THYROTROPIN-RELEASING HORMONE INCREASES THE LEVELS OF C-FOS AND β-ACTIN mRNA IN GH3/B6 PITUITARY TUMOR CELLS

ANDREA S. WEISMAN, ANDRÉE TIXIER-VIDAL, AND DANIELLE GOURDJI
Groupe de Neuroendocrinologie Cellulaire et Moléculaire, U.A. C.N.R.S. 1155,
Collège de France, 11 Place Marcelin Berthelot 75231, Paris Cedex 05, France

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SUMMARY
The effects of thyrotropin-releasing hormone (TRH) on the mRNA levels of c-fos, N-myc, β-actin and prolactin (PRL) were studied in GH3/B6 cells, a rat pituitary cell line. TRH has previously been shown to increase biosynthesis and release of PRL, and to stimulate PRL gene transcription (13,19) in these cells. All experiments were performed on quiescent serum-deprived cells, and under these conditions, addition of TRH stimulated PRL production but did not alter cellular proliferation. Simultaneously, TRH induced time- and concentration-dependent increases in c-fos and β-actin mRNA levels, with peak responses at 30 min and 4 h, respectively, in a dose range from 1 nM to 100 nM. The TRH effects on N-myc mRNA levels were less consistent. Addition of serum to quiescent GH3/B6 cells induced (3-5 fold) increases in c-fos, β-actin and PRL mRNAs which differed in magnitude and kinetics when compared to TRH stimulation. Serum did not alter N-myc mRNA levels. The TRH-induced increases in c-fos and β-actin mRNA may play a role in the secretory response.

Key words: oncogenes; neuropeptide; c-fos; TRH; prolactin cells; secretion.

INTRODUCTION
The hypothalamic neuropeptide thyrotropin-releasing hormone (TRH) stimulates prolactin release and biosynthesis in GH3/B6 cells, a rat pituitary tumor-derived cell line. The latter event has been shown to be a consequence of the increased transcription of the prolactin gene (13,19,25). These effects are mediated via occupation of specific binding sites coupled to the phosphatidylinositol pathway (11). Concomitantly, TRH alters cell shape, membrane traffic and under certain conditions, cell proliferation (4,13).

Recent evidence has indicated that several proto-oncogenes play a role in the proliferation and/or differentiation of numerous cell types, including epithelial cells (14,16,29). The expression of the myc and fos oncogenes which code for nuclear proteins and the actin gene which codes for a structural protein, has been shown to be induced by growth factors (NGF, PDGF, FGF, EGF) and thyroid stimulating hormone (TSH) in both rapidly proliferating and differentiated cells (2,15,17,23). Therefore, we have studied the effects of TRH and serum on proto-oncogene expression in GH3/B6 cells in culture. We demonstrate that TRH increases the levels of c-fos mRNA in a manner that differs from serum, while neither treatment is capable of substantially altering N-myc expression. In addition, TRH and serum also increase the mRNA level of the β-actin gene. The effects of TRH are both time- and concentration-dependent and appear to be temporally related to the activation of the prolactin gene. This work provides the first example of hypothalamic neuropeptide regulation of c-fos gene expression in pituitary target cells.

MATERIALS AND METHODS
Cell culture. GH3/B6 cells, a subclone of the GH3 tumor-derived rat pituitary cell line (13,31), were grown in monolayers in Ham’s F12 medium supplemented with horse serum (15%), fetal calf serum (2.5%), penicillin and streptomycin (50 units/50 μg/ml). 36 h prior to addition of TRH or serum, cells were exposed to a serum-free medium composed of Ham’s F12 containing transferrin (5 μg/ml), selenium (3 × 10⁻⁸ M) and antibiotics (5 units/5 μg/ml) (F12(+)1) to induce quiescence (10). DNA, protein content, and cell number were determined as previously described (19) in 6-day cultures exposed to serum-free medium for 36 h and then stimulated with TRH or serum for 48 h. Medium prolactin was radioimmunoassayed as described (4) and expressed in ng-equivalents of rPRL-RP3 provided by the Hormone Distribution Program of the National Institutes of Health.

RNA preparation and analysis. Incubations with TRH or serum were terminated by trypsinization of cells followed by centrifugation (1500 x g, 5 min) and resuspension of the cell pellet in a guanidium thiocyanate...
solution (4 M GnSCN, 25 mM Na citrate, 1 mM Na-EDTA and 0.1 mM β-mercaptoethanol). RNA was isolated by centrifugation through a CaCl2 cushion (5), precipitated twice with ethanol and quantitated by measurement of absorbance at 260 nm. Total cellular RNA was denatured in a glyoxal (1M)/dimethylsulfoxide (50%) solution and fractionated (10 μg/well) on a 1.2% agarose gel, or used in "dot-blot" experiments following denaturation in 10x SSC containing 7.5% formaldehyde. mRNA (1, 2.5 and 10 μg/dot) was transferred to Genescreen (NEN) membranes and fixed by baking for 2 h at 80°C (28). Hybridizations were performed using the methods of Maniatis et al. (21).

Both cDNA probes v-fos, a PstI/PvuII fragment cloned in PBR322, and N-myc, an EcoRI/BamHI fragment cloned in PBR322, were obtained from Oncor (Gaithersburg, MD). The actin probe, the entire coding sequence of the mouse β-actin gene, was cloned in PAL 41 (1). The prolactin probe, a 823 bp cDNA corresponding to the complete coding sequence (7) cloned in PBR322, was a gift from Dr. J. Martial (Liège, Belgium). All probes were labeled by nick-translation {26) to a specific activity of 0.5-2 x 10^6 dpm/μg. Autoradiography was performed with Kodak X-AR film for 1-14 days at -70°C. Total cellular RNA was denatured in a glyoxal solution and precipitated twice with ethanol and quantitated by measurement of absorbance at 260 nm. Total cellular RNA was denatured in a glyoxal (1M)/dimethylsulfoxide (50%) solution and fractionated (10 μg/well) on a 1.2% agarose gel, or used in "dot-blot" experiments following denaturation in 10x SSC containing 7.5% formaldehyde. mRNA (1, 2.5 and 10 μg/dot) was transferred to Genescreen (NEN) membranes and fixed by baking for 2 h at 80°C (28). Hybridizations were performed using the methods of Maniatis et al. (21).

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RESULTS

TRH treatment (48 h) of quiescent GH3/B6 cells stimulated PRL production but did not alter cell proliferation. In contrast, serum addition slightly increased cell number but did not greatly alter PRL production (Table 1).

To elucidate the possible effects of TRH on N-myc, c-fos and β-actin mRNAs, we first performed a time course study. The levels of N-myc, c-fos and β-actin mRNAs were determined by dot-blot analyses of total RNA extracted from quiescent GH3/B6 cells and cells exposed to 30 nM TRH for 15 minutes to 10 hours (Fig. 1). Hybridization studies revealed signals of variable intensities for all genes under control conditions (untreated cells) (Fig. 1A). Treatment with TRH resulted in a rapid increase in the c-fos mRNA signal by 15 min (the shortest duration tested), which reached a peak at 30 min to 1 h. This increase (Fig. 1B) (compared to control cells) was observed in three independent experiments, and was followed by a lower but sustained level of expression which eventually declined to control values by 10 h. A slight increase in the N-myc mRNA signal was detected between 15 minutes and 1 hour. A two-fold increase in β-actin mRNA levels occurred at 4 h and thereafter, the signal slowly decreased to control levels.

We then examined the concentration dependence of TRH effects on N-myc, c-fos, β-actin and PRL mRNAs. Quiescent GH3/B6 cells were exposed to TRH in a range of concentrations from 1-100 nM for 30 min (Fig. 2). TRH treatment induced an increase in gene expression for c-fos, β-actin and prolactin; maximal increases were 3-fold, 4-fold and 3-fold, respectively, over control values. It is important to note that 80-90% of the maximal increase in mRNA levels was achieved in the same dose range for the three mRNA species. In contrast, the levels of N-myc mRNA signal did not increase in response to TRH in a dose-dependent fashion, although a small increase was observed at 1 and 10 nM (Fig. 3).

Total RNA obtained from TRH-treated cells and control cells was then analyzed using the Northern hybridization procedure to determine the length of the mRNA transcripts recognized by the probes in our study (Fig. 3). When blots were hybridized with the c-fos specific probe, very little mRNA was detected in the sample from control cells. However TRH stimulation resulted in the appearance of prominent bands (larger than 10-fold increase) which migrated as a 2.2 kb species having the same molecular weight as reported previously for c-fos mRNA (15). The discrepancy in the levels of c-fos expression in the dot-blot and Northern analyses is possibly the result of a higher background in dot-blot methodology, although these differences are well within the range of variability of responses currently observed in similar studies. In the case of N-myc, a 3.0 kb mRNA transcript was observed in both control and TRH treated-cells in which a slight increase was observed at 1 and 10 nM. Under identical experimental conditions (data not shown) β-actin mRNA migrated as a 2.2 kb species, and PRL mRNA as a 1.0 kb species. These molecular weights are identical to those previously published (7,19).

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<td>EFFECTS OF TRH AND SERUM TREATMENT ON CELL GROWTH AND PROLACTIN PRODUCTION*</td>
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*GH3/B6 cells were grown and treated as described in Methods. At the end of the 36 hour exposure to F12 (i+1) (time 0 of treatment) cell number was 457 ± 17.3 x 10^3 cells/well; protein was 253 ± 23 μg/10^6 cells; medium prolactin was 5.64 ± 0.48 μg/10^6 cells. The data represent the mean ± SD of triplicate wells from a representative experiment.

*These values were obtained after subtracting the amount of prolactin produced prior to addition of TRH or serum.