32, 33) and inversely related to the amount of iron accumulated in the cells (19–22). The correlation with cell growth is at least in part explained by the absolute requirement for iron of ribonucleotide reductase, *i.e.* an essential enzyme for conversion of ribonucleotides to their deoxy derivatives (34, 35). The iron modulation has been clearly demonstrated by experiments of iron load or chelation in a variety of cell types (19–22).

Our studies confirm that TrfRs are not detectable in fresh peripheral blood human monocytes. However, *in vitro* maturation of these cells to macrophages is linked with a gradual rise of the number of TrfRs. The maturation is apparently not associated with cell proliferation as indicated by the very low level of [^{3}H]thymidine incorporation throughout the culture period.

Addition of iron salts to the culture of monocytes-macrophages enhances their Trf-binding capacity in a time- and concentration-dependent manner. Scatchard analysis (36) revealed that the increase is mediated by a rise in the number of receptors rather than an alteration in the ligand-receptor affinity. Furthermore, iron treatment induces an early rise of the TrfR RNA level over control values, thus accounting for the higher TrfR synthesis in iron-treated macrophages.

These phenomena are associated with a marked increase of the intracellular iron concentration as indicated by the rise of the intracellular ferritin content over control level.

It is therefore concluded that in human monocytes maturing *in vitro* to macrophages, the intracellular iron level is directly related to the TrfR RNA level and hence to the synthesis and number of receptors.

The effect of iron is highly specific in that its addition does not affect expression of other membrane markers. In particular, iron treatment does not modulate the expression of the monocytic membrane antigens recognized by Leu-M3, Leu-M5, and Tec-M1 mAbs. Furthermore, the addition of iron salts does not modify the size and phagocytic capacity of macrophages.

As mentioned previously, experiments on a variety of cell types (*i.e.* fibroblasts (19), leukemic cell lines (20), mitogen-stimulated T lymphocytes (21), and hepatoma cells[2]) indicate that the expression of TrfR is modulated by iron through a negative feedback via the intracellular iron level. Indeed, TrfR synthesis is markedly reduced by iron addition although strongly enhanced by treatment with chelators (20, 22, 37). Furthermore, the fluctuations of the number of receptors are inversely correlated with those of the intracellular ferritin content and therefore of the intracellular iron level (38).

We suggest that TrfR expression is modulated by the intracellular iron level through a negative feedback in cells requiring iron for their growth or other functions, whereas a positive feedback operates in macrophages and possibly other iron storage cells. In the presence of elevated amounts of extracellular iron, the increase of TrfR may allow macrophages to store large amounts of the metal, whose toxic effect is neu-

tralized by simultaneous synthesis of elevated levels of intracellular ferritin. Studies are in progress to identify possible alterations of this macrophage iron storage mechanism in diseases characterized by abnormal iron accumulation.

The differential mechanisms regulating TrfR expression in macrophages *versus* other cell types are obviously mediated by alternative molecular pathways. In erythroleukemic lines, RNA transcription experiments in isolated nuclei suggested that the negative feedback is at least in part mediated by regulation at post-transcriptional level (39). Owen and Kühn (40) and Müllner and Kühn (41) reported that in mouse fibroblasts, expression of human TrfR cDNA constructs is regulated by the intracellular iron level. In this system, sequences within the 3′-noncoding region, which form a stem-loop structure predicted by a computer algorithm, are required for the iron-dependent regulation of receptor expression, whereas the presence of the TrfR promoter region is apparently not necessary (41). The 3′ sequences modulate the cytoplasmic TrfR RNA half-life, whereby iron deprivation increases mRNA stability, whereas an opposite effect is elicited by iron supplementation (42). Thus, strong evidence indicates that the regulation of TrfR gene expression is largely mediated through post-transcriptional mechanisms. Iron treatment in cultured monocytes-macrophages initially induces a rise of TrfR expression at both RNA and protein levels, thus suggesting modulation via transcriptional and/or post-transcriptional mechanisms. At later culture times, iron induces a further rise of TrfR synthesis, which is associated with fluctuations of the Trf RNA level. Studies are currently in progress in an attempt to clarify the molecular mechanism underlying these phenomena.

Our results indicate that cultured monocytes-macrophages acquire the capacity to synthesize large amounts of ferritin, which are more elevated than in other cell types. This phenomenon is directly related to a marked increase in both H- and L-ferritin RNA levels, thus indicating a transcriptional and/or post-transcriptional activation of ferritin genes during *in vitro* monocyte-macrophage maturation.

The synthesis of H- and L-ferritin in the culture monocytes-macrophages is further stimulated by addition of iron salts. Since this phenomenon is associated with only a moderate rise of corresponding RNA species, it is apparent that iron modulates ferritin chain expression via translational mechanisms. In this regard, Casey and co-workers (42, 43) recently showed that an iron-responsive sequence in the 5′-untranslated region of the ferritin messenger RNA mediates iron-dependent control of its translation.

These studies indicate that *in vitro* maturation of monocytes to macrophages is linked to activation of the genes for TrfR and ferritin chains, apparently at transcriptional and/or post-transcriptional level. Iron treatment causes a further rise of both TrfR and ferritin chain synthesis; the former is apparently mediated via transcriptional and/or post-transcriptional modulation, whereas the latter is by a translational mechanism.

In conclusion, it is apparent that the intracellular iron level exerts a negative feedback on TrfR expression in cells requiring iron for their proliferation or other functions, in contrast to the positive feedback observed in macrophages and possibly other iron storage cells. This positive feedback accounts for the iron storage capacity of human macrophages under normal conditions, whereas its malfunction may play a role in iron storage diseases.

Acknowledgments—We are grateful to Dr. I. Trowbridge and R. Cortese for the generous gift of the cDNA probes encoding TrfR and H- and L-ferritin, respectively. We extend our thanks to C. Miracco, P. Alessandri, and M. Ganci for secretarial assistance.

REFERENCES

1. Werb, Z. (1987) in *Basic and Clinical Immunology* (Sites, D. P., Stobo, J. P., and Wells, J. V., eds) pp. 96–106, Appleton-Century-Crofts, Norwalk, CT
2. Zucherman, S. H., Ackerman, S. K., Douglas, S. D. (1979) *Immunology* **38,** 401–411
3. Maoz, H., Polliack, A., Barak, V., Biran, S., Giloh, S., and Treves, A. J. (1986) *Int. J. Cell Cloning* **4,** 167–185
4. Nathan, C. F., Murray, H. W., and Cohn, Z. A. (1980) *N. Engl. J. Med.* **303,** 622–630
5. Johnston, R. B. (1978) *Fed. Proc.* **37,** 2759–2771
6. Lands, W. E. M. (1979) *Annu. Rev. Physiol.* **41,** 633–692
7. Huebers, H. A., and Finch, C. A. (1987) *Physiol. Rev.* **67,** 520–582
8. Fillet, G., Cook, J. D., and Finch, C. A. (1974) *J. Clin. Invest.* **38,** 392–396
9. Noyes, W. D., Bothwell, T. H., and Finch, C. A. (1960) *Br. J. Haematol.* **6,** 43–55
10. Musson, R. A. (1983) *Am. J. Pathol.* **111,** 331–342
11. Haskill, S., and Becker, S. (1982) *J. Reticuloendothel. Soc.* **32,** 273–279
12. Andreesen, R., Osterholz, J., Bodemann, H., Bross, K. J., Costabel, U., and Löhr, G. W. (1984) *Blut* **49,** 195–202
13. Hamilton, T. A., Weiel, J. E., and Adams, D. O. (1984) *J. Immunol.* **132,** 2285–2290
14. McGowan, S. E., Murray, J. J., and Parrish, M. G. (1986) *J. Lab. Clin. Med.* **108,** 587–595
15. Alvarez-Hernández, X., Felstein, M. V., and Brock, J. H. (1986) *Biochim. Biophys. Acta* **886,** 214–222
16. Hamilton, T. A., Gray, P. W., and Adams, D. O. (1984) *Cell. Immunol.* **89,** 478–491
17. Teatle, R., and Honeysett, J. M. (1988) *Blood* **71,** 1590–1595
18. Hirata, T., Bitterman, P. B., Mornex, J.-F., and Crystal, R. G. (1986) *J. Immunol.* **136,** 1339–1345
19. Ward, J. H., Kushner, J. P., and Kaplan, J. (1982) *Biochem. J.* **28,** 19–26
20. Louache, F., Testa, U., Pelicci, P., Thomopoulos, P., Titeux, M., and Rochant, H. (1984) *J. Biol. Chem.* **259,** 11576–11582
21. Pelosi, E., Testa, U., Louache, F., Thomopoulos, P., Slavo, G., Samoggia, P., and Peschle, C. (1986) *J. Biol. Chem.* **261,** 3036–3042
22. Testa, U., Louache, F., Titeux, M., Thomopoulos, P., and Rochant, H. (1985) *Br. J. Haematol.* **60,** 491–502
23. Golde, D. W., Bersch, N., Quan, S. G., and Lusis, A. J. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 593–597
24. Testa, U., Thomopoulos, P., Vinci, G., Titeux, M., Bettaieb, A., Vainchenker, W., and Rochant, H. (1982) *Exp. Cell Res.* **140,** 251–260
25. Bonner, W. M., and Laskey, R. A. (1974) *Eur. J. Immunol.* **46,** 83–88
26. Chomczynsky, P., and Sacchi, N. (1987) *Anal. Biochem.* **162,** 156–159
27. Thomas, P. S. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 5201–5205
28. McClelland, A., Kühn, L. C., and Ruddle, F. H. (1984) *Cell* **39,** 267–274
29. Costanzo, F., Santoro, C., Colantuoni, V., Bensi, G., Raugei, G., Romano, V., and Cortese, R. (1984) *EMBO J.* **3,** 23–27
30. Luzzago, A., Arosio, P., Iacobello, C., Ruggeri, G., Capucci, L., Brocchi, E., De Simone, F., Gamba, D., Gabri, E., Levi, S., and Albertini, A. (1986) *Biochim. Biophys. Acta* **872,** 61–71
31. Testa, U. (1985) *Curr. Top. Hematol.* **5,** 127–161
32. Trowbridge, I. S., and Ouray, M. B. (1981) *Proc. Natl. Acad. Sci. U. S. A.* **78,** 3039–3043
33. Pelosi-Testa, E., Testa, U., Samoggia, P., Salvo, G., Camagna, A., and Peschle, C. (1986) *Cancer Res.* **46,** 5330–5334
34. Thelander, L., and Gräslund, A. (1983) *J. Biol. Chem.* **258,** 4063–4066
35. Kucera, R., Brown, C. L., and Paulus, H. (1983) *J. Cell. Physiol.* **117,** 158–168
36. Scatchard, G. (1949) *Ann. N. Y. Acad. Sci.* **51,** 660–672
37. Mattia, E., Rao, K., Shapiro, D. S., Sussman, H. H., and Klausner, R. D. (1984) *J. Biol. Chem.* **259,** 2689–2692
38. Jacobs, A. (1985) *Curr. Top. Hematol.* **5,** 25–62
39. Testa, U., Pelosi-Testa, E., Mavilio, F., Petrini, M., Sposi, N. M., Petti, S., Samoggia, P., Montesoro, E., Giannella, G., Bottero, L., Camagna, A., Salvo, G., Isacchi, G., Habetswallner, D., and Peschle, C. (1987) *J. Recept. Res.* **7,** 355–375
40. Owen, D., and Kühn, L. C. (1987) *EMBO J.* **6,** 1287–1293
41. Müllner, E. W., and Kühn, E. W. (1988) *Cell* **53,** 815–825
42. Casey, J. L., Hentze, M. W., Koeller, D. M., Wright Caughman, S., Rouault, T. A., Klausner, R. D., and Harford, J. B. (1988) *Science* **240,** 924–928
43. Casey, J. L., Di Jeso, B., Rao, K., Klausner, R. D., and Harford, J. B. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85,** 1787–1791