Plant Physiol. (1994) 104: 263–270

Posttranscriptional Regulation of Ferritin during Nodule Development in Soybean[1]

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During soybean (*Glycine max*) nodule development, induced ferritin mRNA concentration remains elevated while the protein concentration decreases 4- to 5-fold (M. Ragland and E.C. Theil [1993] Plant Mol Biol 21: 555–560). Investigation of posttranscriptional regulation of nodule ferritin during development showed that ferritin mRNA was efficiently translated based on polyribosome size in vivo, protein synthesis (0.8% of total protein) in vitro, and protein synthesis in intact nodules. Ferritin, a plastid protein, was processed in both immature and mature nodules. In chimeric mRNA, soybean ferritin mRNA sequences blocked the function of the iron regulatory element (IRE), the *cis* regulatory element of animal ferritin mRNA; the IRE regulates chimeric animal mRNAs. The absence of translational regulation of ferritin in plants contrasts with ferritin regulation in animals. Thus, ferritin regulation has diverged during evolution, whereas structure of the mature protein has been conserved. Ferritin in mature soybean nodules is apparently regulated after translation, possibly in analogy with such plastid proteins as chlorophyll-binding proteins D_1, CP43, LHCI, and LHCII, the small subunit of ribulose-bisphosphate carboxylase, and apoplastocyanin. An autocatalytic mechanism observed in vivo for degradation of plastid protein D_1 and in vitro for pea ferritin during iron release could explain the ferritin decreases in mature nodules.

Gene transcription is a major site of regulation during development and differentiation. However, examples of posttranscriptional regulation during development include changes in translation, mRNA turnover, and protein turnover (Klaff and Gruissem, 1991, 1992; Lobreaux and Briat, 1991; Shipton and Barber, 1991; White and Thompson, 1991; Hershko and Ciechanover, 1992; Hunt et al., 1992). Evidence for posttranscriptional regulation of ferritin during nodule development was recently obtained when the induced level of ferritin mRNA was observed to remain high in mature soybean (*Glycine max*) nodules (DAI 21) after the ferritin protein level declined 4- to 5-fold from the levels in immature nodules (DAI 12) (Ragland and Theil, 1993).

Posttranscriptional regulation of ferritin has been extensively studied in animals (reviewed in Theil, 1990a, 1993; Klausner et al., 1993). Ferritin mRNA is very stable in animals and posttranscriptional regulation can be accounted for by the effects on translation of the conserved *cis* RNA regulatory element, the IRE. The possibility that the posttranscriptional regulation of ferritin observed previously during nodule development (Ragland and Theil, 1993) was due to translational control was examined here by studying the size of polyribosomes translating ferritin, rates of translation of ferritin mRNA in heterologous cell-free extracts, and ferritin synthesis in nodules. The results show that translation of soybean ferritin mRNA induced by nodulation was unchanged during development, indicating that changes in nodule ferritin protein content are posttranslational and could be due to increased ferritin turnover or autocatalytic degradation in mature nodules. Ferritin degradation has previously been observed in germinating pea seeds (Lobreaux et al., 1993).

MATERIALS AND METHODS

Inoculation and Cultivation of Soybeans and Collection of Nodules

Seedlings of *Glycine max* (var Bragg) were inoculated 3 d after germination with *Bradyrhizobium japonicum* MN110 and cultivated in a phytotron as previously described (Ragland and Theil, 1993). Nodules were collected at 12 and 21 DAI, frozen immediately in liquid nitrogen, and stored at −80°C until analysis; previous studies showed that the ferritin mRNA content increased 7- to 25-fold over 8-DAI uninoculated controls at DAI 8 and at DAI 12 and 21 (Ragland and Theil, 1993). Ferritin mRNA was induced in soybean leaves of plants grown for 16 d in iron-deficient medium by soaking mature trifoliate leaves overnight in an aqueous solution of 500 μM Fe-EDTA. Dot blot analysis (Ragland and Theil, 1993) of RNA from treated and untreated leaves showed that ferritin mRNA had increased 5-fold in iron-treated leaves.

Isolation of RNA, Polyribosome Isolation, and in Vitro Synthesis of RNA from DNA Templates

Total RNA was isolated from soybean tissue using guanidine isocyanate (Berger and Chirgwin, 1989). Poly(A)$^+$ RNA was isolated from total RNA using oligo(dT)-cellulose as previously described (Shull and Theil, 1982) except that commercially available microcolumns were used (Quik columns from Stratagene).

Polyribosomes were isolated as described by Klaff and

[1] Supported in part by the U.S. Department of Agriculture (grant 9103274) and the National Institutes of Health (grant DK20251).

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Abbreviations: BP, binding protein; DAI, days after inoculation; EP, extension peptide; IRE, iron regulatory element.

Gruissem (1991) by grinding tissue with a solution of 0.2 M Tris-Cl (pH 9), 0.2 M KCl, 35 mM $MgCl_2$, 25 mM EGTA, 0.2 M Suc, 1% Triton X-100, 2% polyoxyethylene-10-tridecylether, 0.5 mg/mL heparin, and 100 mM β-mercaptoethanol, from which debris was removed by centrifugation at 10,000*g* and 4°C for 5 min. The clarified extract was layered on a 15 to 55% Suc gradient in 40 mM Tris-Cl, pH 8.0, 20 mM KCl, 10 mM $MgCl_2$, 0.5 mg/mL heparin, and centrifuged at 4°C for 90 min at 150,000*g*. Ten fractions were collected, and after addition of SDS to 0.5%, the RNA was isolated by phenol extraction and alcohol precipitation (Ragland et al., 1990). Ferritin mRNA concentration was determined by hybridization of the RNA with labeled sof-35 cDNA (Ragland et al., 1990) and globin mRNA by hybridization to Lb23c (kindly provided by V.P.S. Verma) as previously described (Ragland and Theil, 1993). Equal amounts (A_{260}) of labeled RNA from each fraction were compared. Hybridization analysis of RNA in the extracts used for polyribosome fractionation showed that the amount of ferritin and globin mRNA was the same as that extracted by guanidine isothiocyanate (Ragland and Theil, 1993).

Plasmids encoding natural (IRE+) frog ferritin cDNA, IRE− frog ferritin cDNA, soybean ferritin cDNA, and IRE+ soybean ferritin cDNA have been previously described (Dix et al., 1992). Synthesis of capped mRNA by transcription from linearized cDNA used procedures described before (Dix et al., 1992).

Protein Synthesis of Ferritin in Vitro and in Nodule Suspension

Translation of poly(A)$^+$ RNA in vitro transcripts in rabbit reticulocyte lysates and labeling protein with [^{35}S]Met followed the procedures of Shull and Theil (1982) modified by Dix et al. (1992). When in vitro transcripts were used, TCA precipitates were analyzed directly. When poly(A)$^+$ RNA was used, total protein synthesis was measured by analyzing TCA precipitates, but ferritin synthesis was analyzed in immunoprecipitates prepared as described by Shull and Theil (1982); 10 μL of polyclonal rabbit antiserum to purified soybean ferritin (Ragland and Theil, 1993), added to protein synthesis reaction mixtures with 7.5 μg of carrier soybean seed ferritin, was sufficient to precipitate all the ferritin. Analysis of immunoprecipitates on SDS-polyacrylamide gels, followed by autoradiography, showed a single subunit size of 30 kD, the size of the subunit precursor (Fig. 1). All data were corrected for nonspecific immunoprecipitation (no added RNA). All radioactivity in the immunoprecipitate in the autoradiogram could be eliminated by adding excessive amounts of unlabeled ferritin before adding antibody.

Ferritin synthesis in nodules was measured using suspensions of freshly picked, washed nodules in an aqueous solution of [^{35}S]Met that was incubated for 1 h at room temperature (Klaff and Gruissem, 1991). After washing with 10 mM Met, the nodules were ground in 1 mL of a solution of 100 mM Tris, pH 7.2, 10% Suc, 5 mM EDTA, 2 mM PMSF, and 40 mM β-mercaptoethanol. Debris was removed by centrifugation for 3 min at 10,000*g* and the radiolabeled ferritin was immunoprecipitated; no immunoprecipitable ferritin remained in the debris. Labeled proteins were immunoprecipitated as described above for proteins synthesized in rabbit reticulocyte lysates. Analysis of the immunoprecipitates by electrophoresis in SDS-polyacrylamide gels showed a single subunit of 28 kD, the processed size (Fig. 1).

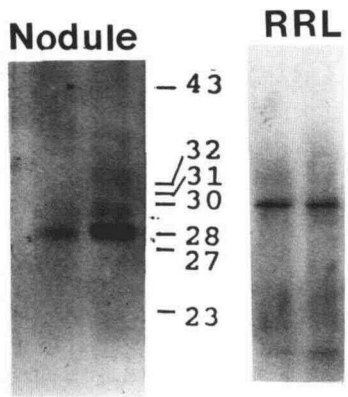

Figure 1. Soybean ferritin synthesized in nodules or in rabbit reticulocyte extracts. Ferritin labeled by [^{35}S]Met, incorporated under the direction of nodule poly(A)$^+$ mRNA added to rabbit reticulocyte extracts, or incorporated into protein in nodules was immunoprecipitated and analyzed by autoradiography after electrophoresis in SDS gels calibrated by standard proteins. Note the presence of the 30-kD ferritin precursor synthesized in rabbit reticulocyte lysates (RRL) and the mature 28-kD subunit synthesized in nodules indicating natural processing/transport to plastids. Equal amounts of ^{35}S-labeled total protein were analyzed from nodules or from poly(A)$^+$ RNA translation in RRL. Peptide sizes were the same at DAI 12 and 21. Nodule: left, DAI 12; right, DAI 21. RRL: left, DAI 12; right, DAI 21.

RESULTS

Distribution of Ferritin mRNA in Polyribosomes of Young and Mature Soybean Nodules

During nodule development in soybean, ferritin mRNA accumulates early (DAI 8) and remains elevated in mature nodules (DAI 21) (Ragland and Theil, 1993). Ferritin protein is highest at DAI 12, when nodules are still immature (low globin and heme) (Ragland and Theil, 1993). However, by DAI 21, when soybean nodules are mature and have attained maximum rates of acetylene reduction and globin (mRNA, protein) concentration, ferritin protein concentration has decreased 4- to 5-fold (Ragland and Theil, 1993). To determine if the low amount of nodule ferritin protein at DAI 21 is due to a block in polyribosome binding to ferritin mRNA, the distribution of ferritin mRNA was determined among polyribosomes of nodule extracts fractionated by centrifugation in Suc gradients. In animals, low amounts of ferritin with abundant ferritin mRNA can be related to a specific block in ribosome binding to ferritin mRNA (Zahringer et al., 1976; Dickey et al., 1988). Extracts from immature soybean nodules (12 DAI) were analyzed for comparison.

The distribution of soybean nodule ferritin mRNA was the same at DAI 12 and DAI 21 (Table I), and is centered around fractions 6 and 8 in the lower half of the Suc gradient. Thus, there is no major change in the rate of initiation of translation to account for the developmental decrease in ferritin protein

observed between immature (DAI 12) and mature (DAI 21) nodules. Moreover, fractions 1 to 3, which have no ribosomes, and fraction 4, which has monosomes, had no detectable ferritin mRNA, indicating that all the nodule ferritin mRNA molecules were being translated in both immature and mature nodules. Disrupting the polyribosomes by incubating the extracts with 20 mM EDTA at 37°C for 15 min before application to the Suc gradient did shift the ferritin mRNA to fractions 1 to 4.

Nodule globin mRNA distribution among polyribosomes was also analyzed for comparison to the ferritin mRNA distribution, since developmental changes in globin contrast with ferritin. For example, the amount of globin protein increases 2.5-fold between DAI 12 and 21 when ferritin decreases 4- to 5-fold, and the amount of globin mRNA increases 4.2-fold (Ragland and Theil, 1993). In spite of the differences in accumulated globin and ferritin protein at DAI 21, globin mRNA was found to be associated with the same size polyribosomes as ferritin mRNA. All globin mRNA was engaged in translation at both stages of nodule development (Table I), even though at DAI 12 the amount of globin mRNA and protein were much lower than at DAI 21 (Ragland and Theil, 1993).

Protein Synthesis in Vitro Directed by Ferritin mRNA Extracted from Immature and Mature Nodules

Since the 4- to 5-fold decrease in ferritin protein observed between 12- and 21-DAI nodules could not be explained by either decreases in ferritin mRNA concentration (Ragland and Theil, 1993) or by a decrease in nodule ribosome binding to ferritin mRNA (Table I), ferritin synthesis in rabbit reticulocyte extracts was analyzed [using poly(A)$^+$ RNA extracted from immature (DAI 12) and mature (DAI 21) nodules] to determine if nodule ferritin mRNA was equally functional in all aspects of translation. A heterologous extract was used to minimize the possibility that *trans* factors in plant extracts might modulate the function of ferritin mRNA at the two stages of nodule development. The product of translation of nodule ferritin mRNA in rabbit reticulocyte extracts had an apparent size of 30 kD (Fig. 1), which is the size of the unprocessed ferritin subunit precursor (van den Mark, 1983; Ragland et al., 1990). The specificity of the immunoprecipitation is indicated by the fact that all of the radioactivity (Fig. 1, RRL) in the precipitate was eliminated by adding excess unlabeled ferritin.

Synthesis of protein under the direction of poly(A)$^+$ RNA was determined for total protein (TCA-precipitable) and for ferritin (immunoprecipitable by soybean ferritin antiserum). Incorporation of [^{35}S]Met into total protein was comparable for both immature (DAI 12) and mature (DAI 21) nodules (Table II). Synthesis of ferritin was identical at the two stages of nodule development and accounted for approximately 0.8% of the protein synthesized (Table II). Since the ferritin mRNA in immature and mature nodules appears to be equally functional, the decrease in the amount of ferritin protein in DAI-21 nodules cannot be explained by a change in nodule ferritin mRNA function.

RNA from iron-treated soybean leaves was tested for comparison. [Dot blots of poly(A)$^+$ RNA from iron-treated and untreated leaves showed a 5-fold increase in ferritin mRNA due to iron.] The amount of ferritin synthesized by ferritin mRNA from nodules was comparable to that in iron-treated leaves (Table II). Since iron induces both ferritin mRNA and protein in plant tissue (Lescure et al., 1991), the data in Table II indicate that nodule development appears to have an effect on nodule ferritin gene expression that is quantitatively similar to that of iron on leaf ferritin gene expression.

Ferritin Synthesis in Mature and Immature Nodules

Although nodule ferritin mRNA from immature (DAI 12) and mature (DAI 21) nodules appeared to initiate translation equally efficiently (Table I) and to be elongated and terminated with equal efficiency in heterologous cell-free extracts (Table II), the possibility remained that developmentally reg-

Table I. *Distribution of ferritin and globin mRNAs among polyribosome fractions of immature and mature nodules*

Nodules were collected and native ribosomes extracted as described in "Materials and Methods"; the amount and size of ferritin and globin mRNA isolated was indistinguishable from that isolated using denaturing conditions (guanidine isothiocyanate), as judged by RNA blotting. Preparation of the Suc gradients and hybridization of the RNA isolated from each fraction is described in "Materials and Methods." Fractions 1 through 3 are the ribosomal supernatant, and fraction 4 is the monosome peak as judged by A_{260}. Nodule extracts from two sets of plants, independently inoculated and cultivated, gave equivalent results. Note the similarity of polyribosome size for both ferritin and globin mRNAs in young (DAI 12) and mature (DAI 21) nodules; the amount of ferritin mRNA was relatively constant during development, but globin mRNA increased 2.5-fold (Ragland and Theil, 1993).

Stage of Development	Fraction Number									
	1	2	3	4	5	6	7	8	9	10
	Ferritin mRNA									
12 DAI	−	−	−	−	+	+	+	+	−	−
21 DAI	−	−	−	+	+	+	+	±	−	−
	Globin mRNA									
12 DAI	−	−	−	−	+	+	+	+	−	−
21 DAI	−	−	−	+	+	+	+	−	−	−

Table II. *Function of ferritin mRNA (translation in vitro in rabbit reticulocyte lysates) extracted from immature and mature soybean nodules*

Poly(A)$^+$ RNA extracted from nodules or from iron-treated leaves used for comparison was translated in rabbit reticulocyte lysates, using [^{35}S]Met as a label as described in "Materials and Methods." Labeled ferritin was precipitated with soybean ferritin antiserum. The data from immunoprecipitates were corrected for nonspecific radioactivity by subtracting radioactivity precipitated from lysates incubated without added RNA. Specificity for ferritin was shown by adding unlabeled ferritin and eliminating immunoprecipitated counts. Note the similarity of ferritin synthesis rates at both stages of development as well as in iron-treated leaves.

Stage of Development	Total Protein Synthesis	Percent Ferritin Synthesis
	cpm μg^{-1} RNA h^{-1} × 10^{-5}	*100 × (cpm ferritin/cpm total protein)*
Nodules 12 DAI (immature)	17.1 ± 6.9	0.89 ± 0.23
Nodules 21 DAI (mature)	15.1 ± 10.8	0.80 ± 0.37
Leaves (iron-treated)	8.61 ± 4.4	0.72 ± 0.21

ulated *trans* factors alter the rate of elongation or termination of nodule ferritin mRNA to diminish ferritin synthesis in mature nodules.

To test for the presence of nodule factors that developmentally regulate ferritin synthesis, nodules from plants at DAI 12 and 21 were incubated in aqueous [^{35}S]Met for 1 h at 25°C before analysis of the labeled proteins (see "Materials and Methods"). Since isolated nodules incorporated approximately three times as much radioactivity as nodules detached from root fragments after incubation, nodules were detached from roots before incubation. Ferritin synthesized in nodules was fully processed, as indicated by the size of the labeled subunit (28 kD) shown in Figure 1.

Synthesis of total protein was similar in DAI-12 and -21 nodules: 108,000 ± 39,000 and 70,000 ± 27,000 cpm mg^{-1} protein h^{-1}, respectively, for nodules from three independent experiments. The greater variability in total protein synthesis of DAI-21 nodules, however, may indicate that rates of protein synthesis decline in older nodules. Ferritin synthesis was similar but slightly higher at DAI 21 compared with DAI 12 (1.42 ± 0.40) (Table III), indicating that developmental decreases in ferritin synthesis do not account for the decrease in ferritin protein at DAI 21. Densitometry of the Coomassie blue-stained immunoprecipitate of nodule ferritin showed a 5-fold decrease in ferritin protein from DAI 12 to 21 (Table III), which confirms the decrease in ferritin protein concentration previously observed during nodule development (Ragland and Theil, 1993). Attempts to measure possible developmental differences in ferritin turnover rate were inconclusive. On the one hand, the amount of radioactivity incorporated for shorter pulses was too low to measure accurately for the amount of nodule tissue conveniently available. On the other hand, after a 5-h "chase" with unlabeled Met, there was no change in the radioactivity of nodule ferritin. Such results indicate either that the turnover is too slow to measure in 5 h or that the metabolism of the nodule suspension was declining.

Two types of experiments were performed to investigate developmentally regulated nodule ferritin degradation. In the first, [^{35}S]ferritin synthesized in DAI-21 nodule suspensions was mixed with extracts of DAI 12 and 21 nodules. Specific degradation sites in ferritin (conversion from 28–21 kD) were observed. However, the degradation was not developmentally specific, since the cleavage was the same at DAI 21 and 12. In the second experiment, we tested the hypothesis that at DAI 21 there would be more apoferritin than at DAI 12, because storage iron had been used for heme or nitrogenase; apoferritin might be degraded more readily than ferritin, since enhanced sensitivity to degradation of pea seed apoferritin has been previously observed (Laulhere et al., 1989). However, when degradation of soybean seed apoferritin and holoferritin by nodule extracts was compared, no difference in degradation was observed. It is possible that observation of developmentally regulated degradation of ferritin in nodules requires more natural nodule/plastid architecture than can be achieved in simple nodule extracts, as has been suggested for apoplastocyanin (S. Merchant, personal communication). In the future, conditions for more active and stable nodule suspensions will be developed to examine the stability and turnover of nodule ferritin.

Translation of Soybean Ferritin mRNA with the Known *cis* RNA Regulatory Element, the IRE

Two independent sets of observations indicate that ferritin synthesis is regulated before or after translation in plants: (a) iron induction of ferritin synthesis in cultured soybean can be fully accounted for by increased transcription (Lescure et al., 1991), which contrasts with iron induction of ferritin in

Table III. *Synthesis of ferritin (incorporation of [^{35}S]Met) compared with ferritin accumulation in soybean nodules during development*

Nodules were incubated in aqueous [^{35}S]Met and protein was extracted, immunoprecipitated with soybean ferritin specific antiserum, and analyzed by electrophoresis in SDS-polyacrylamide gels as described in "Materials and Methods." Note the 5-fold difference in the amount of accumulated nodule ferritin in young (12 DAI) and mature (21 DAI) nodules, as previously observed (Ragland and Theil, 1993) compared with relatively similar rates of ferritin synthesis at the two stages of development. The results are the average of data from two sets of plants, inoculated and cultivated independently, with the error shown as the SD.

Developmental Stage	[^{35}S]Met Incorporation (Density of Autoradiogram of Nodule Ferritin Immunoprecipitate)	Ferritin Accumulation (Density of Coomassie Blue Stain of Nodule Ferritin Immunoprecipitate)
12 DAI (immature)	672 ± 76[a]	359 ± 4
21 DAI (mature)	965 ± 135[a]	76 ± 10
21 DAI:12 DAI	1.42 ± 0.40[b]	0.21 ± 0.04

[a] Total protein synthesis (predominantly globin) was also similar at 12 and 21 DAI, i.e. 108 ± 3.9 and 70 ± 27 cpm × 10^{-3} mg^{-1} protein h^{-1}. [b] In a preliminary experiment (Fig. 1), incorporation of [^{35}S]Met appeared to be even higher at DAI 21 than at DAI 12. However, since there was no data equivalent from Coomassie blue staining, the ^{35}S data were not included in calculating the ratio.

animals (reviewed in Theil, 1990a, 1993; Klausner et al., 1993); (b) translation of soybean nodule ferritin mRNA was unchanged during development (Tables I–III). To determine if features of soybean ferritin mRNA itself inhibit specific translational regulation, the animal IRE was fused to soybean ferritin cDNA and the translation of the in vitro transcripts was analyzed in the rabbit reticulocyte lysate, which contains the endogenous IRE-specific negative regulator, the IRE-BP (Dickey et al., 1988; Walden et al., 1988). Previous studies showed that the IRE fused to reporter sequences such as chloramphenicol acetyltransferase or human growth hormone conferred translational regulation on the mRNA (Aziz and Munro, 1987; Hentze et al., 1987).

The IRE conferred essentially no specific translational regulation on soybean ferritin mRNA (Table IV) in rabbit reticulocyte lysates; neither positive nor negative control (Dix et al., 1992) was conferred on soybean ferritin mRNA by the IRE. Synthesis in vitro was similar for soybean ferritin mRNA with or without the IRE. Moreover, synthesis was linearly related to the soybean ferritin mRNA concentrations tested whether or not the IRE was present. Translation of frog mRNA with or without the IRE included for comparison provided a sharp contrast to soybean ferritin mRNA (Table IV).

The two types of IRE-regulated translation can be seen in the frog mRNA, i.e. positive or enhanced rates at high mRNA concentrations and negative or repressed (IRE-BP-dependent) regulation at low mRNA concentrations (see Dix et al., 1992). For example, for natural frog ferritin mRNA (IRE+) at high mRNA concentrations (between 9 and 27 nM mRNA), translation rates were linear and much higher (>40×) than for frog ferritin mRNA minus the IRE, demonstrating the enhancing effect of the IRE on IRE-BP-independent translation rates (Dix et al., 1992). Translation of natural ferritin mRNA was also slightly higher (3×) than that of soybean ferritin mRNA. On the other hand, at low mRNA concentrations, where the IRE-BP is in excess, frog ferritin synthesis was disproportionately low, indicating negative control or repression by the IRE-BP; if there were no repression by the IRE-BP, synthesis rates should be about 30×10^{-5} cpm $(25\ \mu L)^{-1}$ h^{-1} rather than 13×10^{-5} cpm $(25\ \mu L)^{-1}$ h^{-1} (Table IV). In vivo, the combined effects of release from negative control and positive control of ferritin mRNA can produce a 50-fold increase in ferritin accumulation in animal cells (Shull and Theil, 1982).

In animal ferritin mRNA the IRE assumes a very specific conformation (Wang et al., 1990; Harrell et al., 1991; Dix et al., 1993), which has the predicted secondary structure of a hairpin loop. When computer programs (Jaeger et al., 1990) that predict the hairpin loop of the isolated IRE and the IRE in animal ferritin mRNA were used to predict structure in the 5′ untranslated region of the chimera of the frog ferritin IRE and soybean ferritin mRNA, the IRE hairpin loop was not predicted to form. Rather, two other hairpin loops were predicted that involve the transit peptide sequence as well as the IRE; the crucial CAGUG of the IRE hairpin loop was part of a stem in the IRE and soybean ferritin mRNA chimera. Since even minor alterations of the hairpin loop, such as a G/A mutation, prevent IRE-BP binding and alter translational control in animal mRNA (Dix et al., 1993), the altered structure of the IRE sequence in the chimeric ferritin mRNA, at least in the computer prediction, can explain the absence of translational regulation. In spite of the high level of sequence conservation among the coding sequences of plant and animal ferritins (Ragland et al., 1990), sequences specific to plant ferritin mRNA appear to prevent IRE function.

DISCUSSION

Translational control is a well-known mechanism of posttranscriptional regulation of ferritin synthesis in animals (reviewed in Theil, 1990a, 1993; Klausner, 1993). In contrast, although less studied, only transcriptional control of ferritin has been observed in plants (Lescure et al., 1991; Lobreaux et al., 1993). The maintenance of ferritin mRNA in mature

Table IV. *Translation of soybean ferritin mRNA with and without the IRE, the animal cis translational regulatory element*

In vitro transcripts (see "Materials and Methods") were translated in rabbit reticulocyte lysates at low RNA concentrations (3 nM), where the IRE-BP (negative regulator) is in excess, and at high concentrations of RNA, where the IRE-BP is saturated and has little effect on translation rate (Dix et al., 1993).

In Vitro Transcript	Protein Synthesis		
	[mRNA] < IRE-BP[a] [RNA] = 3 nM	[mRNA] < IRE-BP[a] [RNA] = 9 nM	[mRNA] < IRE-BP[a] [RNA] = 27 nM
	cpm $(25\ \mu L)^{-1}\ h^{-1} \times 10^{-5}$		
Native soybean ferritin (IRE−)	11 ± 6	56 ± 18	118 ± 20
IRE + soybean ferritin	13 ± 8	38 ± 20	100 ± 33
Native animal (frog) ferritin (IRE+)	13 ± 4	110 ± 26	353 ± 70
IRE− frog ferritin	ND[b]	ND	8 ± 3

[a] IRE-BP = RNA binding protein specific for the IRE *cis* RNA element (Klausner et al., 1993; Theil, 1993). The IRE-BP is an endogenous component of rabbit reticulocyte lysates. [b] ND, Not detectable.

(DAI 21) soybean nodules, when protein concentrations declined 4- to 5-fold (Ragland and Theil, 1993), indicated the possibility of translational control in the highly specialized nodule, which requires large amounts of iron for nitrogenase and leghemoglobin. In animals, translational control of ferritin is particularly dramatic in cells that have high iron concentrations, such as cells of the liver (Zahringer et al., 1976), which store iron for the entire animal, and red blood cells, which store iron for hemoglobin (Shull and Theil, 1982). Moreover, during nodulation, induction of ferritin mRNA and protein preceded heme and globin accumulation, just as in red blood cells (Ragland and Theil, 1993), further supporting a hypothesis of translational regulation of ferritin mRNA in nodules. Finally, conservation of the IRE sequence among animal ferritin mRNAs is very high, about 97%, which is greater than among the coding regions, where sequence conservation is nevertheless very high (65–85%) (Theil, 1990a, 1990b). In fact, conservation of coding sequence between plant and animal ferritins is comparable to that among animal ferritins (reviewed in Theil and Hase, 1993).

The results in nodules showed similar translation of ferritin mRNA in both DAI-12 and -21 nodules. For example, polyribosome sizes for ferritin mRNA were similar whether ferritin protein accumulated or not (Table I). Thus, the initiation of translation of ferritin mRNA was normal in both mature and immature nodules. Moreover, ferritin mRNA was fully functional in all steps of translation, at different stages of nodule development, as judged by translation of nodule poly(A)$^+$ RNA in rabbit reticulocyte lysates (Fig. 1, Table II); note that nodule ferritin mRNA was the same size during development as well (Ragland and Theil, 1993). In addition, ferritin synthesis in nodules was the same in mature and immature nodules, indicating that *trans* factors do not block ferritin synthesis in mature nodules (Table III). Ferritin subunits synthesized in the nodule suspensions were also processed the same in both mature (DAI 22) and immature nodules (DAI 12) (Fig. 1), eliminating the possibility that developmental changes in processing led to increased degradation of ferritin. If translational repression of ferritin mRNA is the explanation of the low amount of ferritin protein in mature (DAI 21) nodules, it is independent of the methods of detection used. More likely, the posttranscriptional regulation of ferritin during nodule development occurs after translation. Developmental degradation of ferritin has been previously described for different parts of germinating pea seeds; degraded fragments were actually observed in the radicle, but not in other parts of the seed (Lobreaux and Briat, 1991).

Ferritin precursors, synthesized in the cytoplasm of plants, are transported to the plastid with concomitant removal of a transit peptide (van der Mark, 1983; Lescure et al., 1991). Changes in the stability of other plastid proteins after translation in the cytoplasm and transport to the plastid are known for Chl-binding proteins (LHCI and LHCII) (Apel, 1979; Bennett, 1981), the small subunit of Rubisco (Schmidt and Mishkind, 1983), and plastocyanin (Merchant and Bogorad, 1986; Hill and Merchant, 1992). The concentration of Chl-binding proteins D_1 and CP43, synthesized in the plastid, is also controlled after translation by regulation of protein turnover (Greenberg et al., 1987; Mullet et al., 1990). In each case, changes in the concentration of an interacting plastid component are associated with increased breakdown of the protein, such as photoinhibition of Chl synthesis in the case of Chl-binding proteins D_1, CP43, LCHI, and LCHII; chloramphenicol inhibition of Rubisco large subunit synthesis in the case of Rubisco small subunit; or, in the case of copper deficiency for plastocyanin, transport of apoplastocyanin to the plastid, where copper is bound (Merchant and Bogorad, 1986; Li et al., 1990). Ferritin also appears to acquire metal ions inside the plastid, since the iron core of pea ferritin has the high phosphate content characteristic of ferritin mineralized in bacteria (Wade et al., 1993); bacteria share with plastids a high phosphate content compared with the cytoplasm of plants and animals. The release of iron during development might enhance ferritin degradation, since lack of copper enhances apoplastocyanin degradation (Merchant and Bogorad, 1986).

The plastid in nodules is a storage organ that accumulates starch as well as iron stored in ferritin (Ko et al., 1985). During nodule development iron incorporation reaches maximum values before maturity and full hemoglobinization of the nodule (Ragland and Theil, 1993). In mature nodules, when storage iron has been consumed for heme, ferritin protein concentration declines, indicating that the absence of plastid storage iron could relate to ferritin breakdown. A mechanism may be related to the EP, a plant-specific extension at the N terminus in the mature ferritin protein (Ragland et al., 1990), which undergoes autocatalytic degradation during iron release in vitro (Laulhere et al., 1989). The EP in plant ferritin may also trigger autocatalytic degradation in plastids when storage iron is released during nodule development. Interestingly, autocatalysis is also responsible for degradation of the apoform of Chl-binding protein D_1 (Greenberg, 1988; Shipton and Barber, 1991).

The apparent absence of translational control of ferritin in plants is puzzling given the high conservation of ferritin among plants and animals during evolution and the apparent existence of an ancient progenitor (Ragland et al., 1990; Theil and Hase, 1993). Although conservation of the IRE, when it occurs, is higher (97%) than that of the coding region (65–85%), the IRE sequence is absent in soybean ferritin cDNAs (Ragland et al., 1990; Lescure et al., 1991) and in known soybean and maize ferritin genes (Lobreaux et al., 1993; D. Proudhon, J.F. Briat, and E.C. Theil, unpublished data).

Moreover, function of the animal IRE was blocked in a chimera with soybean ferritin mRNA (Table IV), although the IRE sequence functions in other chimeric mRNAs such as chloramphenicol acetyltransferase and human growth hormone (Aziz and Munro, 1987; Hentze et al., 1987). A major conserved difference between plant and animal ferritin mRNAs is the presence of the transit peptide and EP sequences in plant ferritin mRNAs (Ragland et al., 1990; Lobreaux and Briat, 1993), which were apparently acquired during evolution after plants and animals diverged. (Although transit peptide and EP sequences are not as highly conserved as the IRE in terms of primary sequence, it is clear that some higher-order structure, at least in the peptide, is conserved. Whether a corresponding structure in the mRNA is also conserved is a subject for future investigation.)

The absence of the IRE in plant ferritin mRNAs could

suggest that the IRE was also acquired by animals after plants and animals diverged. However, the IRE-BP is a member of an ancient protein family, the aconitases (Rouault et al., 1991; Haile et al., 1992; Kennedy et al., 1992), which is common to plants and animals; this suggests that the IRE could also have been present before plants and animals diverged. If such were the case, the IRE may have been lost when plants acquired the transit peptide sequence in ferritin, since the IRE has little functional effect on the translation of plant ferritin (Table IV). It is tempting to speculate that the apparent contradiction of conservation of the coding region in plant and animal ferritins and divergence of posttranscriptional regulatory mechanisms is related to a blocking of IRE-dependent translational regulation by plant-specific ferritin sequences.

ACKNOWLEDGMENT

The authors are grateful to Jiangzhon Wei for the analysis of the predicted secondary structure of the IRE in the chimeric soybean ferritin mRNA.

Received June 14, 1993; accepted September 21, 1993.
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