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MULTIPLE MECHANISMS OF IRON-INDUCED FERRITIN SYNTHESIS IN HeLa CELLS

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SUMMARY: Iron administration to HeLa cells stimulates the accumulation of H-subunit and L-subunit rich isoferritins at similar extent. The increase in both types of isoferritins is accompanied by an increase in the amount of messenger RNAs specific for H and L subunits. The increase in the amount of these messenger RNAs, which occurs in the nucleus as well as in the cytoplasm, is proportionately lower than the increase in the protein. These results, together with analysis of transcription in isolated nuclei indicate the existence of a mechanism of transcriptional control of ferritin synthesis, associated to the translational control described so far.

Ferritin, an iron containing protein present in the tissues of various species, plays a major role in intracellular iron storage and detoxification (1). The heterogeneity and complexity of this protein is mainly due to the assemblage in various proportion of two subunit types, called H and L, into the multimeric shell (1). Each tissue and body fluid has characteristic isoferritins profile which can be modified during development, iron loading and other pathological conditions including malignancy (2-4).

H and L subunits are the products of separate mRNAs which are coded by distinct gene families (5-8).

The induction of ferritin by iron is well documented in a whole variety of tissues and in cultured cells. Several studies on rat liver have reported that

ABBREVIATIONS: FeNTA, ferric nitrilo triacetate; Tris, tris (hydroxymethyl)-aminomethan; NP-40, nonidet P-40; Hepes, 2 4-(2-Hydroxyethyl)-1-piperazinyl -ethane sulfonic acid; PMSF, phenilmethane sulphonyl fluoride; DTT, dithiothreitol; BSA, bovine serum albumin; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol-bis(ß-amino-ethyl ether)N,N,N',N'-tetraacetic acid.

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iron preferentially stimulates the synthesis of the more basic isoferritin types (rich in L subunits) relatively to the acidic ones (rich in H subunits) (9-12). HeLa cells also respond to iron supplementation by increasing apoferritin content (13).

In both rat liver and HeLa cells actinomycin treatment fails to prevent the stimulatory effect of iron indicating that the mechanism of iron induction of ferritin synthesis is mainly situated at the translational level (9, 13, 14). However, previous work did not analyze specifically the different classes of isoferritins or the single subunits, and was based on the determination of ferritin mRNA by a functional assay in cell-free systems using antibodies for the liver ferritin.

The development of specific radioimmunoassays for H subunit rich isoferritins (HRI) and L subunit rich isoferritins (LRI) together with the isolation of cloned cDNAs for the H and L subunits has provided us with more sensitive tools to analize the mechanisms of induction of ferritin synthesis by iron in HeLa cells.

MATERIALS AND METHODS

Cell and cell culture. HeLa cells were grown at confluent monolayer in Eagle's minimal essential medium, and the cells were refed with fresch medium without or with 2 mM FeNTA prepared as described (15). At various times the cells were removed from the dish with a rubber policeman and stored at -70°C in PBS buffer (150 mM NaCl, 3 mM KCl, 8 mM NaH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$, pH 7.4) containing 10% glycerol (the cells were prepared at the Istituto Zooprofilattico della Lombardia, Brescia, Italy).

Characterization of HRI and LRI. HeLa cells were homogenized in 2-5 volumes of Tris-HCl, pH 7.4, and heated for 5 min at 75°C. The clarified solutions were then analyzed with an anti-heart immunoradiometric assay (2A4-IRMA) based on a monoclonal antibody specific for HRI (16), and an anti liver immunoradiometric assay (HLF-IRMA) based on a polyclonal antibody which detects LRI (17).

Subcellular fractionation and RNA extraction. All operations were carried out at 4°C. Cells were washed several times in PBS buffer. The cellular pellet was used either for extraction of total cellular RNA or for preparation of nuclei and cytoplasm. For this purpose 20 x 10^6 cells were resuspended in 2.5 ml of RSB (10 mM NaCl, 10 mM Tris-HCl, pH 7.6, 3 mM $MgCl_2$) containing 0.5%. NP-40 and 1 mM PMSF, and homogenized in a loosely fitted Dounce homogenizer followed by centrifugation at 2000 x g for 2 min. RNA was extracted from the supernatant (cytoplasm) and from the nuclear pellet as reported (18). The purity of nuclear preparation was monitored with a light microscope after staining with 0.5% Toluidine blue. Total cellular RNA was extracted as described for nuclear RNA.

Transcription rate analysis. Washed cells were homogenized in 0.25% NP-40 in Buffer A (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 15 mM Tris-HCl, pH 7.5, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 0.5 mM EGTA, 14 mM ß-mercaptoethanol) and spun for 2 min at 2000 x g. Nuclear pellet was washed twice in buffer A and once in buffer B (50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 0.5 mM DTT, 10% BSA, 25% gly

cerol) and resuspended in buffer B. 5 x 10^6 nuclei were incubated at 25°C for 20 min in a 100 ul reaction mixture containing: 12.5% glycerol, 50 mM Hepes, pH 8.0, 5 mM $MgCl_2$, 1 mM $MnCl_2$, 2 mM DTT, 150 mM NH_4Cl, 0.5 mM each of ATP, GTP, CTP, 200 uCi of ^{32}P-UTP (600 Ci/mmole) and 25 U of RNasin.
^{32}P labeled RNA was extracted according to (19) with minor modifications and hybridized to excess cloned plasmid DNA immobilized on nitrocellulose filters as previously described (20).

Analysis of specific sequences. Northern blot analysis and hybridization with ^{32}P labeled DNA probes of total, cytoplasmic and nuclear RNA, were performed as described (21). The amount of RNA in each lane was corrected by the amount of rRNA as determined by hybridization of pXCR7 probe to the same filter.
The probes for H and L subunits of human ferritin were full length cDNAs cloned in pBR322 and were kindly provided by Dr. R. Cortese.
The chicken ß-actin cDNA clone and the pXCR7 clone for rDNA of Xenopus laevis were generous gift of Dr. J. Gurdon and Dr. I. Bozzoni respectively.
The probes were labeled by nick translation with ^{32}P-dCTP according to (22).

RESULTS

Amount of LRI and HRI in iron-treated HeLa cells. HRI were detected by a 2A4 monoclonal antibody while LRI quantitation was performed with a polyclonal antibody. The specificity of the antibodies and the characteristics of these immunoradiometric assays have been described elsewhere (16, 17). Fig. 1 shows the time course of HRI and LRI induction in HeLa cells after addition of 2 mM FeNTA. The increase over the control at 24 hrs is 12.5 and 17 times for HRI and LRI, respectively. The time course as well as the extent of stimulation are similar for both types of isoferritins. The change of the medium alone does not influence the amount of isoferritins (data not shown).

H and L subunit mRNAs concentration in total cellular RNA. Total RNA was isolated from HeLa cells after incubation for 24 hrs with FeNTA and the relative amount of specific sequences was determined by Northern blot analysis using H and L subunit cDNAs. Fig. 2 shows that in iron treated cells, the relative amount of H and L subunit mRNAs increases over the control two and three times respectively. However HRI and LRI accumulate to a higher degree than H and L subunit mRNAs. The discrepancy between increase in mRNAs and increase in proteins confirms that other factors, in addition to the availability of mRNAs, are responsible for the induction of ferritin synthesis by iron.

H and L subunit mRNAs concentration in nuclear and cytoplasmic RNA. Nuclear and cytoplasmic RNA were isolated from HeLa cells after incubation for different times in the presence of FeNTA and the relative amount of H and L subunit mRNAs was determined. Fig. 1 shows that in the nucleus the amount of mRNA for both L

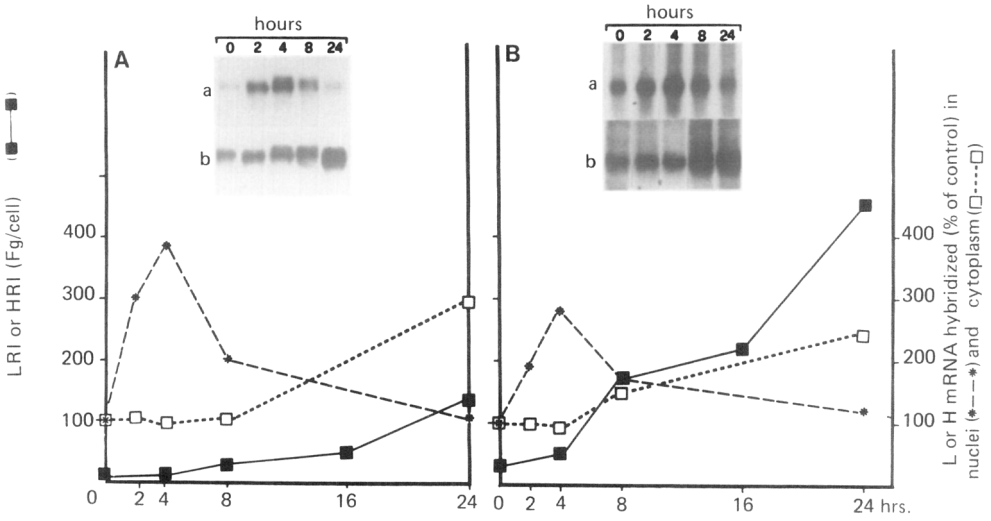

Fig. 1. A: Effect of time of incubation with iron. Amount of LRI (■—■) and relative concentration of L subunit mRNA in nuclei (*---*) and cytoplasms (□---□) HeLa cells were grown at confluent monolayer. The medium was replaced with medium containing 2 mM FeNTA, and after 0, 2, 4, 8, 16 and 24 hrs, cells were harvested and assayed as described in the text. Amounts of specific sequences were calculated by densitometric scanning of autoradiograms. The insert shows Northern blot analysis with L specific probes. Radioactivity visualized by autoradiography. 10 ug of nuclear (a) or cytoplasmic (b) RNA were loaded.

B: As Fig. 1 referred to HRI and H subunit mRNA.

and H ferritin subunits is significantly enhanced at 4 hrs (4- and 3-times over the control, respectively) and returns to control levels at 24 hrs; in the cy-

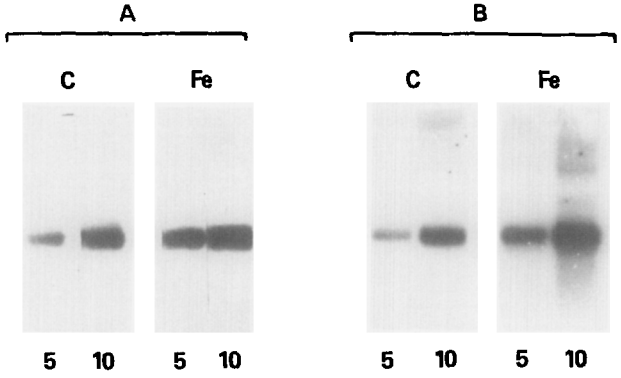

Fig. 2. Effect of iron on the relative amount of L and H subunit specific mRNAs in total cellular RNA. Northern blot analysis with L (panel A) and H (panel B) subunit specific probes. 5 and 10 ug of total RNA were loaded. Radioactivity visualized by autoradiography. HeLa cells were grown at confluent monolayer and refed with fresh medium without (C) or with 2 mM FeNTA (Fe). After 24 hrs cells were harvested ana assayed as described in the text.

toplasm a significant increase is found only at 24 hrs (3- and 2.5-times over the control, respectively). For both mRNAs the extent of induction is greater and more prompt in the nucleus than in the cytoplasm. As for total RNA the increase is slightly more pronounced for L than H subunit mRNA in both cellular compartments.

An increase in the concentration of ferritin specific mRNAs in nuclei seems to suggest a nuclear site of action of iron. Increased transcription and/or increased stability could be responsible for the higher amount of ferritin subunits mRNAs. To distinguish between these two possibilities we examined endogenous nuclear run on transcription in HeLa cells incubated in the presence of FeNTA.

Transcription rate analysis. To assay transcription rates of ferritin mRNAs the nuclei of HeLa cells were isolated and nascent RNA was labeled by chain elongation in the presence of ^{32}P-UTP; the labeled RNA was hybridized to plasmid DNA fixed on nitrocellulose filters, and RNase-resistant signals were detected by autoradiography. The complementarity between H subunit mRNA and 28 S rRNA (23) results in a high non specific background and does not allow a correct analysis of the transcription rate of H subunit mRNA; therefore we limited our investigation to L subunit mRNA. In comparing different samples, equal amounts of labeled nuclear RNA were used so that differential transcription rates could be assessed. Fig. 3 shows that the transcription rate of L subunit mRNA increases

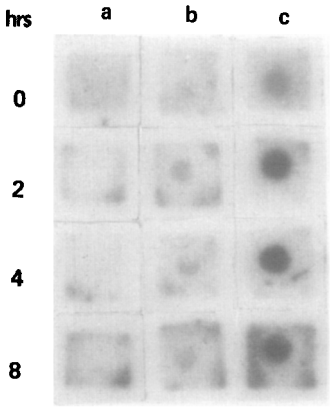

Fig. 3. Effect of iron on the transcription of L subunit mRNA. ^{32}P-labeled nuclear RNA was hybridized to pBR322 (a), actin (b), L subunit (c) specific probes as described in the text. Radioactivity visualized by autoradiography. HeLa cells were grown and incubated for 0, 2, 4 and 8 hrs in the presence of FeNTA as described for Fig. 1.

significantly relatively to control two hrs after addition of FeNTA without further increase at 4 and 8 hrs of treatment. Therefore the stimulation of L subunit mRNA transcription could explain the enhanced steady state concentration. Transcription of actin mRNA, used as a control, showed a minor increase possibly due to the replacement of the culture medium. Since, to the best of our knowledge, iron does not influence actin synthesis and the addition of fresh culture medium does not interfere with the synthesis of mRNAs for ferritin subunits, the increased transcription of L subunit mRNA seems specifically induced by iron.

DISCUSSION

Ferritins synthesis is stimulated by iron in a wide variety of cellular systems. Previous reports suggest that in mammalian cells iron exerts its action by altering the translational efficiency and distribution in the cytoplasm of ferritin mRNAs rather than by stimulating their synthesis (1, 14, 24). On the contrary the iron-dependent regulation of phytoferritin synthesis in bean leaves is correlated to an increased amount of translatable mRNA (25).

This is the first study, to the best of our knowledge, on the effect of iron on ferritin synthesis in human cultured cells using direct measurements of mRNA levels by cDNA probes. Our results show that in iron loaded HeLa cells both HRI and LRI increase at similar extent, while in liver LRI appear to be preferentially stimulated by the metal (26). The increase in isoferritins is accompanied by an increase in mRNA specific for H and L subunits suggesting the existence of a transcriptional control of ferritin synthesis by iron. Moreover the discrepancy between levels of mRNAs and proteins confirms the presence and the importance of a translational control. Besides the indirect experimental data, obtained with the use of actinomycin D, one reason to rule out a possible transcriptional control of ferritin synthesis was the theoretical consideration that direct contact of iron could be harmful to the nuclei (27). However, heavy metals have been reported to enter the nucleus and to stimulate transcription of metallothionein genes (28); on the other hand the effect of iron on the nuclei needs not to be a direct one. The increase of ferritin mRNAs in the nucleus and the increased synthesis of L subunit mRNA in isolated nuclei indicate that iron can really act at the nuclear level enhancing the rate of transcription of ferritin genes; however additional effect on mRNA processing and stability cannot be excluded.

In conclusion, iron-dependent regulation of ferritin synthesis appears to be more complex than previously hypothesized and possibly different in various cellular systems.

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