Iron Induction of Ferritin Synthesis in Soybean Cell Suspensions

Dominique Proudhon, Jean-François Briat, and Anne-Marie Lescure*
Laboratoire de Biologie Moléculaire Végétale, CNRS-URA 57, Université Joseph Fourier (Grenoble 1), BP 53X, F-38041 Grenoble Cedex, France

ABSTRACT

In animal cells specialized for iron storage, iron-induced accumulation of ferritin is known to result from a shift of stored mRNA from the ribonucleoprotein fraction to polysomes. Previous reports with bean leaves suggested that in plants iron induction of ferritin synthesis would result from a regulation at the transcriptional level (F van der Mark, F Bienfait, H van der Ende [1983] Biochem Biophys Res Commun 115:463-469). Soybean (Glycine max, cv Mandarin) cell suspension cultures have been used here to support these findings. Ferritin induction is obtained by addition of Fe-citrate to the culture medium. A good correlation is found between cellular iron content and the amount of ferritin accumulation. This protein accumulation corresponds to an increase of in vitro translatable ferritin mRNA. Addition of 4 micrograms actinomycin D per milliliter to the cultures inhibits completely in vivo RNA synthesis, whereas protein synthesis was poorly affected, at least for 24 hours. During the same time, this concentration of actinomycin D strongly inhibits the iron-induced synthesis of ferritin. These results show that in soybean cell cultures, the mechanism of regulation of ferritin synthesis in response to iron does not result from recruitment of preexisting mRNA. They confirm that in plant systems, ferritin synthesis results from increased transcription of the corresponding genes.

Ferritins constitute a widespread group of proteins in eukaryotic cells. They maintain iron in a soluble form and they protect cells against the toxic effects of iron overload. In animal cells, two types of ferritins have been identified (18), based on whether the iron is stored for other cells (specialized-cell ferritins) or used for intracellular purposes (housekeeping ferritins). Specialized-cell ferritin is the most abundant and as a consequence has been extensively studied. Studies have shown that iron-induced accumulation of ferritin in cells specialized for iron storage does not result from changes in the concentration of ferritin mRNA species, but from the recruitment of stored mRNA (14). It has recently been shown that in such cells, iron regulates ferritin mRNA translation through a 28 bp segment of its 5' untranslated region (1, 6, 10). In contrast, housekeeping ferritins, which are found in small amounts in most cell types, have been little studied. In a recent work, Dickley et al. (5) showed that iron induces a change in relative amounts of the housekeeping ferritin mRNA indicating that the level of regulation differs from that described for specialized cell ferritins. In this case, iron appears to influence either the transcription or the stability of ferritin mRNA. The molecular mechanism of this regulation is not known. Plant ferritins have been identified from both dicoyleldons (4, 15, 20) and monocotyledons (8). They appear to be coded by nuclear DNA and plastid localized (22). Little is known about the regulation of their synthesis. The only work dealing with this regulation was by van der Mark et al. (21). Their results indicated that the ferritin content in bean leaves with different iron status correlated with the contents of translatable ferritin mRNA, suggesting a regulation at the transcriptional level.

To further investigate the regulation of ferritin synthesis in plants, we have developed a physiological system using soybean cell suspension cultures. Such cultures, containing undifferentiated cells, respond rapidly and in a homogenous way to changes in iron supplies. This physiological system appears to be well adapted to investigate the transcriptional regulation of plant ferritin synthesis.

MATERIALS AND METHODS

Cell Cultures

Soybean cell cultures (Glycine max cv Mandarin, line Sbe4) were a gift of Dr. J. P. Jouanneau (CNRS, Gif sur Yvette, France). Cultures were grown at 30°C in the dark in B5 medium with 5 μM 2,4-D and 0.2% casein hydrolysate as described by Leguay and Jouanneau (9). This medium contains 100 μM FeNa2 EDTA. For protein and RNA extractions, aliquots of cells were washed with 20 mM KCl, 5 mM EDTA and were blotted and frozen in liquid nitrogen. Frozen cells were kept at -70°C until use.

Cell growth was followed by measuring the PCV according to Leguay and Jouanneau (9).

Protein Analyses

Protein extractions from frozen cells were carried out according to Nechustai and Nelson (13). Protein concentrations were determined according to Bradford (3). Electrophoresis of proteins under denaturing conditions was done in 12.5% polyacrylamide-SDS gels according to Laemmli (7). Immunoblots were performed as previously described (8), using a pea seed antiferritin (8) and anti-rabbit IgG sheep immunoglobulins coupled with peroxidase (Biosys).

1 Abbreviation: PCV, packed cell volume.
Cellular Iron Concentration Measurements

Defined quantities of cells were mineralized according to the Beinert's procedure (2). Iron concentration was measured by absorbance of Fe$^{3+}$-orthophenanthroline (0.02%) at 510 nm and pH 6.0 (50 mM acetic acid-NaOH buffer) using an excess of dithionite crystals as reducer.

Extraction and in Vitro Translation of RNA

RNA was extracted from frozen cells as described previously (11). Poly(A$^+$) RNA was separated on oligo dT-cellulose (Pharmacia, type 7) according to Maniatis et al. (12). RNA samples were translated in a mRNA dependent rabbit reticulocyte cell-free system (Promega, Biotech) in the presence of $[^{35}$S]methionine (1000 Ci/mmole, 50 µCi per sample), according to the supplier's informations.

Immunoprecipitations and Analysis of the Cell Free Translation Products

The products synthesized were immunoprecipitated with the antiserum raised against pea seed ferritin (8) in the presence of *Staphylococcus aureus* cells (Pansorbin, Calbiochem) as described by van der Mark et al. (22). Precipitated proteins were electrophoresed on 12.5% polyacrylamide-SDS gels. Stained polyacrylamide gels were soaked for 15 min in Amplify (Amersham), dried, and fluorographed at −70°C.

RESULTS

Iron Induced Accumulation of Ferritin in Soybean Cell Suspensions

In preliminary experiments, we tried to induce ferritin synthesis by increasing the amounts of Fe-EDTA (100–500 µM) raised in the culture medium. Immunoblot analysis using the antiserum raised against pea seed ferritin failed to detect ferritin in the protein extracts from these cells (not shown). However, this antiserum has been shown to cross-react with ferritin purified from soybean seeds (8).

Failure to induce ferritin synthesis in the presence of 500 µM Fe-EDTA could result from the high stability constant of this iron ligand. Therefore, we tried to induce synthesis with iron-citrate which has a weaker stability constant (17). Cells grown in the standard medium (100 µM Fe-EDTA) until stationary phase were transferred into three media: (a) in the presence of 500 µM Fe-citrate, (b) in the presence of 100 µM Fe-citrate, and (c) without any iron supply. The cell generation times in the three media were similar at least for three doublings (Fig. 1). Cells were collected after two doublings (72 h). The cellular iron content in each condition was determined by mineralization of defined quantities of cells. The results, reported in Table I, show that the cellular contents of iron increase from 0.8 to 11.8 nmol iron per mg dry weight in response to changes of iron levels in the culture medium.

The relative amounts of ferritin in the proteins extracted from the three cell samples were examined by immunoblot analysis (Fig. 2A). The results clearly showed that the accumulation of ferritin correlated with the amount of iron. No ferritin was detected in the absence of iron, whereas two polypeptides of 28 and 26.5 kD, corresponding to the bands of the soybean ferritin control, were observed in the presence of iron. The amount of ferritin present in lane 1 (500 µM Fe-citrate) is about twice the amount of ferritin present in lane 2 (100 µM Fe-citrate). There is a good correlation between the amounts of ferritin synthesized in the presence of 500 and 100 µM Fe citrate and the corresponding cellular levels of iron (11.8 and 6.9, Table I). No ferritin was measured in the extract of cells grown in iron depleted medium. The lowest amount of ferritin detectable by this method is 4 ng. Therefore, in the absence of iron, the amount of ferritin present in the cellular protein extracts is lower than 0.1 µg/mg.

Relative Amounts of Ferritin mRNA in Cells Grown in the Absence or in the Presence of 500 µM Fe-Citrate

Equal amounts of RNA extracted from cells grown for 72 h with 500 µM or without Fe-citrate, were translated in a reticulocyte system, in the presence of $[^{35}$S]methionine. Translation products were immunoprecipitated with the antiserum raised against pea seed ferritins and analyzed by electrophoresis and fluorography (Fig. 2B). Lane 1 corresponds to the RNAs from cells grown in the absence of iron: no translatable ferritin mRNAs were observed in this sample. Lane 2 corresponds to the translation products of RNAs from cells grown with 500 µM Fe-citrate: an immunoprecipitated radioactive polypeptide of 32 kD is observed in this sample. The size of

![Figure 1. Growth curves of soybean cell suspensions in 500 µM Fe-citrate medium (●); in 100 µM Fe-citrate medium (○) and in iron-depleted medium (□). PCV, Packed cell volumes of 5 mL suspension fractions.](image-url)
Kinetics of Iron Induction of Ferritin Synthesis

Cell suspensions constitute a convenient system to study more accurately the kinetics of accumulation of ferritin and of corresponding mRNAs after iron addition. Cells previously grown in standard conditions were transferred into 500 μM Fe-citrate medium. Aliquots were collected after 0, 12, 24, and 48 h of growth and total protein and RNA were extracted from each sample. Immunoblot analysis of the proteins (Fig. 3A) shows that ferritin was detectable in the protein extracts after 12 h of culture in Fe-citrate medium. The ferritin content then increased during 48 h. In another experiment (not reported here), it was observed that the cellular content of ferritin decreased after 48 h of induction. Using a titration curve established with defined amounts of purified soybean ferritin and 125I-protein A, we have estimated the amounts of ferritin present in lane 4 (48 h) to 200 ng. This corresponded to 5 μg of ferritin per mg of total protein. No ferritin was

Figure 3. Kinetics of ferritin and translatable ferritin mRNA iron inductions. A, Immunoblot analysis of proteins extracted from cells grown for 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), and 48 h (lane 4) in the presence of 500 μM Fe-citrate. Forty μg proteins were analyzed in each case. Lane 1 corresponds to 1 μg ferritin purified from soybean seeds. B, Autoradiography of the immunoprecipitates of in vitro translation products. Ten μg RNA extracted from cells grown for 0 h (lane 1), 12 h (lane 2), 24 h (lane 3), and 48 h (lane 4), were translated in vitro. The translation products were immunoprecipitated and analyzed as in Figure 2B.

Figure 4. A, RNA synthesis in cell suspensions in the presence of actinomycin D. Cells were labeled with 3H]uridine (1 μCi/mL) in the presence of various amounts of actinomycin D: 0 μg/mL (□); 0.04 μg/mL (●); 0.4 μg/mL (○); and 4 μg/mL (△). The radioactivity incorporated into TCA precipitates from 2 mL cell suspensions was measured in each case after 0, 1, 2, 3 h of culture. B, Protein synthesis in cell suspension cultures in the presence of actinomycin D. Cells were labeled with 35S]methionine (1 μCi/mL) in the presence of 0 μg/mL actinomycin D (□); 0.04 μg/mL (●); 0.4 μg/mL (○); and 4 μg/mL (△). The radioactivity incorporated into TCA precipitates from 2 mL fractions of cell suspension were measured after 5, 24, and 48 h of culture.
detected by this method at zero time (lane 1). Equal amounts of RNA extracted at the various times of culture were translated in a reticulocyte system. The products of translation were immunoprecipitated and analyzed as previously. Figure 3B shows that ferritin mRNA is detectable after 12 h (lane 2), reached a maximum at 24 h (lane 3) and then decreased at 48 h (lane 4). These results indicate that the maximum accumulation of translatable ferritin mRNA precedes that of the protein.

**Effect of Actinomycin D on Ferritin Accumulation in the Presence of Iron**

To confirm that the accumulation of ferritin in response to iron addition results from a de novo synthesis of ferritin mRNA and not from activation by iron of stored preexisting ferritin mRNAs, induction was done in the presence of actinomycin D, an inhibitor of transcription.

Preliminary experiments were undertaken to determine the concentration of actinomycin D needed to inhibit completely RNA synthesis with low effect on protein synthesis. To test the effect of increasing concentrations of actinomycin D on RNA synthesis, cells were grown in the presence of [3H]uridine. After 2 h labeling, various concentrations of actinomycin D were added. Radioactivity incorporated into trichloroacetic acid precipitates was used as a measure of RNA synthesis. Figure 4A shows that in the presence of 4 μg actinomycin D per mL, the RNA synthesis was completely inhibited after a 1-h incubation.

[35S]methionine incorporated into trichloroacetic acid precipitates was used as a measure of total protein synthesis. Cells were grown in the presence of 1 μCi [35S]methionine per mL. After 8 h of labeling, various concentrations of actinomycin D were added. Figure 4B shows that after 24 h the inhibition of protein synthesis by 4 μg actinomycin D per mL is only of 30%. After this time, a stronger inhibition was observed. In the next experiments iron induction of ferritin synthesis in the presence of 4 μg per mL actinomycin D was measured 24 h after the addition of Fe-citrate.

Two cell suspensions were grown for 48 h in the absence of iron. Four μg per mL actinomycin D were then added to only one culture and both cultures were supplemented with iron-citrate 500 μM. The relative amounts of ferritin synthesized in both conditions after 24 h were examined by immunoblot analyses (Fig. 5). The accumulation of ferritin observed in the control culture (lane 1) was strongly inhibited in the presence of actinomycin (lane 2). This strong inhibition of ferritin synthesis in the presence of the drug, supports the hypothesis that ferritin accumulation results from de novo mRNA synthesis.

**DISCUSSION**

Van der Mark’s experiments (20,21) with bean leaves showed that the addition of excess Fe-EDTA to normal secondary bean leaves resulted in a 4-fold increase of ferritin content. This increase was correlated with similar increase of the translatable ferritin mRNA contents, suggesting a control at the transcriptional level. These results were in opposition with those obtained in animal cells by Schull and Theil (16). In tadpole reticulocytes, a 40-fold increase of ferritin synthesis after iron induction was not correlated with any significant differences between the levels of ferritin mRNA in control or iron-induced cells, determined by translation in a wheat germ system. Since these reports, intensive work on animal cells specialized in iron storage have shown that the rapid response to iron was achieved by a shift of stored mRNA from the ribonucleoprotein fraction to the polysomes (translational shift) (14). Therefore, at first sight, van der Mark’s experiments suggested that plants had a different mechanism for the regulation of ferritin syntheses. However, in a recent review, Theil (19), underlines that it is not completely assured that the changes in translatable ferritin mRNA in bean leaves could not be attributed to activation by iron of a stored inactive ferritin mRNA pool.

The physiological system we have developed with soybean cell suspension cultures allows further investigation of this question. We have shown that addition of 500 μM Fe-citrate to normal cells (100 μM Fe EDTA), results in a large induction of ferritin accumulation. No ferritin is detected in the protein extract of normal cells, which indicates that the level of ferritin is lower than 0.1 μg per mg of protein. After 48 h of induction, the ferritin level reaches 5 μg per mg of protein. This about 50-fold increase is comparable to that described in specialized animal cells. The comparison between the kinetics of accumulation of ferritin and that of its translatable mRNA in response to iron shows a good correlation between both increases. This induction is fast since both protein and mRNA are detected already 12 h after the iron supply. The speed of this response allowed us to test the synthesis of ferritin in the presence of actinomycin D in conditions where RNA synthesis was completely inhibited, whereas protein synthesis was only weakly affected. In these conditions, we clearly show that the iron induction of ferritin accumulation is inhibited by actinomycin D. Therefore, the ferritin synthesis appears dependent on de novo mRNA synthesis and not on the recruitment of a preexisting inactive mRNA pool.

Hybridization experiments with cloned plant ferritin cDNA probes would bring definitive response to this problem. However, in the absence of such probes, our results with actinomycin D confirm those obtained by van der Mark and strongly suggest that in both plant systems described, bean leaves and soybean cell cultures, the mechanism of regulation...
of ferritin synthesis in response to iron overload does not occur at a translational level. The regulation is more likely to be of the same type as that found in housekeeping animal cells (5), where iron influences either the transcription or the stability of ferritin mRNA, although the 50-fold increase in ferritin level we observed in soybean cells is much higher than what has been reported for housekeeping animal cells (18). However, it remains possible that in other types of plant cells more specialized in iron storage, such as seed tissues, a regulation at the translational level would occur.

Experiments are in progress in our laboratory to isolate plant ferritin genes. This will allow us to compare their structures with those of animal ferritin genes already established and to further investigate the regulatory mechanism(s) of iron induction of ferritins in plant cells.

ACKNOWLEDGMENTS

We thank Dr. Jean-Pierre Laulhère for iron measurements and for helpful advice during this work. The skillful technical assistance of Hélène Pesey is greatly appreciated.

LITERATURE CITED