Journal of Cellular Biochemistry 44:107–115 (1990)

Iron Regulation of Ferritin Gene Expression

Hamish N. Munro

USDA Human Nutrition Research Center on Aging at Tufts, Boston, Massachusetts 02111; Division of Toxicology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

Ferritin is a ubiquitous iron-storage protein found in the cells of animals, plants, molds, and bacteria which it protects from toxic intracellular levels of iron. Ferritin stores iron within a hollow protein shell formed by subunits of two types, H and L. The 5′ untranslated regions of the two subunit mRNAs contain an almost identical 28-nucleotide sequence which regulates translation by binding to a specific cell sap protein. When cell iron level is low, this repressor protein obstructs translation of stored ferritin mRNAs, whereas increased iron levels release this protein, thus permitting extensive ferritin subunit synthesis to respond rapidly. Similar motifs in the 3′ untranslated region of transferrin receptor mRNA interact with this protein to regulate breakdown of the mRNA and thus change the receptor population. Finally, transcription of the H and L genes can be independently increased by iron and other factors. In the case of iron, synthesis of the L-mRNA is increased preferentially since ferritin shells with a preponderance of L-subunits store iron more efficiently. Thus regulation of ferritin synthesis at the translational and transcriptional levels and by transferrin receptor mRNA abundance at the level of breakdown provide a coordinated mechanism for protecting cells against the effects of excess iron.

Key words: iron regulation, iron storage, ferritin, H and L subunits, mRNA, translational control, transcriptional control, transferrin receptor

In the mid-1970s two experiences focussed my attention on the importance of trace metals. First, I became chairman of a National Academy of Sciences committee to revise the Recommended Dietary Allowances for some 40 nutrients. Our report [1] provided not only tentative requirements for a number of trace elements but also included levels of intake likely to be toxic if habitually exceeded. This precaution can be related to my second relevant experience of this period, namely, exploration of the role played by the iron storage protein ferritin in protecting cells against excessive intracellular levels of iron. In response to iron administration to rats, synthesis of liver ferritin was largely accelerated by translational control of mRNA availability through a

Abbreviations: IRE, iron responsive element; IRE-BP, IRE-binding protein.

Received May 31, 1990; accepted June 11, 1990.

novel iron-responsive mechanism [2], an observation subsequently confirmed and extended with the more powerful techniques of molecular biology as described below. Thus we enter the last decade of this century with a clear indication that the molecular biology of the trace metals can lead to a better understanding of the basis of their long-term toxic actions and the mechanisms which living organisms have developed to protect themselves.

FERRITIN STRUCTURE AND GENE REPRESENTATION

As reviewed elsewhere [3], ferritin occurs in the cells of animals, plants, molds, and bacteria, where it protects cell structures against peroxidation from excessive levels of iron. The iron is stored within the protein shell of ferritin, which is fabricated in vertebrates from 24 subunits of two types, H ($\sim$21,000 M_r) and L ($\sim$19,000 M_r), with expression of a third subunit in the bullfrog. The proportions of H and L subunits vary between molecules even in the same cell. Isoferritins rich in the L-subunit appear to favor deposition of iron within the shell, while those rich in H subunits occur in the contractile cells of the heart and are seen in cells during differentiation. The advantage of having ferritin rich in H-subunits under these latter circumstances remains obscure.

Figure 1 (upper diagram) displays the general features of the H and L mRNAs of the rat. Although the open reading frames of H and L agree in only 55% of their amino acid sequences, Chou and Fasman analysis [4] suggests that they have the same five alpha helices, thus ensuring that H and L subunits can be used interchangeably to construct the ferritin protein shell. This retention of a common secondary structure suggests that the H and L subunits arose from a single ancestral gene, a conclusion strengthened by examination of the structure of the expressed H and L genes (see below). In addition to these features of the open reading frames, attention is drawn to the long 5′ untranslated regions of both H and L mRNAs. There is little homology between the 5′ untranslated regions of these two mRNAs except near the cap region, where the same 28-nucleotide sequence is present in both H and L mRNAs. The same motif is also present in the H and L mRNAs of human, rabbit, mouse, chicken, and frog [3], indicating that this sequence must have a special function to be conserved during evolution.

Figure 1 (lower diagram) also shows the expressed genes for H and L in the rat genome. Although the rat DNA contains some 20 copies of the L-sequence and 5 of the H-sequence, only one gene appears to be expressed for each of the subunits, the other gene copies being processed pseudogenes. The intron-exon patterns of these two genes are interesting. Both display four exons (thick lines), three of which correspond to the first three alpha-helices while the fourth includes the fourth alpha-helix and also the short fifth helix. These exons for H- and L-subunits show a close relationship in size, especially the two middle exons which are identical in nucleotide length. Based on similar data for the mouse, human and chicken [3], this is also true across species. Thus these observations confirm the common origin of the two subunits during the course of evolution.

REGULATION OF FERRITIN SYNTHESIS IN RESPONSE TO IRON

Regulation of mRNA populations can be achieved either by changes in transcription, by altering the rate of breakdown of mRNA, or by varying the availability of

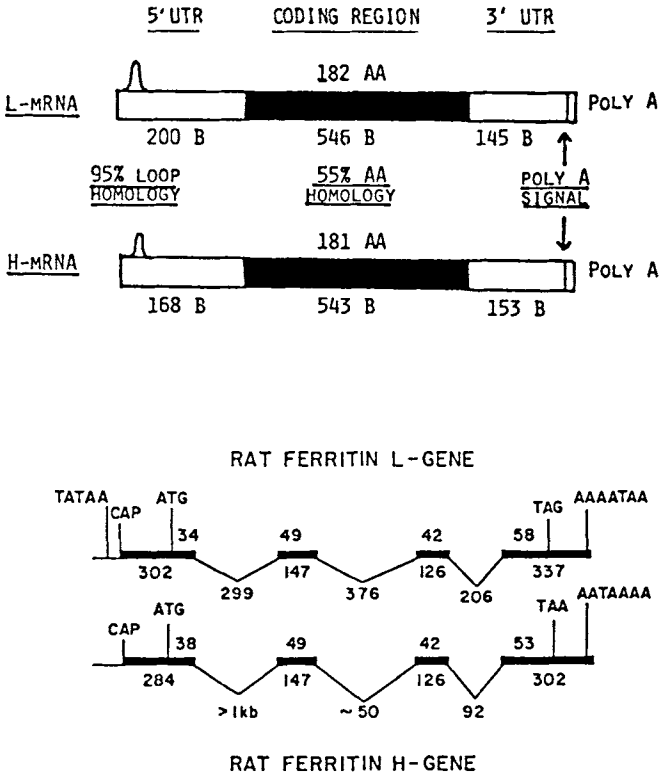

Fig. 1. **Upper diagram:** Comparison of the number of nucleotide bases (B) and amino acids (AA) of the coding regions of rat ferritin L- and H-subunit mRNAs. Also shown are the features of the 5′ and 3′ untranslated regions with a loop and stem structure at the same position in the 5′ regions of both mRNAs. **Lower diagram:** Comparison of the genomic structures of the expressed rat ferritin L- and H-subunit genes. The exons are indicated by thick bars and the introns by thin lines. Note the close coincidence of the lengths of the corresponding H and L exons but not the introns. The location of the TATA box, initiating methionine codon (ATG), termination codons (TAG, TAA), and polyA polymerase (AATAA) are indicated. (Reprinted with permission from Munro et al. (1988) Hemochromatosis 526:117, 121, Annals of the New York Academy of Sciences, New York.)

pre-formed mRNA for translation. We begin with translational control of ferritin subunit synthesis from mRNA since the mechanism is novel and furthermore provides the best-authenticated example of translational regulation.

Translational Control of Ferritin Synthesis

When a large dose of iron is injected into a rat, there is a transient increase in synthesis of ferritin protein in the liver [5]. Since this response is not suppressed by prior administration of actinomycin D, it was attributed to activation by iron of a store of inactive ferritin mRNAs [2,5]. In 1976, we [2] confirmed this speculation by finding a large pool of ferritin mRNAs in the liver cell sap of the untreated rat. Iron administration caused this pool to be translocated to the polyribosome fraction of the cells for active translation. It was suggested that administered iron activates a mechanism that removes a specific protein blocking translation of ferritin mRNA when the iron

content of the liver is below a critical level. The result of increasing cellular iron above the critical level is to activate ferritin mRNA translation in an attempt to control toxic actions of the iron.

With the advent of the era of molecular biology, we set about exploring this mechanism for regulating translation in response to changes in the supply of a specific nutrient (Fig. 2). First, we confirmed the presence of a pool of H and L ferritin mRNAs lying dormant in the liver cell sap by separating the postmitochondrial fraction of liver homogenates on sucrose gradients into fractions which were tested by Northern blotting for H and L mRNA content [6]. In rats not receiving iron, most of the two species of ferritin mRNA was found in the free mRNA fraction of the gradient, whereas within a few hours of giving iron to rats, this pool was nearly empty and the polyribosome fraction showed extensive deposits of these two mRNAs. A similar series of events followed iron treatment of rat hepatoma cells in tissue culture [7]. In this case, hemin was also used as an iron source and caused a considerable transfer of ferritin mRNAs from the inactive status to the polyribosomes. The presence of the iron chelator desferrioxamine in the medium prevented the response to hemin, but this was not the case for another chelator, diethylaminetriamine penta-acetic acid (DPTA), which does not cross cell walls. It was concluded that

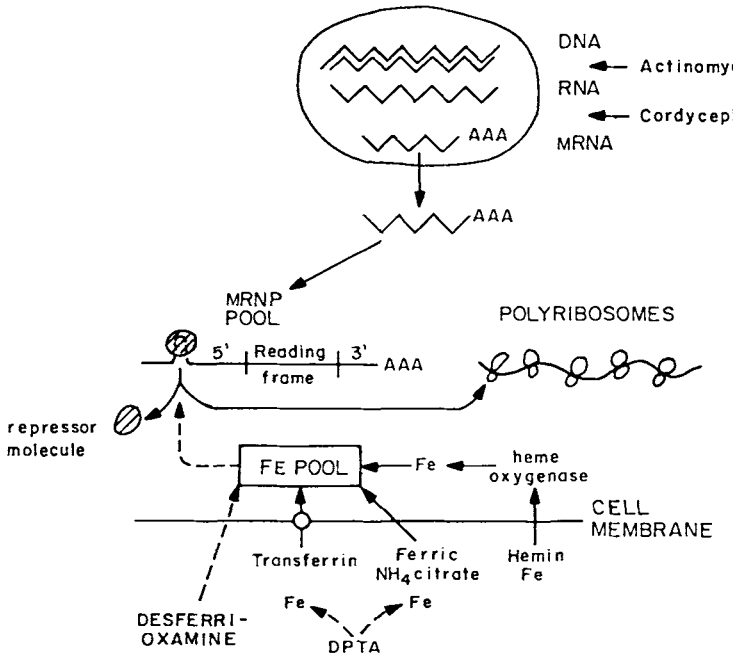

Fig. 2. Factors involved in regulation of ferritin subunit mRNAs. These include the supply of mRNA from the nucleus, segregation of inactive ferritin mRNA, and its release for polyribosome function when excess iron enters the cell. This is determined by the effect of the entering iron on the free iron pool which can be restricted by chelation with desferrioxamine which enters the cell, but not with DPTA, which cannot enter. Note that iron can come from transferrin, inorganic salts, or hemin, the latter through the action of heme oxygenase. (Reprinted with permission from Munro et al. (1988) Hemochromatosis 526:117, 121, Annals of the New York Academy of Sciences, New York.)

hemin acts as a source of free iron released from the hemin by the action of heme oxygenase. Other opinions in the literature [8] claim that heme is itself the immediate form of iron controlling ferritin mRNA translation. Based on a recent series of experiments [9], we conclude that heme is not the major regulator.

As discussed earlier, the 5′ untranslated regions of both H and L mRNAs carry almost identical 28-nucleotide sequences (iron-responsive elements, IRE) which are conserved over a wide range of animal species, thereby suggesting a specific function which could be involved with availability of the stored mRNAs. In order to test this hypothesis, constructs were made [10] with the open reading frame of the ferritin L-subunit replaced by that of the bacterial enzyme chloramphenicol acetyl transferase (CAT). This chimera and deletion constructs were then introduced into rat hepatoma cells in culture by retrovirus-mediated gene transfer. When the medium of the culture was enriched with iron, cells containing the complete chimera responded with increased synthesis of CAT, whereas those receiving a chimera lacking the first 67 nucleotides of the 5′ untranslated region, including the 28-nucleotide sequence, lost the ability to regulate CAT output in response to iron. Klausner and colleagues [11] report experiments with the human H-subunit that are in agreement with this finding.

As discussed in further detail later, the 28-nucleotide sequence serves as the *cis*-element or IRE which is recognized by a cytoplasmic protein, the *trans*-element or IRE-binding protein. Computer analysis shows that this area of the 5′ untranslated region can form a stem-loop structure in which six nucleotides (CAGUGU for the L mRNA, CAGUGC for the H-mRNA) provide the single-stranded loop while the remaining nucleotides form the double-stranded stem. Klausner et al. [12] report various experimental mutations in this structure, some of which alter its capacity to regulate in response to iron. We [13] have also examined the relationship between the secondary structure of the stem-loop and its structural tolerance in binding the repressor protein. First, the two-dimensional folding of the sequence containing the conserved 28-nucleotide segment was projected in the form of a stem-loop motif using a recent computer program (Fig. 3). Then the same segment of 5′ RNA was subjected to attack by specific restriction enzymes that act on single-stranded RNA (dotted line arrows in Fig. 3), or on double-stranded RNA (solid arrows in Fig. 3). This analysis confirmed that the computer program reflected the single stranded/double stranded pattern identified by the enzymes. The response to variations in this basic sequence was evaluated by competition curves between the native sequence and the mutant sequence in a protein-binding assay. In this way, it was shown that reversing the loop CAGUGU reduced binding capacity 50-fold, while deletion of GUG from the loop also caused a large reduction in protein-binding capacity. Binding capacity was also diminished 50-fold by inserting an additional CA across the base of the loop, thus increasing its size or by adding the nucleotide pair CG into the stem adjacent to the loop, thus increasing the stem length. In contrast, substitution of G for the last U of the loop had little effect on protein binding, in agreement with the change over a range of species in the same U of the L-subunit for a C in the H-subunit sequence which performs as well as the L-subunit motif. Next, attention was turned to the stem. An experiment was performed in which the first three stem nucleotides on one side were replaced by nucleotides not capable of forming a double strand. The loop therefore gained a number of nucleotides; this enlarged structure was no longer able to recognize the binding protein efficiently. However, if the original nucleotides of the complementary strand of the stem were now replaced by nucleotides compatible with

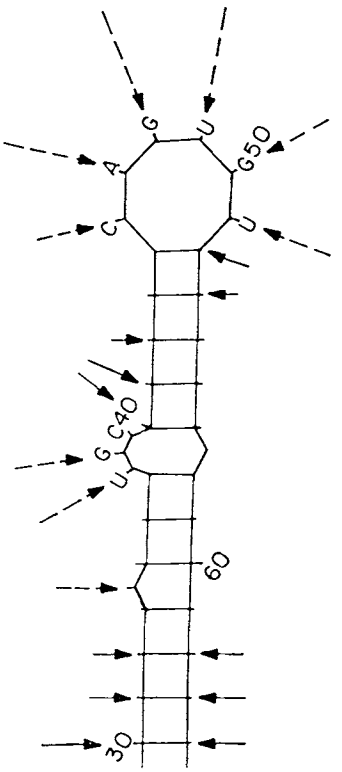

Fig. 3. Structure map of the 28-nucleotide sequence (IRE) in the 5′ untranslated region of the rat L-ferritin mRNA. The figure summarizes experimentally determined sites of single-strand enzymic cleavage (broken arrows) and double-strand cleavage (solid arrows). The numbers refer to nucleotide locations downstream from the cap. The conserved 28-nucleotide sequence (IRE) begins at nucleotide 35 and extends to nucleotide 62.

forming a double strand with the first set of substituted bases, binding capacity was restored even though the double-stranded area no longer had the sequence of the stem in the native motif. Finally deletion of an unpaired C in the stem resulted in a several-hundred-fold reduction in binding, indicating that the distortion of the stem by this unpaired nucleotide probably provides a mandatory structural recognition feature involving the tertiary profile of the stem-loop area. On the other hand, in another publication [14] we show that substitution of A for C at this point does not have this drastic effect on regulatory protein binding, thus supporting the conclusion that deletion of a nucleotide must be having an effect on tertiary structure, whereas substitution by another nucleotide does not necessarily have this consequence. Finally, it has been demonstrated [15–17] that the mRNA for the transferrin receptor includes five copies of a similar stem-loop motif in its 3′ untranslated region and that the same cytoplasmic protein binds to these, in this case regulating mRNA breakdown. Thus iron metabolism is under two sites of regulation involving the same motif, namely, ferritin control by translation and transferrin receptor abundance through rate of breakdown. Each of these contributes to defending the cell against the toxic effects of excessive iron.

The repressor protein that functions as a *trans*-acting factor binding specifically to the 28-nucleotide *cis*-element (IRE) in the 5′ untranslated region of both the L-mRNA and H-mRNA of ferritin was next investigated [18]. With the aid of gel retardation of ^{32}P-labeled 5′ untranslated RNA to detect complexes formed with protein in cell sap from rat liver, heart and hepatoma cells, it was found that two such complexes could be identified in the case of rats not given iron. After the donor rat or cells in culture had been given iron, one of these complexes diminished or disappeared, whereas the other binding protein appeared to increase, especially noticeable in the cells in culture. The ^{32}P-labeled RNA that was bound to both of these complexes was then shown to include the 28-nucleotide motif. Following UV-cross-linking the binding protein was estimated to be 87 kDa in size. Other investigators [19–21] have subsequently confirmed that a protein of this magnitude is the specific repressor protein and one group using gel retardation have found two such proteins present in mouse cells [22] but not in samples from other species (rat was not included).

Klausner and colleagues [23] have examined the specific binding protein in human cells and have shown by Scatchard analysis that it exists in two states, one having high affinity for the 28-nucleotide motif (K_d 10–30 nM), whereas the other has a low affinity (K_d 2–5 nM). Changes in oxidation-reduction state of the SH groups on the protein are associated with changing the low affinity (oxidized) form to the high affinity (reduced) form and vice versa. This switching of affinities and of SH groups is responsive to iron excess and conversely to iron depletion by chelators; the mechanism of the response to available iron is unclear. We [14] have applied well established quantitative receptor-ligand binding methods to identify the characteristics of three interconverting cytoplasmic pools of the binding protein (Fig. 4). First, at high cell concentrations of iron, a pool of low affinity inactive protein (R_i) accumulates, accompanied by release of ferritin mRNA which now becomes free for translation. The SH groups of this low-affinity form are oxidized but become reduced when the iron content of the cell diminishes. The protein now assumes a stable high affinity form (R_a*), and is available to interact with the 28-nucleotide sequence of the ferritin mRNAs or other mRNAs bearing the same motif. This forms a third pool, namely, the endogenous complex of protein and mRNA. The reduced form with high affinity can also be generated in vitro by adding beta-mercaptoethanol to the oxidized form of the protein, but the product apparently lacks a covalent modification which stabilizes it in vivo. This implies that another in vivo factor must be involved in the stabilization of the active form of binding protein.

Transcriptional Control of Ferritin

Translational control of ferritin synthesis involves utilization of dormant mRNAs for H and L subunits present in the cytoplasm. Both mRNAs have similar IRE motifs and thus the relative amounts of the two subunits formed as a result of activation should reflect the proportions of the two mRNAs stored in the cytosol. However, a single injection of iron to rats results in a preferential increase in L-subunits which form ferritin protein shells rich in this subunit [24]. Such L-rich shells are associated with a greater capacity to take up iron [24]. White and Munro [25] have undertaken a study of the contributions of transcription and translation to the impact of iron on ferritin synthesis. Run-off assays of transcription rate showed that a large dose of iron

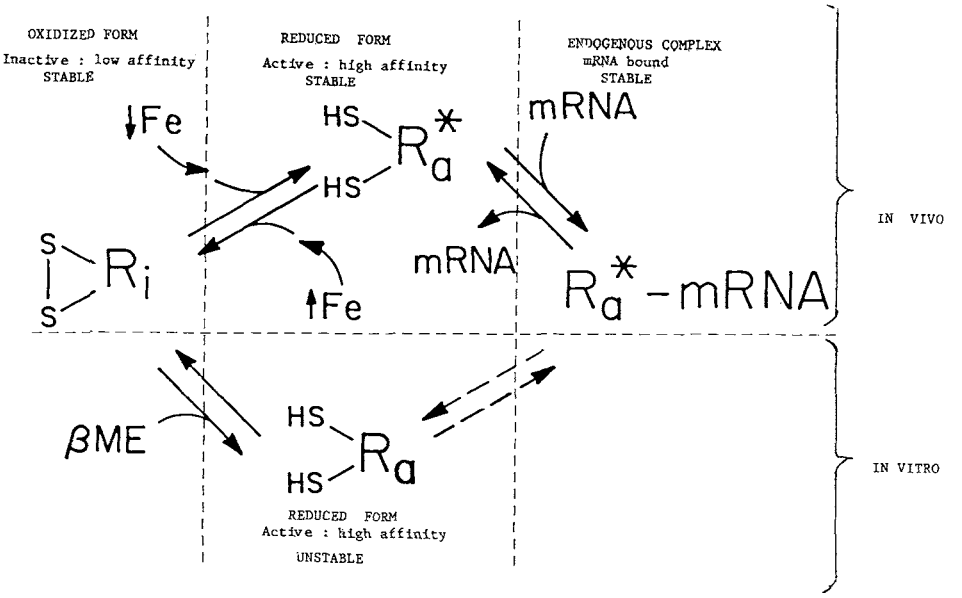

Fig. 4. Scheme for interconversions of the pools of IRE-binding protein (IRE-BP) in response to cytosolic levels of iron. When the iron content of the cytosol is high, IRE-BP occurs as an inactive form (R_i) with low affinity for the IRE motif, thus allowing ferritin subunit synthesis to proceed. The sulfhydryl groups of R_i are in the oxidized state. In response to a reduction in cytosolic iron level, the R_i can be converted in vivo to a high affinity active protein (R_a*) with reduced sulfhydryl groups. Since this form binds tightly to the 28-nucleotide IRE, it prevents translation of the ferritin mRNAs and is thus converted to the third pool, the endogenous complex made up of R_a* and mRNA. Note that by adding thiol reductants the first pool (R_i) can also be converted in vitro to an active form (R_a) which however differs from Ra* in its instability. (Diagram kindly provided by Dr. Hugh A. Barton.)

given to rats caused an increase in liver cell transcription of L-mRNA. It was maximal at 2 hr after iron dosage, whereas the transcription rate of the H-mRNA was not significantly increased by the iron treatment. This preferential acceleration in L-mRNA synthesis showed up next in the ribonucleoprotein pool of messages which eventually caused an increase in the L/H ratio in the polyribosome-associated messages. Thus the effects of iron on transcription and translation provide a mechanism for generating a rapidly increasing pool of subunits with the added flexibility of changing the ratio of L to H subunit populations.

PROSPECTS FOR FUTURE RESEARCH

Inorganic trace elements are a class of nutrients, many of which are not only required for normal cell function but also carry the hazard of toxic actions at excessive intakes. Iron represents an extensively studied inorganic element that, taken in excess, can damage cells by peroxidation, against which elegant control mechanisms have evolved. The basis for this protective action involves at least two processes responsive to a shared common motif located in the 5′ or 3′ untranslated areas of mRNA; these processes are, first, storage of excess iron in ferritin and, second, exclusion of iron uptake into cells through reduction in abundance of receptors for iron-charged

transferrin. The mechanisms have now been worked out in considerable detail, but a number of questions remain to be resolved. Although there is evidence that ferritin shells rich in L-subunits favor iron storage, it is still unclear what function isoferritins mostly made from H-subunits perform except to note that cell differentiation increases the abundance of H-mRNA. Another area likely to expand is the regulation of abundance of the trans-acting repressor protein binding to the iron-responsive sequence, which may also turn out to be responsive to iron at the transcription level. Ultimately, the details of regulatory mechanisms involving different inorganic elements will differ, while an understanding of how they serve the needs of the organism depends on a knowledge of the relevant physiological functions.

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