Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 1787–1791, March 1988
Biochemistry

Two genetic loci participate in the regulation by iron of the gene for the human transferrin receptor

(gene regulation/iron metabolism/gene transfer)

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Communicated by Hamish N. Munro, November 23, 1987 (received for review October 14, 1987)

ABSTRACT **Iron regulation of the human transferrin receptor gene was examined in murine cells transformed with chimeric constructs containing the human transferrin receptor gene's promoter and either the structural gene for bacterial chloramphenicol acetyltransferase or the human transferrin receptor cDNA. The activity of the transferrin receptor gene's promoter with the heterologous indicator gene was found to be ≈3-fold higher in cells treated with the iron chelator desferrioxamine than in cells treated with the iron source, hemin. A higher degree of iron regulation was seen in the expression of the human transferrin receptor cDNA driven by its own promoter. The receptor cDNA under the control of the simian virus 40 early promoter was also iron-regulated. Several human transferrin receptor transcripts differing in their 3′ end were produced in the murine cells regardless of the promoter used, with the shorter transcripts being relatively unregulated by iron. Deletion of cDNA corresponding to most of the 3′ untranslated portion of the mRNA for the receptor ablated the iron regulation. We conclude that at least two genetic elements exist for the regulation of the transferrin receptor gene by iron. One has its locus in the DNA upstream of the transferrin receptor gene's transcription start site, and the other is dependent upon the integrity of the sequences in the 3′ end of the gene.**

Eukaryotic cells acquire iron through the endocytosis of diferric transferrin (Tf) bound to its high-affinity cell-surface receptor (1–3). The expression of transferrin receptors (TfR) in proliferating cells is regulated by iron availability. Chelation of intracellular iron by desferrioxamine leads in K562 cells to an increase in expression of TfR mRNA, and provision of iron results in decreased TfR mRNA expression (4–6). Isolated nuclei from desferrioxamine-treated K562 cells incorporate more [α-^{32}P]UTP into TfR-specific RNA than do nuclei from hemin-treated cells (6). However, the magnitude of the observed difference in nuclear transcription rates (estimated at 3- to 5-fold) was less than the difference in TfR mRNA levels (often >20-fold) in identically treated intact cells.

Here we report that the gene for chloramphenicol acetyltransferase (CAT) under the control of the promoter region of the gene for human TfR (TfR promoter) in murine cells is regulated by manipulations of iron availability to a degree similar to that seen in the aforementioned nuclear run-off experiments. We present evidence for an additional iron-responsive element residing in sequences corresponding to the 3′ untranslated portion of the TfR mRNA.

MATERIALS AND METHODS

Plasmids. The constructs −1700/+66CAT and −4300/+13CAT were prepared by isolation of the *Eco*RI–*Xba* I fragment and the *Hin*dIII–*Eco*RV fragment, respectively, of the TfR genomic clone λTR4 (7). These fragments were ligated via linkers in the *Hin*dIII site of the promoterless plasmid pSV0cat (8).

The TfR minigene construct 1, TRmg1, was prepared by ligation of the *Eco*RI–*Eco*RV fragment (−1700/+13) of λTR4 and the *Eco*RV–*Bam*HI fragment of the TfR cDNA clone pcDTR1 (9) with the vector pUC8, which had been cut with *Eco*RI and *Bam*HI. The construct TRmg6 was derived from TRmg1 by cutting with *Eco*RI (−1700) and *Nru* I (−95) followed by fill-in and ligation. The constructs TRmg2 and TRmg3 were also derived from TRmg1. A *Bcl* I–*Bam*HI fragment containing the early and late polyadenylylation signal sequences was isolated from simian virus 40 (SV40) DNA (Bethesda Research Laboratories) and inserted into either the *Bam*HI site of TRmg1 (to yield TRmg2) or between the *Bgl* II and *Bam*HI sites of TRmg1 so that the 3′ untranslated portion [≈2 kilobases (kb)] of the TfR cDNA was deleted (to yield TRmg3). The construct SVcDTR was prepared by a ligation of the 2.6-kb *Eco*RI–*Hin*dIII fragment of pSV2cat (8) with the 4.9-kb *Eco*RV-*Bam*HI fragment of pcDTR1.

Plasmids were amplified in *Escherichia coli* strain DH5 (Bethesda Research Laboratories) by using spectinomycin (International Biotechnologies, New Haven, CT). Plasmids were prepared by the alkaline $NaDodSO_4$ method (10) with the inclusion of an ammonium acetate protein precipitation prior to CsCl gradient centrifugation. Plasmids were treated with DNase-free RNase prior to transfection into murine fibroblasts. All plasmids were assessed by restriction site mapping for the arrangement and orientation of the fragments composing them.

Transfection of Murine Cells and Selection of Stable Transformants. Mouse B6 cells, a thymidine kinase-negative (tk^-) derivative of L cells, were the gift of R. Padmanabhan and B. Howard (National Institutes of Health). Cells maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum were plated at a density of 5×10^5 per 100-mm culture dish 24 hr prior to transfection. Calcium phosphate precipitates of plasmid DNA were made as described (8) with a 50:1 molar ratio of relevant plasmid to pHSV106 (Bethesda Research Laboratories) containing the herpes simplex *tk* gene. Precipitated DNAs were added to the culture medium, and 16 hr later cells were given fresh DMEM containing 10% fetal bovine serum. After an additional 30 hr, selection medium (containing 0.1 mM sodium hypoxanthine, 0.4 μM aminopterin, and 16 μM thymidine) was added. After 14 days, colonies of tk^+ cells were pooled and maintained in the selection medium.

Assessment of TfR Biosynthesis. Cells were incubated for 2 hr in 3 ml of methionine-free medium containing 100 μCi of

Abbreviations: Tf, transferrin; TfR, transferrin receptor; CAT, chloramphenicol acetyltransferase; TRmg1–TRmg6, TfR minigene constructs 1–6; SV40, simian virus 40; tk, thymidine kinase; TfR promoter, promoter region of the gene for TfR.

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Tran ^{35}S-label per ml (1 Ci = 37 GBq; ICN). The cells were scraped into ice-cold lysis buffer (0.5% Triton X-100/300 mM NaCl/50 mM Tris·HCl, pH 7.6/10 μg of leupeptin per ml/0.1 mM phenylmethylsulfonyl fluoride). TfR was isolated as described (11) by using either the monoclonal antibody B3/25 (12) together with protein-A agarose (Bethesda Research Laboratories) or by affinity purification with human Tf (Miles Scientific, Naperville, IL) that had been coupled to CNBr-Sepharose (Pharmacia). Samples were analyzed by autoradiography after electrophoresis in $NaDodSO_4$/polyacrylamide gels cross-linked with Acryl-Aide (FMC, Rockland, ME) using the buffer system of Laemmli (13).

RNA Isolation and Analyses. Total cellular RNA was prepared by the guanidinium isothiocyanate method (10). Cytoplasmic RNA was prepared from the postnuclear supernatant fluids after lysis of cells with Nonidet P-40. RNA samples were subjected to electrophoresis in 1% agarose/formaldehyde gels and analyzed by standard blot-hybridization procedures (10).

[^{3}H]Uridine Labeling of RNA. Cells were treated for 8 hr with 6 mM sodium butyrate in growth medium prior to addition of either 50 μM hemin or 50 μM desferrioxamine. After an additional 15 hr, the cells were radiolabeled in medium containing the same agents and 100 μCi of [5,6-^{3}H]-uridine (45 Ci/mmol; Amersham) per ml. [^{3}H]RNA was isolated by the guanidinium isothiocyanate method. The specific activities of the RNA preparations were unaffected by the iron manipulations. RNA (50 μg) was hybridized to a plasmid containing the CAT gene immobilized on strips of Biodyne nylon membranes (ICN). The hybridized RNA was washed five times with 0.6 M NaCl/0.06 M sodium citrate, pH 7, at 50°C and then quantitated by liquid scintillation counting of the strips.

RESULTS

CAT Gene Constructs Under the Influence of the TfR Promoter Region Display Regulation by Iron. Murine L tk$^-$ cells were stably transformed with plasmids that contained the structural gene for CAT downstream of fragments of the TfR promoter region (Fig. 1). In cells transformed with −1700/+66CAT [numbering relative to the TfR mRNA start site (14)] or with −4300/+13CAT, the level of CAT RNA was 2- to 3-fold higher after desferrioxamine treatment than after treatment with hemin (Fig. 2*A*). Cells transformed with pSV2cat displayed no iron effect on the level of CAT RNA. In these experiments CAT RNA expression was increased by treatment with sodium butyrate, which has been shown to alter expression of certain genes (15). Although the absolute levels of expression in cells not treated with butyrate were approximately 1/10th those in butyrate-treated cells, the extent of iron regulation was similar to that obtained in butyrate-treated cells (data not shown).

We confirmed the iron responsiveness of the TfR promoter region by measuring [^{3}H]uridine incorporation into CAT RNA in −4300/+13CAT cells (Fig. 2*B*). Kinetics of approach to steady-state labeling were similar in hemin- and desferrioxamine-treated cells, and the steady-state incorporation level was estimated to be 2.8-fold higher in cells treated with the iron chelator.

These experiments reveal transcriptional regulation of the TfR promoter with a range of regulation approaching 3-fold. However, the magnitude of this iron response is insufficient to account for the extent of the the regulation of TfR mRNA levels observed (6) upon manipulation of chelatable iron of cells in culture (often >20-fold). This suggested that portions of the TfR gene other than the upstream sequences might participate in iron regulation.

TfR cDNA Constructs Are Highly Regulated by Iron. To determine whether elements within the TfR cDNA were involved in iron regulation, we prepared murine cell lines transformed by using TRmg constructs containing the human TfR cDNA ligated in its native position downstream of the TfR promoter region (see Fig. 1) and assessed iron regulation of human TfR biosynthesis (Fig. 3*A*). Human Tf-Sepharose was used to affinity-purify the [^{35}S]TfR. Human Tf-Sepharose is recognized by both human TfR and mouse TfR, but the receptors from the two species can be distinguished by gel electrophoresis because of a slight difference in mobility (3). Murine cells transformed with TRmg1 (Fig. 3*A*) or TRmg6 (Fig. 3*B*) produced human TfR that was highly regulated in its biosynthesis by iron. Regulation was most apparent in immunoprecipitates with B3/25, which does not recognize the mouse TfR. The lower band seen in these gels represents a biosynthetic precursor of the mature human TfR (12).

FIG. 1. Schematic representation of chimeric genes involving the human TfR gene. In the TfR receptor–CAT gene constructs, the solid bar represents TfR genomic DNA derived from the genomic clone λTR4 (7). The CAT structural gene is represented by the open box. Nomenclature reflects the ends of the TfR gene sequences numbered relative to the TfR mRNA start site (14). Closed boxes in the TRmg constructs also represent DNA fragments of λTR4, whereas the open boxes here represent all or part of the TfR cDNA derived from the clone pcDTR1 (9). Numbers in parentheses represent the 5′-most nucleotide of the TfR gene contained within the construct. The striped bar in the construct SVcDTR represents the early promoter region of SV40. The striped bars at the 3′ end of constructs TRmg2 and TRmg3 represent an SV40 fragment containing a polyadenylylation signal. TRmg3 differs from TRmg2 in that the 3′-most 2 kb of the TfR cDNA has been deleted.

When the TfR cDNA was linked to the TfR promoter, the magnitude of iron regulation of human TfR in mouse cells was similar to that of the endogenous mouse TfR. Added to the findings of Fig. 2, this result strongly suggested that the transcribed region of the TfR gene participates in iron regulation. To test this conclusion, we stably transformed B6 cells with plasmic SVcDTR in which the human TfR cDNA was placed downstream of a heterologous promoter (see Fig. 1). In these cells, expression of the human TfR was undetectable unless the cells were treated with sodium butyrate (Fig. 4*A*). When butyrate was used to induce human TfR expression in SVcDTR cells, its biosynthesis was also highly regulated by iron (Fig. 4*B*).

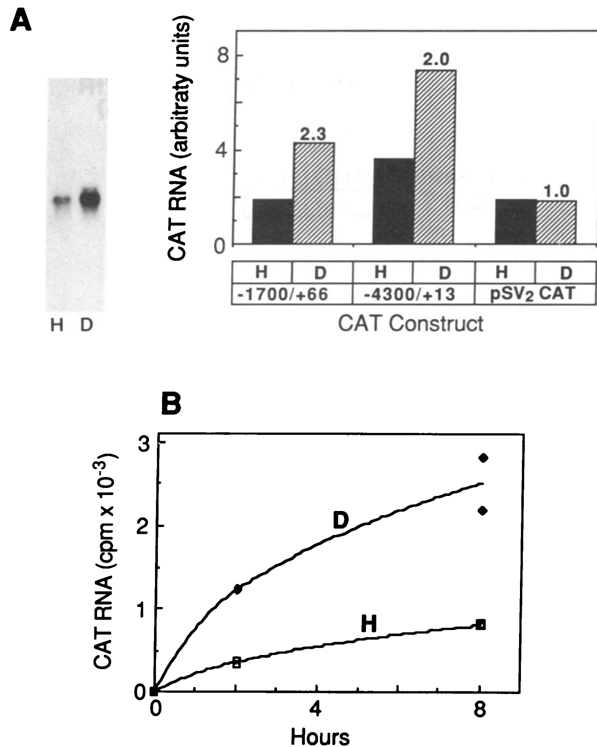

FIG. 2. Iron regulation of accumulation and synthesis of CAT RNA in cells transformed with TfR promoter–CAT gene constructs. Murine tk$^-$ cells were transformed with the indicated plasmids. Cells were treated with 6 mM sodium butyrate for 8 hr and then with either 50 μM hemin (H) or 50 μM desferrioxamine (D) for 16 hr. Cytoplasmic RNA was isolated from the $-1700/+66$CAT cells, and the content of CAT RNA was assessed by blot-hybridization analysis with a ^{32}P-labeled CAT GenBlock (Pharmacia) as probe. The resultant autoradiograph was quantitated by scanning densitometry (*A Left*) revealing a 2.8-fold higher level of CAT RNA in the desferrioxamine-treated cells (▨) than in cells treated with hemin (■) (D/H = 2.8. In a separate experiment, total RNA samples from the indicated transformants were similarly quantitated for CAT RNA content (*A Right*) after treatment with hemin (■) or desferrioxamine (▨). The D/H ratio for each transformant in this experiment is shown above the hatched bars. (*B*) [^{3}H]Uridine (100 μCi/ml) was used to label cellular RNA in cells transformed with $-4300/+13$CAT. Total RNA was isolated and hybridized to an immobilized plasmid containing the CAT gene. Results from duplicate hybridizations are shown. Values obtained with [^{3}H]RNA from similarly treated tk$^+$CAT$^-$ cells have been subtracted.

Iron Regulation of TfR Gene Expression Correlates with the Presence of the 3′ End of the TfR cDNA. When RNA was isolated from either TRmg1 cells or SVcDTR cells, multiple human TfR transcripts were observed (Fig. 4*C*). In K562 cells (6) and in mouse L cells (data not shown) the TfR transcript is 4.9 kb, similar to the largest of the human transcripts in the mouse transformants. All of the human TfR transcripts in the transformants were polyadenylylated as judged by their binding to an oligo-dT column (data not shown). That the TfR transcripts in the transformants differed from one another in their 3′ end was established by comparison of hybridization with the full-length TfR cDNA (Fig. 4*C Left*) and with a probe corresponding to the 3′-most 2 kb of the TfR cDNA (Fig. 4*C Right*). In the latter case, the hybridization pattern was heavily weighted toward the 4.9-kb transcript, indicating that the smaller transcripts were lacking some or all of the mRNA sequences that are homologous to this 3′ probe.

It was apparent that the 4.9-kb transcript was much more highly regulated than were the shorter transcripts. Whereas the total signal in desferrioxamine-treated TRmg1 cells was

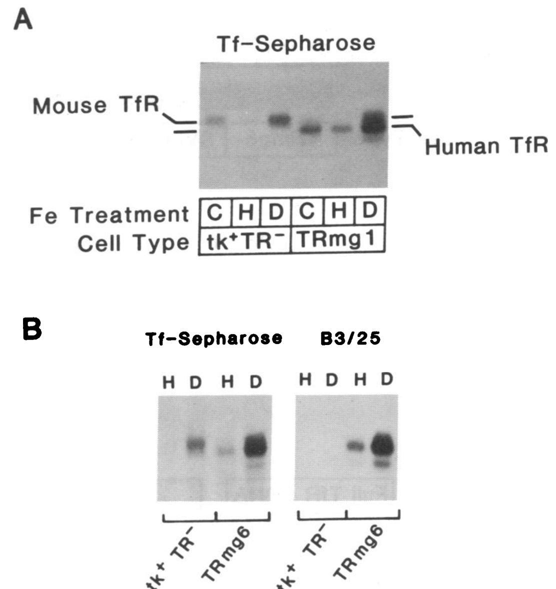

FIG. 3. Iron regulation of TfR biosynthesis in cells transformed with TRmg constructs. Cells were treated for 16 hr with either 50 μM hemin (lanes H) or with 50 μM desferrioxamine (lanes D) and are compared with untreated control cells (lanes C). Cellular protein was labeled with [^{35}S]methionine. Equal amounts of CCl_3COOH-precipitable radioactivity were exposed to either Tf-Sepharose (*A* and *B Left*) or to the monoclonal antibody B3/25 and protein A-agarose (*B Right*). Samples were analyzed by $NaDodSO_4$/PAGE, and the relevant portions of the resultant autoradiographs are shown. The tk$^+$ TR$^-$ cells are murine L tk$^-$ cells transformed with pHSV106 and contain no portion of the gene for the human TfR.

estimated by densitometry to be increased <10-fold over that for the same cells treated with hemin (D/H < 10), the D/H value for the 4.9-kb transcript in these cells was determined to be >35. The D/H value for the 4.9-kb transcript in the SVcDTR cells was estimated to be ≈10. This difference between TRmg1 and SVcDTR cells in the magnitude of iron regulation of the 4.9-kb transcript may be a reflection of the iron-responsive transcriptional element.

Our conclusion that the 3′ untranslated portion of the TfR transcript is a major locus of iron regulation was initially based upon this lower degree of iron regulation of transcripts lacking this region. To test this conclusion more directly, we prepared a construct termed TRmg3 from which we had excised the 3′-most 2 kb of the TfR cDNA (see Fig. 1). We introduced downstream an SV40 polyadenylylation signal sequence on the possibility that this would simplify the pattern of transcripts. For comparison, we prepared TRmg2, which contained the full-length cDNA with the same downstream SV40 polyadenylylation signal. Murine transformants of TRmg2 or TRmg3 produced human TfR of identical size, consistent with the fact that both plasmids contained cDNA corresponding to the entire translated region of the TfR mRNA. The biosynthesis of human TfR in TRmg2 cells was highly regulated by iron (Fig. 5*A*) as had been seen with the two previous plasmids that contained the full-length cDNA (TRmg1 and TRmg6). The TfR biosynthesis in TRmg3 cells appeared to be unregulated by iron when assessed in this way.

An analysis of the RNA transcripts produced by TRmg2 and TRmg3 cells is shown in Fig. 5*B*. The TRmg2 cells continued to produce smaller transcripts in addition to the 4.9 kb that was highly iron-regulated. The TRmg3 cells produced a single human TfR transcript the size of which was consistent with the truncation of the 3′ end of the TfR cDNA in the parental plasmid. The level of this transcript in

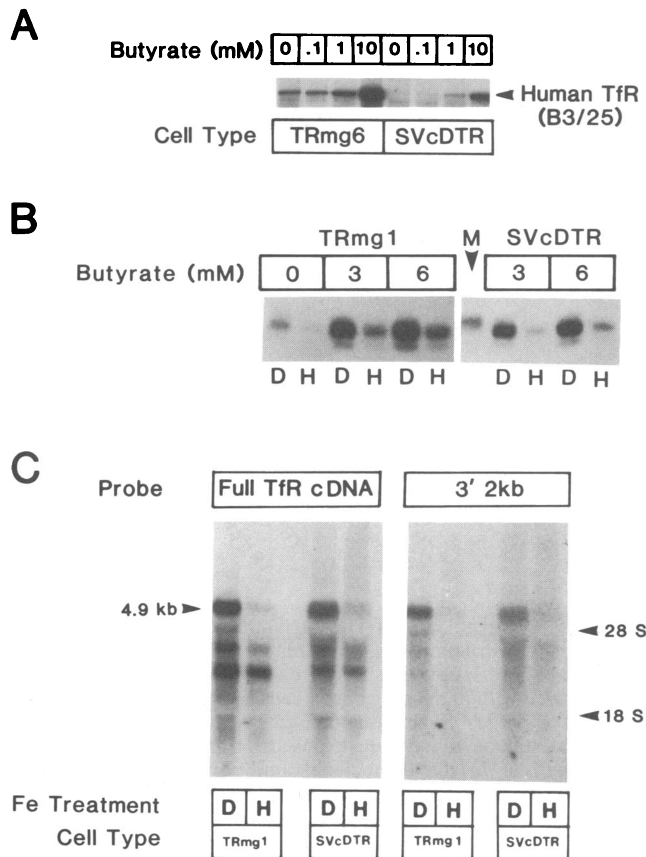

FIG. 4. Iron regulation of TfR gene expression in TRmg constructs having either the TfR promoter or the early promoter of SV40. (*A*) Cells transformed with either the construct TRmg6 or with SVcDTR were treated with increasing concentrations of sodium butyrate for 8 hr prior to protein metabolic labeling, and an equal amount of CCl_3COOH-precipitable radioactivity was immunoprecipitated with B3/25. The samples were analyzed by $NaDodSO_4$/PAGE, and the relevant portion of the resultant autoradiograph is shown. (*B*) Cells transformed with either TRmgl or SVcDTR were treated with the indicated concentrations of sodium butyrate for 8 hr before treatment with either 50 μM desferrioxamine (lanes D) or 50 μM hemin (lanes H) for 16 hr. TfR biosynthesis was assessed as described above. The lane designated M contains metabolically labeled mouse TfR isolated via Tf-Sepharose from tk^+ TfR^- cells. (*C*) RNA was isolated, and 10 μg was electrophoresed, transferred to nitrocellulose, and hybridized with ^{32}P-labeled probe corresponding to the full TfR cDNA (left set of lanes). After autoradiography, this probe was removed in H_2O at 95°C, and the nitrocellulose was rehybridized (right set of lanes) with a ^{32}P-labeled probe corresponding to the 3'-most 2 kb of the TfR cDNA (*Bgl* II–*Bam*HI fragment of pcDTR1). No hybridization of either probe to mouse TfR mRNA occurred under the conditions used.

the TRmg3 cells appeared to be unchanged after 16 hr of treatment with either hemin or desferrioxamine.

DISCUSSION

The understanding of the mechanisms by which specific genes are regulated in response to various stimuli remains a central goal of modern biology. Much insight has been gained concerning the regulation of genes at the level of initiation transcription (16). Less is understood concerning regulation at points distal to transcription initiation, though ample evidence exists for such regulation. Evidence has been put forward for control of specific gene expression at the levels of transcription elongation (17), modulation of nuclear transcript stability (18), cytoplasmic mRNA stability

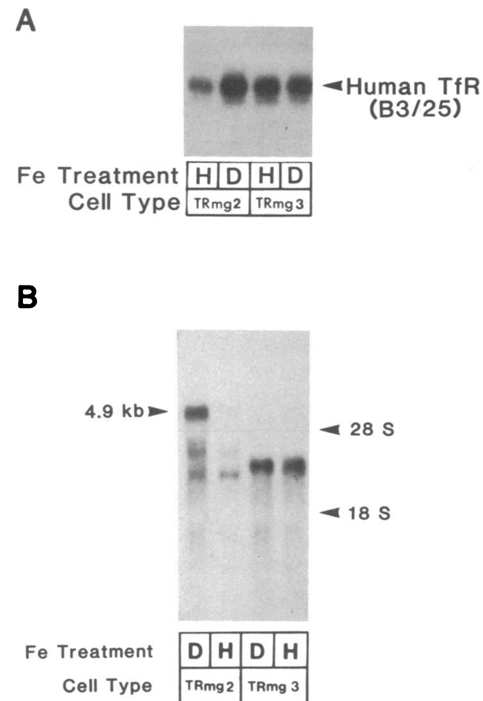

FIG. 5. Iron regulation of TfR gene expression requires the 3' 2 kb of the TfR cDNA. Treatments with sodium butyrate and desferrioxamine (lanes D) or hemin (lanes H) were as described in Fig. 2. Immunoprecipitation and analysis of human TfR were performed by using B3/25 and protein A-agarose (*A*). After electrophoresis, the RNA was transferred to nitrocellulose and hybridized to a ^{32}P-labeled probe corresponding to the full TfR cDNA (*B*).

(19, 20), and translation (21). Not surprisingly, diverse regions of the genes in question have been implicated in these diverse regulatory processes.

More than one mechanism mediated by more than one gene region can be operative within a single gene or with a single stimulus. Exemplifying this phenomenon is glucocorticoid regulation of hormone-responsive genes. Evidence has been presented for transcriptional activation of responsive genes (22), for stabilization of their transcripts (23), and for modulation of translation (24). Our results suggest that at least two elements of the TfR gene are involved in the regulation of TfR expression by iron availability. Previously, we had shown that nuclei from desferrioxamine-treated K562 cells incorporated more [α-^{32}P]UTP into TfR mRNA than did nuclei from hemin-treated cells (6). In the present study, we have examined the iron regulation of chimeric gene constructs following integration into murine fibroblasts. A CAT construct containing the TfR promoter with only 13 bp corresponding to nucleotides of the native TfR transcript was regulated ≈3-fold by iron manipulations (Fig. 2).

The magnitude of the iron regulation of our TfR promoter–CAT gene constructs was similar to that observed in K562 nuclear run-offs (6) but was insufficient to account for the iron-dependent alterations of TfR mRNA levels that occur in cultured cells. In instances where the level of mRNA for a gene is altered (as with TfR), regulation beyond the level of initiation of transcription often has been inferred from an inability to account for the magnitude of the changes in RNA level by the directly measured increase in transcription rate. For example, prolactin causes a 13-fold increase in casein mRNA levels through a 2- to 4-fold increase in transcription and a marked increase in casein mRNA stability (25).

We observed that a construct in which expression of the TfR cDNA was driven by a heterologous promoter (SVcDTR) was also iron-regulated (Fig. 4). The constructs −4300/+13CAT and SVcDTR have no TfR gene sequences in common, indicating that at least two regions of the TfR gene are involved in iron regulation. The construct −4300/+13CAT contains an iron-responsive transcription element. The second region mediating iron responsiveness was identified by the loss of iron regulation after a deletion of plasmid DNA corresponding to the 3′ untranslated portion of the TfR mRNA. Our inspection of the published sequence of the 3′ end of the TfR cDNA (26) revealed multiple copies of the sequence element proposed to be responsible for the short half-lives of mRNAs of several other rapidly regulated genes (27), suggesting that one possible mechanism for regulation would be an iron-dependent modulation of TfR mRNA stability.

In addition to our nuclear run-off experiments in K562 (6), others have examined in like fashion TfR transcription rates within activated T cells (28) and in HL60 cells treated with dibutryl cAMP (29). Activation of T cells results in increased TfR gene expression, whereas cAMP treatment of HL60 cells results in decreased TfR gene expression. The corresponding changes in transcription rates that were observed in both instances are consistent with a transcriptional component to these regulations of TfR gene expression. Based upon constructs similar to those described here, Owen and Kühn (30) have recently concluded that the 3′ untranslated portion of the TfR gene is required for mRNA regulation by iron. No evidence for a transcriptional iron-responsive element was presented by Owen and Kühn (30). Interestingly, they observed that deletion of the 3′ untranslated sequences did not eliminate the decrease in TfR gene expression that accompanied fibroblast growth arrest by serum deprivation. Pelosi et al. (31) have also suggested a distinction between iron regulation of TfR and the increase in TfR gene expression that is observed upon activation of T lymphocytes. Multiple levels of regulation mediated by distinct regions of the TfR gene might be used to differentially respond to more than one stimulus or might be utilized to achieve a magnitude of response greater than that which could be attained using only one regulatory mechanism.

We thank Dr David Sabatini for his suggestion regarding the use of sodium butyrate and Drs. David Koeller and Matthias Hentze for their critical reading of this manuscript and helpful suggestions.

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