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THYROTROPIN STIMULATES TRANSCRIPTION FROM THE FERRITIN HEAVY CHAIN PROMOTER

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SUMMARY: Thyrotropin (TSH) is the primary hormone regulating the activity of the thyroid gland. We have recently shown that TSH stimulates H-ferritin mRNA levels in rat thyroid. Ferritin plays a key role in determining the intracellular fate of iron. The induction of ferritin synthesis by iron in liver is regulated both at transcriptional and translational levels. Here we present evidence that the mechanisms by which TSH regulates the mRNA levels are mediated by a diffusible product acting in trans on its own promoter. In fact, the H-ferritin promoter mediates increased CAT activity in response to hormone induction. Our results identify transcription as an important regulatory step of TSH action. They suggest that TSH induces expression of the ferritin gene, and that continuous protein synthesis is required to maintain basal ferritin gene expression in the absence of hormone.

Ferritin is a ubiquitous protein necessary for intracellular iron storage and detoxification. The protein consists of 24 subunits of heavy and light chains. Some cells (hepatocytes, macrophages and embryo red cells) are specialized for iron storage. Ferritin regulation is mediated mainly by changes in the intracellular distribution of mRNA, at least in cells specialized for iron storage. However, mechanisms of regulation could vary depending on the cell type: ferritin synthesis may depend more on mRNA concentration in some cell types and on mRNA utilization in others (see references 1 and 2 for reviews). Synthesis of protein subunits is regulated, at least in part, posttranscriptionally. The mechanisms by which ferritin synthesis is induced by iron have been extensively investigated, and recent studies have demonstrated translational regulation of ferritin expression in rat liver in response to iron (3, 4). However, little is known about other agents that can induce ferritin expression, or about the mechanisms controlling these differences in mRNA levels.

By differential screening of a thyrotropin-(TSH) stimulated rat thyroid cDNA library we have recently cloned the 3' end of rat ferritin heavy-chain cDNA (5). In the present investigation we have used that probe to study the

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levels of ferritin expression in response to TSH in two rat thyroid epithelial cell lines and in the thyroid gland.

Rat thyroid FRT-L5 cells express *in vitro* the main differentiated functions of the thyroid gland (iodide uptake, and thyroglobulin synthesis and secretion). Thyrotropin is a prerequisite for cell proliferation. It is, moreover, known to be the prime regulator of thyroid cells since it stimulates almost all the thyroid-specific differentiated functions. We have shown that TSH stimulates ferritin mRNA levels *in vitro* and *in vivo* (5). These results were subsequently confirmed by Cox et al. (6). Viral transformation by acute retroviruses is followed by the block of the expression of the differentiated phenotype (7). Viral cell transformation, in contrast, results in decreased mRNA levels (8).

Here we present evidence that enhanced ferritin expression depends, at least partially, on transactivation of the ferritin promoter. Thus, it appears that this is an excellent system with which to study hormone-dependent regulation of the transcription of ferritin in epithelial cells. Interestingly, hormone induction enhances the expression of ferritin at the same time as the differentiated phenotype.

MATERIALS AND METHODS

Cells: The isolation, growth and properties of FRTL-5 cells have been described elsewhere (9). Cells were grown in Coon's modified Ham's F12 medium supplemented with 5% calf serum (Gibco) and a 6-hormone mixture including bovine TSH (10^{-10}M) (Sigma). When cells approached confluency they were split at a ratio of 1:3 in the absence of TSH. Eight days later TSH was added again at the same concentrations.

Isolation of RNA and RNA filter hybridization: Total RNA was extracted from cultured cells as described previously (10). RNA was dissolved in 2.2 M formaldehyde; 50% formamide; 1X MOPS buffer (20 mM Morpholino-propanesulfonic acid, 5mM sodium acetate, 1mM EDTA, pH 7.5) for 15 min at 55°C, electrophoresed and transferred to nitrocellulose paper (Schleicher & Schuell). Hybridizations were performed as previously described (8). The filters were probed also with rat B-actin cDNA (kindly provided by Dr. Bruce Paterson) as a control of RNA integrity. The northern blotting data are examples of at least three independent RNA preparations. Cyclohexamide treatment of cells was performed as previously described (11).

Transfections: Quiescent, serum starved FRTL-5 cells (10^6) were transfected in duplicate with 20ug of plasmid DNA with the calcium phosphate precipitation method (12). Four hours later the medium was removed and the cells were treated with 15% glycerol. After one minute the glycerol was removed by washing two or three times with fresh medium, after which the cells were incubated at 37°C; either pure TSH (10^{-10}M) or the 6H mixture was then added to the stimulated samples and the cells were incubated for 36 hrs. For each plasmid, lysates from an equal number of cells were assayed for Chloramphenicol Acetyltransferase (CAT) activity (13). The B-actin-CAT construct was a gift of (14). The 400 bp H-ferritin promoter-CAT construct was a gift of (15).

RESULTS AND DISCUSSION

Transcriptional controls on ferritin heavy chain promoter have been described in HL60 cells stimulated to differentiate with dimethylsulphoxide (DMSO) or tetra-decanoyl-phorbol-acetate (TPA) (16). On the other hand, experiments conducted on mouse fibroblasts suggest that the major part of iron induction of ferritin synthesis is attributable to translational controls residing in the 5' leader region of that gene (13). Here we demonstrate that in the TSH-stimulated thyroid cells H-ferritin mRNA levels depend on the continuous synthesis of a specific gene product (or products) rather than on an increased stability of the messenger RNA. Cyclohexamide superinduces mRNA levels by two mechanisms, i.e. by inhibiting the shut-off of transcription, and by prolonging the half-life of the mRNAs. Therefore cyclohexamide was added to quiescent serum-starved, FRT-L5 cells, and 120 min later, the cells were stimulated with TSH, after which RNA was isolated at various times. Under these conditions the overall protein synthesis is reduced by 95% (11). The inhibition of protein synthesis did not stabilize the basal levels of ferritin in the absence of TSH (fig. 1, compare lanes 1, 2 and 3). Moreover, inhibition of protein synthesis caused a further reduction in ferritin mRNA basal levels (fig. 1, lanes 1 and 4; and table I). These experiments suggest that a limiting amount of a gene product is required either for messenger stability or for transcription initiation rate.

By using a recombinant plasmid in which the Chloramphenicol Acetyltransferase (CAT) mRNA is placed under the control of a 400-base pairs (bp) human fragment containing the promoter of the ferritin H-chain gene (HfepCAT), we investigated whether or not the exogenous human promoter is

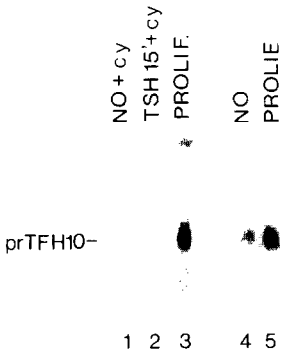

Fig 1 Northern blot analysis of H ferritin mRNA in cyclohexamide treated FRT-L5 cells. 10ug of total RNA was separated on 1% agarose gel and probed with ^{32}P labeled pRTFH10 cDNA insert. Lane 1) RNA from quiescent cells treated for 120 min with 10 ug/ml of cyclohexamide. Lane 2) RNA from the same cells as in lane 1) to which TSH has been added for 15 min. Lane 3) RNA from proliferating cells grown in the presence of 6H mixture (including TSH) and 5% serum. Lane 4) RNA from quiescent cells. Lane 5) the same as in lane 3) but from a separate experiment.

TABLE I

PER CENT H FERRITIN mRNA LEVELS

1) FRT L5	prolif.	100
2) FRT L5	6H-starved	43
3) FRT L5	6H-starved + cyclo	18

1) prolif.: proliferating cells grown with serum and the 6H mixture.
2) 6H-starved: quiescent cells serum- and hormone-starved for 4 days; the hormone mixture includes TSH.
3) 6H-starved + cyclo: quiescent cells as in 2) incubated in the presence of cyclohexamide for 120 minutes.

The hybridization levels were quantified by integration of the peak areas of the densitometric scanning and are expressed as per cent of levels found in the chronically stimulated cells.

sensitive to TSH induction in the FRT-L5 cells. The cells grown at 1/3 confluency were deprived of serum and hormones for 4 days, and then the HfepCAT was transfected by calcium phosphate precipitation. Four hours after DNA addition, one set of cells was stimulated with TSH, and a second set of cells remained in starved growth conditions. As control, a parallel experiment was conducted in which the CAT gene was transfected under the control either of the viral Rous sarcoma virus (RSV) promoter or the human B-actin promoter.

Figure 2 shows the results of the CAT assay. The addition of TSH had little or no effect on RSV or actin promoter. In contrast, TSH caused a more

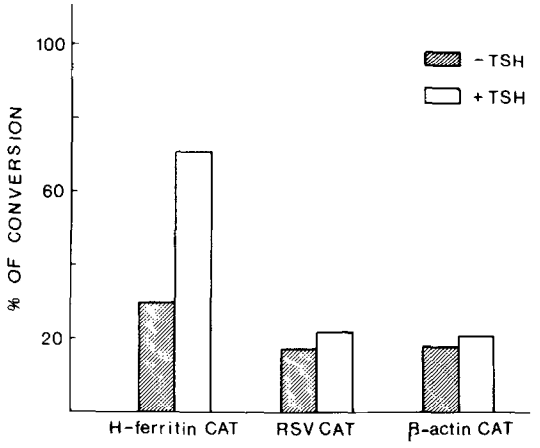

Fig 2 The human ferritin heavy chain promoter is sufficient to transfer TSH-dependent regulation to an heterologous CAT gene. H-Fe-CAT (left), RSV-CAT (center), and B-actin-CAT (right) constructs were transiently expressed in FRT-L5 cells as described in Material and Methods. In the histogram, the CAT activity levels are reported in arbitrary units per 10^6 cells. Those levels result from the mean obtained in four independent experiments.

than two-fold increase in the expression of the H fer-CAT construct more than two fold. Addition of the 6H mixture instead of TSH to the starved cells did not change appreciably the relative induction of fer-CAT activity (data not shown).

H-chain ferritin transcription is regulated in a hormone-dependent fashion in rat thyroid gland and in two thyroid epithelial cell lines. We have, in fact, recently demonstrated that steady state levels of ferritin H chain mRNA levels are induced in thyroid gland a few hours after TSH addition (5).

Studies on HL60 (16) and Friend erythroleukemia cells (17) have revealed a transcriptional response of ferritin chains following addition of inducers of differentiation. More recently, iron has been shown to induce an increase in mRNA accumulation and synthesis of ferritin chains (18).

Here we show that TSH induction of H ferritin mRNA levels acts, at least partially, via its own promoter. The transfection experiments demonstrate, in fact, that TSH stimulates CAT activity when transcription of the gene is controlled by a human H-ferritin promoter, but not when it is controlled by a viral or B-actin promoter. The 5' not transcribed flanking sequences of human and rat H-ferritin share a large sequence homology (19). This supports the notion that, in our experiments, the heterologous human H ferritin promoter stimulated CAT expression via a mechanism similar to that of the endogenous ferritin promoter. Ferritin mRNA stability in quiescent thyroid cells is not enhanced when protein synthesis is inhibited. In contrast, the transcription levels found in the quiescent cells are further depressed when these cells are treated with cyclohexamide (table I).

It is difficult to establish the biological meaning of TSH induction of ferritin, TSH being both a competence growth factor (12) and a general inducer of the differentiated functions of thyroid cells. The addition of either forskolin or dibutyril-cAMP (data not shown and ref. 5) reproduces exactly the TSH induction of ferritin levels; thus cell proliferation is not a prerequisite for ferritin stimulation.

Finally, it is noteworthy that also transferrin receptor levels (20) are stimulated by TSH in FRTL-5 cells, thus supporting the idea that mechanism operating in this system is different from that found in the specialized cells where transferrin receptor and ferritin synthesis are inversely correlated in response to iron (21, 22).

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