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Iron regulation of transferrin receptor mRNA levels requires iron-responsive elements and a rapid turnover determinant in the 3′ untranslated region of the mRNA

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Post-transcriptional regulation of transferrin receptor mRNA levels by iron is mediated by a portion of the 3′ untranslated region (UTR) of the mRNA. We have previously shown that a 678 nucleotide fragment of the 3′UTR contains the regulatory element(s). Within this region are five RNA structures which resemble the iron-responsive element (IRE) in the 5′ untranslated region of the ferritin mRNA which is regulated translationally by iron. The IREs from the ferritin and transferrin receptor mRNAs compete in an *in vitro* assay for interaction with a cytoplasmic protein; the activity of this IRE-binding protein is dependent upon the iron status of the cells. Based on further deletion analysis reported here, the sequences required for iron regulation of the transferrin receptor have been limited to 250 nucleotides which we have produced synthetically and cloned. This sequence, which contains three IREs, is capable of producing iron-dependent regulation of transferrin receptor levels. Removal of the three IREs from the synthetic element results in loss of iron regulation. Moreover, deletion of a single cytosine residue from each of the three IREs in the synthetic regulatory element eliminates high-affinity binding to the IRE-binding protein *in vitro* and results in low levels of iron-independent transferrin receptor expression, consistent with production of a constitutively unstable mRNA. These data indicate that the ability of the mRNA to interact with the IRE-binding protein is required for regulation of transferrin receptor mRNA levels by iron. Certain other deletions within the regulatory region which do not affect the *in vitro* interaction between the transferrin receptor RNA and the IRE binding protein result in relatively high levels of iron-independent transferrin receptor expression, consistent with production of a constitutively stable mRNA. Collectively, our data support a model for transferrin receptor regulation in which the interaction between the IRE-binding protein and the transferrin receptor mRNA can protect the transcript from rapid degradation that is mediated by a rapid turnover determinant within the regulatory region.

Key words: iron-responsive element/mRNA stability/transferrin receptor/untranslated region

Introduction

Iron is an essential but potentially toxic nutrient for mammalian cells. Cellular uptake and storage of iron is therefore carefully controlled. The rate of internalization of iron by cells is determined by the level of cell-surface expression of the transferrin receptor (TfR), which brings iron into the cell via endocytosis of diferric transferrin (Dautry-Varsat *et al.*, 1983; Klausner *et al.*, 1983). The level of available iron within the cell in turn regulates the level of TfR biosynthesis; TfR biosynthesis is increased when available cellular iron levels are low and decreased when iron levels are high (Pellicci *et al.*, 1982; Ward *et al.*, 1982; Mattia *et al.*, 1984). This regulation is accomplished by altering the amount of TfR mRNA (Rao *et al.*, 1986). We (Casey *et al.*, 1988a,b) and others (Owen and Kühn, 1987; Müllner and Kühn, 1988) have previously mapped the region of the TfR gene required for post-transcriptional iron regulation to sequences within the 3′ untranslated region (UTR) of the mRNA. Our analysis of a 678 nucleotide (nt) region encompassing the regulatory sequences showed that it contains five potential RNA structures which closely resemble the iron responsive element (IRE) found in the 5′UTR of the ferritin mRNA (Casey *et al.*, 1988b). The IRE in the ferritin mRNA 5′UTR has been shown to be necessary and sufficient for the full range of iron-dependent regulation of translation of ferritin (Hentze *et al.*, 1987; Caughman *et al.*, 1989). This regulation of ferritin biosynthesis is opposite in direction to that of the TfR. Thus, when iron is scarce more TfR and less ferritin are made, whereas when iron is in excess, less TfR and more ferritin are produced. In this way cellular iron homeostasis is maintained.

An RNase protection and electrophoretic mobility shift assay has recently been used to demonstrate that a cytosolic protein binds to the ferritin IRE (Leibold and Munro, 1988) and that the binding activity in cell lysates depends upon the iron status of the cells (Rouault *et al.*, 1988). The 3′UTR of the TfR RNA has been shown to compete with the ferritin IRE for the IRE binding protein (IRE-BP) in this assay (Koeller *et al.*, 1989). Moreover, it has been shown by direct binding studies that two of the five IREs within the 678 nt regulatory region of the TfR mRNA are bound by the IRE-BP *in vitro* with high affinity. As isolated entities, the other three IREs also interact with the IRE-BP, but with somewhat lower apparent affinities (Koeller *et al.*, 1989).

We have previously suggested that the TfR mRNA is protected against rapid degradation by the binding of the IRE-BP to IREs in the TfR mRNA 3′UTR (Casey *et al.*, 1988b). In this report, we show that a cloned 250 nt synthetically made sequence which contains three IREs is capable of producing iron-dependent regulation of TfR levels. Single base deletions within each of the three IREs in the regulatory element eliminate interaction with the IRE-BP and result in low, unregulated levels of TfR expression, consistent with

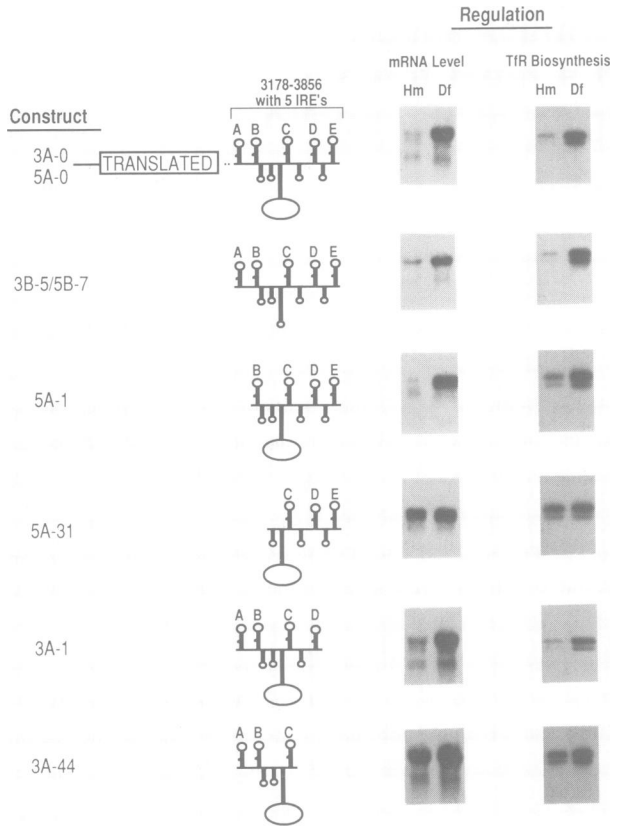

Fig. 1. Deletion analysis of the 678 nt regulatory region of the TfR mRNA. Plasmids from which various segments of the regulatory region were removed were stably transfected into mouse tk^- fibroblasts. Regulation of TfR by iron was assessed by treating cells with 6 mM sodium butyrate and either 100 μM hemin (Hm) or 100 μM desferrioxamine (Df) overnight. For mRNA analysis cytoplasmic RNA was prepared at the end of the treatment period; 5 μg was separated on an agarose–formaldehyde gel, transferred to nylon membranes and hybridized with a ^{32}P-labeled human TfR cDNA probe. For TfR biosynthesis, cells were labeled for 2 h with [^{35}S]methionine in methionine-free media; after labeling, cells were lysed and human TfR was immunoprecipitated from equal TCA-precipitable amounts of lysates with the monoclonal antibody B3-25 (Boehringer-Mannheim) and Protein A coupled to agarose (Bethesda Research Laboratories). Immunoprecipitated TfR was separated on an SDS–polyacrylamide gel. The schematic diagrams refer to the proposed structure of the 678 nt iron regulatory region within the TfR 3′UTR (Koeller *et al.*, 1989). The translated region, though not shown, is present in all constructs. The five IREs are labeled A–E (Casey *et al.*, 1988b; Koeller *et al.*, 1989). Deleted segments have been omitted from the schematic. 3A-0 and 5A-0 are constructs that contain the full 678 nt regulatory region. The TfR sequences deleted from the other plasmid constructs are as follows: 3B-5/5B-7, 3390–3606; 5A-1, 2404–3280; 5A-31, 2404–3327; 3A-1, 3792–end; 3A-44, 3732–end.

rapid mRNA degradation. Regulation-disrupting deletions which do not affect IRE-BP binding, or which remove the IRE structures entirely, produce high levels of expression, consistent with a more stable mRNA.

Results

Our laboratory has proposed a structure for the 678 nt iron-regulatory region of the TfR mRNA which includes five IRE-like structures as well as additional structural elements (Casey *et al.*, 1988b; Koeller *et al.*, 1989). The structure of this region is shown schematically in Figure 1 (in constructs 3A-0 and 5A-0). Briefly, the structure consists of two IRE-containing regions brought together by a relatively long central base-paired region. The more 5′ region contains two IREs (designated A and B) whereas the more 3′ region contains three IREs (designated C–E). Additional non-IRE stem-loop structures are also contained in both regions. Both regions are very similar in sequence to corresponding regions found within the chicken TfR mRNA (Chan *et al.*, 1989; Koeller *et al.*, 1989). In contrast, the regions of the chicken and human TfR 3′UTRs between the two halves of the central base-paired region and those outside the 678 nt fragment have negligible sequence similarity.

We had previously noted that two non-overlapping restriction fragment deletions within the 678 nt region each eliminated regulation (Casey *et al.*, 1988b). To define more narrowly the sequences required for iron regulation of the human TfR mRNA, portions of the 678 nt regulatory region of the TfR cDNA were removed by progressive *Bal*31 exonuclease digestion from either the 5′ or 3′ end. Plasmid constructs resulting from these deletions were transfected into mouse thymidine kinase negative (tk^-) fibroblasts along with the herpes simplex virus tk gene to obtain cells stably expressing the human TfR constructs. Certain of these TfR deletion constructs are shown schematically in Figure 1, along with the observed regulation by iron of TfR mRNA levels and the corresponding biosynthesis of the TfR protein. There was good correlation in all constructs between mRNA level and TfR biosynthetic rate. Removal of 215 nt between the two halves of the central base-paired region had little effect on the degree of iron regulation (construct 3B-5/5B-7, Figure 1). The region deleted in 3B-5/5B-7 has virtually no sequence similarity with the chicken TfR mRNA, whereas the two remaining segments of the regulatory region are very similar to the chicken transcript in sequence and predicted structure (Chan *et al.*, 1989; Koeller *et al.*, 1989).

Removal of IRE A and sequences 5′ of it (Construct 5A-1) also had virtually no effect on regulation. However, further *Bal*31 deletion through IRE B and 8 nt 3′ of it (construct 5A-31) abolished regulation even though three IREs remain. In the 3′ portion of the regulatory region, the removal of IRE E and sequences 3′ of it did not significantly affect the degree of iron regulation (construct 3A-1). However, further deletion of sequences 5′ of IRE E (construct 3A-44) destroyed iron regulation. Deletion constructs 5A-31 and 3A-44 each retain three of the five IREs present in the full 678 nt regulatory region, yet did not produce mRNAs which were regulated by iron. These data suggest that iron regulation requires a particular combination of IREs or the combination of one or more IRE and non-IRE sequences.

Having shown that those sequences 5′ of IRE B, 3′ of IRE D, and between the two halves of the central base-paired region were dispensible in terms of iron regulation, we synthesized DNA oligonucleotides corresponding to the rest of the regulatory region of the human TfR (see Materials and methods). The resultant synthetic element includes IREs B, C and D (see Figure 2). As assessed by computer-based predictions of RNA secondary structure (Züker and Steigler, 1981), the RNA from the synthetic element would be expected to fold into a structure containing three characteristic IRE stem-loops.

The synthetic element was cloned into a TfR cDNA plasmid 45 nt 3′ of the translation stop codon to give the construct designated TRS-1. We observed iron regulation

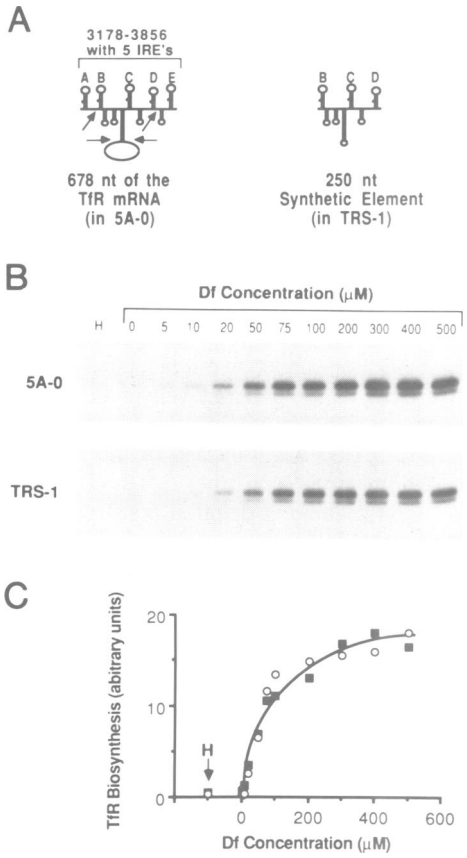

Fig. 2. Construction and iron regulation of a synthetic regulatory element. (**A**) Schematic representation of the synthetic regulatory element. The arrows indicate the endpoints of segments of the 678 nt regulatory region which are included in the synthetic regulatory element. (**B**) Comparison of the iron regulation conferred by the synthetic regulatory element in construct TRS-1 and the 678 nucleotide regulatory region in construct 5A-0. Mouse fibroblasts stably transfected with either 5A-0 or TRS-1 were treated overnight with 6 mM sodium butyrate and either desferrioxamine at the indicated concentrations, or 50 μM hemin (H). TfR biosynthesis was assessed as in Figure 1. (**C**) Quantitation by densitometric scanning of autoradiograph in (B) (■) 5A-0, (○) TRS-1.

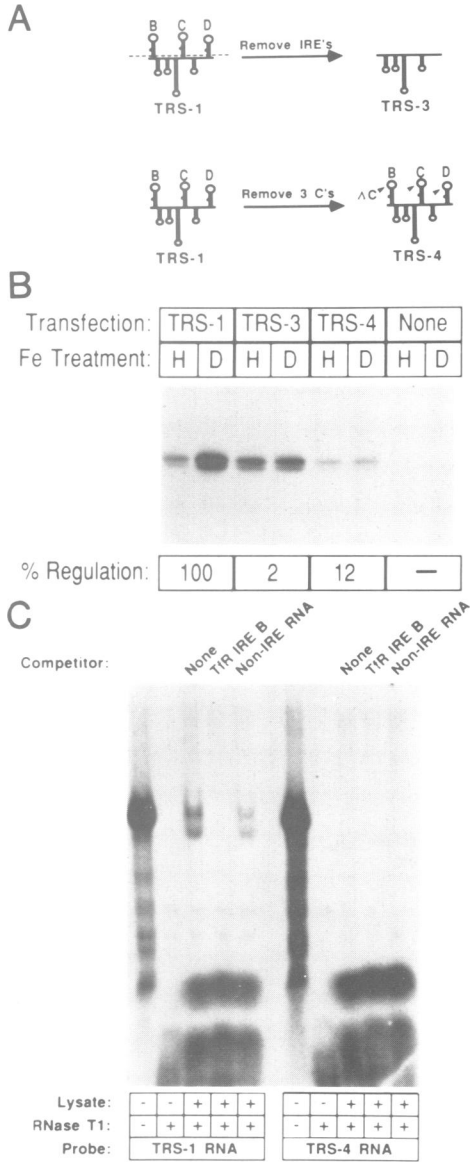

Fig. 3. Effects of deletion of the IREs on regulation and band-shift activity. (**A**) Schematic representation of synthetic regulatory elements containing deletions of IRE sequences. All IRE sequences are absent in TRS-3; in TRS-4 ΔC indicates the absence of the C residue from the IRE loop consensus CAGUGX in each of the three IREs. (**B**) Deletion of the IRE sequences abolishes iron regulation of TfR biosynthesis. Mouse fibroblasts were transfected with plasmids TRS-1, TRS-3 or TRS-4 which contain the TfR coding region and synthetic regulatory elements, or a plasmid which contains no TfR sequences. The calcium phosphate precipitates consisted of 40 μg of TfR plasmid and 4 μg of pSV_2CAT (Gorman *et al.*, 1982); each precipitate was divided equally between two plates of cells, one of which subsequently received hemin, the other desferrioxamine. After 16 h of treatment with the DNA-calcium phosphate precipitate, cells were washed and given fresh media containing 6 mM sodium butyrate and either 100 μM hemin (H) or desferrioxamine (D). After an additional 24 h, TfR biosynthesis was assessed as in Figure 1. The calculation of percentage regulation compares the D:H value of a plasmid with that of TRS-1 and defines D:H = 1 for a totally unregulated construct. (**C**) Absence of three nucleotides in the synthetic regulatory element of TRS-4 abolishes band shift activity. The synthetic regulatory region sequences from TRS-1 and TRS-4 were cloned into the RNA transcription vector pGEM3Z. *In vitro* RNA transcripts (0.2 ng) labeled with [^{32}P]GTP (150 Ci/mmol) were incubated with 20 μg of mouse fibroblast cell lysate and 1 μg of unlabeled specific or non-specific competitors, as indicated. The specific competitor was a 35 nt RNA identical to TfR IRE B; the non-specific competitor was the 5′ end of the β-globin mRNA. In the lanes with no lysate or RNase T1, 0.002 ng of probe was added.

of TfR protein biosynthetic levels and mRNA levels produced by TRS-1 in both transiently and stably transfected mouse fibroblasts. When cells stably transfected with TRS-1 were compared to cells transfected with a similar construct containing the full 678 nt regulatory region (5A-0), we found that both the degree of regulation, and the sensitivity to the iron chelator desferrioxamine were indistinguishable for the two constructs (Figure 2). Thus, all sequences necessary for iron regulation of TfR RNA levels are contained within the 250 nt of the synthetic regulatory element.

The synthetic regulatory element contains three IRE structures. We assessed the effects of removing IRE function by either deleting the entire sequences for all three IREs (Figure 3, construct TRS-3) or by deleting from each of the three IREs the cytosine residue that is the most 5′ base of the loop (Figure 3, construct TRS-4). The removal of this base of the CAGUGN consensus IRE loop has been shown to disrupt the ability of an isolated ferritin IRE to bind the IRE-binding protein with high affinity *in vitro* (Rouault *et al.*, 1988). Furthermore, in the context of a 5′UTR this 'ΔC' deletion abolishes the ability to function as a translational IRE (Hentze *et al.*, 1987). Here we observed

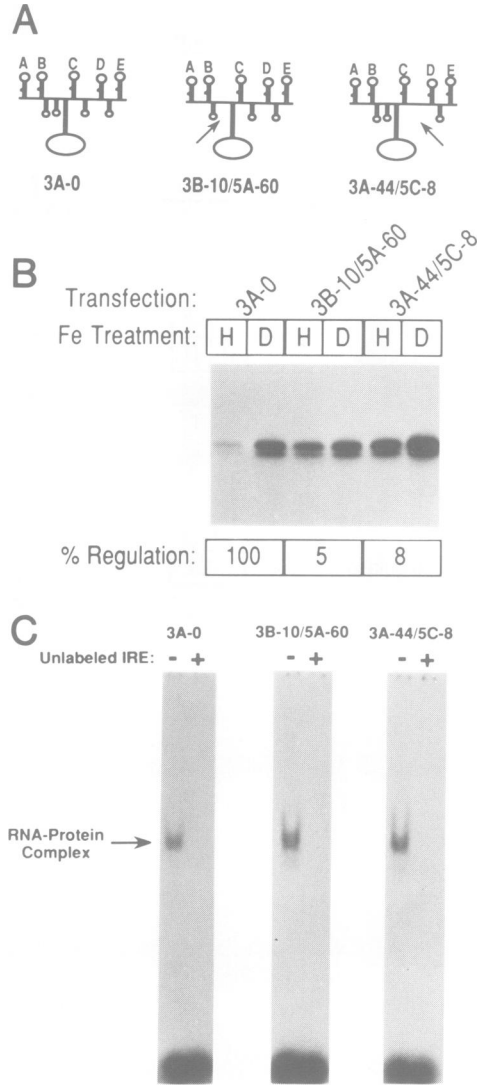

Fig. 4. Deletions not directly affecting the sequences of the TfR IREs. (**A**) Schematic representation of the non-IRE deletion constructs 3B-10/5A-60 and 3A-44/5C-8 compared with the 678 nt regulatory region. Arrows indicate the deleted segments. (**B**) Iron regulation of TfR in non-IRE deletion constructs. Plasmids containing deletions of segments of the regulatory region other than IREs were transfected into mouse fibroblasts and analyzed for iron regulation of human TfR biosynthesis as in Figure 3. The *Xho*I linker sequence CCTCGAGG replaced nucleotides 3359–3382 in construct 3B-10/5A-60 and nucleotides 3732–3748 in construct 3A-44/5C-8. (**C**) Band-shift of RNAs derived from non-IRE deletions. The regulatory region from 3A-0 and the corresponding region from the deletion constructs 3B-10/5A-60 and 3A-44/5C-8 were cloned into the *in vitro* transcription vector pGEM3Z. RNAs produced *in vitro* were assessed for band-shift activity as in Figure 4. Plus sign (+) indicates the addition of the unlabeled competitor IRE as described in Figure 3.

that the removal of this nucleotide from the loop of all three IREs in the synthetic regulatory element destroyed the interaction between the IRE-binding protein and the TfR regulatory element (Figure 3C). The effects of the IRE deletions on iron regulation were assessed by transfecting mouse fibroblasts with the corresponding plasmid constructs and immunoprecipitating [^{35}S]methionine-labeled human TfR produced by these cells after treatment with hemin or desferrioxamine (Figure 3B). As shown above in Figure 2, the biosynthetic rate of the receptor produced by TRS-1 transfection was regulated by iron. However, no regulation was observed for construct TRS-3, from which all IRE sequences had been removed, nor for construct TRS-4, in which none of the mutated IRE structures is capable of functioning as an IRE. The failure to observe iron regulation of TfR biosynthetic rates in cells transfected with either TRS-3 or TRS-4 demonstrates clearly that IREs capable of interacting with the IRE-BP are necessary for iron regulation of TfR mRNA levels.

In the course of our more detailed deletion analysis of the TfR regulatory region, we have found that, in addition to the deletions of IREs, removal of other sequences can diminish or abolish the iron regulation of the TfR mRNA. Two of these deletions are shown schematically in Figure 4, along with the regulation of TfR levels produced from these constructs after a transient transfection. In this experiment, deletion 3B-10/5A-60 has only 5% of the iron regulation of its regulating parent construct 3A-0. This deletion is similar to a non-regulating deletion construct obtained by other workers (Müllner and Kühn, 1988). Deletion 3A-44/5C-8 yields 8% of the regulation of 3A-0. It should be noted that both of these deletions were assessed in the context of the full 678 nt regulatory region containing five IREs. Both of these apparently essential non-IRE sequence elements are contained in the synthetic regulatory element of TRS-1 discussed above. Deletions 3B-10/5A-60 and 3A-44/5C-8 do not affect the interaction between the 678 nt region and the IRE-binding protein (Figure 4C). RNAs produced *in vitro* from constructs having these deletions gave band shifts identical to that given by an RNA derived from a regulating construct. This finding indicates that these deletions do not eliminate iron regulation by virtue of an indirect effect on the ability to form recognizable IREs. Furthermore, RNase T1 fingerprinting of the RNA fragments protected and shifted in the assay (Koeller *et al.*, 1989) were identical for both deletion-derived RNAs and regulating RNA's (data not shown).

Evidence has been presented indicating that the major means of iron regulation of TfR mRNA levels is regulation of TfR mRNA stability in the cytoplasm (Müllner and Kühn, 1988). In our experiments, it appeared that all of the deletions that abolished iron regulation, with the exception of TRS-4, resulted in high levels of TfR expression. In contrast, TRS-4 yielded very low levels of expression. This observation is most likely due to differences in the half-lives of the TfR transcripts. Construct TRS-4 has the same promoter as TRS-1 and differs in only three bases of its nearly 3 kb unspliced transcript. Thus, one would expect the rates of transcription, processing and transport of two mRNAs to be the same. To quantitate levels of expression of different plasmids, constructs were co-transfected with the plasmid pSV_2CAT (Gorman *et al.*, 1982) into mouse fibroblasts and transient TfR and CAT expression assessed at 48 h after transfection. Variations in transfection efficiencies (usually <2-fold) were corrected by normalizing TfR biosynthetic rates to CAT enzyme activities. The only deletion construct which resulted in low, unregulated levels of expression was the construct TRS-4, which contains three mutated IRE structures which do not function as IREs because of the 'ΔC' deletions. The level of TfR expression produced by TRS-4 was even lower than that produced by TRS-1 in transiently transfected cells treated with hemin (Figure 5). The low level of expression in TRS-4 transfected cells remained low even after desferrioxamine treatment (see Figure 3). The other

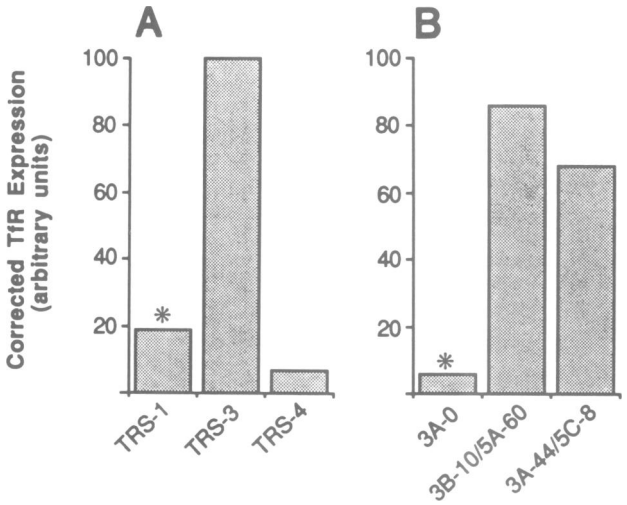

Fig. 5. Relative level of TfR expression from regulating and non-regulating plasmid constructs in transiently transfected mouse fibroblasts treated with hemin. Densitometric scanning of TfR expression as assessed in Figures 3 and 4 was normalized to CAT enzyme activity from expression of the co-transfected pSV_2CAT plasmid. CAT assays were performed as described (Casey *et al.*, 1988a); percentage conversion of chloramphenicol to its acetylated derivatives was well within the linear range (10−20%). The asterisk (*) indicates those constructs (TRS-1 and 3A-0) whose level of TfR expression was iron-responsive.

three non-regulating deletion constructs, including the IRE deletion construct TRS-3, gave high, unregulated levels of TfR expression (Figure 5). The high level of TfR expression produced by these three non-regulating constructs was essentially the same as that seen with the regulating constructs 3A-0 and TRS-1 after treatment with the iron chelator desferrioxamine (data not shown). Collectively, these data are consistent with the production of a constitutively unstable mRNA by the low expressing, unregulated construct TRS-4, and production of a constitutively stable mRNA by the high expressing, unregulated constructs TRS-3, 3B-10/5A-60 and 3A-44/5C-8.

Discussion

When it is considered that the stabilities of different mRNAs in vertebrate cells vary over two to three orders of magnitude, it is evident that mRNA stability plays an important role in the control of gene expression. The half-life of an mRNA not only determines the abundance of the mRNA, but also determines the rate of change in mRNA level in response to stimuli which affect transcription rates (Rodgers *et al.*, 1985). Interest in mRNA stability has increased as a number of genes have been described for which the stability of the mRNA is regulated or tightly controlled (Hunt, 1988; Brawerman, 1989). These systems include tubulin (Yen *et al.*, 1988), vitellogenin (Brock and Sharpiro, 1983), apolipoprotein II (Gordon *et al.*, 1988), a number of lymphokines, cytokines and proto-oncogenes which contain an A-U rich sequence (Caput *et al.*, 1986; Shaw and Kamen, 1986) as well as the transferrin receptor. In most cases which have been described, the 3′UTR of the mRNA contains the *cis*-acting sequences required for control of half-life. Still, the specific sequences and structures responsible for control of mRNA turnover have not been precisely defined and very little is known about cellular *trans*-acting factors which stabilize or destabilize these or any other mRNAs. As opposed to DNA regulatory motifs, in which the sequence of a double-stranded element is the primary determinant of recognition, it is likely that both primary sequence and RNA structure will be important recognition factors in regulatory RNA elements.

Evidence has been presented indicating that iron regulation of TfR mRNA levels occurs by altering the stability of the TfR mRNA in the cytoplasm (Müllner and Kühn, 1988). This regulation is mediated by sequences within a 678 nt region in the 3′UTR of the receptor mRNA (Owen and Kühn, 1987; Casey *et al.*, 1988; Müllner and Kühn, 1988). Here we have shown that 250 nt of this region are sufficient for iron regulation. The TfR mRNA contains five IREs, but no more than three are required since the synthetic 250 nt element (TRS-1) regulates fully but contains just three IREs. IREs are defined as mRNA stem−loop structures which bind a cytosolic protein in an iron-dependent manner such that the binding activity is highest at low intracellular iron concentrations (Rouault *et al.*, 1988; Hentze *et al.*, 1989). A single IRE is present in the 5′UTR of the ferritin mRNA where it mediates iron-dependent regulation of ferritin translation (Aziz and Munro, 1988; Hentze *et al.*, 1988). It is believed that, at low iron concentrations, the high-affinity interaction between the IRE-BP and the IRE in the 5′UTR represses translation of the ferritin mRNA. It is our model that a similar iron-dependent interaction between the IRE-BP and IREs in the TfR mRNA 3′UTR is responsible for iron regulation of TfR mRNA stability (Casey *et al.*, 1988b; Hentze *et al.*, 1988). Specifically, at low iron levels, the high-affinity interaction between the IRE-BP and the TfR mRNA IREs would stabilize the mRNA against the action of a specific nuclease; this nuclease would recognize specific sequences/structures in the regulatory region which would constitute a rapid turnover determinant. At high iron concentrations, the lesser interaction between the IRE and IRE-BP would not be sufficient to protect the mRNA from the action of the nuclease.

To address this model, we abolished IRE function in construct TRS-4 by removing the first cytosine residue in the six-member loop of each IRE in a synthetic regulatory element. A 'ΔC' deletion in the context of a ferritin IRE has been shown previously to destroy the high-affinity interaction between the IRE-BP and the RNA (Rouault *et al.*, 1988). In construct TRS-4, the loss of IRE-BP interaction led to the loss of iron regulation of receptor mRNA levels. With only 3 nt critical to IRE function deleted from the synthetic regulatory region, TRS-4 clearly demonstrates the neccessity of functional IREs for TfR regulation. In transient transfection assays corrected for transfection efficiency, we found that TRS-4 produced low TfR levels, consistent with the production of a constitutively unstable mRNA. Thus the 'ΔC' deletion in TRS-4 abolishes interaction with the IRE-BP, such that even at low iron concentrations the mRNA cannot be protected against the action of the putative ribonuclease.

In contrast to TRS-4, the construct TRS-3 produced constitutively high TfR levels, consistent with the production of a more stable mRNA. As expected, the complete deletion of the three IREs of TRS-1 (resulting in construct TRS-3) abolished iron regulation. Based on the relatively high levels of TfR expression in the TRS-3 transfectants, it appeared that this deletion also eliminated the ability to

form the rapid turnover determinant. It should be noted that, whereas TRS-4 is altered in only 3 of 250 nt, TRS-3 is missing 87 of the 250 nt found in TRS-1. The IRE sequences missing from TRS-3 apparently perform two functions: one is to form IRE stem−loop structures which interact with the IRE-BP when iron is scarce; the other is to participate, in an as yet unknown manner, in the formation of the rapid turnover determinant. RNA from TRS-4 can still perform the latter of these functions but RNA from TRS-3 can neither bind the IRE-BP nor participate in formation of the rapid turnover determinant. The construct TRS-4 thus provides evidence that the TfR mRNA features responsible for IRE-BP interaction and those required for rapid turnover of the TfR transcript can be functionally separated. The constructs 3B-10/5A-60 and 3A-44/5C-8 also address the separation of these functions. In these two constructs, ability to interact with the IRE-BP is retained but the ability to form an effective rapid turnover determinant has been removed. The construct TRS-3 indicates that there is overlap between these functions since both are lost in this construct.

The binding of the IRE-BP to the TfR mRNA might block a specific nuclease attack by direct steric hindrance near the nuclease recognition site or by favoring a secondary structure for the mRNA which is not recognized by the nuclease. The latter possibility is intriguing given that an alternative secondary structure has been proposed for the regulatory region (Müllner and Kühn, 1988) which is very close in predicted energy to the one containing IRE stem−loops which we have suggested (Casey *et al.*, 1988; Koeller *et al.*, 1989). In the structure proposed by Müllner and Kühn (1988), the sequences of the IREs are not in the characteristic IRE stem−loops but are engaged in other base pairing that stabilizes the alternative structure. Might this alternative structure constitute the nuclease target? In this scenario, the binding of the IRE-BP to the IREs would favor the IRE-containing structure in the equilibrium between the two alternative structures and thus diminish the probability of the mRNA being in the target form. The construct TRS-3, which contains no IRE sequences, could form neither structure and, owing to its inability to form the putative nuclease target, would yield a relatively stable unregulated mRNA. In contrast, TRS-4 contains only single base deletions in each of the IREs and would be able to form the hypothetical nuclease target structure but be unable to be protected from the nuclease through interaction with the IRE-BP. The prediction of this combination would be that TRS-4 would be incapable of iron regulation but would produce a constitutively unstable mRNA. This is what we find for TRS-4.

All of our findings are consistent with our working model for the coordinate regulation of the biosyntheses of TfR and ferritin. The single IRE in the ferritin mRNA 5′UTR and one or more of the five IREs in the TfR mRNA 3′UTR interact with a common protein (Koeller *et al.*, 1989). This interaction is favored when iron is scarce (Hentze *et al.*, 1988; Rouault *et al.*, 1988). The effect of interaction of the IRE-BP with the ferritin mRNA is to repress ferritin translation. The effect of a similar interaction with the TfR mRNA is to repress transcript degradation. In this study, we have begun to dissect the relationship between the iron regulatory elements and other sequence/structure elements that result in the short half-life of TfR mRNA. It is the interplay between these two types of functional elements that will explain the iron regulation of mRNA stability.

Materials and methods

Construction of 5′ and 3′ deletion mutants

A derivative of the transferrin receptor minigene plasmid TRmg-2 (Casey *et al.*, 1988a) was used as starting material for all 5′ and 3′ deletions. This derivative, TRmg-2(Nhe), contains an *Nhe*I restriction site in place of the *Hin*dIII site at 3856 (see Casey *et al.*, 1988a for description of numbering). Construct 3A-0 was made by cutting TRmg-2(Nhe) with *Nhe*I (3856) and *Bam*HI (3′ of the end of the cDNA), filling in with the Klenow fragment of *Escherichia coli* DNA polymerase and ligating with T4 DNA ligase. 5A-0 was created from 3A-0 by cutting with *Xba*I (2400) and *Bal*I (3175), filling in with the Klenow fragment of *E.coli* DNA polymerase and ligating. The 5′ deletions were created by cutting 3A-0 with *Bal*I, and digesting for various times with *Bal*31 exonuclease. Pools of DNA digested for different times were cut with *Xba*I, filled in with Klenow, ligated with *Xho*I linkers and cut with *Xho*I. Gel-isolated fragments were ligated and used to transform *E.coli* HB101. The 3′ deletions were created by cutting TRmg-2(Nhe) with *Nhe*I, and digesting with *Bal*31 exonuclease. Pools of DNA digested for various times were cut with *Bam*HI, filled in with Klenow, ligated with *Xho*I linkers and cut with *Xho*I. Gel-isolated fragments were ligated and used to transform *E.coli* HB101. In some cases, 5′ and 3′ deletions were combined (constructs 3B-5/5B-7, 3B10/5A-60 and 3A-44/5C-8). These constructs were created by cutting both the parent 3′ and 5′ deletion constructs with *Eco*RI and *Xho*I. The fragment containing the coding region and promoter sequences was obtained from the 3′ deletion parent; the 5′ parent yielded the fragment containing the 3′ end of the deletion construct. The gel-purified fragments were ligated with T4 DNA ligase and used to transform *E.coli* HB101. Deletion endpoints were determined by dideoxy sequencing (Sanger *et al.*, 1977) of miniprep plasmid DNA or of CsCl banded plasmids.

Construction of plasmids containing synthetic regulatory sequences

Regulatory sequences were synthesized on an Applied Biosystems model 381A synthesizer. For the construct TRS-1, four oligonucleotides were produced, corresponding to nucleotides 3285−3389 and 3663−3788 and the complements of those sequences. For cloning purposes, the 3285−3389 region was synthesized with an *Xba*I restriction site at the 5′ end and an *Xho*I site at the 3′ end; the 3663−3788 region contained an *Xho*I site at the 5′ end and *Spe*I and *Bam*HI sites at the 3′ end. The oligonucleotides were phosphorylated with polynucleotide kinase and ligated with the plasmid 3A-0 which had been previously cut with *Xba*I and *Bam*HI and dephosphorylated with calf intestinal phosphatase. Plasmids were isolated from transformed clones of *E.coli* HB101 and sequenced.

Similar procedures were followed from constructs TRS-3 and TRS-4. TRS-3 was made from two oligonucleotides instead of four. The synthetic oligonucleotides contained: an *Xba*I site, nucleotides 3320−3389; an *Xho*I site, nucleotides 3663−3690, 3722−3759 and 3783−3788; and *Spe*I and *Bam*HI sites. TRS-4 contained the same sequences as TRS-1 except that the C nucleotides at positions 3301, 3704 and 3769 and their complements were omitted.

Assessment of iron regulation

Cell culture, transient and stable transfections, iron treatments and analysis of transferrin receptor biosynthesis and mRNA levels were as described in Casey *et al.* (1988a).

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