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Transcriptional Regulation by Iron of the Gene for the Transferrin Receptor

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Treatment of K562 cells with desferrioxamine, a permeable iron chelator, led to an increase in the number of transferrin receptors. Increasing intracellular iron levels by treatment of cells with either human diferric transferrin or hemin lowered the level of the transferrin receptors. By using a cDNA clone of the human transferrin receptor, we showed that the changes in the levels of the receptor by iron were accompanied by alterations in the levels of the mRNA for the receptor. The rapidity of these changes indicated that the mRNA had a very short half-life. By using an in vitro transcriptional assay with isolated nuclei, we obtained evidence that this regulation occurred at the transcriptional level.

All proliferating cells acquire iron via transferrin (Tf), a serum glycoprotein that binds to a specific receptor on the cell surface. The receptor-Tf complex is internalized by a process of receptor-mediated endocytosis in which the iron is dissociated from the Tf (3). Investigators have previously shown that the levels of intracellular iron regulate the level of expression of the Tf receptor (6, 8, 9, 14). Raising the levels of intracellular iron by treatment of K562 cells (a human erythroleukemic cell line) with either human diferric Tf or ferric ammonium citrate results in decreased levels of the receptor. Conversely, treatment with desferrioxamine, a permeable iron chelator, leads to marked elevations in receptor levels. These changes in cellular content of the Tf receptor through manipulation of intracellular iron levels are caused by altered rates of biosynthesis of the receptor. Corresponding changes in the levels of translatable mRNA for the receptor were measured by in vitro translation (9).

Recently, cDNA clones for the human Tf receptor have been isolated (4, 12). By using one such clone, we showed that the alterations of the biosynthetic rate of the receptor produced by manipulations of iron levels were caused by changes in the levels of the mRNA for the receptor. The modulation of the expression of the Tf receptor gene provided an important example of the specific feedback regulation of a eucaryotic gene. In this regard, the regulation of Tf receptor expression produced by changes in iron levels is analogous to the regulation of low-density lipoprotein receptors produced by cholesterol (11). We showed that cellular control of iron uptake was accomplished, at least in part, through transcriptional control of the synthesis of the Tf receptor message. The rapid adjustment of specific mRNA levels was possible because of the short half-life of this message. We expect that the mechanisms underlying the regulation of this gene will be further elucidated when the genetic regulatory sequences conferring iron sensitivity are determined.

MATERIALS AND METHODS

Chemicals. Human diferric Tf and hemin were purchased from Calbiochem-Behring. La Jolla, Calif. Desferrioxamine was obtained from CIBA-GEIGY Corp., Summit, N.J. Vanadyl-ribonucleoside complex, nick translation kits, and oligo(dT)-cellulose were from Bethesda Research Laboratories, Inc., Gaithersburg, Md. Human placental RNase inhibitor was purchased from Boehringer Mannheim Biochemicals, Indianapolis, Ind.

Cells. 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.). Cells were maintained in the log phase of growth at between 2×10^5 and 5×10^5 cells per ml.

Isolation of RNA. Cells (1×10^8 to 2×10^8) were homogenized at 4°C with a B-type pestle Dounce homogenizer in 4 ml of 10 mM Tris chloride, pH 7.4, containing 10 mM vanadyl-ribonucleoside complex. Isotonicity was restored by the addition of an equal volume of 1.7% NaCl–10 mM Tris chloride (pH 7.4)–6 mM $MgCl_2$ with 10 mM vanadyl-ribonucleoside complex. Nuclei were removed by centrifugation at 600 × *g* for 10 min. To the supernatant was added an equal volume of buffer containing 0.2 M Tris chloride (pH 7.5), 25 mM EDTA, 0.3 M NaCl, and 2% sodium dodecyl sulfate (SDS). Proteinase K was added to achieve a final concentration of 200 μg/ml, and the mixture was incubated at 37°C for 30 min. The mixture was then extracted with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1), and 2.5 volumes of ethanol was added to the aqueous phase to precipitate nucleic acids. The precipitate was recovered by centrifugation at 5,000 × *g* at 0°C for 10 min and was dissolved in 1 ml of 50 mM Tris (pH 7.5)–1 mM EDTA. Vanadyl-ribonucleoside complex and $MgCl_2$ were added to achieve final concentrations of 2 and 10 mM, respectively. RNase-free DNase was added to achieve a final concentration of 10 μg/ml, and the mixture was incubated at 37°C for 30 min. After the incubation, EDTA and SDS were added to achieve final concentrations of 10 mM and 0.2%, respectively. After extraction with phenol-chloroform, the RNA was precipitated with 2 volumes of ethanol in the presence of 0.3 M sodium acetate (pH 5.2). Total RNA was loaded onto an oligo(dT)-cellulose column in 20 mM Tris chloride (pH 7.5)–0.5 M NaCl–1 mM EDTA–0.1% SDS. The column was washed with the same buffer, and the poly(A)$^+$

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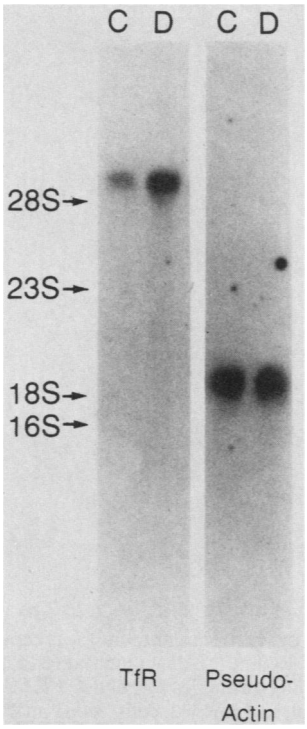

FIG. 1. Induction of Tf receptor mRNA by desferrioxamine. Samples of poly(A)$^+$ RNA (5 μg) from control K562 cells (lane C) and from cells treated with 50 μM desferrioxamine for 24 h (lane D) were electrophoresed in a 0.7% agarose gel. After transfer to nitrocellulose, hybridization was performed with [^{32}P]pCD-TR1 as described in the text. As an indication of specificity, the pCD-TR1 probe was removed from the blot, and the blot was reprobed with a nick-translated pseudo-actin gene. The positions of marker RNAs in the gel are indicated on the left.

RNA was eluted with a buffer containing 10 mM Tris chloride (pH 7.5), 1 mM EDTA, and 0.05% SDS.

Gel electrophoresis and Northern blot analysis. Total RNA (20 μg) or poly(A)$^+$ RNA (5 μg) was electrophoresed at 80 V for 12 to 16 h in a 0.7% agarose gel containing 40 mM MOPS (morpholinepropanesulfonic acid) (pH 7.0), 10 mM sodium acetate, 1 mM EDTA, and 2.2 M formaldehyde. The RNA from the gels was transferred to nitrocellulose paper as described by Maniatis et al. (5). Prehybridization was carried out for a period of 6 to 12 h at 42°C in a solution containing 50% formamide, 4 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium M citrate), 10 mM Tris (pH 7.5), 10% dextran sulfate, 0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll (Pharmacia Fine Chemicals, Piscataway, N.J.), and 20 μg of denatured salmon sperm DNA per ml. Hybridization reactions were carried out for 18 to 24 h in the same buffer at 42°C with 20 to 30 ng of nick-translated pCD-TR1. Nick translations were performed with [^{32}P]dCTP according to the instructions of the manufacturer. Specific activity of the probe was approximately 10^8 cpm/μg. The nitrocellulose filters were washed at 65°C with five 200-ml washes of 0.1× SSC plus 0.1% SDS, dried, and exposed to XAR-5 X-Omat films (Eastman Kodak Co., Rochester, N.Y.) with an intensifying screen for 3 to 8 h.

In vitro nuclear transcription assay. K562 cells were treated with either 50 μM desferrioxamine or 50 μM hemin for a period of 4 h. Cells (1.5×10^8) were suspended in 4 ml of lysis buffer containing 10 mM Tris chloride (pH 7.5), 2 mM $MgCl_2$, 3 mM $CaCl_2$, 3 mM dithiothreitol, and 0.05% Nonidet P-40 for 10 min. Cell lysis was monitored microscopically. To the suspension was added 4 ml of 2 M sucrose in 10 mM Tris chloride (pH 7.5)–5 mM $MgCl_2$–3 mM dithiothreitol, making the final concentration of sucrose 1 M. The suspension was layered over a 4-ml cushion of 2 M sucrose. The nuclei were pelleted at 25,000 rpm in an SW41 rotor at 4°C for 45 min. The nuclei were resuspended in 50 mM Tris (pH 7.5)–5 mM $MgCl_2$–0.1 mM EDTA–40% glycerol at a density of 4×10^8 to 5×10^8 nuclei per ml.

Nuclear transcription assays were carried out with 4×10^7 nuclei in a reaction volume of 200 μl containing 0.1 M KCl, 2 mM $MgCl_2$, 2 mM dithiothreitol, ATP, CTP, and GTP (0.4 mM each), 5 μl of RNasin, and 400 μCi of [α-^{32}P]UTP (3,000 Ci/mmol) at 26°C for 5 min. The reaction was terminated with DNase at 20 μg/ml for 15 min at 26°C. SDS and EDTA were then added to achieve concentrations of 1% and 5 mM, respectively. Proteinase K (300 μg) was added, and the reaction mixture was incubated at 37°C for 60 min and extracted twice with phenol-chloroform in the presence of 100 μg of tRNA as carrier. The labeled RNA was ethanol precipitated three times in the presence of unlabeled, 0.1 mM UTP. The final RNA pellet was dissolved in 100 μl of water.

Between 5×10^6 and 10×10^6 cpm of labeled RNA per filter was hybridized. Each filter was prebound with 0.5 μg of either pCD-TR1 (the cDNA clone carrying the 4.9-kilobase insert of the Tf receptor message) or pBR322 DNA. The hybridization was carried out at 42°C for 3 days in a volume of 1 ml of the same solution as that used in Northern blot hybridization, supplemented with additional tRNA at 150 μg/ml. The filters were washed in the same way as described for Northern blot analysis.

RESULTS AND DISCUSSION

Induction of mRNA by desferrioxamine. We have previously shown that treatment of K562 cells with desferrioxamine is accompanied by a three- to fivefold increase in the biosynthetic rate of the Tf receptor (6, 9). To examine whether this increase was due to increases in the steady-state level of the mRNA for the receptor, we isolated poly(A)$^+$ mRNA from K562 cells that had been treated with 50 μM desferrioxamine, a concentration sufficient to give the maximal effect of the chelator. The level of the specific mRNA was determined by Northern blot analysis. The cDNA clone pCD-TR1 was used as a specific probe (4). Treatment of cells with desferrioxamine for a period of 18 h resulted in a fourfold increase in the levels of the mRNA for the receptor (Fig. 1). In a number of experiments, the range of stimulation varied from three- to sixfold for untreated cells. The effect of desferrioxamine seemed to be specific to the Tf receptor mRNA. Thus, when the pCD-TR1 probe was removed and the blot was reprobed with a nick-translated human pseudo-actin gene (a gift from Takeo Kakunaga), the levels of the mRNA for this gene measured under the two conditions appeared to be identical (Fig. 1).

The increase in mRNA for the receptor occurred quite rapidly and was detectable as soon as 2 h after the addition of desferrioxamine. The maximal increase was reached after approximately 18 h (Fig. 2). If cells are maintained under conditions that alter the rate of biosynthesis of a given mRNA, a new steady-state level of that mRNA will result. Moreover, if the transition between the old and new biosynthetic rates is rapid, then the characteristic time for the system to move one-half of the difference between the old and new steady-state levels is equal to the half-life of the mRNA (10). This type of analysis of the desferrioxamine

modulation time course yielded an estimate for the half-life of the Tf receptor mRNA of approximately 2.5 h (Fig. 2B). This estimate was most likely an overestimate of the mRNA half-life in the presence of desferrioxamine because the desferrioxamine-mediated transition to the new rate of Tf receptor mRNA synthesis was probably not instantaneous. The entry of desferrioxamine into cells has been measured, and maximal intracellular levels are attained only after several hours (K. Bridges, personal communication). We are unaware of the exact intracellular desferrioxamine concentration needed to cause the change in Tf receptor mRNA and of the time course of the transition once this intracellular concentration has been achieved.

Increases in intracellular levels of iron lower the level of mRNA for the receptor. K562 cells that have been treated with human diferric Tf increase their intracellular iron stores, the consequence of which is a decrease in the biosynthetic rate of the Tf receptor (9). This decrease was correlated with a lowering of the levels of the mRNA for the receptor (Fig. 3). Treatment of cells with 50 μg of human diferric Tf per ml led to a 50% reduction in receptor mRNA levels within 2 h with a maximal decrease of 70 to 80% observed after 6 h of treatment.

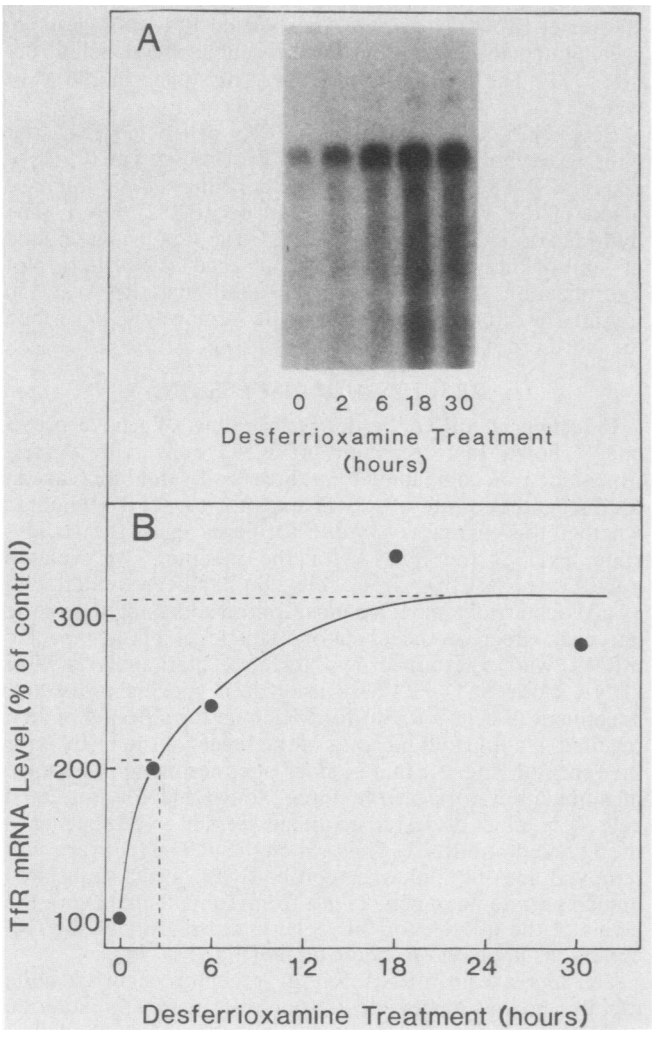

FIG. 2. Time course of induction of Tf receptor mRNA by desferrioxamine. K562 cells were treated with desferrioxamine for 0, 2, 6, 18, and 30 h as indicated. (A) Samples of poly(A)$^+$ RNA (5 μg) were analyzed by Northern blotting as described in the text with [^{32}P] pCD-TR1 as probe. (B) Samples from each RNA preparation were similarly analyzed by slot blot hybridization and were quantitated by scanning densitometry and peak integration. Data are expressed as percentages of the receptor-specific mRNA in untreated cells.

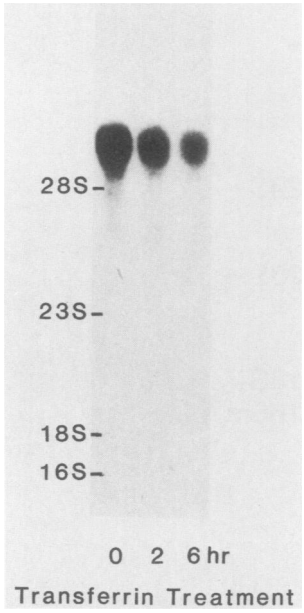

FIG. 3. Effect of Tf treatment on Tf receptor mRNA levels. K562 cells were treated with human diferic Tf (50 μg/ml) as indicated for 2 and 6 h. Samples of poly(A)$^+$ RNA (5 μg) from these cells and from untreated K562 cells were analyzed by Northern blotting as described in the text with [^{32}P]pCD-TR1 as probe.

The extent of the reduction in the mRNA for the receptor is presumably determined by the number of iron atoms that are introduced into the cells and is limited by the Tf cycle. More recently, it has been reported that hemin is much more efficient in delivering iron into cells and that such treatment lowers the expression of Tf receptors (8, 13, 14). We have shown that desferrioxamine is able to completely abolish the effect of hemin or diferric Tf on receptor synthesis, though it is unable to remove iron directly from either iron source (T. Rouault, K. K. Rao, J. Harford, E. Mattia, and R. D. Klausner, J. Biol. Chem., in press). We have concluded that both Tf and hemin deliver iron to an intracellular regulatory iron pool which is accessible to chelation by desferrioxamine. Treatment of K562 cells with hemin for 4 h resulted in a marked drop in Tf receptor mRNA levels. The maximum drop in mRNA levels was reached with 50 μM hemin (Fig. 4A). The decrease in Tf receptor mRNA levels seen after addition of hemin was rapid. Specific message levels decreased to 10% of the levels in control cells as soon as 2 h after the addition of the hemin, and within 4 h the levels were less than 3% of those of control cells (Fig. 4B). The demonstration of an 8- to 10-fold drop in specific message levels in 2 h imposed an upper limit on the half-life of the mRNA of between 35 and 40 min. As this assumed a cessation of all specific receptor mRNA transcription as soon as the drug was added, it is likely that even this relatively short time was an overestimate of the actual half-life. This calculation only related to the mRNA half-life in the presence of the perturbant (hemin in this case). In the

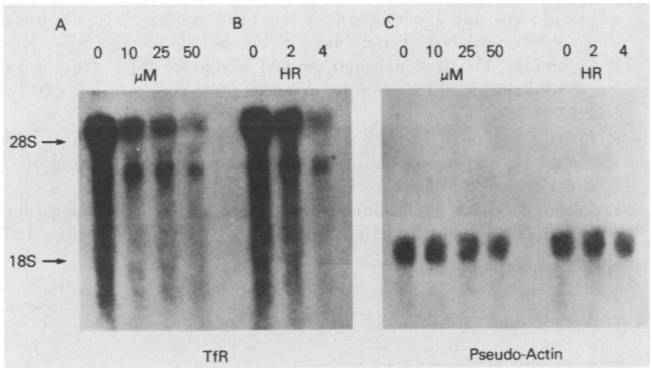

FIG. 4. Effect of hemin on the Tf receptor mRNA. K562 cells were treated as indicated with 10, 25, or 50 μM hemin for 4 h (A) or with 50 μM hemin for 2 and 4 h (B). Samples of total RNA (20 μg) were analyzed by Northern blotting as described in the text with [^{32}P]pCD-TR1 as probe. The pCD-TR1 probe was melted from the blot, and the blot was reprobed with a nick-translated pseudo-actin gene probe (C).

Northern blotting procedure, the band that migrated slightly ahead of the 28S marker was most likely a degradation product of the Tf receptor mRNA (Fig. 4A and B). Treatment of cells with hemin did not have any effect on the levels of actin mRNA when the Northern blots were reprobed with a nick-translated pseudo-actin gene (Fig. 4C).

In vitro nuclear transcription. To determine whether changes in the intracellular iron levels influenced the rate of gene transcription for the human Tf receptor, nuclear transcription or run off experiments were performed. In vitro nuclear transcription assays have been used to study transcriptional control in a number of systems (2, 7). Incorporation of radiolabeled nucleotides by isolated nuclei into specific RNA provides an estimate of the number of polymerase molecules in the process of transcribing a specific gene (1). For this purpose, K562 cells were treated with either 50 μM desferrioxamine or with 50 μM hemin, and nuclei were isolated as described in Materials and Methods. The total transcription rate was linear with respect to time

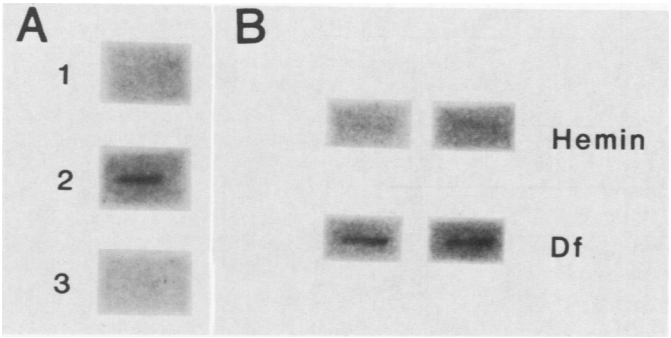

FIG. 5. In vitro nuclear transcriptions. Nuclei (4×10^7) were isolated from K562 cells treated with desferrioxamine for in vitro nuclear transcription as described in the text. The resultant RNAs (5×10^6 cpm) were hybridized to filters bearing either pBR322 (A, filter 1) or pCD-TR1 (A, filter 2). The spent hybridization solution from filter 2 was rechallenged with another filter containing pCD-TR1 (A, filter 3). In a separate experiment, nuclei were isolated from K562 cells that had been treated with either desferrioxamine (50 μM) or hemin (50 μM) as indicated (B). In vitro transcription was carried out as described in the text, and the RNA was hybridized to pCD-TR1 immobilized on filters.

during a period of 45 min. The incorporation of [α-^{32}P]UTP into trichloroacetic acid-precipitable material was identical in both hemin- and desferrioxamine-treated nuclei. The total transcription with both hemin- and desferrioxamine-treated nuclei was inhibited by 70% by 1 μg of α-amanitin per ml. This indicated that 70% of the transcripts were directed by RNA polymerase II in both nuclear preparations. After in vitro transcription for 5 min at 25°C, radiolabeled RNA preparations from hemin- or desferrioxamine-treated cells were hybridized to filters bearing either the cDNA clone for the Tf receptor (pCD-TR1) or for pBR322 DNA. There was a dramatic difference in the levels of specific hybridization between the transcripts of cells treated with desferrioxamine and those treated with hemin (Fig. 5). This result strongly suggested that manipulation of intracellular levels of iron was transcriptionally controlled. In a separate experiment (Fig. 5A), we showed that the hybridization was exhaustive by rechallenging spent hybridization medium with a second filter bearing pCD-TR1. In this case, no additional hybridization was observed. Nuclei from cells treated with desferrioxamine were employed in the in vitro transcription (Fig. 5A). Panel A represents an experiment distinct from that shown in panel B, and thus the two cannot be directly compared. We have not shown results from in vitro transcription which used nuclei from untreated cells. All available evidence suggests that treatments with desferrioxamine or hemin result in a range of the intracellular iron levels of the cells. The intracellular iron levels of untreated cells vary with the availability of iron in the growth medium. In general, the rate of transcription in nuclei from untreated cells was somewhat lower than that in cells which were treated with desferrioxamine but was much higher than that seen in nuclei from hemin-treated cells. These experiments demonstrated that a high level of control existed at the transcriptional level for gene coding for the human Tf receptor. A short half-life of the mRNA provided for rapid changes in the steady-state level of this message. Current work is aimed at elucidating the transcriptional control elements underlying this phenomenon.

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