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Effects of Alterations in Cellular Iron on Biosynthesis of the Transferrin Receptor in K562 Cells

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Treatment of K562 cells, a human erythroleukemia cell line, with desferrioxamine raised the levels of the receptor for transferrin (Tf) two- to threefold over that of the control cells. The levels of receptor were reduced by at least 50 and 35% of that of the control in cells treated with diferric Tf and ferric ammonium citrate, respectively. These changes were of total cellular receptors with no alteration in the proportion of receptors found on the cell surface. The half-lives of the receptor were identical in cells treated with desferrioxamine, diferric Tf, or ferric ammonium citrate. Cells metabolically labeled with [^{35}S]methionine showed a 2.5-fold increase in the rate of receptor synthesis when treated with desferrioxamine and a 35 and 65% decrease when treated with ferric ammonium citrate and diferric Tf, respectively. In vitro translations of polyadenylated mRNA isolated from cells incubated with desferrioxamine showed a 2.5-fold increase in translatable mRNA for the receptor, whereas treatment of cells with ferric ammonium citrate and diferric Tf resulted in a 25 and 50% reduction, respectively, in translatable mRNA for this receptor.

Iron is an essential constituent of all cells. It is required for the normal functioning of a wide array of cellular enzymes, including those of the respiratory pathway (2, 4, 6). Cells receive iron via the receptor-mediated endocytosis of iron-transferrin (Tf). Large amounts of iron can be stored in cells in ferritin and as hemosiderin. The iron needs of cells vary and are high in fetal, erythropoietic, and rapidly proliferating cells. Concomitant with these needs is a high level of expression of Tf receptors in placental cells, reticulocytes, and proliferating cells (1, 8, 10). It is not known exactly how cells regulate their iron uptake, but clearly the modulation of the number of Tf receptors can play a role in such regulation. Recently, Bridges et al. (Fed. Proc. **42**:2193, 1983) and Mattia et al. (9) showed that K562 cells can alter Tf receptor numbers in response to intracellular iron chelation by desferrioxamine. The locus of this regulation was shown to be at the level of mRNA for the receptor (9). Ward et al. (12) reported a down regulation of Tf receptors in HeLa cells that were treated with iron salts. In this paper we report the extension of our studies on the regulation of Tf receptor biosynthesis in K562 cells. We show that delivering iron to cells results in a twofold decrease in specific synthesis of the Tf receptor. A decrease in the levels of mRNA is responsible for this drop. Furthermore, we compare the efficacy of Tf with that of ferric ammonium citrate in lowering receptor biosynthesis and establish the range of receptor regulation achievable with iron depletion or iron introduction without causing observable cellular toxicity.

MATERIALS AND METHODS

Chemicals and cells. Human diferric Tf was purchased from Calbiochem-Behring, La Jolla, Calif. Desferrioxamine was obtained from CIBA-GEIGY Corp., Summit, N.J. Protein A-Sepharose CL-4B was purchased from Pharmacia Inc., Piscataway, N.J. L-[^{35}S]methionine (1,190 Ci/mmol) was from Amersham Corp., Arlington Heights, Ill. Aprotinin and phenylmethylsulfonyl fluoride were obtained from Sigma Chemical Co., St. Louis, Mo. Vanadyl ribonucleoside complex was from Bethesda Research Laboratories, Inc., Gaithersburg, Md., and rabbit reticulocyte lysate was from Amersham. Oligodeoxythymidylate-cellulose was from Bethesda Research Laboratories. Human placental RNase inhibitor was from Boehringer Mannheim Biochemicals, Indianapolis, Ind. Human erythroleukemia K562 cells were grown in RPMI 1640 medium with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid) supplemented with 10% heat-inactivated fetal bovine serum (GIBCO Laboratories, Grand Island, N.Y.) and L-glutamine. Cells were maintained in the log phase at 2×10^5 to 5×10^5 cells per ml. Ferric ammonium citrate was prepared as previously described (3).

Surface binding assay of human Tf receptor. K562 cells were treated with desferrioxamine, human diferric Tf, or ferric ammonium citrate at a concentration of 50 μM, 50 μg/ml, or 6 μg/ml, respectively, for 18 h. The cells were grown to a density of 3×10^5 to 5×10^5 cells per ml, collected, and washed in RPMI 1640 medium containing 0.1% bovine serum albumin (BSA) at 4°C. They were reincubated at 37°C for 15 min before being chilled to 0°C in RPMI 1640–0.1% BSA, in which they were finally resuspended to a density of 10^7 cells per ml. ^{125}I-diferric human Tf (specific activity, 1,000 cpm/ng) or ^{125}I-OKT9 (mouse monoclonal anti-Tf receptor antibody; specific activity, 2,000 cpm/ng), prepared as described previously (6), was added to a final concentration of 10 μg/ml. Incubations were carried out for 30 min at 0°C, and 2×10^6 cells were spun through a cushion of dibutyl phthalate as previously described (6). The radioactivity associated with the cell pellet was determined in a Beckman 5500 gamma counter. Nonspecific binding was

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determined for each assay by incubation with a 100-fold excess of unlabeled diferric Tf or OKT9.

Determinations of total cell-associated Tf receptors. K562 cells were treated as above for 18 h before they were washed with RPMI 1640–0.1% BSA at 0°C. They were warmed to 37°C for 15 min and rechilled to 0°C, and for each assay, 2×10^6 cells were solubilized in 0.2 ml of TBS (10 mM Tris [pH 7.4], 0.14 M NaCl) containing 0.1% Triton X-100. ^{125}I-diferric Tf (5 μg) was added, and the volume was brought to 0.5 ml with TBS–0.1% Triton X-100. The binding was carried out at room temperature for 10 min, following which an equal volume of 60% saturated ammonium sulfate (pH 7.4) was added. The precipitate was kept on ice for 5 min and then collected by filtration over GF/C filters (Whatman, Inc., Clifton, N.J.) and washed four times with 1 ml of 30% saturated ammonium sulfate (pH 7.4) containing 0.8% BSA. The radioactivity was counted in a Beckman 5500 gamma counter. Nonspecific binding was determined for each assay by the inclusion of a 100-fold excess of unlabeled diferric Tf.

Uptake of ^{59}Fe-diferric human Tf. K562 cells were treated with desferrioxamine, diferric Tf, or ferric ammonium citrate as described above for 18 h. The cells were washed, warmed to 37°C for 15 min, and resuspended at a density of 10^7 cells per ml in RPMI 1640–0.1% BSA. ^{59}Fe-diferric Tf (specific activity, 7 cpm/ng), prepared as described previously (6), was added to a final concentration of 30 μg/ml and incubated at 37°C for 90 min. Cells (2×10^6) were spun through a cushion of dibutyl phthalate as previously described (6), and the cell-associated radioactivity was determined in a Beckman 5500 gamma counter whose windows were set to detect 95% of the radioactivity of a ^{59}Fe standard.

Pulse-labeling of Tf receptor. Log-phase growing cells (5×10^5 cells per ml) were treated with desferrioxamine, diferric human Tf, or ferric ammonium citrate at a concentration of 50 μM, 50 μg/ml, or 6 μg/ml, respectively, at 37°C for 4 h. Cells (10^6) were washed with 5 ml of one-tenth-normal methionine-containing RPMI 1640 medium (1.5 mg of methionine per liter), incubated for 15 min in 1 ml of the same medium, and then labeled with 200 μCi of [^{35}S]methionine for 20 min. The cells were then washed three times with ice-cold RPMI 1640–0.1% BSA before they were lysed for immunoprecipitation.

Immunoprecipitation of receptor from K562 cells. Labeled cell pellets were solubilized in TBS containing 1% Triton X-100, 0.2 mM phenylmethylsulfonyl fluoride, and 0.5 trypsin inhibitory unit of aprotinin per ml (final volume, 0.25 ml) for 30 min at 4°C. After centrifugation for 2 min at 4°C in an Eppendorf microcentrifuge, 100 μl of the supernatant was removed and mixed with 15 μl of mouse monoclonal anti-Tf receptor antibody (OKT9; Ortho Diagnostics, Inc., Raritan, N.J.) and incubated overnight at 4°C. Protein A-Sepharose was saturated with rabbit anti-mouse immunoglobulin G (IgG) by incubation for 2 h at 4°C. This IgG-saturated protein A-Sepharose (200 μl) was added to the OKT9-receptor complex and tumbled for 1 h at 4°C. The complexes were centrifuged and washed three times with 1 ml of TBS containing 0.5% sodium deoxycholate, 1% Triton X-100, and 1 mg of BSA per ml. Proteins were released from the Sepharose pellet by boiling it for 10 min in electrophoresis sample buffer.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis and autoradiography. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out by the method of Laemmli (7), using 12.5% polyacrylamide gels. The gels were fixed and dried for autoradiography after impregnation with 1 M sodium salicylate. Kodak X-Omat AR5 film was exposed to the gels for 12 to 38 h at −70°C, and the amount of receptor was quantitated with a Hoeffer densitometer.

Degradation rates of Tf receptor. K562 cells were treated with diferric human Tf or ferric ammonium citrate at 50 or 6 μg/ml, respectively, for 18 h. The cells were labeled at a density of 5×10^5 cells per ml with 200 μCi of [^{35}S]methionine per ml in low-methionine RPMI 1640 medium for 30 min at 37°C. The cells were then washed three times in full-methionine RPMI 1640 medium with 10% fetal bovine serum and resuspended to a density of 2.5×10^5 cells per ml in growth medium. After initial incubation at 37°C for 2 h, equal volumes of cells were taken at 4, 8, 12, and 24 h and processed as described above for immunoprecipitation of the receptor.

Isolation of total cellular RNA and poly(A)$^+$ mRNA. K562 cells were grown to a density of 2.5×10^5 cells per ml. Desferrioxamine, diferric Tf, or ferric ammonium citrate was added to 10 100-ml flasks at 50 μM, 50 μg/ml, or 6 μg/ml, respectively, and the cells were incubated at 37°C for 24 h. The cells were harvested and washed twice with cold phosphate-buffered saline. Total RNA and then polyadenylated [poly(A)$^+$] mRNA were isolated as previously described (9).

In vitro translation and immunoprecipitation. Poly(A)$^+$ mRNA from control and treated cells (5 μg from each) was translated in a rabbit reticulocyte lysate containing 100 mM KCl, 50 μM amino acids minus methionine, 150 μCi of [^{35}S]methionine, and 50 U RNase inhibitor in a total reaction volume of 100 μl. The reaction was carried out at 30°C for 90 min. After incubation, the reaction volume was brought to 200 μl with translation buffer (10 mM Tris hydrochloride [pH 7.4], 0.15 M NaCl, 10 mM EDTA, 0.5% Triton X-100, 2% methionine). Preimmune rabbit serum (10 μl) was added and incubated at room temperature for 30 min. Protein A-Sepharose equilibrated in translation buffer was added, incubated for 10 min at room temperature, and removed by centrifugation. To the supernatant was added 10 μl of either preimmune rabbit serum or immune rabbit serum directed against the human Tf receptor. The volume was then brought to 1 ml with translation buffer, sodium dodecyl sulfate was added to a final concentration of 0.1%, and the mixture was incubated overnight at 4°C. Precipitation of immune complexes was accomplished with protein A-Sepharose as described above, except that the washes were performed with translation buffer plus 0.1% sodium dodecyl sulfate. The amount of product translated was linear as a function of mRNA concentration over the range of 1 to 10 μg used in this study.

RESULTS

Effect of iron and desferrioxamine on surface and total receptors. The effects of ferric ammonium citrate, diferric Tf, and desferrioxamine on the levels of surface receptors were determined by the binding of ^{125}I-diferric Tf and ^{125}I-OKT9, a monoclonal antibody against the human Tf receptor. Briefly, K562 cells were treated with ferric ammonium citrate, diferric Tf, or desferrioxamine for a period of 18 h. None of these agents altered the viability or the growth rates of the cells. Binding measurements were performed at 0°C (Fig. 1A). Introduction of iron as ferric ammonium citrate and diferric Tf reduced the number of surface receptors by 35 and 50%, respectively. Identical results were obtained when either OKT9 or diferric Tf was used to quantitate surface receptors. Desferrioxamine, by chelating intracellu-

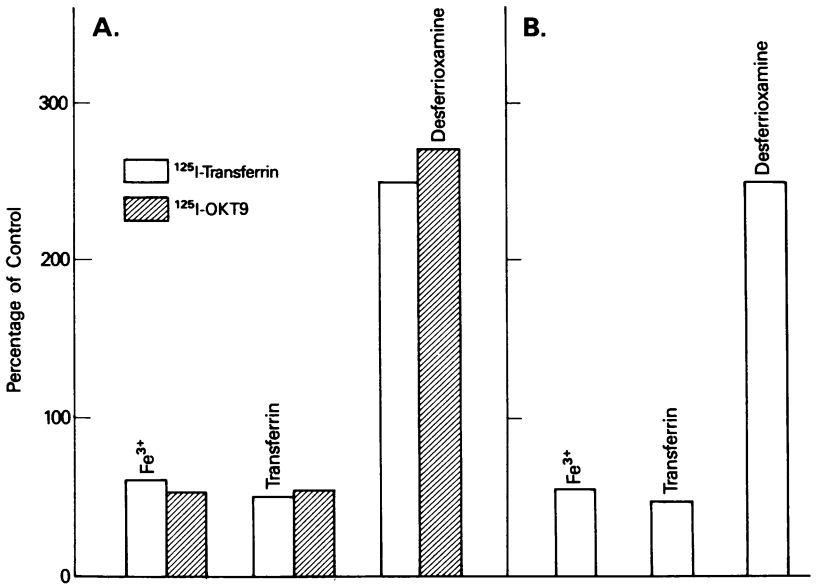

FIG. 1. (A) Measurement of surface Tf receptors. K562 cells were treated with ferric ammonium citrate, diferric Tf, or desferrioxamine for 18 h, and binding measurements were performed on 2×10^6 cells at 4°C with ^{125}I-Tf and ^{125}I-OKT9. The specific activities of ^{125}I-Tf and ^{125}I-OKT9 were 1,000 and 2,000 cpm/ng, respectively. Saturation binding yielded 1.5×10^5 surface receptors per cell in control cells. (B) Measurement of total cell receptors. Cells treated with ferric ammonium citrate, diferric Tf, or desferrioxamine for 18 h were assayed for total cellular receptors with ^{125}I-Tf as described in the text.

lar iron, raised the level of the surface receptors about 2.5-fold over that of the control. Diethylenetriamine pentaacetic acid, an iron chelator that is unable to enter cells, did not have any effect on the levels of the surface receptors (data not shown).

We measured the level of total cell receptors when K562 cells were treated with the three agents as described above. Treatment of cells with ferric ammonium citrate and diferric Tf lowered the level of total receptors by 35 and 50%, respectively, paralleling the decrease in surface receptors (Fig. 1B). Similarly, desferrioxamine treatment raised the level of total receptors about 2.5-fold over that of the control. Thus, no alteration in receptor distribution was seen.

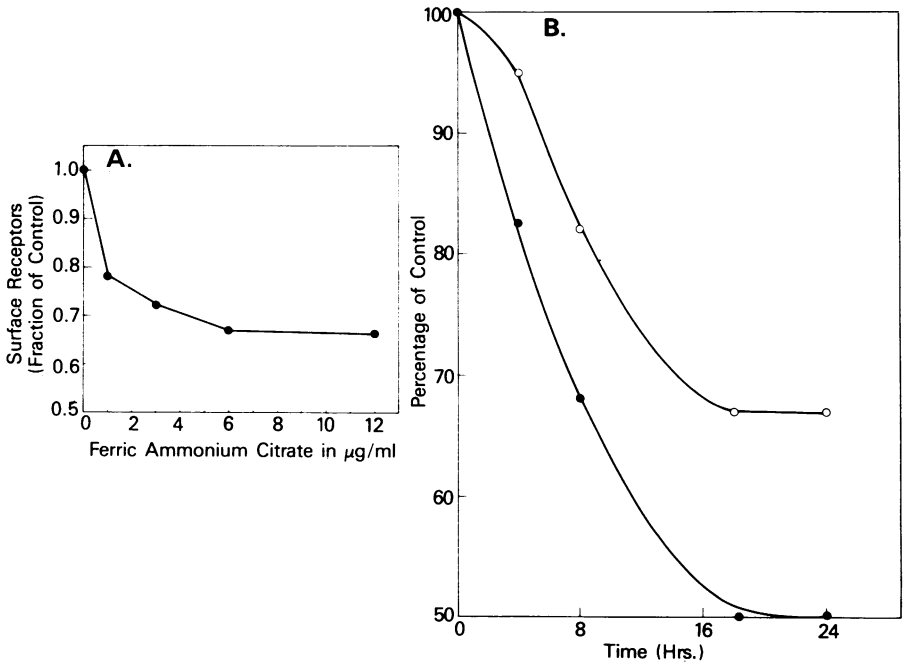

FIG. 2. (A) Measurement of surface receptors with increasing concentrations of ferric ammonium citrate. K562 cells were treated with ferric ammonium citrate at concentrations of 1, 3, 6, and 12 μg/ml for 18 h. Surface receptors were measured at 4°C at saturation binding with ^{125}I-OKT9 as described in the legend to Fig. 1. (B) Measurement of surface receptors with time. Cells were treated with ferric ammonium citrate (○) and diferric Tf (●) for 4, 8, 18, and 24 h, and surface receptors were measured at 4°C with ^{125}I-OKT9.

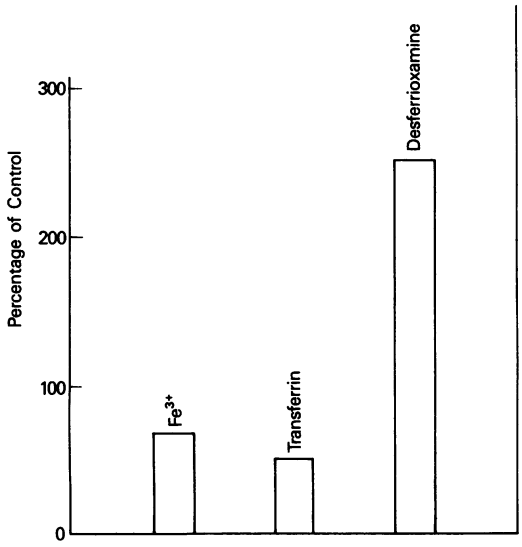

FIG. 3. Uptake of ^{59}Fe-Tf by K562 cells. Cells were treated with ferric ammonium citrate, diferric Tf, or desferrioxamine for 18 h. The cells were allowed to take up ^{59}Fe-Tf at 37°C for 90 min, and cell-associated ^{59}Fe was determined as described in the text. Control cells accumulated 6,389 cpm of ^{59}Fe over 90 min, which represents a rate of uptake of 2.2×10^6 atoms of Fe per cel per h or six to seven complete cycles of total surface receptor per cell per hour.

The effects of ferric ammonium citrate and Tf upon the level of surface Tf receptors shown in Fig. 1 represent the maximal effects of each reagent. A titration of ferric ammonium citrate revealed a plateau in the diminution of Tf receptors that was reached at a concentration of about 6 μg/ml (Fig. 2A). Above 15 to 20 μg/ml the ferric ammonium citrate was toxic to the cells as reflected in a decreased growth rate. Tf was able to deliver a stronger iron signal to these cells, leading to a greater decrease in receptor number. A decrease in the level of the receptors was seen relatively rapidly upon the addition of these agents, with a maximal effect reached between 10 and 18 h of incubation (Fig. 2B).

The distribution of Tf receptors in the cell changes during the uptake of Tf (5, 6). We examined the redistribution of receptors in K562 cells treated with the regimens described above. In all cases, Tf induced a 50% reduction in the ratio of surface to total receptors during the steady-state uptake of ligand at saturating Tf concentration. The maximum steady-state level of Tf association with the cell at 37°C was taken to reflect the size of the total cycling receptor pool. Treatment of K562 cells with iron as ferric ammonium citrate or diferric Tf reduced to identical extents the level of surface receptors as measured at 0°C and the maximum steady-state association of Tf with the cells at 37°C. Similarly, treatment with desferrioxamine elevated both the binding at 0°C and the maximum steady state obtained at 37°C. Since the amount of iron that is taken up by cells is limited by the number of cycling receptors, we measured the accumulation of ^{59}Fe in K562 cells when the cells were pretreated with ferric ammonium citrate, diferric Tf, or desferrioxamine for 18 h. Treatment of cells with ferric ammonium citrate and diferric Tf reduced the amount of ^{59}Fe that was cell associated by 35 and 50%, respectively (Fig. 3). Treatment with desferrioxamine increased the amount of ^{59}Fe accumulated into cells 2.5-fold over that of the control. The uptake of ^{59}Fe under all these conditions was linear with time.

Effect of iron and desferrioxamine on biosynthesis of the receptor. An alteration in the number of receptors per cell could arise due to changes in biosynthesis, degradation, or both. We determined the degradation rates of the receptor under conditions in which cells were treated with either ferric ammonium citrate or Tf as described above. The degradation rates were virtually identical, with a half-life of degradation of 8 h (Fig. 4). Cells treated with desferrioxamine showed the same half-life (ca. 8 h).

We determined the biosynthetic rates of the Tf receptor in K562 cells treated with ferric ammonium citrate, diferric Tf, and desferrioxamine (Fig. 5). The effect of these agents on the biosynthesis of the receptor was seen as early as 2 h after treatment. Both ferric ammonium citrate and diferric Tf lowered the biosynthetic rates by 35 and 65% of that of the control, whereas desferrioxamine elevated the biosynthetic rate 2.5- to 3.5-fold over that of the control, closely paralleling the effects of these agents on the number of cellular receptors as measured by the ^{125}I-Tf binding experiments. None of the treatments had a significant effect on either the amount or pattern of total protein synthesis.

Effect of iron and desferrioxamine on mRNA levels for the Tf receptor. To determine whether the changes in the rates of biosynthesis of the receptor were reflected in changes in the levels of translatable mRNA for the receptor, we isolated poly(A)$^+$ mRNA from cells treated with ferric ammonium citrate, diferric Tf, and desferrioxamine. The yields of poly(A)$^+$ mRNA under all these conditions were identical. The poly(A)$^+$ mRNA was translated in a rabbit reticulocyte lysate translation system, and the Tf receptor was immunoprecipitated with a rabbit polyclonal antiserum (Fig. 6). The polyclonal antiserum immunoprecipitated an 80,000-dalton protein that was not precipitated with a preimmune serum. Poly(A)$^+$ mRNA from cells directed equivalent amounts of total protein synthesis regardless of the treatment of the cells. Furthermore, the patterns of total proteins synthesized, as judged by polyacrylamide gel electrophoresis, were identical. However, translation of mRNA from cells treated with ferric ammonium citrate and diferric Tf lowered the

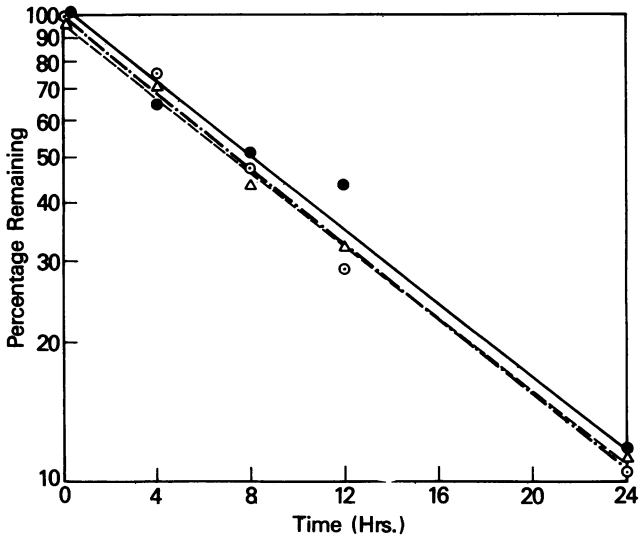

FIG. 4. Measurement of half-life of Tf receptor in K562 cells. Cells were treated with ferric ammonium citrate or diferric Tf for 18 h. The cells were then labeled with [^{35}S]methionine and chased for 4, 8, 12, and 24 h. The cells were processed as described in the text. Symbols: ⊙, control; ●, ferric ammonium citrate; △, Tf.

specific receptor synthesis by 30 and 50%, respectively, of that of the control, whereas translation of mRNA from desferrioxamine-treated cells resulted in a two- to threefold enhancement of specific receptor synthesis over that of the control. The amount of receptor synthesized was linear with respect to the concentration of mRNA added over the range of 1 to 10 μg.

DISCUSSION

We recently reported that the biosynthesis of the Tf receptor can be specifically enhanced by treating cells with the iron chelator desferrioxamine (9; Bridges et al., Fed. Proc. **42**:2193, 1983). We furthermore presented evidence that treatment with this drug results in an increase in intracellular levels of mRNA coding for the receptor. In this paper we present similar evidence that the biosynthesis of the receptor can be specifically inhibited by two manipulations that deliver iron to cells. This study was undertaken to assess whether such biosynthetic regulation could explain the observation of Ward et al. (12) that treatment of HeLa cells with iron salts led to a reduction in the level of surface Tf receptors. K562 cells clearly exhibited the same phenomenon. The loss of surface receptors as judged by ^{125}I-Tf binding in cells that had been treated with diferric human Tf could be artifactual due to the blocking of surface receptors. For this reason, we used two monoclonal antibodies against the receptor, neither of whose binding is blocked by Tf. Quantitative binding assays with the antireceptor antibody confirmed the loss of surface receptors induced by Tf and ferric ammonium citrate. We never observed alterations in the distribution of Tf receptors in the cell by any manipulation of cellular iron. Thus, changes in total receptor numbers always paralleled changes in the surface receptors.

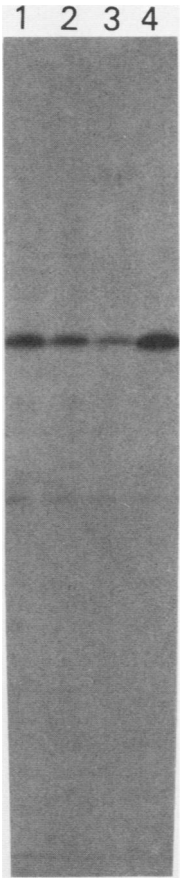

FIG. 5. Biosynthetic rates of Tf receptor. K562 cells were treated with ferric ammonium citrate, diferric Tf, or desferrioxamine for 4 h. Cells (10^6) were labeled with 200 μCi of [^{35}S]methionine for 20 min, and receptors were immunoprecipitated and processed as described in the text. Lanes: 1, control lane; 2, ferric ammonium citrate; 3, Tf; 4, desferrioxamine.

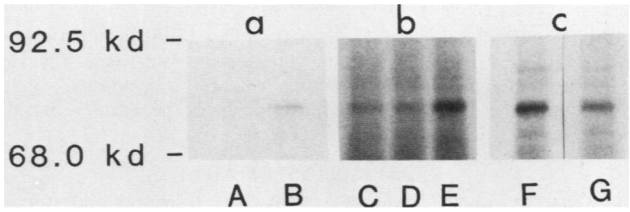

FIG. 6. In vitro translation of Tf receptor with poly(A)$^+$ mRNA from K562 cells. Poly(A)$^+$ mRNA was isolated from K562 cells that were treated with ferric ammonium citrate, diferric Tf, or desferrioxamine for 18 h. Poly(A)$^+$ mRNA was translated in a rabbit reticulocyte lysate system, and the receptors were immunoprecipitated as described in the text. (a) Poly(A)$^+$ mRNA translated and immunoprecipitated with preimmune rabbit serum (lane A) and immune rabbit serum (lane B). (b) Specific immunoprecipitated protein from control (lane C), ferric ammonium citrate (lane D)-, and desferrioxamine (lane E)-treated cells. (c) Immunoprecipitation of the Tf receptor from in vitro translation of poly(A)$^+$ mRNA derived from control (lane F) and Tf (lane G)-treated cells. kd, Kilodaltons.

It is clear that the effect of added iron is to lower receptor numbers by reducing the rate of biosynthesis. No change in the rate of receptor turnover was seen. Furthermore, the magnitude of the effect on biosynthetic rates could entirely explain the magnitude of the resulting receptor levels. Thus, there is no reason to propose inactivation or sequestration of receptors, or both. The kinetics of the change in total receptors are also consistent with the kinetics of the alterations in biosynthesis. Thus, maximal changes in biosynthesis were seen within 4 to 6 h, and the new steady state was achieved by about 18 h, compatible with a 6- to 8-h half-life of the receptor in these cells.

The details of intracellular iron distribution remain a mystery. We know that cytosolic ferritin is the site to which the majority of iron is delivered from Tf (6). However, we do not know what pool or pools of iron determine the regulation of Tf receptor biosynthesis. That this pool(s) cannot be determined by measuring the total cellular iron content was demonstrated by Ward et al. (12) in studies of the iron regulation of Tf receptor expression in HeLa cells. In this regard, it is interesting that the total amount of radioactive iron accumulated by cells from ferric ammonium citrate may be no less than that accumulated from Tf (data not shown), yet the Tf was capable of delivering a signal leading to a greater diminution of receptor biosynthesis than was seen with the iron salt alone. We do not know what amount of iron was delivered to the cells bound to bovine Tf in the serum in the presence of the soluble iron salt. However, a recent report demonstrated that this ferric salt can provide for cellular iron requirement in the absence of any exogenous Tf (11). Only a more complete understanding of the detailed distribution of iron within cells will allow us to more precisely define the regulatory pool of this metal.

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