Transferrin receptor mRNA interactions contributing to iron homeostasis

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ABSTRACT

The transferrin receptor is the primary means of iron importation for most mammalian cells and understanding its regulatory mechanisms is relevant to hematologic, oncologic, and other disorders in which iron homeostasis is perturbed. The 3' UTR of the transferrin receptor mRNA contains an instability element that is protected from degradation during iron depletion through interactions of iron regulatory proteins (IRPs) with five iron-responsive elements (IREs). The structural features required for degradation and the site of IRP binding required for *in situ* protection remain unclear. An RNA-CLIP strategy is described here that identifies the predominant site of IRP-1 interaction within a nontransformed primary cell line. This approach avoided complications associated with the use of elevated concentrations of protein and/or mRNA and detected interactions within the native environment of the mRNA. A compensatory mutagenesis strategy indicates that the instability element at minimum consists of three non-IRE stem-loops that can function additively, suggesting that they are not forming one highly interdependent structure. Although the IREs are not essential for instability, they enhance instability when IRP interactions are inhibited. These results are supportive of a mechanism for a graded response to the intracellular iron resulting from a progressive loss of IRP protection.

Keywords: endonuclease; iron homeostasis; iron-responsive elements; iron regulatory proteins; transferrin receptor

INTRODUCTION

In most organisms the coordination chemistry and redox potential of iron in its ferrous and ferric forms are essential for several key processes including DNA replication, energy generation, and oxygen transport. Conversely, high concentrations of iron can generate reactive oxygen species through the Fenton reaction, which have been associated with an increased incidence of a wide range of pathologies including oncologic and neurodegenerative disorders (for review, see Silva and Faustino 2015; Bogdan et al. 2016; Gozzelino and Arosio 2016). As a result, homeostatic mechanisms are required to ensure that sufficient but nontoxic levels of iron are maintained at both the cellular and whole organism levels.

The import, export, utilization, and storage of iron in mammalian cells are largely regulated through the interaction of iron regulatory protein-1 (IRP-1) and iron regulatory protein-2 (IRP-2) with mRNAs that contain an iron-responsive element (IRE). The function of some IREs can also be altered through a direct interaction with Fe^{2+} (Ma et al. 2012). The canonical IRE has a hairpin loop structure with a bulged C five base pairs from a hairpin loop containing

the CAGWGH consensus sequence, where W is A or U and H is A, C, or U (for review, see Kuhn 2015). The IRE-binding site of IRP-1 is occluded under iron-replete conditions by an iron-sulfur cluster, and the disassembly of this cluster under iron-deplete conditions is believed to be a major mechanism of regulating the RNA binding of IRP-1. However, IRP-1 can also be regulated through ubiquitin-dependent degradation mediated by FBXL5, an iron-responsive E3 ligase (Salahudeen et al. 2009; Vashisht et al. 2009). In contrast, IRP-2 does not appear to be regulated through iron-sulfur cluster assembly but is instead regulated primarily through the FBXL5 ligase. In addition to iron, the IRE-binding of both IRPs can be influenced by hypoxia, nitric oxide, oxidative stress, and phosphorylation (for review, see Anderson et al. 2012). The IRE-IRP system is essential, as indicated by the embryonic lethality of mice lacking both IRPs (Smith et al. 2006; Galy et al. 2008).

The IRE-IRP interaction can function to either modulate mRNA translation or stability, depending on the location of the IRE. When the IRE is within the 5' UTR, IRP binding

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blocks translation through inhibition of small ribosomal subunit recruitment to the cap complex (Muckenthaler et al. 1998). The mRNAs containing a well-characterized functional IRE within the 5' UTR include those encoding the following: the ferritin light and heavy chains, which are the major iron-storage proteins; ferroportin, the only identified cellular exporter of iron; erythroid 5-aminolevulinic synthase, which catalyzes the initial step of heme biosynthesis; and HIF2 α , a major modulator of the hypoxic response (for review, see Anderson et al. 2012). In contrast, the transferrin receptor (TFRC) mRNA contains five IREs within the 3' UTR, and IRP interactions in this region impact mRNA stability. The 3' UTR that is essential and sufficient for iron-responsive mRNA stability has been minimized to a 245-nucleotide (nt) region that contains three of the five IREs, and IRP binding to these sites has been proposed in some manner to inhibit access to an endonuclease (Casey et al. 1988; Mullner and Kuhn 1988). Despite this mechanism having been proposed over 25 years ago, the means through which IRP binding stabilizes the TFRC mRNA, the nature of the endonuclease recognition element, and the identity of the endonuclease remain unclear. Here, we identify the major site of the IRP-1 interaction occurring with the TFRC mRNA in situ, and show that the instability element consists of three 5-bp hairpin loops that can function additively. The study expands upon an earlier model for iron-responsive regulation and provides a mechanism for a graded response to a loss of IRP protection.

RESULTS

RNA-CLIP identifies sites of in situ IRP-1 interaction with the TFRC mRNA

An RNA-CLIP strategy (Ule et al. 2003) was adapted to identify the IRP-1 interactions occurring with the TFRC mRNA in situ (Fig. 1A). Human umbilical vein endothelial cells (HUVECs), which are nontransformed primary cells, were exploited for the in situ crosslinking. The approach detected interactions within the native environment of the TFRC mRNA and avoided the detection of low affinity interactions potentially forming in cell lines that over-express either the IRPs or TFRC mRNA. The polyclonal antibody used for

FIGURE 1. Identifying the in situ sites of IRP-1 interaction with the TFRC mRNA. (A) The RNA-CLIP strategy used to identify sites of IRP-1 interaction. The immunoprecipitation step enriched for IRP-1 and associated crosslinked RNAs relative to unrelated RNA binding proteins (RBP). (B) The anti-IRP-1 polyclonal antibody exploited for the RNA-CLIP immunoprecipitates IRP-1-IRE complexes but not IRP-2-IRE complexes. One picomol of each recombinant IRP was UV crosslinked to a radiolabeled 43-nt IRE transcript for direct loading on the gel (pre) or incubation with either anti-IRP-1 polyclonal or control rabbit IgG antibody that had been bound to magnetic beads. The nonbound complexes in the supernatant (sup) and the complexes eluted from the beads, by heating in gel-loading buffer after two washes in lysis buffer, are indicated. Gels are representative of three sets of reactions. (C) IRP-1 interacting sites identified by the RNA-CLIP. Upper case letters represent the most abundant CLIP sequences and lower case the longest 3' and 5' extensions present within the nine libraries. (D) The TFRC mRNA level in HUVECs is iron-responsive. Cells were treated with either DFO or FAC for 14 h prior to the isolation of total RNA. Both RPL4 and POLR2A were used as qRT-PCR reference amplicons.

the coimmunoprecipitation (step 5 of Fig. 1A) was raised against full-length human IRP-1. Although the anti-IRP-1 polyclonal quantitatively binds crosslinked IRP-1-IRE complexes, the avidity is not sufficient to recover any detectable IRP-2-IRE complexes from the immunoprecipitation (Fig. 1B). As a result, the RNA-CLIP strategy should only identify IRP-1 interactions. Nine CLIP libraries were prepared for high-throughput sequence analysis from cells grown under the following conditions: three under 20% O₂ in the presence of ferric ammonium citrate (FAC), an iron source; and three each under 20% and 3%–5% O₂ in the presence of deferoxamine (DFO), an iron chelator. The reduced O₂ condition was chosen because it had earlier been reported to suppress IRP-1 and induce IRP-2 (Meyron-Holtz et al. 2004).

Over 37,000 sequence-reads from the nine RNA-CLIP libraries were mapped to the TFRC gene (Table 1). Since the RNA-CLIP fragments are relatively short (40–50 nt),

TABLE 1. Number of mapped sequence-reads from cells grown in DFO or FAC and 20% or 3%–5% oxygen^a

	IRE	DFO ^b (20% O ₂)	FAC ^c (20% O ₂)	DFO–FAC (20% O ₂)		DFO ^d (3–5% O ₂)	DFO (20% O ₂)– DFO (3%–5% O ₂)	
				<i>P</i> -value	<i>q</i> -value		<i>P</i> -value	<i>q</i> -value
TFRC (total reads)		30,560	64	0.005	0.047	6865	0.58	0.63
	A	0	0			0		
	B	0	0			0		
	C	30,559	64			6837		
	D	0	0			5		
	E	1	0			23		

^aValues were obtained from three independent CLIP libraries prepared for each of the indicated conditions.^bThe relative TFRC/RPL4 mRNA level is 0.03 ± 0.01 .^cThe relative TFRC/RPL4 mRNA level is 0.0009 ± 0.0003 .^dThe relative TFRC/RPL4 mRNA level is 0.03 ± 0.01 .

it was possible to unambiguously identify the crosslinked IRE within each of the sequences, and all of the TFRC sequences are centered on an IRE (Table 1; Fig. 1C). Even though the TFRC mRNA contains five IREs within the 3' UTR, designated A through E in the 5'–3' direction, the predominant crosslinked site is to IRE C under all conditions tested (Table 1). A minor fraction (<0.1%) of the RNA crosslinks was also identified at IREs D and E.

Approximately 500 times more sequence-reads were obtained from the DFO treatment under 20% O₂ as compared to the corresponding FAC treatment (Table 1). This reached statistical significance as indicated by both the *P*- and *q*-values for the comparison of the six RNA-CLIP libraries calculated using Galaxy software (Giardine et al. 2005; Blankenberg et al. 2010; Goecks et al. 2010). It is consistent with there being more IRP bound to the TFRC mRNA during iron-depletion since the relative number of sequence-reads under each condition is, in part, a function of the extent of IRP interaction. It also correlates with the TFRC mRNA abundance being greater with the DFO treatment, which was determined by qRT-PCR of total RNA using both RPL4 and POLR2A as normalization amplicons (Fig. 1D). However, the difference in sequence-reads obtained from DFO-treated cells grown in 20% O₂ from those grown in 3%–5% O₂ is well short of statistical significance (Table 1). This indicates that, within the sensitivity of the assay, the extent and site of IRP-1 interaction with the TFRC mRNA does not significantly differ with the two O₂ concentrations.

An assay to identify key elements required for TFRC mRNA instability

The RNA-CLIP study suggests that IRP-1 binding to IRE C is a means through which the instability element of the TFRC mRNA could be protected from degradation during iron depletion. To gain insight into the identity of the instability element, a luciferase assay was developed to facilitate the comprehensive mutagenesis of the previously minimized 245-nt

TFRC 3' UTR (Fig. 2). The 245-nt sequence was cloned 3' of the firefly luciferase (FL) coding sequence of the pmirGLO vector and transfected into mouse fibroblasts (L-M, TK⁺). This cell line was chosen because it had been exploited for much of the pioneering work on the iron-responsive stability of the TFRC mRNA. After transfection, FL activity was measured and normalized to the renilla luciferase (RL) activity encoded on the same vector. Conditions that stabilize the FL mRNA through the promotion of IRP interactions should be reflected by an increase in the FL/RL ratio.

To initially test the assay, the FL/RL enzyme ratio for the wt TFRC was compared to that of a large deletion (Δ 31–108, pFL #26), which was earlier demonstrated to result in the loss of iron-responsive instability (mutation pTR-82 in Mullner and Kuhn 1988). During iron depletion, IRP binding to the wt TFRC sequence is expected to protect the mRNA from degradation and as a result the wt stability should approximate that of the mutation. Consistent with this, there is not a significant difference in the FL/RL enzyme ratios for the wt and Δ 31–108 mutation in the presence of DFO (Fig. 2B). In contrast, the FL/RL of the wt is reduced by approximately one-third relative to the nonresponsive control during iron repletion, and this difference remains relatively constant up to 48 h of FAC treatment (Fig. 2B). The difference is consistent with the wt sequence being less stable than the Δ 31–108 mutation as a result of the functional endonuclease site in the wt not being protected by IRP binding during iron repletion.

To test whether changes in the FL/RL enzyme ratio are indicative of changes in mRNA levels, the FL/RL ratios for the enzyme activity and mRNA levels were measured for a series of 10 mutations within the 245-nt TFRC minimized sequence (Table 2). The FL/RL values for each mutation were normalized to those of the wt. There is a good correlation ($R^2 = 0.95$) between changes in the enzyme activity and mRNA levels (Fig. 2C), indicating that the FL/RL luciferase measurement can be used as a surrogate for detecting changes in mRNA abundance. Since all of the described

FIGURE 2. The luciferase assay used to identify sites within the TFRC mRNA that are required for degradation during iron repletion. (A) The previously minimized TFRC 3' UTR that supports iron-dependent regulation (Casey et al. 1989) was cloned into the 3' UTR of the FL gene within the pmirGLO vector. (B) The effect of iron depletion and repletion on the FL enzyme activity of wt FL-TFRC mRNA and the $\Delta 31-108$ deletion, which earlier was demonstrated to be nonresponsive to iron (Mullner and Kuhn 1988); the FL activity was normalized to the corresponding RL value. Transfected mouse fibroblasts were treated with either 100 μ M DFO or 100 μ g/mL FAC for the indicated times prior to the luciferase assays. (C) The change in FL/RL for enzyme activity correlates well with the change in FL/RL for mRNA abundance. FL/RL values are relative to the wt and were measured after 14 h of treatment with 100 μ g/mL FAC. Statistical significance was analyzed by two-tailed Student's *t*-test. (***) $P < 0.001$, (ns) not significant: $P > 0.05$. All error bars represent $\pm$ SEM of at least three biological replicates.

TFRC mutations are in the context of the same promoter, reporter, and vector, it is also a reasonable initial assumption that the mutations altering mRNA abundance result from changes in stability rather than changes in transcription. The relative half-lives for two of the key RNAs measured in the presence of actinomycin D are consistent with this assumption (Fig. 3).

Non-IRE sequences influencing luciferase activity and mRNA abundance

A compensatory mutagenesis strategy was used to probe the 245-nt TFRC minimized sequence for structural features required for endonuclease function. An advantage over earlier studies that relied predominantly on deletional analysis is that it is possible to distinguish between disruptions of a predicted helix from more indirect structural changes. Cells transfected with plasmids encoding TFRC sequence variants were main-

tained under iron-replete conditions to minimize IRP binding and increase the probability that observed effects are reflective of changes in the endonuclease interaction. The major results from the mutagenesis of the non-IRE sequences are summarized in Table 2 and Figure 3A. These results are briefly discussed below using earlier established convention, where available, for labeling of the individual TFRC mRNA structural features (Horowitz and Harford 1992; Schlegel et al. 1997):

Stem-loop I

The importance of helix I to mRNA stability is supported by the increased mRNA abundance resulting from disruption of the helix with the C54G substitution (pFL #1) and subsequent restoration to wt level by the compensatory (C54G + G48C) mutation (pFL #2). Substitutions within the loop sequence (49–51 CCU) also increased the FL (pFL #3), further supporting a critical role for the stem-loop.

Stem-loop II

In contrast to stem-loop I, helix II appears to be nonessential for endonuclease function as large deletions and substitutions within this region have no significant impact on FL relative to the wt sequence (pFL #4 and #5).

Nucleotides 69–75

Deletions or substitutions within the nt 69–75 region can be made with no significant increase in FL (pFL #6–8). However, complete deletion of nt 60–73 (pFL #9) increased FL, indicating that some type of minimal constraint exists between loops I and III.

Stem-loop III

Stem-loop III is essential as disruption of the helix with a C78G substitution (pFL #10) increased FL and the compensatory (C78G + G86C) mutation (pFL #11) restored it to the wt level. This is also indicated by substitutions within the loop sequence (pFL #12), which increased both FL activity and mRNA levels. The importance of this structure is consistent with an earlier 24-nt deletion (mutation 3B-10/5A-60 in Casey et al. 1989) and a 47-nt deletion (pTR-93 in Mullner and Kuhn 1988) that encompassed the region and resulted in a loss of iron regulation. The substitution of Us at nt

TABLE 2. Non-IRE sequences influencing the FL/RL enzyme activity and mRNA abundance^a

pFL	Structural element	Mutation (mut) ^b	mut(FL/RL)/wt(FL/RL)		
			Enzyme activity	<i>P</i> -value ^c	mRNA abundance
1	Stem-loop I	C54G	1.4 ± 0.1 (<i>n</i> = 3)	0.02	(rel. to pFL 1)
2		C54G + G48C	0.9 ± 0.1 (<i>n</i> = 3)		
3		49–51 CCU	1.4 ± 0.1 (<i>n</i> = 6)		
4	Stem-loop II	Δ60–64 + 65–69 AAAAG	0.98 ± 0.03 (<i>n</i> = 4)	ns	(rel. to pFL 6)
5		94–97 UUAG	1.01 ± 0.01 (<i>n</i> = 3)		
6		Δ69–71 + Δ73	1.03 ± 0.05 (<i>n</i> = 3)		
7	Nucleotides 69–75	72–73 GC	1.11 ± 0.03 (<i>n</i> = 4)	0.014	(rel. to pFL 6)
8		74–75 UU	0.9 ± 0.1 (<i>n</i> = 3)		
9		Δ60–73	1.3 ± 0.1 (<i>n</i> = 3)		
10	Stem-loop III	C78G	1.4 ± 0.1 (<i>n</i> = 3)	0.009	(rel. to pFL 10)
11		C78G + G86C	1.0 ± 0.1 (<i>n</i> = 3)		
12		81–82 GU	1.4 ± 0.1 (<i>n</i> = 3)		
13	Nucleotides 89–90	89–90 UU	1.05 ± 0.04 (<i>n</i> = 3)	ns	(rel. to pFL 14)
14	Stem-loop IV	Δ99–133	0.97 ± 0.05 (<i>n</i> = 3)		
15		Δ99–147	0.93 ± 0.01 (<i>n</i> = 3)		
16	Nucleotides 179–189	U180A + A182U	0.96 ± 0.04 (<i>n</i> = 3)	0.003	(rel. to pFL 21)
17		184–190 CCCCCC	1.3 ± 0.1 (<i>n</i> = 3)		
18		183–185 UUU	1.1 ± 0.1 (<i>n</i> = 3)		
19	Stem-loop V	185–187 UUG	0.97 ± 0.03 (<i>n</i> = 3)	2 × 10 ^{−5}	(rel. to pFL 17)
20		186–189 UGUU	0.7 ± 0.1 (<i>n</i> = 5)		
21		190–191 CC	1.20 ± 0.02 (<i>n</i> = 3)		
22	Stem-loop V	190–191 CC + 205–206 GG	0.8 ± 0.1 (<i>n</i> = 4)	0.003	(rel. to pFL 21)
23		195–201 UAAC	1.15 ± 0.03 (<i>n</i> = 3)		
24		197–200 AAAC	1.14 ± 0.04 (<i>n</i> = 3)		
25	Neg control	207–209 UUG	1.0 ± 0.1 (<i>n</i> = 3)	0.002	(rel. to pFL 20)
26		Δ31–108	1.6 ± 0.2 (<i>n</i> = 15)		

^aTransfected cells were treated with 100 μg/mL FAC for 14 h prior to the assays.^bNucleotide positions correspond to the minimized TFRC mRNA of Figure 3A; substituted sequence is indicated to the right of the numbered position(s).^c(ns) Not significant: *P* > 0.05.

89–90, which potentially could have extended the stem-loop through pairing with A74 and A75, failed to significantly impact FL (pFL #13).

Stem-loop IV

Stem-loop IV and the immediate 3′ single stranded sequence (nt 99–147) can be deleted with no significant effect on FL, suggesting that the region is not required for the TFRC endonuclease (pFL #14 and #15).

Nucleotides 179–189

Major changes can be made within the nt 179–189 region that do not increase FL (pFL #16, #18–20). This is relevant because it was earlier proposed that nt 184–190 contain a recognition element for the TFRC mRNA endonuclease (Binder et al. 1994). The earlier proposal was based on the mapping of an endonuclease cleavage site (3′ of G184 in Fig. 3A) and the stabilization of the mRNA that resulted from the substitution of nt 184–190 (GAACAAG) with cytidines (mutation TRS-1C₇ in Binder et al. 1994). The effect of the polycytidine substitution at nt 184–190 was reproduced in the luciferase assay

(pFL #17). However, smaller mutations covering most of the sequence (nt 184–189) did not increase FL (pFL #18–20), and the substitution of nt 186–189 with UGUU actually had the opposite effect (pFL #20). Both results argue against the nt 184–189 sequence containing the major endonuclease recognition site. The apparent stimulation of endonuclease activity by pFL #20 is consistent with the stabilization of the helix of loop V through pairing of U186 with A209, G187 with C208, and U188 with A207 (Fig. 3A). Other substitutions, though, that are predicted to increase the stem-loop V stability did not impact the FL (pFL #25), suggesting that more complex interactions could also be involved.

Stem-loop V

The 3′-most nucleotide of the earlier polycytidine substitution at nt 184–190 overlaps with stem-loop V at nt 190 (mutation TRS-1C₇ in Binder et al. 1994), making it possible that the observed stabilization resulted from the disruption of the stem-loop. To test this possibility, helix V was disrupted through a CC substitution at nt 190–191 (pFL #21). The substitution increased FL to a similar extent as the nt 184–190 polycytidine substitutions while the compensatory mutation

FIGURE 3. Three 5-bp hairpin loops have an additive effect on instability. (A) Summary of mutagenesis study (Tables 2, 3); green, sites of mutations that destabilize mRNA; red, sites of mutations resulting in stabilization; gray, sites of mutations with no significant effect. The endonuclease cleavage site was earlier proposed to be 3' of G184 (arrow; Binder et al. 1994). *Inset* shows the UAAC tetraloop substitution in IRE C. (B) Predicted secondary structure of the RNA optimized for instability; green, mutations that are destabilizing. (C) Sequential mutagenesis of the three non-IRE stem-loops results in an additive stabilization of the opt RNA. Values are relative to the wt RNA (dashed line). Luciferase was measured after 14 h treatment with 100 μ g/mL FAC. (D). The opt RNA within transfected mouse fibroblasts has decreased stability relative to the wt sequence. Fourteen hours after the transfections, cells were transferred into fresh iron-replete media for 2 h prior to the addition of 5 μ M actinomycin D. Total RNA was isolated at the indicated time points and mRNA levels assayed by qRT-PCR. The FL mRNA was normalized to the corresponding RL mRNA, and the FL/RL ratio for the zero hour actinomycin D point was set as 100%. Statistical significance was analyzed by two-tailed Student's *t*-test. (ns) Not significant; $P > 0.05$. Unless indicated otherwise, all error bars represent $\pm$ SEM of three biological replicates.

(190–191 CC + 205–206 GG) decreased mRNA abundance back close to wt levels (pFL #22). Mutations within the loop also increased FL (pFL #23–24), further indicating the importance of this structure. The importance of the stem-loop is also supported by an earlier deletion that encompassed the region and resulted in a loss of iron regulation (3A-44/5C-8 in Casey et al. 1989).

In summary, three major non-IRE structures appear to impact mRNA abundance during iron repletion: stem-loops I, III, and V. All three stem-loops have 5-bp helices that share some sequence similarity. Helices II and IV are dispensable,

and nt 180–189 is not consistent with being an endonuclease recognition element (Fig. 3A).

Mutations suggestive of residual IRP interactions during iron-repletion

The mutagenesis is suggestive that sites within the IREs, earlier well defined as critical for IRP interactions during iron depletion (for review, see Kuhn 2015), also appear to maintain some IRP interactions during iron repletion (Table 3). The CAGWGH IRE hairpin loop is critical for IRP

TABLE 3. IRE sequence influencing the FL/RL enzyme activity^a

pFL	Structural element(s)	Mutation (mut) ^b	mut(FL/RL)/wt(FL/RL)	P-value ^c	
27	IRE B	ΔC25	0.87 ± 0.10 (n = 5)	ns	(rel. to pFL 27)
28		25–29 UAAC	0.82 ± 0.07 (n = 6)		
29	IRE C	ΔC161	0.80 ± 0.02 (n = 3)	ns	(rel. to pFL 29)
30		161–166 UAAC	0.77 ± 0.04 (n = 4)		
31	IRE D	ΔC155	0.76 ± 0.04 (n = 3)	ns	(rel. to pFL 32)
32		ΔC226	1.0 ± 0.1 (n = 3)		
33		226–231 UAAC	1.0 ± 0.1 (n = 4)		
34		211–215 AAAAA	0.8 ± 0.1 (n = 3)		
35	IRE B + C	ΔC25 + ΔC161	0.55 ± 0.03 (n = 4)	ns	(rel. to pFL 35)
36		25–29 UAAC + 161–166 UAAC	0.7 ± 0.1 (n = 6)		
37	IRE B + C + D	ΔC25 + ΔC161 + ΔC226	0.44 ± 0.05 (n = 7)	0.003	(rel. to pFL 35)
38		25–29 UAAC + 161–166 UAAC + 226–231 UAAC	0.83 ± 0.05 (n = 7)	<10 ^{−6}	(rel. to pFL 37)
39		25–29 UAAC + 161–166 UAAC + 229–231 GUU	1.08 ± 0.04 (n = 4)	0.0003	(rel. to pFL 38)
40		ΔC25 + 161–166 UAAC + 226–231 UAAC	0.75 ± 0.06 (n = 3)	ns	(rel. to pFL 38)
41		25–29 UAAC + ΔC161 + 226–231 UAAC	0.69 ± 0.12 (n = 3)	ns	(rel. to pFL 38)
42		25–29 UAAC + 161–166 UAAC + ΔC226	0.58 ± 0.06 (n = 3)	0.0004	(rel. to pFL 38)
43		ΔC25 + ΔC161 + 226–231 UAAC	0.62 ± 0.01 (n = 3)	0.0005	(rel. to pFL 37)

^aTransfected cells were treated with 100 μg/mL FAC for 14 h prior to the assays.^bNucleotide positions correspond to the minimized *TFRC* mRNA of Figure 3A; substituted sequence is indicated to the right of the numbered position(s).^c(ns) Not significant: *P* > 0.05.

interaction, and deletion of the 5' C of this loop in either IRE B (pFL #27) or IRE C (pFL #29) decreased FL under iron-replete conditions, consistent with a loss of residual IRP protection resulting in the further degradation of the mRNA. Substitution of individual IRE hairpin loops with a UAAC tetraloop (inset, Fig. 3A), which energetically stabilizes hairpins (Molinari and Tinoco 1995), also decreased FL to the same extent (compare pFL #27 with #28 and #29 with #30). This suggests that the decrease is not a simple consequence of the IRE stem or overall structure being energetically less stable with the 5' C loop deletions. A requirement for the bulged C of the IRE is another hallmark of an IRP interaction, and deletion of this position in IRE C (C-155) decreased FL (pFL #31), consistent with a loss of IRP binding. Residual IRP binding is expected, at least to some extent, as the equilibrium between the IRP-bound and unbound forms of the IRE is unlikely to go to completion in either direction; this is supported by the IRP interactions detected under high iron conditions by the RNA-CLIP (Table 1). However, the possibility that these mutations are also stimulating endonuclease activity in some manner cannot be eliminated.

Mutations to IRE D behave very differently from the corresponding changes made to IREs B and C. Deletions or substitutions to the CAGWGH hairpin loop had no significant impact on FL (pFL #32–33). This is consistent with the site not interacting with an IRP during iron repletion or alternatively the interaction could occur but is redundant for endonuclease protection. Although mutation of hairpin loop D by itself does not impact FL activity, the mutation becomes significant (*P* = 0.003) when both IREs B and C have also been mutated (contrast pFL #35 and #37). This suggests that the hairpin loop of IRE D can interact with an IRP, as previously

demonstrated in vitro (Koeller et al. 1989), but the interaction is either inhibited or not essential in the context of functional IRE B and C sequences. However, mutation of the lower helix of IRE D does result in a minor FL decrease in the context of wt IRE B and C (pFL #34). This lower helix is also protected from in vitro chemical modification by IRP-1 (Schlegel et al. 1997), which is suggestive of the possibility of some type of interaction at this site.

IRE sequence-dependent destabilization during iron repletion

Although the destabilization resulting from the IRE mutations could be partly attributed to a simple loss of residual IRP interaction occurring during iron repletion, there also appear to be more complex sequence-specific effects. The FL is significantly increased when UAAC tetraloop substitutions are made to the hairpin loops of all three IREs as compared to point deletions (ΔC25, ΔC161, and ΔC226) to the same loops (Table 3, contrast pFL #37 and #38). The result was unexpected since both types of mutations are sufficient to inhibit IRP interactions and behave similarly when made to any single IRE, as already indicated (compare pFL #27 with #28, #29 with #30, #32 with #33). Even though the UAAC tetraloop has minimal interactions in other systems (Zhao et al. 2012), one possibility for the different behavior of the multiple tetraloop substitutions is that they are indirectly inhibiting endonuclease activity through unintended structural changes. However, non-tetraloop substitutions to the hairpin CAGWGH sequence of IRE D actually have a greater increase in FL (contrast pFL #38 and #39), making it less likely that tetraloop-specific interactions or the

increased stem-loop stability associated with the tetraloops are inhibiting endonuclease activity.

An alternative possibility for the behavior of the two types of loop mutations is that the AGWGH part of the IRE hairpin loops in some manner facilitates the endonuclease activity. The effect would be dependent upon potential IRP interactions with all three IREs being inhibited. To test this possibility, the UAAC tetraloop substitution of IRE D, which has no effect in the context of the wt IRE B and C sequences (pFL#33), was made in the context of the C25 and C161 deletions (pFL #43). If the C25 and C161 deletions unmask a destabilizing property within the AGWGH part of the loops by inhibiting IRP interactions, the UAAC tetraloop substitution in IRE D would be predicted to now have a stabilizing effect. The observed increase in FL of the UAAC substitution in IRE D relative to the corresponding 5' C deletion (Δ C226) is consistent with the prediction (contrast pFL #37 with #43). Although mutation of the AGWGH sequences in IREs B and C do not have a significant effect when mutated individually (contrast pFL #38 with #40–41), mutation of both together result in a similar change in FL as the IRE D substitution (contrast pFL #38 with #42–43). The results are suggestive of a destabilizing role for the AGWGH loops that only becomes possible with the loss of IRP interactions and also suggest that there is some redundancy to the effect.

An RNA optimized for instability

The mutagenesis (Tables 2, 3) enabled the 3' TFRC mRNA sequence to be optimized for instability (Fig. 3B). The optimized sequence (opt) includes the 5' C deletion within each of the three IRE hairpin loops (pFL # 37), substitutions within the lower stem of IRE D (pFL # 34) and minor changes near stem-loops III and V (pFL #8 and #22). The opt mRNA also contains a deletion of nt 99–147 (pFL #15), encompassing stem-loop IV and the flanking 3' sequence, which was found not to impact FL. The combination of these changes resulted in a decreased FL of 7.8-fold relative to the wt sequence and 2.8-fold relative to the triple hairpin loop deletions (pFL #37) alone (Fig. 3C).

The time course for the decay of the wt and opt RNAs was followed in mouse fibroblast cells treated with 5 μ M actinomycin D (Fig. 3D). The first-order rate constants were obtained from the slopes of the plots, and the half-life of the opt RNA determined to be $\sim$ 2 h while that of the wt is 5 h. The greater instability of the opt mRNA is consistent with the decreased FL enzyme activity (Fig. 3C). Despite considerable effort, no evidence was found that the opt RNA is able to catalyze its own cleavage (not shown), analogous to several natural ribozymes.

The non-IRE instability elements can function additively

Stem-loops I, III, and V were sequentially mutated in the context of the opt RNA (Fig. 3C). Since IRP binding to all

three IREs has been disabled in the opt RNA, performing the mutagenesis in this context has the advantage of unambiguously eliminating potential complications resulting from residual IRP interactions. Sequential mutagenesis of stem-loops I, III, and V resulted in a sequential increase in the relative FL from 0.15 to 1.5, consistent with a sequential increase in mRNA stability (Fig. 3C). The FL of the above-stated triple mutation approximates that of the earlier Δ 31–108 mutation (Table 2, pFL #26), which has a complete loss of iron-responsive stability (mutation pTR-82 in Mullner and Kuhn 1988). The additive effect of the mutations suggests that the stem-loops can function independently of each other as opposed to being required to form one highly interdependent destabilizing structure.

DISCUSSION

RNA-CLIP identified the TFRC mRNA sequence interacting with IRP-1 within a nontransfected primary cell line. A major advantage of the approach is that it avoided the detection of interactions that are potentially physiologically irrelevant but favored when elevated concentrations of protein and/or RNA are used for either in vitro or in situ studies. Since the cross-linking occurs in the native environment of the mRNA, the CLIP strategy also avoided in vitro complications resulting from incorrect folding and the detection of interactions that are normally masked in situ through protein interactions or RNA secondary and tertiary interactions. A limitation of the CLIP approach, however, is that the relatively low expression of the endogenous IRP-1 and the inefficient crosslinking, as well as other potential biases could have resulted in some interactions not being represented. The possibility that there are species or cell-specific differences in the IRP-1 binding also cannot be eliminated. Nevertheless, RNA-CLIP identified IRE C as the major site of IRP-1 interaction from an analysis of nine independent libraries containing 37,489 mapped TFRC sequence-reads, all of which encoded an IRE. Unfortunately, several earlier in vitro and in situ studies exploited a TFRC construct that contained an inadvertent mutation within the IRE C hairpin loop, corresponding to G3921A in Figure 1C (Koeller et al. 1989; Binder et al. 1994; Schlegel et al. 1997). This would have inhibited the IRP interaction at IRE C and may have increased the relative importance of other sites.

The mutagenesis indicates that the instability element at minimum includes three 5-bp stem-loops that share some sequence similarity within the stems (I, III, and V, Fig. 3). An earlier substitution within nt 184–190, that stabilizes the mRNA and was proposed to directly inhibit the endonuclease recognition site (Binder et al. 1994), is more consistent with the disruption of stem-loop V (Table 2, pFL #17–22). Sequential mutagenesis of the three critical loops has an additive destabilizing effect indicating that the loops are unlikely to be forming one highly interdependent structure (Fig. 3C). It is suggestive of a potential mechanism for a graded

response to the intracellular iron in which the loops become sequentially unhindered in response to changes in IRP protection, however this still remains to be tested. The study also provides some of the biochemical tools that will facilitate the identification of the endonuclease.

Several mutations were identified that are suggestive of IRP interactions occurring with the *TFRC* mRNA even during prolonged treatment with a relatively high concentration of an iron source (Table 3). The bulged C and hairpin loop of the IRE are the two major hallmarks of an IRP interaction and the mutagenesis of either of these sites on IRE C has a destabilizing effect during iron repletion, consistent with a loss of IRP protection. Mutations were also identified at sites within the hairpin loop of IRE B that appear to destabilize the mRNA. Although IRP interactions with IRE B were not detected by the RNA-CLIP analysis (Table 1), IRE B was also found not to be required for iron regulation in an earlier mutagenesis study of the full-length *TFRC* 3' UTR (mutation pTR-80 in Mullner and Kuhn 1988). The discrepancy with the mutagenesis study described here may have resulted from the use of a highly minimized *TFRC* construct where the relative importance of IRE B could have been increased. Alternatively, it is also possible that IRP-2 is interacting with IRE B in situ, which could not have been detected by the RNA-CLIP study since it was specific for IRP-1 interactions (Fig. 1B). Although the two IRPs have considerable overlap in their binding specificities, each also has unique specificities (Henderson et al. 1996; Anderson et al. 2013). A further possibility for the discrepancy is that mutations to IRE B could be in some manner stimulating the endonuclease activity rather than inhibiting residual IRP interactions.

Several of the IRE mutations are suggestive that the AGWGH part of the IRE loops stimulate endonuclease activity during iron repletion. This is most apparent when an RNA containing point deletions to the 5' C in the CAGWGH loop of all three IREs (Δ C25, Δ C161, and Δ C226) is compared with an RNA in which all three loops had been completely substituted (Table 3, contrast pFL #37 with #38). Even though both sets of mutations should disrupt IRP interactions, the point deletions to the 5' Cs resulted in a greater decrease in the FL, consistent with decreased stability. The difference was even greater with non-tetraloop substitutions (contrast pFL #42 with #38–39). The results are suggestive that the AGWGH part of the IRE hairpin loops may have a role in destabilizing the mRNA in the absence of IRP interactions. It would also be consistent with the decreased stability associated with an earlier mutation containing deletions to the 5' C of all the three IRE hairpin loops (mutation TRS-4 in Casey et al. 1989). Although the AGWGH sequences augment instability, they are not essential for degradation as the RNA with tetraloop substitutions at all three IRE loops is still unstable during iron repletion (contrast pFL #38 and #26). This is unlike the combined mutation of the three non-IRE loops (I, III, and V) that approximates the effect of the non-responsive Δ 31–108 mutation (Fig. 3C).

MATERIALS AND METHODS

Optimization and adaptation of the RNA-CLIP methodology to IRP-1

The RNA-CLIP libraries were prepared similarly to those described earlier (Licatalosi et al. 2008; Wang et al. 2009; Konig et al. 2011). However, the low abundance and poor crosslinking efficiency of the endogenous IRP-1 relative to the RNA-binding proteins of other RNA-CLIP experiments required several adaptations. The optimization was facilitated through the use of synthetic radiolabeled IRE-containing transcripts and recombinant IRP-1. N-terminal His₆ tag human IRPs were expressed in *Escherichia coli* as previously described (Gray et al. 1993) and purified by Q-Sepharose and nickel-chelate chromatography followed by dialysis against 25 mM HEPES pH 7.6, 150 mM KOAC, 1.5 mM MgCl₂, and 5% glycerol. Radiolabeled IRE containing RNAs were transcribed by T7 RNA polymerase from the corresponding DNA template (Milligan and Uhlenbeck 1989). The IRE transcript used for most steps of the optimization consisted of nt 19–61 of the *FTL* mRNA (NM_000146.3).

Crosslinking of HUVECs for RNA-CLIP

The HUVECs were obtained commercially from Lonza (C2517A) and were grown in EGM-2 media on 10-cm gelatin-treated plates for up to five passages. Cells were treated with either 100 μ M DFO or 15 μ g/mL FAC for 14 h. Cells grown in 3%–5% O₂ were placed in modular incubator chambers (Billups-Rothenberg) and flushed with 95% N₂, 5% CO₂ for 15 min prior to sealing. The cells were 70%–80% confluent at harvesting and appeared healthy, as assessed by visualization at 200 $\times$ magnification; however, higher concentrations of FAC, commonly used for other cell lines, appeared to cause death. After treatment, media was removed and replaced with 5 mL ice-cold PBS. The plates were placed on ice and irradiated three times with 400 mJ/cm² using a Stratilinker 1800 (Stratagene) at 254 nm. The PBS was removed and replaced with 2.8 mL fresh PBS and the cells harvested with a cell scraper and transferred into two 1.5 mL tubes and pelleted for 1 min at 13,000g at 4°C. Cell pellets were resuspended in 600 μ L lysis buffer (50 mM HEPES pH 7.5, 40 mM KCl, 1 mM MgCl₂, and 0.05% Tween) containing 0.1 mM CaCl₂, protease inhibitor cocktail (Calbiochem), and 12 units RNase inhibitor (Life Technologies, AM2690). Cells were sonicated on ice with 30 one second 30 kHz pulses over 1 min and then treated with 1.5 units of RNase I (Life Technologies) and 5 units of Turbo DNase (Life Technologies) at 37°C for 3 min with occasional mixing. The solution was cooled on ice and spun at 100,000g at 4°C for 20 min.

Preparation of the RNA-CLIP libraries

The ultracentrifuge supernatant from the cell lysates was precleared by incubating with magnetic beads coupled to Protein A (Life Technologies) that had been bound to normal rabbit IgG (Santa Cruz Biotechnology). After incubation for 15 min, the cleared lysate was transferred to 50 μ L magnetic beads that had been bound to a rabbit polyclonal antibody generated against human IRP-1 (Abnova, H00000048-D01) and resuspended in 30 μ L 10% BSA.

After incubation for 1 h at 4°C, four successive washes were performed: 0.1% SDS in dH₂O, lysis buffer, 1 M KCl in lysis buffer and lysis buffer alone. While on the column, the RNA was sequentially dephosphorylated with T4 polynucleotide kinase, ligated at the 3' end with a 5' adenylated (rAPP) DNA adapter (5'-rApp/TGGAATTCTCGGGTGCCAAGG/3'-ddC), and labeled at the 5' end with P³²-γ-ATP. After washing the beads with 0.1% SDS in dH₂O, and twice with lysis buffer, the cross-linked RNA-IRP complexes were eluted from the beads by heating at 65°C for 10 min in 20 μL 1× Nupage gel loading solution (Life Technologies). The RNA-protein complexes were resolved on a 4%–12% NUPAGE gel (Life Technologies), transferred to nitrocellulose and exposed to film at –80°C for 12 h. Nitrocellulose slices corresponding approximately to 110–150 kDa size range were excised and the RNA released by proteinase K digestion and phenol/chloroform extraction. The 5' adapter RNA (5-amino 6 carbon linker/GUUCAGAGUUCUACAGUCCGACGAUCGG) was added at this point as a carrier. After ethanol precipitation and resuspension, the 5' adapter was ligated and the RNA subsequently purified by gel electrophoresis. Gel slices corresponding to ~90–110 nt (40–60 nt of RNA + 49 nt adapters) were excised, eluted, and precipitated.

RT-PCR amplification of libraries for high-throughput sequence analysis

The RT primer (CCTTGGCACCCGAGAATTCCA) was added as a carrier during the RNA elution from the 90- to 110-nt gel slices. Reverse transcription was with Sensiscript (QIAGEN), which has a high affinity RNA binding site. The PCR primers correspond to Illumina RNA PCR primer RPI and RNA PCR primer, indexes 1–13 (RPI1–RPI13); the indexed primers contain barcodes that permit multiplexing during the high-throughput sequencing. PCR products were purified for sequence analysis using AMPure beads (Beckman Coulter). Single read (1 × 50 cycles) sequencing was performed on an Illumina MiSeq system at the University of Minnesota Genomics Center. Up to six libraries were multiplexed in a single run with approximately 6 million reads obtained for the pool and 97% of bases with a quality score >Q30, indicating an accuracy of >99.9%. Sequences were aligned with the human genome (hg19) and analyzed using Galaxy software (Giardine et al. 2005; Blankenberg et al. 2010; Goecks et al. 2010) at the University of Minnesota Supercomputer Institute.

Luciferase assay of TFRC mutations

The TFRC constructs were synthesized at GenScript and cloned into the pMIRGLO luciferase vector (Promega). Mouse L-M (TK-) fibroblasts (ATCC, CCL-1.3) were grown in DMEM (Life Technologies, 11995–065) containing 10% fetal bovine serum (FBS) and plated at 2000 per well in a 96 well plate and transfected 8 h later with 30 ng of vector using 0.3 μL lipofectamine 2000 (Life Technologies). Media was replaced 14 h after transfection. DFO or FAC was added 8–10 h later and the plates incubated a further 12–14 h as indicated. Dual-Glo (Promega) reagents were used to assay luciferase activity and quantification was done with a luminometer (Tecan). A minimum of three independent transfections were performed for each TFRC construct, and each transfection was assayed in triplicate.

Quantification of changes in mRNA abundance

Total RNA was prepared from cells using RNeasy columns (QIAGEN). The RNA was subsequently digested in a 40 μL volume with 4 units of RNase free DNase (Promega) for 20 min at 37°C. After phenol–chloroform extraction and ethanol precipitation, the RNA was resuspended in dH₂O. cDNA was synthesized from 25–75 ng of the total RNA annealed with 50 ng of a random decamer. Each reverse transcription reaction contained 70 units of Superscript III (Life Technologies) in a 5 μL volume with 0.5 mM deoxynucleotide triphosphates in 1× Superscript buffer. The reactions were at 25°C for 10 min, followed by 42°C for 50 min. After heat inactivation at 95°C for 10 min, 15 μL of 1 mg/mL *E. coli* M600 tRNA (Roche) was added to each tube. One microliter of each cDNA was amplified in a 10 μL volume within 96-well plates using a Sybr Green mix and a StepONE Plus thermal cycler (Life Technologies). The thermal profile consisted of an initial 10 min 95°C denaturation followed by 40 cycles of a 15 sec 95°C denaturation and a 1 min 65°C extension. Minus reverse transcriptase controls were run for all samples and consistently indicated <1% genomic DNA contamination in nontransfected samples and <3% DNA in samples that had been transfected. The PCR primers (Table 4) for the qRT-PCR were designed with Primer Express software (Life Technologies), and the amplification efficiencies were all close to 95%. The ΔΔC_q method was used to quantify changes in the mRNA amplicons relative to the reference amplicons. All values were calculated from at least three independent sets of biological replicates, which were each assayed in triplicate.

TABLE 4. Amplicons used for qPCR

Amplicon	Species	NCBI reference	Location	PCR primers
POLR2A	Human	NM_000937.4	3492–3573	AATGCCACGCTGCTCTTCA CCCCACTGAGCCGAAACTC
RPL4	Human	NM_000968.3	1065–1119	ACCATGCGCCGGAACA CCACCCGGAGCTTGTGATT
TFRC	Human	NM_001128148.1	4301–4387	AGAGTCCCTGAAGGTCTGACA CTCACGGAGCTTCGAACATTATC
Firefly (pmirGLO)	-	FJ376737	7117–7188	AACCATGACCGAGAAGGAGATC CCACCGCGCAGCTTCTT
Renilla (pmirGLO)	-	FJ376737	2227–2293	ATGCGGCGGCTGCATA GATGCGATGTTTCGCTTGGT

Quantification of changes in mRNA stability

The half-lives of the opt and wt FL-*TFRC* mRNAs were measured in the presence of 5 μ M actinomycin D. Mouse fibroblasts were plated at 65,000 cells per 3.5 cm diameter plate and transfected 8 h later. Fourteen hours after the transfections, cells were transferred into fresh iron-replete media for 2 h prior to the addition of actinomycin D. Stock actinomycin D solutions ($\sim$ 300 μ M) were prepared in DMSO and the concentration determined in dH₂O using an extinction coefficient of 21,900 at 441 nm. RNA was harvested at the indicated time points (Fig. 3D) as described for the other qRT-PCR with the exception that additional phenol-chloroform extraction and ethanol precipitation steps were included prior to the DNase treatment. RNA for each time point was quantified by qRT-PCR from three replicate plates with the exception of the 4 h wt point, for which one of the samples was lost during preparation.

Statistical analysis

Statistical significance was analyzed by a two-tailed Student's *t*-test.

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