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TSH REGULATION OF FERRITIN H CHAIN MESSENGER RNA LEVELS IN THE RAT THYROIDS

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Ferritin heavy chain mRNA steady state levels are increased by thyrotropin both in vivo and in two independent thyroid derived permanent cell lines. Maximum induction was achieved 48 hours after thyrotropin addition in the same conditions in which all the thyroid differentiated functions were stimulated.
Thyrotropin stimulation of the levels of ferritin heavy chain mRNA seems to be mediated by cyclic AMP since it mimicks the hormone induction.

TSH is known to be the prime regulator of thyroid cells since it stimulates almost all the thyroid-specific differentiated functions (iodide uptake,thyroglobulin synthesis and thyroid hormone secretion) (1). This hormone is, however, strictly required for the thyroid differentiated cells to proliferate.

We have previously demonstrated that thyroglobulin mRNA levels are regulated by TSH both in vivo and in vitro (2). The question arises whether other functions are stimulated by TSH at the mRNA level. The availability, in our laboratory, of two independently isolated rat thyroid cell lines (FRTL-5 and PC), that express in vitro the main differentiated functions of the thyroid gland(3,4), provided an excellent system to answer this question.

By differential screening of a TSH stimulated thyroid cDNA library, we identified a cDNA clone (pRTFH 10), specific for a 0.9 kb mRNA species whose accumulation in rat thyroid is under TSH control. The DNA sequence analysis of

The abbreviations used are: TSH,thyrotropin; $(Bu)_2$-cAMP,dibutyryl cAMP;PTU, propylthiouracil, S.D., standard deviation.

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this clone revealed a high degree of homology with the human ferritin heavy (H) chain (5). The steady state levels of this mRNA are increased by TSH both *in vivo* as well as in the two thyroid cell lines,FRTL5 and PC, and the TSH effect is mimicked by a cAMP analog, dibutiryl cAMP.

MATERIALS AND METHODS

Animals A group of male Wistar rats (approx 250 g) were artificially made hyperthyroid by daily injections of T4(10 ug/100g body weight/day for 10 days of treatment). A second group was not treated and a third group was given the antithyroid drug PTU, for 8 days, in order to raise the TSH concentration in their serum(2,6).

Cells The isolation,growth and properties of FRTL-5 (3) and PC (4) cells have been described. Cells were grown in Coon's modified Ham's F12 medium supplemented with 5% calf serum(Gibco) and a 6 hormones mixture including bovine TSH($1x10^{-10}$ M) (Sigma). When cells approached confluency they were split at a ratio of 1:3 in the absence of TSH. After 8 days TSH or $(BU)_2$-cAMP (Sigma) were readded at the noted concentrations.

cDNA library and screening procedures The cDNA synthesized from the polyA$^+$ fraction of the RNA obtained from rat thyroid glands was cloned in the PstI site of pBR322 by poly(dC)-poly(dG) tailing (7). Individual colonies were grown on nitrocellulose filters (Schleicher & Schuell). Duplicate filters (8) were probed with (^{32}P)-cDNA synthesized by RNA : a)from the thyroids of T4 treated rats; b)from the thyroids of PTU treated rats. Bacterial colonies showing differential hybridization signals were picked and re-screened.

Isolation of RNA Cells were washed twice with cold phosphate buffered saline, treated with 6M guanidine isothiocyanate and removed from plates with a teflon policeman. RNA was separated by centrifugation through a cesium chloride cushion (9).

Total RNA from rat tissues was prepared as described(10).

RNA filter hybridization RNA was dissolved in 2.2M formaldeide/ 50%formamide/ 1x MOPS buffer(20 mM Morpholino-propanesulfonic acid,5mM Sodium Acetate,1mM EDTA,pH7.5) for 15 min. at 55°, electrophoresed and transferred to nitrocellulose paper(Schleicher & Schuell) or spotted using a blotting manifold (Bethesda Research Laboratories) (11). Hybridizations were performed as previously described (2).

All the RNA dot blot hybridizations were probed with rat β-actin cDNA (kindly provided by Dr. Bruce Patterson) in order to compare the level of hybridization to the quantity of RNA actually spotted.

DNA sequencing Sequence analysis was done by the dideoxy chain termination method (12).

Statistics Results of all experiments are expressed as the mean$\pm$ SD of replicate experiments and analysis.

RESULTS AND DISCUSSION

Four thousand clones from a rat thyroid cDNA library were screened with labeled cDNAs synthesized from two mRNA populations: a)from TSH-deprived rat thyroids and b)from TSH-stimulated rat thyroids (see Materials and Methods). About 20 clones with differential hybridization signal in the two cases were

detected and all of them showed a stronger signal when the cDNA from PTU treated animals was used as probe.

The clones were purified, their DNAs radio-labeled and probed in Northern blot analysis with the same two mRNA preparations used for the screening. Four cDNA clones identified thyroglobulin mRNA, one out of the 16 remaining, named pRTFH10, gave a stronger signal with TSH stimulated thyroid mRNA, suggesting that its levels are positively regulated in vivo by TSH.

This cDNA clone identified, by Northern blot analysis, an abundant mRNA species, about 0.9 Kb long, present in the total RNA preparations from thyroid, liver, brain and spleen (Fig.1) as well as kidney, heart and muscle (data not shown).

To identify the gene product of pRTFH10 cDNA we performed a DNA sequence analysis of its 250 base pairs insert. Fig.2 shows the DNA sequence of this clone and the high degree of homology with the COOH terminus of the H chain of human ferritin cDNA (5). This establishes that the isolated cDNA clone corresponds to rat ferritin H chain. The derived amino acid sequence corresponds (with minor variations) to the previously reported sequence of a

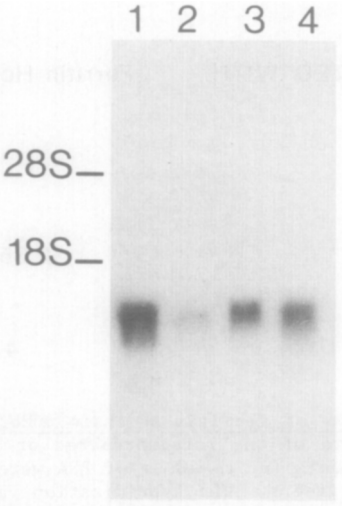

Fig.1 Northern blot analysis of pRTFH10 mRNA in several rat tissues 10 ug of total RNA was separated on 1% agarose gel and probed with labeled pRTFH10 cDNA insert. Lane 1 was RNA from rat thyroid, lane 2 from rat brain, lane 3 from liver and lane 4 from spleen.
The arrows indicate the 28S and 18S rRNA's position on the stained gel.

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                                        Ser
pRTFH10        CATTACCTGAATGAGCAGGTGAAATCCATTAAAGAACTGGGTGACCACGTGACCAACTTA
                                      *   *     *                       *
Human      500 CATTACCTGAATGAGCAGGTGAAAGCCATCAAAGAATTGGGTGACCACGTGACCAACTTG
Ferritin       HisTyrLeuAsnGluGlnValLysAlaIleLysGluLeuGlyAspHisValThrAsnLeu
H chain

                                       Met
               CGCAAGATGGGAGCCCCTGAATCTGGCATGGCAGAATATCTCTTTGACAAGCACACCCTG
                                 *  *        *   *
           560 CGCAAGATGGGAGCGCCCGAATCTGGCTTGGCGGAATATCTCTTTGACAAGCACACCCTG
               ArgLysMetGlyAlaProGluSerGlyLeuAlaGluTyrLeuPheAspLysHisThrLeu


                  HisGly   Asp
               GGACACGGTG   ATGAGAGCTAA       gctgacgtccccaag  gccatgtgactt
                 *  *   ***    *      *******   * **    **  ** ****    *
           620 GGAGACAGTGATAATGAAAGCTAAgcctcgggctaatttcccatagccgtggggtgac t
               GlyAspSerAspAsnGluSerStop


               tcatggtcactgaggcagtgcatgcatgctg agctgcctttatctttctata gtt
                 *      **                 * ** * *      *   *    *
           679 tcctggtcaccaaggcagtgcatgcatgttggggtttcctttaccttttctataagtt
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Fig.2 Homologie between the nucleotide sequence of pRTFH10 insert and the human ferritin H chain mRNA. Capital letters indicate the coding region present in the cDNA sequence. The numbers above the sequence designate the position in the coding region of human ferritin H chain mRNA.

peptide derived from cyanogen bromide digestion of the rat liver ferritin H subunit (13).

We assessed the TSH specific regulation of ferritin H chain mRNA in vivo. Fig.3 shows the level of hybridization detected with total RNA extracted from

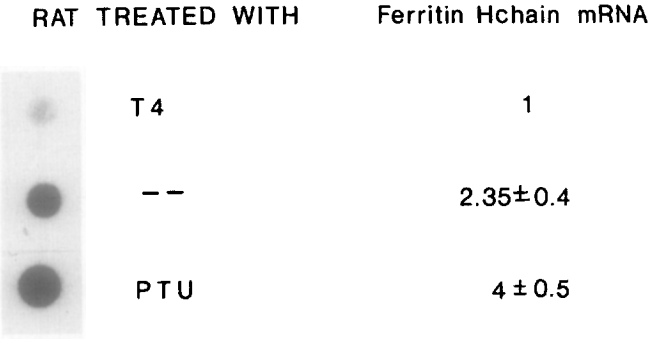

Fig.3 In vivo stimulation of ferritin H chain mRNA levels Total RNA was extracted from the thyroid of the rats,untreated or treated as described in the text. Increasing amounts (up to 10 ug)of RNA were dot spotted and probed with prFH10 labeled cDNA insert. The hybridization values were normalized by integration of the peak areas of the densitometric scanning and are expressed relatively to the level of the T4 treated rats,arbitrarily sets to 1. The results exposed are the means ± SD determined for each RNA concentration in 3 independent experiments.
In the left part of the table is shown a dot blot hybridization made with the different RNA preparations.

thyroid glands of normal rats, T4-treated rats and PTU-treated rats using the pRTFH 10 labelled insert as probe. TSH levels are decreased by T4 treatment and increased by PTU treatment (see Materials and Methods). An increased TSH level in the serum was accompanied by an increase of ferritin H mRNA in the thyroid, and viceversa.

The same treatment did not affect the steady state mRNA levels detected in several other tissues (data not shown).

The effect of TSH on ferritin H mRNA levels was analysed in the two rat thyroid differentiated cell lines ,FRTL-5 and PC. Cells were TSH-starved for 8 days before the hormone was readded to the medium. The cells were cultured for 48 hours, total RNA was extracted and ferritin H chain mRNA concentration was determined by dot blot hybridization. Using the same experimental conditions we have previously detected a 2 fold increase in thyroglobulin mRNA levels (2). Fig.4a shows that a two fold increase of ferritin H chain mRNA in PC cells could be detected 48 hours after the hormone readdition, reaching the same level of chronically stimulated cells (Fig.4a). Similar results have been obtained with FRTL-5 cells (data not shown).

In Fig.4b the kinetics of this process are shown in FRTL-5 cells. TSH raised the ferritin H chain mRNA levels about 1.7 fold in 6 hours but maximal induction, 2.3 fold, occurred at 72 hours and it remained constant after longer stimulation. No further increase of ferritin H chain mRNA level was observed at higher TSH levels, up to $1x10^{-8}$ M (data not shown).

The complex TSH regulation in these cells involves cAMP as second messenger, thus iodide uptake, as well as thyroglobulin gene expression, are TSH dependent cAMP mediated processes (14,15). We have therefore investigate whether also ferritin H chain mRNA levels are induced through the effect of cAMP as are the above mentioned differentiated functions.

We performed the same experiment shown in Fig.4a . Cells were TSH starved for 8 days and subsequently $(Bu)_2$-cAMP $1x10^{-3}$ M or $1x10^{-4}$ M was added to the culture medium . A comparable increase in ferritin H chain mRNA was reached when cells were treated with either $1x10^{-10}$ M TSH or $1x10^{-3}$ M $(Bu)_2$-cAMP

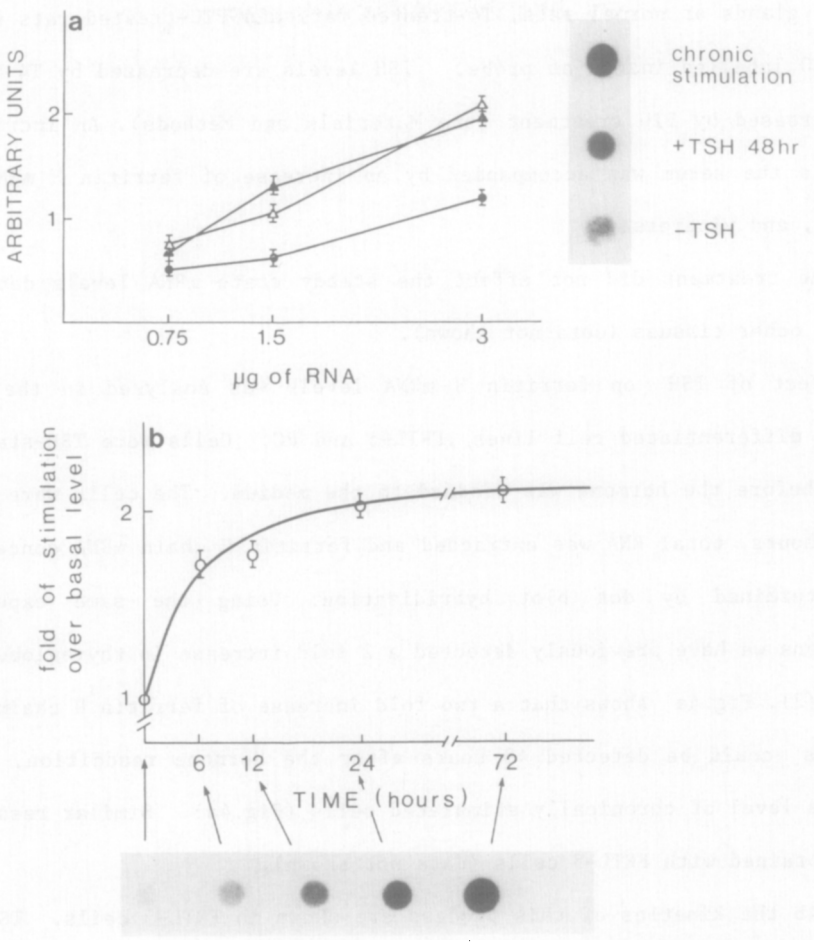

Fig.4 In vitro ability of TSH to increase the accumulation of ferritin H chain mRNA. Actively growing cells were plated in culture medium depleted of TSH and maintained in this conditon for 8 days. TSH ($1x10^{-10}$ M) was readded to the medium at the time 0 for the indicated numbers of hours. Total RNA was extracted at each time and ferritin H chain mRNA level was determined by dot blot hybridization as described in the legend to Fig.3:
a)PC cells coltured without TSH (●-●),with TSH for 48 hours (▲-▲) and cronically stimulated (▲-▲) by the hormone;
b)FRTL-5 cells:time course of ferritin H chain mRNA increase.
The experimental points reported in the figure are the means ± SD determined for each RNA concentration in 3 independent experiments as described in the legend to fig.3. In both a) and b) representative dot blots are reported.

(Fig.5). At the same cAMP concentrations used also thyroglobulin mRNA stimulation has been previously detected (16). Furthermore, cAMP mimics the effects of TSH both on endogenous c-myc and c-fos mRNAs levels in FRTL-5 cells (17,18).

Ferritins are a heterogeneous family of ubiquitous proteins which are able to sequester and detoxify intracellular iron in eukaryotic cells. Each molecule contains different amounts of the H and L chains which are responsible for the

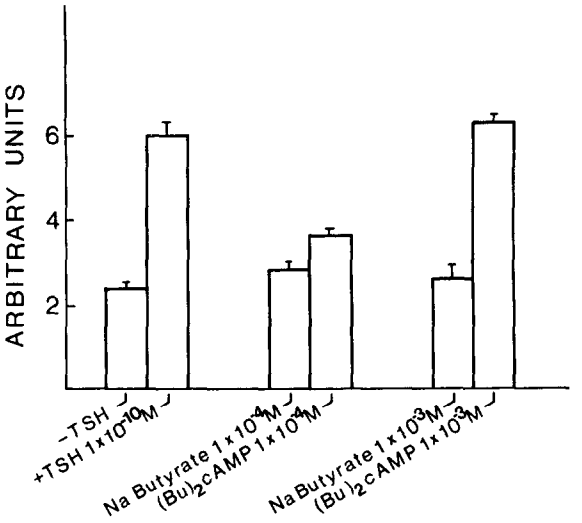

Fig.5 Ferritin H chain mRNA induction by cAMP. FRTL-5 cells were starved for TSH as described in the legend to Fig.4. Both TSH or $(Bu)_2$-cAMP at the indicated concentrations were readded to the medium for a 48 hours time. Sodium butirrate was also readded to the medium at the same concentration as $(Bu)_2$-cAMP.
Total RNA was extracted in each cases and ferritin H chain mRNA level determined by dot blot hybridization as described in the legend to Fig.3.

protein heterogenity. The induction of ferritin by iron has been demonstrated in various tissues and cultured cells (19). Translational regulation as well as changes in the intracellular distribution of the specific mRNA, have been hypothesized as playing a major role in ferritin regulation in response to iron induction(20). In HL-60 human promyelocytic cell line induced to terminal differentiation by DMSO, a dramatic increase in H and L chains mRNA accompanies the induced differentiation (21) : that is the only case so far reported of mRNA steady state regulation for the two ferritin subunits.

In the thyroid cells TSH has been demonstrated to be the major inducer of differentiated functions ; in this system we showed an increase of ferritin H chain mRNA accumulation in response to TSH addition both in vivo and in vitro, through a mechanism involving cAMP as second messenger. These data suggest, in analogy with HL-60 cells system, that in thyroid cells the TSH dependent stimulation of the differentiated phenotype is accompanied by an increased H ferritin mRNA steady state level.

Recently we observed that ferritin H chain mRNA is repressed in Kirsten Murine Sarcoma Virus transformed rat thyroid cells (22). In these cells viral trasformation is followed by a block in the expression of the thyroid epithelium differentiated functions (23): this observation strenghtens the hypothesis of a correlation between the H ferritin mRNA levels and the degree of differentiation of the cells under analysis.

Whether the TSH induction of ferritin mRNA in thyroid cells reflects an increase of transcriptional activity or mRNA stability, remains to be established.

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