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Position Is the Critical Determinant for Function of Iron-Responsive Elements as Translational Regulators

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At least two groups of eukaryotic mRNAs (ferritin and erythroid 5-aminolevulinate synthase) are translationally regulated via iron-responsive elements (IREs) located in a conserved position within the 5′ untranslated regions of their mRNAs. We establish that the spacing between the 5′ terminus of an mRNA and the IRE determines the potential of the IRE to mediate iron-dependent translational repression. The length of the RNA spacer rather than its nucleotide sequence or predicted secondary structure is shown to be the primary determinant of IRE function. When the position of the IRE is preserved, sequences flanking the IRE in natural ferritin mRNA can be replaced by altered flanking sequences without affecting the regulatory function of the IRE in vivo. These results define position as a critical *cis* requirement for IRE function in vivo and imply the potential to utilize transcription start site selection to modulate the function of this translational regulator.

The biosynthesis of the iron storage protein ferritin and of erythroid 5-aminolevulinic acid synthase (eALAS), an enzyme involved in the major iron utilization pathway of the human body, is translationally determined by cellular iron status (1, 6, 7, 8a, 15, 17, 28, 33, 34, 40; unpublished observations). Previous work established that the interaction between an iron-responsive element (IRE) contained in the 5′ untranslated region (UTR) of an mRNA and a specific cytoplasmic IRE-binding protein, IRE-BP, results in translational repression of the mRNA in vivo and in vitro (2, 4, 9, 36). Iron regulation of mRNA translation results from iron-dependent control of the binding activity of IRE-BP (11, 18, 24).

The presence of an IRE in the 5′ UTR of ferritin mRNA has been established as a necessary *cis* requirement for translational control of ferritin expression (4, 15). However, the conditions under which the presence of an IRE in the 5′ UTR of an mRNA suffices for iron control are less clearly defined. When the complete 5′ UTR of a ferritin cDNA was fused to human growth hormone (hGH) or chloramphenicol acetyltransferase protein-coding regions or when a synthetic oligodeoxyribonucleotide encoding a ferritin IRE was cloned into the 5′ UTR of the hGH or chloramphenicol acetyltransferase gene, expression of the indicator proteins was rendered iron responsive, suggesting that the presence of an IRE in the 5′ UTR was sufficient for regulation (1, 9, 15, 17). However, introduction of one or more nonfunctional IRE mutants between the 5′ end and the intact IRE of an hGH indicator construct renders hGH expression unresponsive to iron (9). On the basis of this finding, the position of the IRE was implicated as a possible critical determinant of IRE function. Apart from positional requirements, the regions flanking the IRE in ferritin mRNA also seemed to be functionally important (13, 38). Since this suggestion was derived from in vitro studies of IRE-BP binding to ferritin mRNA, we evaluated the contribution of the flanking regions to IRE function as a translational regulator in vivo.

In this report, we define the *cis* requirements for IRE function as a translational iron regulator in vivo and show that (i) the IRE must be located close to the 5′ terminus of the mRNA, (ii) increasing the length of the spacer between the 5′ end of the mRNA and the IRE reduces the range of iron regulation, (iii) the primary nucleotide sequence and predicted secondary structure of the spacer appear noncritical if the affinity of the IRE for IRE-BP is preserved, and (iv) sequences flanking the 5′ and 3′ ends of the IRE do not affect iron regulation mediated by the IRE, provided that the position requirement is met. Thus, for an IRE motif which can bind IRE-BP with high affinity, its position is the critical determinant for function as a translational iron regulator in vivo.

MATERIALS AND METHODS

Plasmid constructions and nomenclature. hGH indicator plasmids belonging to an F, T, or L series were used. L-series plasmids L1-GH and L3-GH have been described previously (17) and contain natural human ferritin H-chain 5′ UTR sequences linked to the hGH reporter (see Fig. 5). F-series plasmids contain a synthetic human ferritin H-chain IRE; T-series plasmids include the synthetic transferrin receptor (TfR)-C IRE from human TfR mRNA. The number in all F and T (but not L) plasmids refers to the number of nucleotides between the 5′ end of the mRNA and the first invariant nucleotide, the unpaired 5′ C residue between top and bottom helix (Fig. 1), and is used to indicate the position of the IRE (9). Constructs F17 (called Fer-GH in reference 7) and T20 (called TfR C in reference 3) have been described elsewhere. F64 and T67 were derived from F17 and T20 by insertion of a double-stranded 47-mer oligonucleotide spacer (synthesized with an Applied Biosystems DNA synthesizer) into the *Bam*HI site immediately 5′ of the IRE. The sequence and orientation of the spacer were confirmed to be 5′-GGATCAAAACAAAACAAAGGCCTCTCGAGTTC GAAGTTAACGATATCGGATCC-3′. F44, F48, and F54 were derived from F64 by removal of *Xho*I-*Bam*HI, *Xho*I-*Eco*RV, and *Hpa*I-*Bam*HI restriction fragments (see also Fig. 3), blunt ending with the Klenow fragment of DNA polymerase, and religation; the constructs were confirmed by DNA sequencing. F usl and F ΔC were derived from F64 by removal of nucleotides between the *Xho*I and *Bam*HI

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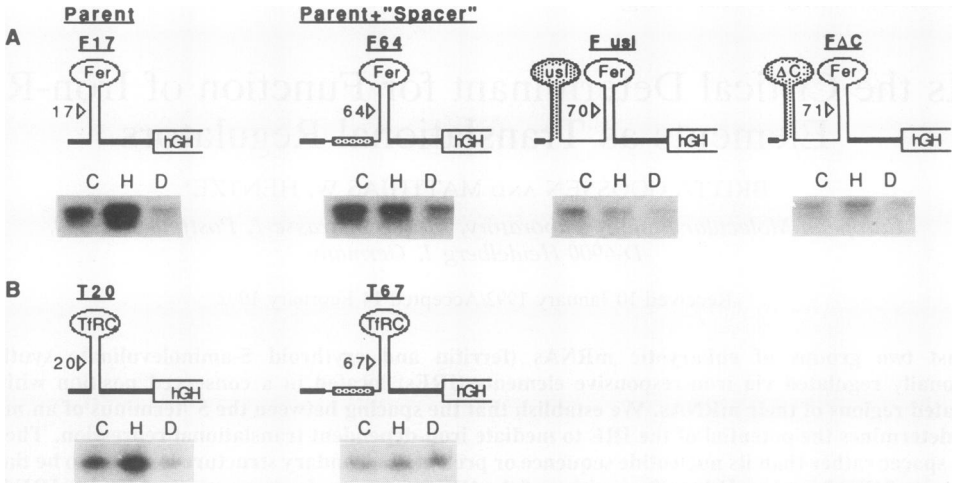

FIG. 1. Position dependence of IRE function. B6 cells were transfected and assessed for iron regulation as described in Materials and Methods. Each arrowhead indicates the position of the C residue used to assign the position of the IRE; the stippled regions (not drawn to scale) highlight the nature of the spacer inserts. Quantitatively immunoprecipitated hGH is shown. Lanes: C, untreated control cells; H, cells treated with hemin as an iron donor; D, cells treated with the iron chelator desferrioxamine. The reproducibility of the effect of cellular iron perturbations on the transfected cells was confirmed by immunoprecipitation of endogenous ferritin (not shown). (A) Transiently transfected B6 cells; (B) stably transfected B6 cells.

sites and insertion of synthetic oligonucleotides bearing appropriate linkers. The spacer sequences were confirmed to be 5′-GGATCAAAACAAAACAAAGGCCTCTCGAGT GTTCTTTGTCAAGGGACCTTGAGGGATCC-3′ for F usl and 5′-GGATCAAAACAAAACAAAGGCCTCTCGAGT GCTTCAAAGTGCTTGGACCTTGAGGGATCC-3′ for F ΔC.

Cell culture, transfections, and assessment of iron regulation. Transient expression experiments using 0.5×10^6 HeLa cells or murine B6 fibroblasts and 15 μg of plasmid DNA were performed as described previously (10). Stable transformants were generated by cotransfection of the hGH indicator plasmid with the herpes simplex virus thymidine kinase gene into murine Tk^- B6 fibroblasts and subsequent selection in Dulbecco modified Eagle medium containing hypoxanthine, aminopterin, and thymidine (HAT medium). Stable cell lines were maintained in HAT medium.

Transfected cells were incubated at 37°C for 4 h with 100 μM hemin or 100 μM desferrioxamine (Sigma, Munich, Germany), washed twice with methionine-free medium, and labeled with 20 μCi of [^{35}S]methionine (Amersham Buchler, Braunschweig, Germany) per ml for 2 h at 37°C. Iron regulation was assessed by quantitative immunoprecipitation from equal amounts of ^{35}S-labeled polypeptides with polyclonal anti-hGH antibodies (National Hormone and Pituitary Program, Baltimore, Md.) or polyclonal antiferritin antibodies (Boehringer Mannheim, Indianapolis, Ind.), analysis by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis, and autoradiography. Accurate quantitation was achieved by analysis with a Compaq Phosphor Imager equipped with Molecular Dynamics Image Quant software, version 3.0.

Generation of in vitro transcripts. A ^{32}P-labeled RNA probe of the ferritin IRE (specific activity, approximately 3.2×10^6 cpm/μg) was generated by the method of Milligan et al. (25), using cloned T7 RNA polymerase; the sequence of the DNA template for transcription of the ferritin IRE was 5′-GGGATCCGTCCAAGCACTGTTGAAGCAGGATC CCTATAGTGAGTCGTATTA-3′. Unlabeled competitor RNAs were generated from single-stranded synthetic DNA templates or from double-stranded polymerase chain reaction (PCR) products by the method described above. For ferritin IRE and ΔC IRE, the following single-stranded templates were used: ferritin IRE (as described above) and ΔC IRE (same as ferritin IRE, but with omission of the G underlined in the sequence presented above). Double-stranded DNA templates representing the 5′ UTRs of F17, F48, F64, T20, F usl, and F ΔC were generated by PCR from the corresponding plasmids used for transfections. The 5′ primer included a T7 promoter followed by 22 nucleotides including the authentic 5′ end of the corresponding mRNA (primer sequence, 5′-TAATACGACTCACTATAGGGACCC GCAGGGCCGGGGATC-3′). The 3′ primer corresponded to the opposite strand of the invariant 5′ UTR sequences preceding the hGH AUG translation start codon (primer sequence, 5′-ACCCTGAGTGGTTCGGGGAGT-3′). Synthetic DNA oligonucleotides and labeled and unlabeled in vitro transcription products were purified by gel electrophoresis (10 or 15% polyacrylamide-bisacrylamide [20:1], 8 M urea) and eluted by the crush-and-soak method (30).

Gel retardation assays. Detergent extract was prepared from HeLa cells as previously described (19). Equal aliquots of cellular extract (15 μg of protein) were incubated in a total volume of 15 μl with 20,000 cpm of probe at 22°C; 1.5% (vol/vol) 2-mercaptoethanol and unlabeled competitor RNA were added to the extract prior to the addition of probe. After 30 min, heparin (3 mg/ml) was added for 10 min. Analysis of RNA-protein complexes by nondenaturing gel electrophoresis and autoradiography was performed as previously described (18, 24).

RESULTS AND DISCUSSION

As shown in Table 1, ferritin and eALAS IREs are located 16 to 42 nucleotides downstream from the cap structure of the mRNAs. For the apparent exception listed in Table 1 (26), it is unclear whether the reported cDNA sequence

TABLE 1. Conserved position of IREs in ferritin and eALAS mRNAs[a]

Protein	Sequence:	Position[b]	Reference
Ferritin			
Human H chain	G UUUCCUG C UUCAA CAGUGC UUGGA CGGAACCC	34 (−42)	5, 16
Human L chain	G UCUCUUG C UUCAA CAGUGU UUGAC GAACAGAU	33	31
Rat H chain	G UUUCCUG C UUCAA CAGUGC UUGAA CGGAACCC	36	27
Rat L chain	G UAUCUUG C UUCAA CAGUGU UUGGA CGGAACAG	37	23
Mouse H chain	G UUUCCUG C UUCAA CAGUGC UUGAA CGGAACCC	36	22, 39
Chicken H chain	G GUUCCUG C GUCAA CAGUGC UUGGA CGGAACCG	39	35
Xenopus laevis H chain	A GUUCUUG C UUCAA CAGUGU UUGAA CGGAACCC	36	21
	A GUUCUUG C UUCAA CAGUGU UUGAA CGGAACCT	174	26
Rana H chain	A GUUCUUG C UUCAA CAGUGU UUGAA CGGAACCC	31 (−42)	8
eALAS			
Human	C AUUCGUU C GUCCU CAGUGC AGGGC AACAGGAC	16	6
Murine	C UUUGGUU C GUCCU CAGUGC AGGGC AACAGGAC	16	32

[a] Nucleotide sequences and positions of 11 IREs from six different species and three different mRNAs are listed. Only IREs from mRNAs with known transcription start sites were selected. Invariant sequence characteristics of the IREs are in boldface.

[b] Number of nucleotides from the experimentally determined transcription start site to the 5'-most invariant unpaired C residue of the IRE (shown in boldface).

exactly corresponds to the ferritin mRNA and whether expression of that mRNA would be regulated by iron (note that an independent isolate of the same cDNA contains a cap-proximal IRE [21]). The evolutionary conservation of IRE position and previously reported experimental results (9) suggested that IRE function was position dependent. To address this question in detail and to evaluate the generality of the position effect, three RNA spacers of similar lengths but different sequences and predicted secondary structures were introduced between the 5' end and the IRE of F17 mRNA to create F64, F usl, and F ΔC, respectively. Figure 1A demonstrates that iron regulation of F17 is affected by introduction of a ΔC IRE spacer (construct F ΔC) (a result expected from our previous findings [9]), by an unrelated stem-loop secondary structure spacer with a similar stability but otherwise unrelated to an IRE (construct F usl), or by an unstructured spacer (construct F64). To extend these results further and to establish that the position effect is not an unusual property of the particular IRE contained in the F-series constructs, we also evaluated the effect of the unstructured spacer (contained in F64) on translational regulation mediated by a TfR mRNA-derived IRE. Consistent with previous observations (3), the range of iron regulation (H [hemin]/D [desferrioxamine] ratio) conferred by the TfR IRE (T20) was smaller than the H/D ratio of construct F17 (Fig. 1); the reason for this difference is unknown. As predicted by the response of F17 to spacer insertion (F64), iron regulation of T20 is completely abolished in derivative T67. The data shown in Fig. 1 are representative of several independent experiments in transiently and stably transfected murine B6 fibroblasts and transiently transfected HeLa cells. We noted that the relative quantitative effect of spacer insertions was variable, and HeLa cells appeared to be less sensitive to the position effect than were B6 cells (not shown). Interestingly, in each experiment, the three different spacer constructs gave consistent results regarding the magnitude of the position effect. Thus, cell type- or growth condition-dependent mechanisms which can diminish or enhance the position effect may exist. In summary, we conclude that increasing the distance between the mRNA cap structure and the IRE affects IRE function irrespective of the primary sequence or the secondary structure of the spacer and independent of the IRE used as a translational regulator.

These results permitted two opposing mechanistic interpretations. Either IRE function was affected by spacer insertion because the spacers interfered with binding of IRE-BP to the IRE, or an IRE/IRE-BP complex could form but failed to repress translation. The finding that different types of spacers had similar effects and that both ferritin and TfR IREs were similarly affected by spacer insertion argued against the first interpretation. To address this critical question experimentally, we analyzed binding of HeLa cell IRE-BP to the IREs in their natural context within the different constructs by gel retardation assays. We devised a PCR-aided approach (Fig. 2A) to ensure faithful representation of the 5' UTRs of the actual hGH indicator mRNAs by the in vitro transcripts used in the gel retardation assays. Figure 2B confirms that the assay detects differences in the IRE-binding affinities between an IRE which can confer Fe regulation in vivo (feIRE; lanes 5 to 8) and a point mutant, ΔC IRE, which cannot (lanes 1 to 4) (14, 29). As shown in Fig. 2C, IRE-BP binding to the structurally intact IREs contained in the 5' UTRs of F64 (lanes 6 to 10) and F ΔC (lanes 16 to 20) transcripts is comparable to the binding seen for parent F17 (lanes 1 to 5). F usl RNA (lanes 11 to 15) repeatedly competed somewhat more strongly for IRE-BP than did the other three transcripts. However, such a possible difference in apparent affinity does not correlate with the similar translational phenotypes of F64, F usl, and F ΔC seen in Fig. 1. All competitor transcripts were gel purified to exclude the possibility that shorter, nonrepresentative transcripts participated in the competition analysis. Consequently, we suggest that IRE position determines IRE function by modulating the efficiency of translational repression by a high-affinity IRE/IRE-BP complex.

An experimental strategy complementary to the approach used for Fig. 1 and 2 was used for the analyses shown in Fig. 3 and 4. In these assays, instead of changing the sequences and structures of spacers with comparable lengths, we varied the position of the IRE in small increments by using internal unique restriction sites within the unstructured F64 spacer (Fig. 3A). The results shown in Fig. 3B demonstrate that the range of iron regulation (the H /D ratio) decreases as the distance of the IRE from the 5' end of the indicator mRNA increases. The regulation of ferritin expression was determined as an internal control and showed only minor variations. The observed position effect is confirmed when hGH regulation is corrected for differences in ferritin regulation (Quant. corr. in Fig. 3B). Similar results were obtained

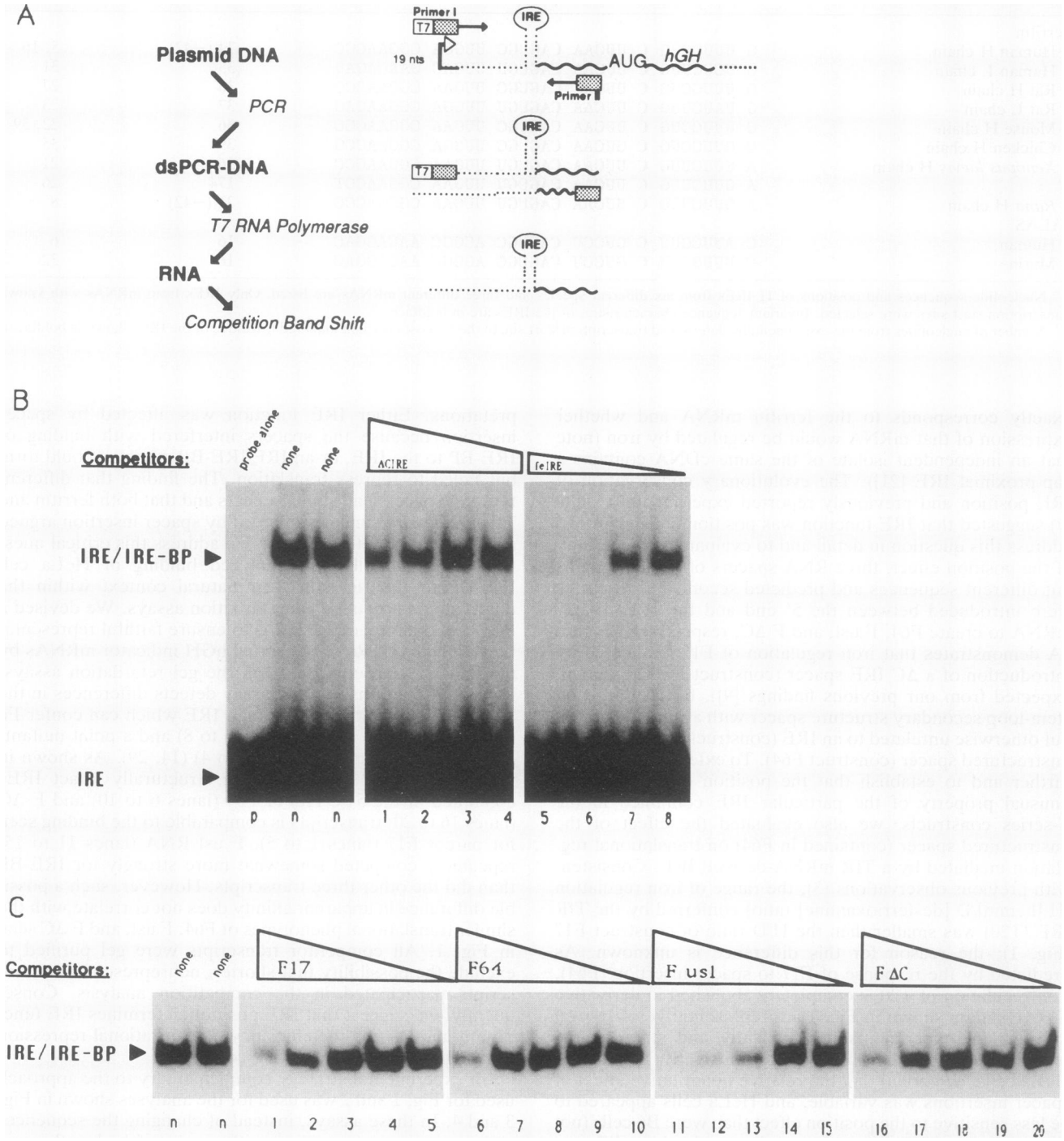

FIG. 2. Equal binding of IRE-BP to IREs in constructs analyzed in Fig. 1. (A) Schematic representation of the PCR-aided method to generate competitor transcripts used for panel C and Fig. 4 (see also Materials and Methods). dsPCR-DNA, PCR-amplified double-stranded DNA; nts, nucleotides. (B and C) Analyses of 15 μg of detergent cell extract in the presence or absence of unlabeled competitor transcripts with a ^{32}P-labeled IRE probe as described in Materials and Methods. Arrowheads indicate the positions of specific IRE/IRE-BP complexes or unbound IRE probe. (B) Competitors were generated as described by Milligan et al. (25). The competitor RNA concentrations are 500, 300, 30, and 3 ng per assay for ΔC IRE (lanes 1 to 4) and 3, 0.6, 0.12, and 0.024 ng per assay for feIRE (lanes 5 to 8). (C) Competitors were generated as illustrated in panel A. Competitor transcript concentrations were 45 ng per assay for F17 (lane 1), 65 ng per assay for F64 (lane 6), and 70 ng per assay for F usl (lane 11) and F ΔC (lane 16) for molar adjustment of size differences; the decrement is by a factor of 5. The region of the gel showing excess free probe was cut off.

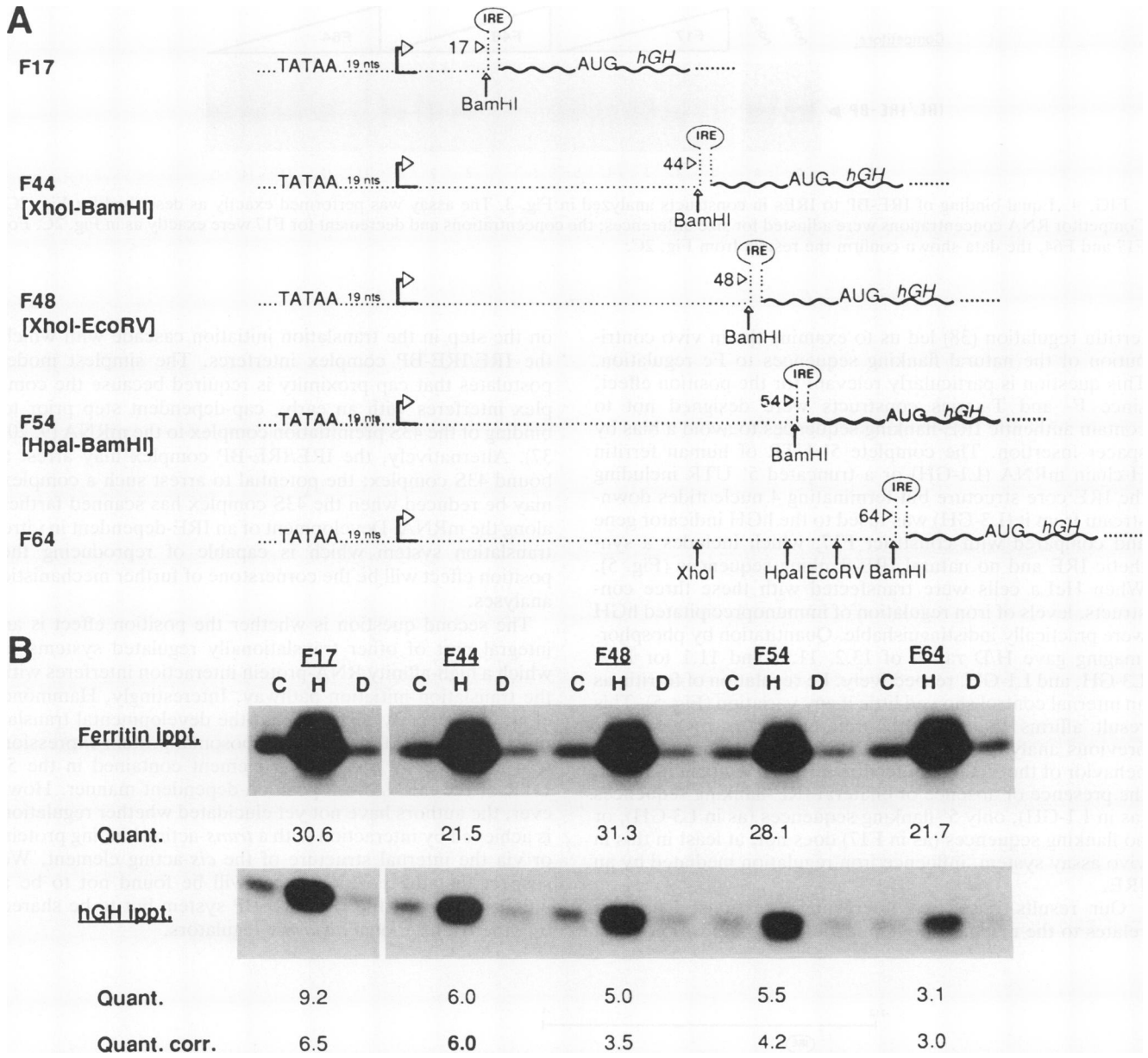

FIG. 3. Evidence that increasing the distance of the IRE from the 5′ end of the mRNA negatively affects IRE function. (A) Constructs used. F44, F48, and F54 were derived from F64, by using unique restriction sites within the F64 spacer. The restriction fragments shown in parentheses were removed, and the resulting plasmid was religated. nts, nucleotides. (B) Assessment of iron regulation of the constructs in transiently transfected HeLa cells, performed as described for Fig. 1. Cells were untreated (lanes C) or treated with hemin (lanes H) or desferrioxamine (lanes D). Accurate quantitation of iron regulation was achieved by using the Compaq Phosphor Imager equipped with Molecular Dynamics Image Quant software, version 3.0. Data represent the range of iron regulation between the H and D points (Quant.). Values for iron regulation of hGH expression corrected for the range of regulation of endogenous ferritin as an internal control are also given (Quant. corr.). Similar results were obtained from transiently or stably transfected B6 fibroblasts (not shown). ippt., immunoprecipation.

from transiently or stably transfected murine B6 fibroblasts (not shown). The data do not allow us to postulate a linear, inverse relationship between the cap distance of the IRE and the range of iron regulation. Conceivably, unrecognized higher-order RNA structures might contribute to the observed effect (hence referred to as position effect rather than distance effect). As demonstrated in Fig. 1, a contribution of experimentally designed secondary structure was not apparent when F64, F usl, and F ΔC were compared.

When tested by the PCR-aided method, the 5′ UTRs of F17, F48, and F64 display, as expected, similar IRE-BP binding characteristics (Fig. 4). These findings confirm and extend the biological scope of the position effect: minor changes in ferritin mRNA transcription start sites (8) could serve to short-circuit or modulate the function of the IRE/IRE-BP system. Similarly, expression systems utilizing the regulatory capacity of the IRE should attempt to introduce the IRE in a cap-proximal position of the target mRNA.

Suggestions that an integrated structure consisting of the ferritin IRE and its natural flanking sequences participated in

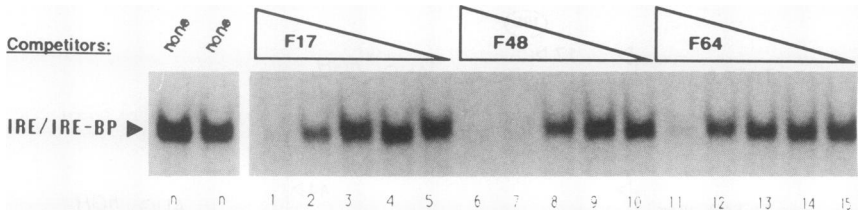

FIG. 4. Equal binding of IRE-BP to IREs in constructs analyzed in Fig. 3. The assay was performed exactly as described for Fig. 2C. Competitor RNA concentrations were adjusted for size differences; the concentrations and decrement for F17 were exactly as in Fig. 2C. For F17 and F64, the data shown confirm the results from Fig. 2C.

ferritin regulation (38) led us to examine the in vivo contribution of the natural flanking sequences to Fe regulation. This question is particularly relevant for the position effect, since F- and T-series constructs were designed not to contain authentic IRE-flanking sequences to avoid a bias by spacer insertion. The complete 5′ UTR of human ferritin H-chain mRNA (L1-GH) or a truncated 5′ UTR including the IRE core structure but terminating 4 nucleotides downstream from it (L3-GH) was fused to the hGH indicator gene and compared with construct F17, which includes a synthetic IRE and no natural IRE-flanking sequences (Fig. 5). When HeLa cells were transfected with these three constructs, levels of iron regulation of immunoprecipitated hGH were practically indistinguishable. Quantitation by phosphorimaging gave H/D ratios of 13.2, 11.1, and 11.1 for F17, L3-GH, and L1-GH, respectively. Fe regulation of ferritin as an internal control showed little if any variation (Fig. 5). This result affirms that F17, the reference construct for our previous analyses (Fig. 1 and 3), faithfully reproduces the behavior of the 5′ UTR of ferritin mRNA. We conclude that the presence or absence of bilateral IRE-flanking sequences (as in L1-GH), only 5′-flanking sequences (as in L3-GH), or no flanking sequences (as in F17) does not, at least in this in vivo assay system, influence iron regulation mediated by an IRE.

Our results raise two interesting questions. The first relates to the mechanistic implications of the position effect on the step in the translation initiation cascade with which the IRE/IRE-BP complex interferes. The simplest model postulates that cap proximity is required because the complex interferes with an early, cap-dependent step prior to binding of the 43S preinitiation complex to the mRNA (9, 20, 37). Alternatively, the IRE/IRE-BP complex may arrest a bound 43S complex; the potential to arrest such a complex may be reduced when the 43S complex has scanned farther along the mRNA. Development of an IRE-dependent in vitro translation system which is capable of reproducing the position effect will be the cornerstone of further mechanistic analyses.

The second question is whether the position effect is an integral part of other translationally regulated systems in which a high-affinity RNA-protein interaction interferes with the translation initiation pathway. Interestingly, Hammond et al. (12) recently reported that the developmental translational regulation of mouse S16 ribosomal protein expression was mediated by a *cis*-acting element contained in the 5′ UTR of the mRNA in a position-dependent manner. However, the authors have not yet elucidated whether regulation is achieved by interaction with a *trans*-acting binding protein or via the internal structure of the *cis*-acting element. We suspect that the position effect will be found not to be a unique feature of the IRE/IRE-BP system but to be shared by other translational *cis*/*trans* regulators.

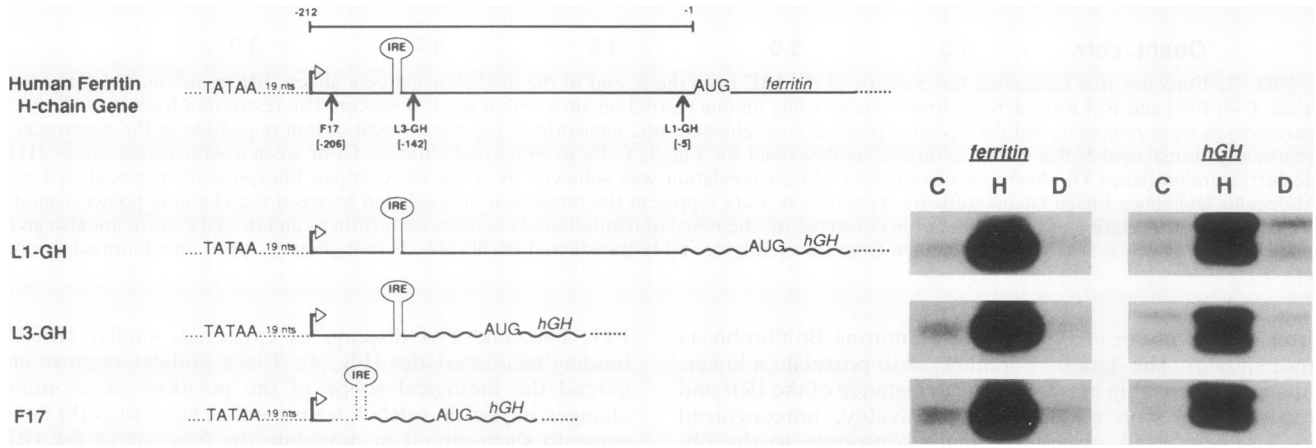

FIG. 5. Evidence that human ferritin H-chain IRE-flanking sequences do not participate in iron regulation in vivo. Iron regulation of a construct including almost the entire natural 5′ UTR of human ferritin H-chain mRNA (L1-GH) was compared with that of construct L3-GH, which exhibits a deletion of almost all natural ferritin sequences 3′ from the IRE, and with F17, which contains virtually no natural 5′ UTR ferritin sequences. Iron regulation of hGH and endogenous ferritin expression in transiently transfected HeLa cells were assessed as described for Fig. 1. Cells were untreated (lanes C) or treated with hemin (lanes H) or desferrioxamine (lanes D). nts, nucleotides.

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