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Noncoding 3′ sequences of the transferrin receptor gene are required for mRNA regulation by iron

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The cell-surface receptor for transferrin mediates cellular uptake of iron from serum. Transferrin receptor protein and mRNA levels are increased in cells treated with iron chelating agents, and are decreased by treatment with iron salts or hemin. Here we report that expression of human transferrin receptor cDNA constructions in stably transfected mouse fibroblasts is regulated both by the iron chelator, desferrioxamine, and by hemin. We found that sequences within the 3′ noncoding region are required for the iron-dependent feedback regulation of receptor expression, whereas the presence of the transferrin receptor promoter region is not necessary. Regulation by iron is observed when transcription is initiated at either the SV-40 early promoter or the transferrin receptor promoter, but deletion of a 2.3 kb fragment within the 2.6 kb 3′ noncoding region of the cDNA abolishes regulation and increases the constitutive level of receptor expression. Furthermore, the 3′ deletion does not affect the decrease in receptors which is observed in response to growth arrest, indicating that transferrin receptor expression is controlled by at least two distinct mechanisms.

Key words: transferrin receptor/gene expression/noncoding 3′ region/iron metabolism/mRNA regulation

Introduction

Intracellular iron is supplied by endocytosis of diferric transferrin bound to its specific cell surface receptor (Morgan, 1981; Newman *et al.*, 1982). The role of iron in controlling transferrin receptor (TR) expression has been demonstrated in several different cell types. Chelating intracellular iron leads to an increase in surface receptors, while receptor expression is reduced in cells treated with hemin or iron salts (Bridges and Cudkowicz, 1984; Ward *et al.*, 1984; Rao *et al.*, 1985). These iron-dependent changes in surface receptor levels are accompanied by alterations in receptor mRNA concentrations (Rao *et al.*, 1986). Several studies have shown that receptor expression is also linked to cell proliferation. A decline in receptors is associated with arrest of cell growth, while expression increases during the transition from stationary-phase to exponential growth and proliferating cells maintain high receptor levels (Larrick and Cresswell, 1979; Trowbridge and Omary, 1981; Musgrove *et al.*, 1984; Pelosi *et al.*, 1986). Pelosi *et al.* (1986) have proposed that intracellular iron concentration and cell growth rate regulate expression by different mechanisms, based on the observation that whereas resting T-lymphocytes do not express detectable levels of surface receptors and are unresponsive to chelators, stimulation with interleukin-2 increases receptor expression and permits response to chelators.

The isolation of the human transferrin receptor gene and the full-length human cDNA clone (Kühn *et al.*, 1984) has made it possible to elucidate the mechanisms underlying the control of receptor expression, and to resolve the question of whether iron levels and cell growth rates act through different regulatory mechanisms. To determine whether specific promoter elements are involved in the control of TR mRNA levels we tested the expression of cDNA constructions containing either the SV-40 promoter or the TR promoter with varying lengths of upstream sequences. We had previously observed that deletion of a 2.3 kb segment within the 3′ noncoding region led to increased expression of receptors in transient expression assays (Kühn, 1985). In view of recent evidence that 3′ noncoding sequences may play an important role in controlling gene expression (Kaufman and Sharp, 1983; Treisman, 1985; Yaffe *et al.*, 1985), we also made cDNA constructions which combined the different promoter regions with the deletion in the 3′ noncoding region. When these cDNA constructions were expressed in transfected mouse cells, sequences within the 3′ noncoding region were found to be essential for the response to changes in intracellular iron levels but not for the response to arrest of cell growth. In addition, the presence of the TR promoter was not required for iron-dependent regulation, suggesting that iron modulates transferrin receptor mRNA concentrations at some step occurring subsequent to the initiation of transcription.

Results

Structure of the transferrin receptor cDNA constructions

The human cDNA clone used for transfection experiments, designated pcD-TR1, had been isolated from a cDNA library of the SV-40-transformed human fibroblast line, GM637 (Kühn *et al.*, 1984; Okayama and Berg, 1983). The 5.0 kb cDNA is comprised of 96 bp upstream from the initiation codon, 2280 bp of coding sequences and a 2.6 kb 3′ noncoding region (McClelland *et al.*, 1984). In the original pcD expression vector transcription of the cDNA is controlled by the SV-40 early promoter and addition of the polyA tail is directed by the SV-40 late region polyadenylation signal (Okayama and Berg, 1983). Since we wished to investigate the expression of the transferrin receptor cDNA under the control of its own promoter, we first sequenced a 619 bp segment within the 5′ region of TR gene to determine the location of likely promoter elements (Figure 1). The genomic clone containing this segment, λTR15, had been previously isolated from the human T-cell lymphoma line, MOLT-4 (Kühn *et al.*, 1984). DNA sequencing data revealed the presence of a TATA box at −27 bp from the cap site determined for mRNA from MOLT-4 cells (Schneider *et al.*, 1984), as well as several GC-rich stretches 5′ to the TATA box. In the subsequent transfection experiments described below the ability of this promoter region to direct initiation of transcription was confirmed.

The first 72 bp of pcD-TR1, comprising exon 1, are entirely homologous with the MOLT-4 genomic sequence. However, pcD-TR1 extends 23 bp upstream from the MOLT-4 cap site, to a position only 4 bp downstream from the MOLT-4 TATA

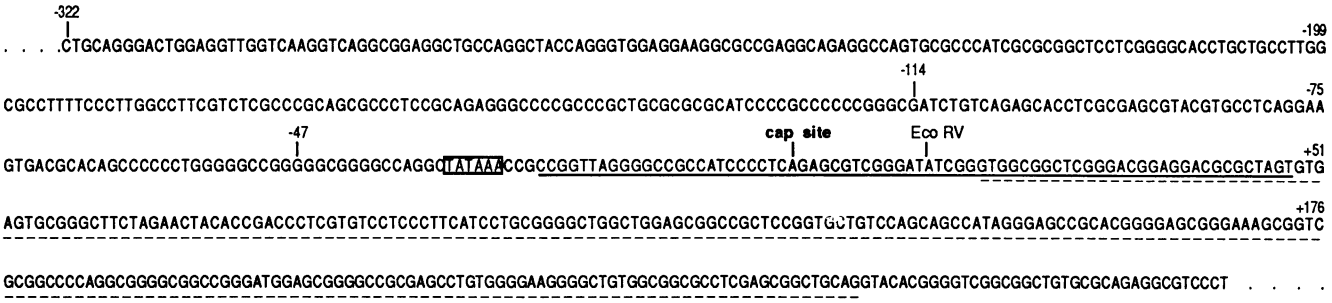

Fig. 1. DNA sequence of the human transferrin receptor promoter region. The nucleotide sequence of a 619 bp segment containing the promoter region, exon 1 and the 5′ portion of the first intron is shown. The cap site of mRNA from MOLT-4 cells is shown at position 1 ± 2 bp (Schneider *et al.*, 1984). A TATA box is located at −27 bp from the cap site. (—) indicates the first exon of pcD-TR1, the cDNA clone from GM 637 cells. (---) indicates the first exon of pTR36, the cDNA isolated from MOLT-4 cells by Schneider *et al.* (1984). The *Eco*RV site, which falls within exon 1 of pcD-TR1, was used to construct the cDNA recombinants containing the transferrin receptor promoter region.

box. This structural difference could be due to the use of alternative promoter elements, as has been described in the case of the α and β globin gene families (Carlson and Ross, 1983; Hess *et al.*, 1985). In addition, a comparison of the genomic sequence with the cDNA clone from GM637 cells, pcD-TR1, and the cDNA clone from MOLT-4 cells, pTR36 (Schneider *et al.*, 1984), revealed that the two cDNA clones differ only within the first exon, whose 3′ boundary is located 211 bp further downstream in pTR36 (Figure 1). We have not investigated the reason for this use of alternative splice sites.

Modifications of the cDNA, pcD-TR1, were made as shown in Figure 2. The SV-40 promoter was replaced by genomic segments extending 1500, 322, 114 or 47 bp upstream from the MOLT-4 cap site and containing the TR promoter. Alternatively, a 3.2 kb *Eco*RV fragment from the TR promoter region was inserted downstream from the SV-40 promoter. In further constructions all but 300 bp of the 3′ noncoding region were deleted by removing two *Xba*I fragments of 2.1 kb and 0.2 kb. The combination of the 3′ deletion with the various promoter regions gave cDNA constructions 1−12. In additional constructions, the 189 kb *Xba*I fragment and a contiguous 208 bp *Xba*I−*Bgl*II fragment were deleted (Figure 2, construction 13), the 2.1 kb *Xba*I fragment was re-inserted at its original location in the opposite orientation (construction 14) or inserted in both orientations 1500 bp upstream from the cap site (constructions 15 and 16). Constructions 17 and 18 contain the promoter and coding region of the HLA-A2 gene upstream from either the *Eco*RV site within the TR 5′ noncoding region of the *Bgl*II site within the TR 3′ noncoding region, respectively.

Iron-dependent regulation of cDNA constructions

The different cDNA constructions were co-transfected with the HSV-tk gene and the HLA-A2 gene into Ltk^- cells, and Ltk^+ cells were selected by growth in HAT medium. Single-cell clones expressing the human receptor were isolated by fluorescence activated cell sorting using the monoclonal anti-human TR antibody, 5E9, in combination with cloning by limiting dilution. A good correlation was found in individual clones between the number of human receptors as determined by [^{125}I]transferrin binding, the amount of fluorescence staining, and the increased uptake of ^{59}Fe-labelled transferrin, indicating that the human receptors were functional in transfected mouse cells (unpublished data).

Regulation of human TR mRNA levels was initially tested by S_1 nuclease mapping of total cytoplasmic RNA from transfected L cell clones and from the human cell line GM637, from which the cDNA had been derived (Figure 3). Cells were treated with hemin or desferrioxamine, an iron chelator (Bridges and Cudkowicz, 1984), using conditions which gave an optimal response without affecting cell-plating efficiency or the cell cycle distribution, as determined by FACS analysis of DNA content (unpublished data). In the stably transfected L cell clones designated E8.2 and E10.2, containing cDNA construction 3 (see Figure 2), human TR mRNA levels increased after treatment with desferrioxamine and were decreased by treatment with hemin. The L cell clone E10.8, containing the same 5′ sequences but with the 3′ deletion (construction 4), responded neither to desferriox-

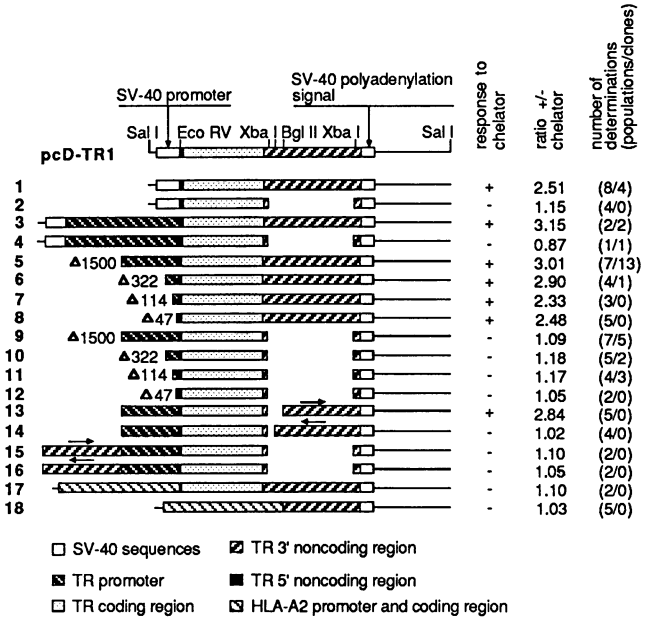

Fig. 2. Regulation of iron chelator of transferrin receptors expressed from TR cDNA constructions. The cDNA clone, pcD-TR1 was modified as shown: a 900 bp *Sal*I−*Eco*RV fragment, containing the SV-40 promoter of the pcD expression vector and 39 bp of exon 1 upstream from the *Eco*RV site, was replaced with genomic segments starting at the *Eco*RV site and extending upstream to positions −47, −114, −322 or −1500 bp from the cap site. Alternatively, a 3.2 kb *Eco*RV genomic fragment containing the transferrin receptor promoter region was inserted at the *Eco*RV site in exon 1, downstream from the SV-40 promoter. Further modifications were made by deleting two *Xba*I fragments of 0.2 kb and 2.1 kb within the 3′ noncoding region. Receptor expression in HAT-resistant cell populations and individual cell clones transfected with the different cDNA constructions was determined by FACS analysis of surface fluorescence. The values shown for constructions 17 and 18 refer to expression of the HLA-A2 antigen. The average ratio of mean fluorescence intensity in cells treated 40 h with 50 μM desferrioxamine and untreated cells is indicated. The standard deviation of these values was ±15% or less.

amine nor to hemin. The endogenous human gene in GM637 cells showed the same pattern of expression as the construction with an intact 3′ region. Expression of the endogenous gene after treatment either with another iron chelator, picolinic acid, or with ferric ammonium citrate, gave the same result as treatment with desferrioxamine and hemin, respectively. The presence of the SV-40 late polyadenylation signal clearly did not interfere with the regulated expression of the cDNA constructions as compared with the endogenous gene.

In further experiments we used the fluorescence activated cell sorter (FACS) to screen independent HAT-resistant cell populations and individual cell lines for regulation of receptor expression in response to treatment with desferrioxamine. Mean surface fluorescence intensity was analysed using the monoclonal antibody against the human transferrin receptor. A compilation of the results obtained from FACS analysis of each of the different cDNA constructions is shown in Figure 2. Figure 4 shows typical profiles generated by the FACS for representative cell populations. cDNA constructions with the 3′ deletion did not respond to desferrioxamine (Figure 4b,e) while constructions with an intact 3′ region responded regardless of whether transcription was directed by the SV-40 promoter (Figure 4a) or the TR promoter (Figure 4c,d) and response was conserved even when all but 47 bp upstream from the cap site were deleted from the TR promoter region (Figure 4d). Surface expression of the co-transfected HLA-A2 gene was not affected by desferrioxamine treatment (Figure 4f).

As shown in Figure 2, re-inserting the 2.1 kb *Xba*I fragment in the opposite orientation at its original location (construction 14) or placing it 1.5 kb upstream from the TR promoter (constructions 15 and 16), did not restore the response to chelator. In addition, in constructions containing the promoter and coding region of the HLA-A2 gene upstream either from the TR cDNA (construction 17) or from the *Bgl*II site within the TR 3′ noncoding region (construction 18), regulation of the HLA-A2 gene by iron chelator could not be detected. However, the 3′ sequences downstream from the *Bgl*II site were able to confer desferrioxamine response to a 3′ deletion mutant of the transferrin receptor cDNA (construction 13).

The average responses to desferrioxamine shown in Figure 2 were calculated from the values for mean relative fluorescence intensity measured in independent determinations. The individual values obtained for constructions 1, 2 and 5 through 12 are plotted in Figure 5. Constructions with an intact 3′ noncoding region responded to treatment with desferrioxamine by an average increase of 2.5-fold in surface fluorescence. A higher average value of mean fluorescence intensities was observed when the 3′ fragment was deleted (36 versus 11), as evident from the graph. Con-

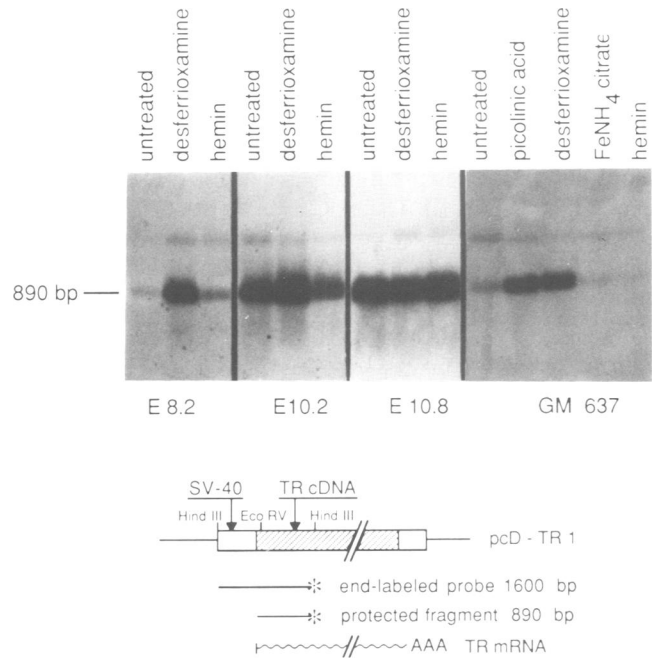

Fig. 3. S_1 nuclease analysis of human transferrin receptor mRNA in transfected cell clones and human GM637 fibroblasts. The end-labelled 1.6 kb probe was hybridized with 50 μg total cytoplasmic RNA isolated from L cell clones E8.2 and E10.2, transfected with the cDNA construction containing the SV-40 promoter and TR promoters in tandem and an intact 3′ region (see Figure 2, construction 3), L cell clone E10.8, transfected with tandem promoter construction and a 2.3 kb 3′ deletion (see Figure 2, construction 4), or the SV-40 transformed human fibroblast cell line, GM637. Transferrin receptor mRNA transcribed from either the endogenous human gene or the transfected human cDNA constructions protected a fragment of 890 bp. Cells were treated as indicated for 40 h with 2 μM picolinic acid, 50 μM desferrioxamine, 10 μg/ml ferric ammonium citrate or 250 μM hemin.

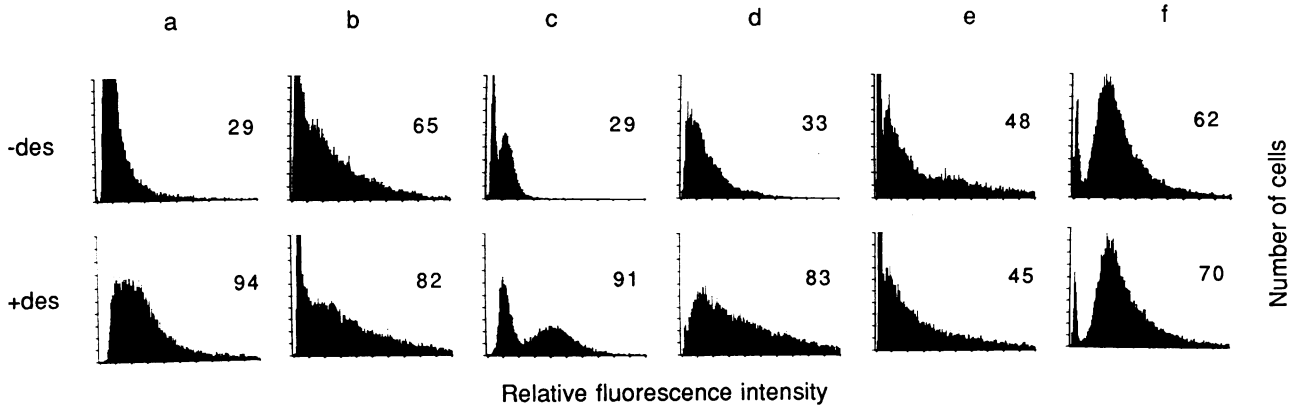

Fig. 4. FACS profiles of human transferrin receptor expression in transfected mouse L cells. Total HAT-resistant L cell populations were analysed by staining with monoclonal antibodies against the human transferrin receptor or HLA-A2 and fluoresceinated goat anti-mouse Ig. Mean fluorescence intensities calculated from the profiles generated by the FACS are given in arbitrary units in the upper right corner of each panel. Cells were untreated (−des) or treated for 40 h with 50 μM desferrioxamine (+des). **Columns** (**a**)-(**e**); expression of the human receptor. Cells were transfected with the following cDNA constructions as specified in Figure 2: (**a**); construction 1, (**b**); construction 2, (**c**); construction 7, (**d**); construction 8, (**e**); construction 12. **Column** (**f**); HLA-A2 expression in duplicate cultures of the cells shown in (c).

sistently higher expression of the 3′ deletion mutants was also seen at the mRNA level (see Figures 3 and 6).

Total cytoplasmic mRNA from the same cell populations shown in Figure 4a,b,d and e was analysed by blot hybridization using a nick-translated probe specific for the coding region of the human transferrin receptor (Figure 6). In cells transfected with constructions carrying an intact 3′ region (lanes 1,2,5,6), a mRNA species with the expected size of 5.1 kb was detected only after treatment with desferrioxamine (lanes 1,5). Expression of the 2.8 kb mRNA species found in cells transfected with 3′ deletion mutations was not affected by treatment with chelator (lanes 3,4,7,8). As a control, aliquots of the same RNA samples as in lanes 1,2 were hybridized with a nick-translated probe specific for mouse β_2-microglobulin (lanes 9,10). The β_2-microglobulin probe hybridizes to two distinct RNA species whose expression is not influenced by desferrioxamine treatment.

Receptor expression in arrested cells

To determine whether the 3′ sequences required for response to chelator were also involved in the regulation of receptor expression in arrested versus proliferating cells, we tested expression of the transferrin receptor cDNA constructions containing the 3′ deletion in NIH/3T3 cells. We had previously determined that these cells could be arrested in the G0/G1 phase of the cell cycle when cultured in 0.4% fetal calf serum, whereas the L cells initially used for transfection experiments could not be arrested by culturing in low serum concentrations.

NIH/3T3 cell clones transfected with cDNA constructions containing the 3′ deletion and 1500, 322 or 114 bp of TR promoter sequences (constructions 9, 10 and 11) were isolated in the same manner described for L cell transfectants. Due to the low levels of expression of constructions with an intact 3′ region we have so far not been able to isolate NIH/3T3 cell clones containing these constructions in repeated transfection experiments. Expression of surface receptors in proliferating and arrested cells, cultured in the presence or absence of desferrioxamine, was tested in several experiments by FACS analysis of surface fluorescence (Figure 7). The 3′ deletion mutants did not respond to treatment with chelator, but cell arrest reduced receptor expression 3- to 10-fold (columns a,b,c). In proliferating cells the endogenous mouse TR gene responded to the iron chelator by a 2-fold increase in receptor levels, while arrested cells expressed only half as many receptors as proliferating cells and were unresponsive to chelator (column d). The mouse H-2 antigens were expressed at the same level under all conditions (column e). Blot hybridization of RNA from the same cell clones as in Figure 7a,c showed that the proportion of the human TR mRNA with respect to total cytoplasmic RNA was reduced in arrested cells (Figure 8A), while levels of mouse β_2-microglobulin remained constant (Figure 8B).

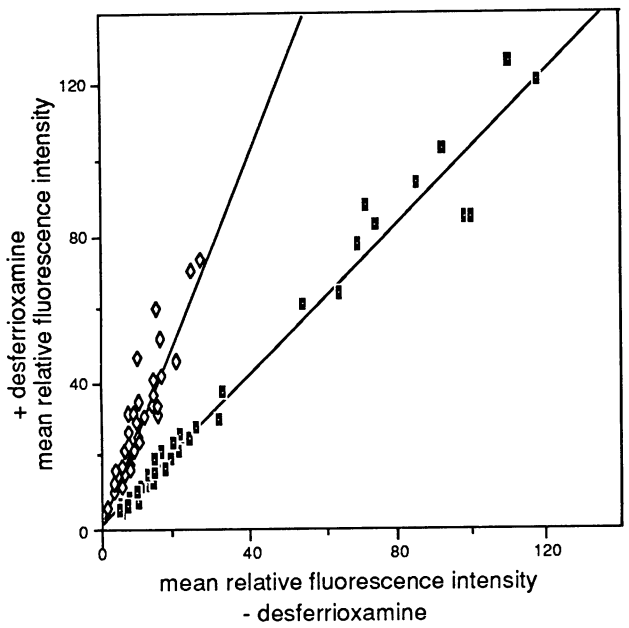

Fig. 5. Effect of desferrioxamine treatment on expression of human transferrin receptor. Data are shown for cells transfected with cDNA constructions 1, 5, 6, 7 and 8, containing an intact 3′ region (◇) or constructions 2, 9, 10, 11 and 12, with the 2.3 kb 3′ deletion (■). Relative mean fluorescence intensities were determined by FACS analysis of untreated cells (−desferrioxamine) or of cells treated with 50 μM desferrioxamine (+desferrioxamine). In the absence of desferrioxamine, the average mean fluorescence intensity in cells containing the 3′ deletion mutations was 36, as compared with 11 in cells containing constructions with an intact 3′ region. The average ratio (slope) of mean fluorescence intensity in desferrioxamine-treated versus untreated cells was 1.02 (r=0.99) for plasmids with the 3′ deletion and 2.5 (r=0.90) for those with an intact 3′ region.

Discussion

The role of transcription rates in regulating eukaryotic gene expression, and the mechanisms which control transcription initiation, have been extensively studied. However, very little is known about the regulatory events that occur subsequent to initiation of transcription, despite the fact that expression of many genes is regulated post-transcriptionally (Johnson *et al.*, 1975; Carneiro and Schibler, 1984; Groudine and Casimir, 1984; Leys *et al.*, 1984; Piechaczyk *et al.*, 1984). The findings presented here argue strongly that control of TR expression by iron belongs to the latter group of regulatory mechanisms. Whereas modulation of transcription rates generally requires the presence of specific elements within the promoter region (for review, see Dynan and Tjian, 1985), expression of the intact transferrin receptor cDNA is regulated by iron when transcription is controlled either by the SV-40 promoter or by the TR promoter with as little as 14 bp upstream from the TATA box. Specific regulation of transcription rates may also involve enhancer elements which can act even when they are located at sites distant from the promoter (Gillies *et al.*, 1983; Banerji *et al.*, 1983). However, we have found no

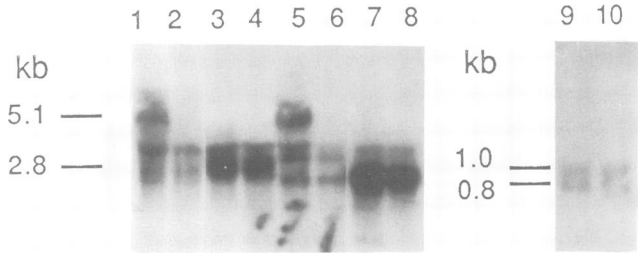

Fig. 6. Blot hybridization analysis of human transferrin receptor mRNA in transfected L cells. Total cytoplasmic RNA from cells grown 40 h in the presence (lanes 1,3,5,7) or absence (lanes 2,4,6,8) of 50 μM desferrioxamine was size-separated, transferred to nitrocellulose and hybridized to a ^{32}P-labelled probe specific for the transferrin receptor coding region. **Lanes 1,2**: cells transfected with cDNA constructions containing the SV-40 promoter and an intact 3′ region; **lanes 3,4**: constructions with the SV-40 promoter and the 2.3 kb 3′ deletion; **lanes 5,6**: constructions with 114 bp of TR promoter sequences and an intact 3′ region; **lanes 7,8**: constructions with 114 bp of TR promoter sequences and the 3′ deletion; **lanes 9,10**: the same RNA as in lanes 1,2, hybridized to a probe specific for mouse β_2-microglobulin.

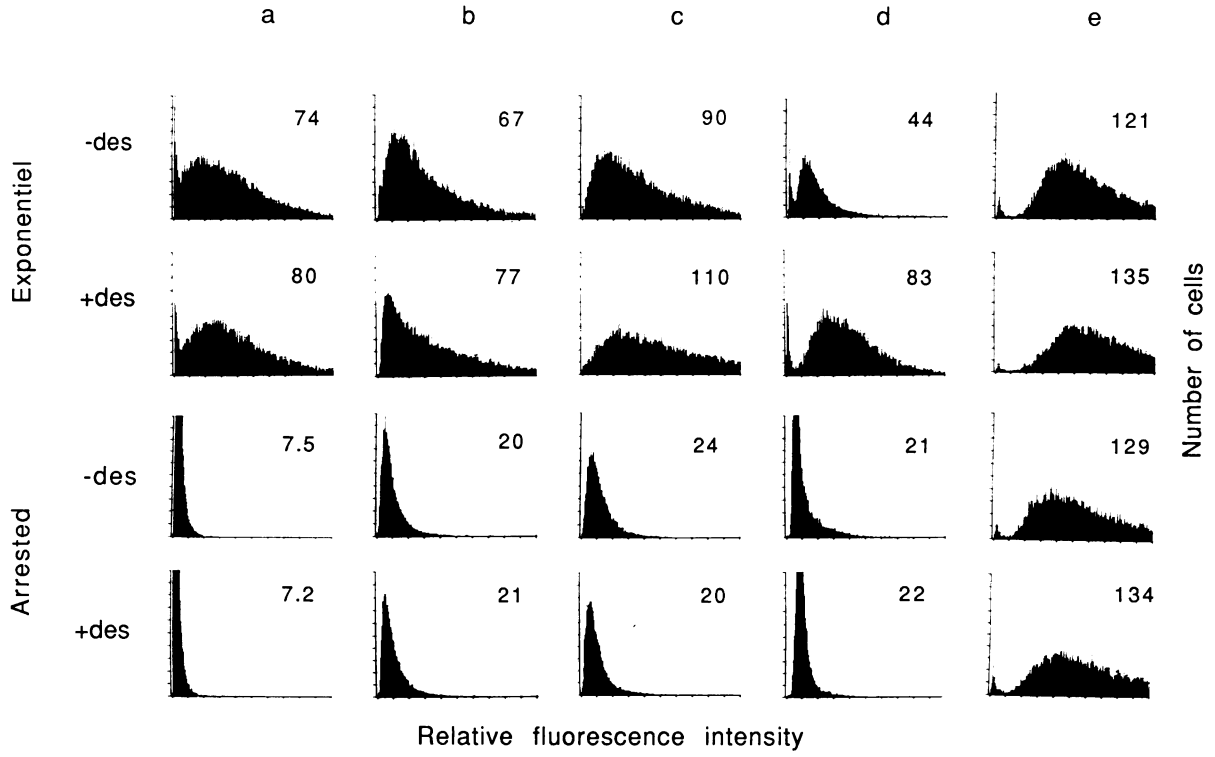

Fig. 7. Transferrin receptor levels in proliferating and arrested NIH/3T3 cells determined by FACS analysis of surface fluorescence. Cells were untreated (−des) or treated for 40 h with 50 μM desferrioxamine (+des). **Columns a-c**; human transferrin receptor expression in NIH/3T3 cells transfected with cDNA constructions containing the 2.3 kb 3′ deletion and 1500 bp, 322 bp or 114 bp of TR promoter sequences, respectively. **Column d**; expression of the endogenous mouse receptor in NIH/3T3 cells. **Column e**; expression of the mouse H-2 antigens in NIH/3T3 cells.

evidence that the transferrin receptor 3′ region exhibits the properties commonly associated with such elements. Inversion as well as deletion of 3′ sequences abolishes iron-dependent regulation, and re-insertion of the deleted sequences upstream from the promoter does not restore response to chelator. Deletion of an enhancer element should lead to a decrease in gene expression, yet whenever cDNA constructions with the critical 2.3 kb deletion were analysed we observed higher average levels of constitutive receptor expression in cells transfected either transiently (Kühn, 1985) or stably. Thus, in view of our results, postulating that iron controls transferrin receptor expression by modulating transcription rates would imply that 3′ noncoding sequences are involved in an iron-dependent repressor activity, and can function only in their appropriate location and orientation. Such a model appears unlikely, since it would also require that both the SV-40 promoter and the TR promoter respond to the repressor effect.

As an alternative to regulation of transcription rates, it is possible that regulation of nuclear or cytoplasmic events occurring subsequent to the initiation of transcription serves to modulate steady-state transferrin receptor mRNA concentrations. For ex- of the developmentally regulated μ/δ immunoglobulin transcription unit (Mather *et al.*, 1984) and of c-myc during the differentiation of HL-60 cells (Bentley and Groudine, 1986). We are currently investigating the possibility that termination of transferrin receptor RNA synthesis is controlled by noncoding 3′ sequences in an iron-dependent fashion. Such regulated termination could yield transcripts which are less stable, or are less efficiently transported from the nucleus, as a result of alterations in the structure of the RNA.

Our data would also be consistent with models in which interaction of specific 3′ sequences with an iron-dependent factor

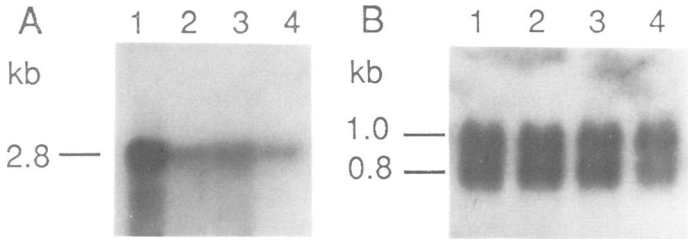

Fig. 8. Human transferrin receptor mRNA levels in arrested and exponentially growing cells. Blot analysis of total cytoplasmic RNA from NIH/3T3 cell clones transfected with cDNA constructions containing 1500 bp (lanes 1,2) or 114 bp (lanes 3,4) of TR promoter sequences and the 3′ deletion. **Lanes 1,3**: exponentially growing cells. **Lanes 2,4**: arrested cells. **A**; hybridization with a ^{32}P-labelled probe specific for the transferrin receptor coding region. **B**; hybridization with a probe specific for mouse β_2-microglobulin.

influences post-transcriptional events such as processing of precursor mRNA, transport, or mRNA degradation. Several recent reports have focused on the role of sequences within the 3′ terminal portion of RNA transcripts in regulating gene expression. For example, the post-transcriptional control of histone mRNA levels during the cell-cycle is controlled by 3′ terminal sequences (Lüscher *et al.*, 1985), probably by directing endonucleolytic cleavage of precursor mRNA (Gick *et al.*, 1986). Analogous sequences within the 3′ noncoding region of a polyadenylated mRNA might serve to generate, by internal cleavage, mRNA species with structural modifications that affect stability.

Multiple polyadenylation sites have been found in many viral and cellular genes, and have been implicated in the control of gene expression (for review, see Nevins, 1983). Kaufmann and

Sharp (1983) have presented data which suggest that growth-dependent variations in dihydrofolate reductase expression involve alternative polyadenylation sites within the 3′ noncoding region. This region of the transferrin receptor mRNA contains two repeats of the AAUAAA hexamer, associated with the recognition site for cleavage and polyadenylation (Proudfoot and Brownlee, 1976), about 1.5 kb upstream from the end of the cDNA (Schneider *et al.*, 1984). In view of the fact that low mol. wt TR transcripts have sometimes been detected (see Figure 6 and Stearne *et al.*, 1985; Rao *et al.*, 1986), the effect of intracellular iron levels on TR mRNA size and stability deserves investigation. The failure of the TR 3′ region to confer iron-dependent regulation to the HLA-A2 gene could be due to inherent differences in the primary or secondary structure of the mRNAs coding for TR and HLA-A2.

The ability of mouse cells to regulate expression of the human cDNA, and the fact that the mouse TR gene also contains a large 3′ noncoding region, imply that the mechanism by which 3′ sequences mediate the response to intracellular iron is conserved in both species.

Our results on the regulated expression of human transferrin receptor cDNA constructions in mouse fibroblasts differ from evidence, reported for K562 cells, that intracellular iron levels influence the transcription rate of the endogenous human TR gene (Rao *et al.*, 1986). However, specialized mechanisms may regulate iron metabolism in these erythroleukemia cells, which are induced to differentiate and to synthesize large amounts of hemoglobin in response to treatment with hemin (Rutherford *et al.*, 1979).

Consistent with the model proposed by Pelosi *et al.* (1986), in which control of transferrin receptor expression operates at more than one level, our results show that at least one major component of the growth-dependent regulation of TR expression involves a mechanism different from the one responsible for iron-dependent regulation. The existence of multiple regulatory mechanisms creates an opportunity for the fine tuning of transferrin receptor expression necessary to assure intracellular iron homeostasis.

Materials and methods

Plasmid constructions and DNA sequencing

Plasmid constructions were made by modifying the full-length human transferrin receptor cDNA clone, pcD-TR1 (Kühn *et al.*, 1984). A unique *Eco*RV site within exon 1 was used for the insertion of a genomic 3.2 kb *Eco*RV fragment from λTR15, which encompasses the TR promoter region and the 5′ end of exon 1 (McClelland *et al.*, 1984). In subsequent cloning steps the SV-40 promoter and various length fragments of the TR promoter were removed from the 5′ end either by restriction enzymes or *Bal*31. 5′ deletions were mapped by dideoxy sequencing (Sanger *et al.*, 1977) of fragments subcloned in M13 cloning vectors. The deletion within the 3′ noncoding region was made by excising the region between the *Xba*I sites.

In a series of cloning steps, a pcD-TR1 fragment beginning at the *Hin*dIII site upstream from the SV-40 promoter and ending at the unique *Bgl*II site within the 3′ noncoding region was replaced by a genomic HLA-A2 fragment starting at a *Hin*dIII site 526 bp upstream from exon 1 and ending at a *Pvu*II site 5 bp downstream from the stop codon (positions 1−3445) (Koller and Orr, 1985). This HLA-A2 fragment was also inserted in the place of a pcD-TR1 fragment beginning at the 5′ *Hin*dIII site and ending at the unique *Eco*RV site within the 5′ noncoding region.

Cell culture

Thymidine kinase deficient L-cells (Ltk^-) were cultured in alpha minimal essential medium supplemented with 10% fetal calf serum. GM637 cells and NIH/3T3 cells were cultured in Dulbecco's modified Eagle medium with 10% fetal calf serum. Where indicated, cells were treated for 40 h with 50 μM desferrioxamine (Desferal, gift of Ciba-Geigy), 10 μg/ml ferric ammonium citrate, 250 μM hemin or 2 μM picolinic acid. Growth arrest of NIH/3T3 cells was achieved by culturing confluent cells for 96 h in 0.4% fetal calf serum.

Transfection and cell sorting

Ltk^- cells (2×10^5/25 cm^2) were co-transfected by calcium phosphate precipitation (Graham and van der Eb, 1973) with 100 ng of the HSV-tk gene (Enquist *et al.*, 1979), 2 μg of the HLA-A2 gene (Koller and Orr, 1985), 2−3 μg of the transferrin receptor cDNA constructions (equimolar amounts of each) and 15 μg of carrier DNA. Selection by medium containing 100 μM hypoxanthine, 0.4 μM aminopterin and 16 μM thymidine (HAT) was applied 48 h later. NIH/3T3 cells were co-transfected in the same way with the transferrin receptor cDNA constructions and 100 ng of plasmid pY3 coding for hygromycin B resistance (Blochlinger and Diggelmann, 1984). Cells were selected with medium containing 200 μg/ml hygromycin B (Lilly).

The monoclonal antibody 5E9 against the human transferrin receptor (Haynes *et al.*, 1981) was used to isolate single-cell clones from total HAT-resistant L cell populations or total hygromycin-resistant NIH/3T3 populations by fluorescence-activated cell sorting followed by limiting-dilution cloning as previously described (Kühn *et al.*, 1983).

Surface fluorescence analysis

Analysis of cell-surface fluorescence staining was performed by a FACS II (Becton Dickinson) as previously described (Kühn *et al.*, 1983) using the following monoclonal antibodies: 5E9 against the human transferrin receptor (Haynes *et al.*, 1981), B9.12.1 against HLA-A2 (Lemonnier *et al.*, 1982), RI7.208 against the mouse transferrin receptor (Trowbridge *et al.*, 1982) or 28-14-8S against mouse H-2 antigens (Ozato and Sachs, 1981). Fluoresceinated second antibodies were either goat anti-mouse Ig (Cappel) or goat anti-rat Ig (Nordic).

RNA isolation and analysis

Total cytoplasmic RNA was isolated according to Favaloro *et al.* (1980) except that macaloid was omitted from the lysis buffer. 25 μg samples were glyoxalated (McMaster and Carmichael, 1977), separated by electrophoresis in 1.2% agarose and transferred to nitrocellulose (Thomas, 1980). Prior to glyoxalation, aliquots were run on a non-denaturing gel and stained with ethidium bromide to assure that the total amount of RNA was the same in each sample. The *Eco*RV−*Xba*I fragment of 2.5 kb containing the entire coding region from pcD-TR1, or the 294 bp *Hha*I−*Eco*RI fragment (positions 23 to 317) of the mouse β_2-microglobulin cDNA clone pβ2-m2 (gift of P.Kourilsky) (Daniel *et al.*, 1983), were labelled with [α-^{32}P]dATP by nick translation, hybridized to filters, washed, and autoradiographed as previously described (Kühn *et al.*, 1984). The stringent wash conditions (0.1 × SSC, 0.1% SDS, 65°C) eliminated cross-hybridization of the human transferrin receptor probe with enodgenous mouse sequences.

End-labelling of the 1.6 kb *Hin*dIII fragment from pcD-TR1, hybridization and digestion with S_1 nuclease was done according to published procedures (Berk and Sharp, 1977; McKnight, 1980). The 25 μl hybridization solution, containing 0.5×10^5 c.p.m. of end-labelled probe and 50 μg total cytoplasmic RNA, was melted at 80°C for 10 min, hybridized for 12 h at 52°C and digested at 30°C for 30 min with 1 U/μl S_1 nuclease in 300 μl. S_1-resistant hybrids were glyoxalated, electrophoresed on 1.6% agarose gels, dried and autoradiographed.

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