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# Onset of Pro-Thyrotropin-Releasing Hormone Gene Expression in Cultured Rat Anterior Pituitary Cells Is Expedited by Dexamethasone[1]

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**The early onset of proTRH gene expression in anterior pituitary (AP) cells in culture and its regulation by dexamethasone (DEX) were investigated. AP cells derived from 15-day-old rats were cultured for up to 4 days in the presence or absence of $10^{-7}$ *M* DEX. TRH peptide levels, which could be detected only after 3 days of culture in control cells, were detectable after 1 day in DEX-treated cells. Levels rose from undetectable (<35 fmol/well/0.2 × $10^6$ cells) to 121 ± 11 fmol/well in control cells and from 59 ± 3 to 2978 ± 88 fmol/well in DEX-treated cells (Day 1 to Day 4; means ± SEM, $n$ = 6). ProTRH mRNA levels as analyzed by *in situ* hybridization showed an excellent correlation with TRH peptide levels: mRNA was already detectable on Day 1 in DEX-treated cells and on Days 2–3 in control cells. DEX stimulated proTRH mRNA levels as determined by Northern blot analysis within 4 h. The half-life of proTRH mRNA was calculated based on a first-order decay model by measuring mRNA levels after addition of 5 μg/ml actinomycin D with or without DEX. The $t_{\frac{1}{2}}$ of proTRH mRNA in control cells was 13.1 ± 2.8 h and was not influenced by DEX treatment (12.5 ± 2.8 h). Since DEX stimulated proTRH mRNA levels acutely without any increase in mRNA stability, we propose that DEX expedites proTRH gene expression in our AP cell culture system by acting at the transcriptional level.** © 1994 Academic Press, Inc.

## INTRODUCTION

Hypothalamic hormones which have been detected in the mammalian anterior pituitary gland (AP) include thyrotropin-releasing hormone (TRH) (1), luteinizing hormone-releasing hormone (2), growth hormone-releasing hormone (3), and somatostatin (3). We have previously identified proTRH-derived peptides in long-term cultures of AP cells (4). The presence of both proTRH mRNA (5) and proTRH-derived peptides (4) in these cultures indicates that endogenous synthesis of TRH occurs in the AP and suggests that TRH may play a paracrine or autocrine role in the regulation of AP function. Accordingly, we have investigated the biological significance of TRH within the AP *in vitro* and its regulation by central and peripheral factors. We recently observed that glucocorticoids dramatically increase TRH production and levels of proTRH mRNA in AP cells in long-term culture in a dose-dependent way (5). Since proTRH gene expression in the AP *in vivo* is minimal to absent it is important to investigate the early onset of gene expression *in vitro* and the mechanisms that control this phenomenon. A recent report showed that glucocorticoid treatment induced growth hormone gene expression in rat fetuses at a stage earlier than that under control conditions (6). Based on our results that a subpopulation of somatotrophs expresses the proTRH gene within the AP (7), we speculated that glucocorticoids would also expedite the appearance of proTRH mRNA. Therefore, we studied the time course through which glucocorticoids increase proTRH mRNA levels and examined whether this is a direct effect on proTRH gene transcription. We report here that the proTRH gene is rapidly expressed after plating of AP cells and that dexamethasone (DEX) accelerates the appearance of proTRH mRNA and the formation of TRH peptide in this culture system. Furthermore, DEX treatment increases proTRH gene expression acutely and since this effect is not associated with any alteration in mRNA stability we propose that it is mediated through an effect on gene transcription.

## MATERIALS AND METHODS

### *Cell Culture and Radioimmunoassay*

Anterior pituitaries from 15-day-old male rats were aseptically collected and dispersed in neutral protease

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(Sigma) as described previously (4). Cells were plated at 1000 cells/mm$^2$ on 16- and 64-mm$^3$ miniwells for peptide determinations and Northern blot analysis, respectively. For *in situ* hybridization, cells were plated on eight-chamber permanox Lab-Tek tissue culture slides (Nunc, Inc., Napierville, IN). Cells were cultured in a modified L-15/DMEM medium containing 10% fetal calf serum; the medium was replaced 2 days after plating. DEX was used at $10^{-7}$ *M* in 0.02% ethanol. Control cells were treated with vehicle only. For TRH radioimmunoassay, pituitary cells were extracted in 1 *N* acetic acid. Samples were boiled for 10 min, sonicated, and spun at 2,000*g* and the supernatant was lyophilized. The samples were reconstituted in RIA buffer and assayed for TRH as previously described (4). The detection limit of the TRH RIA was 35 fmol per well of $0.2 \times 10^6$ cells and 1 ml medium.

*In Situ Hybridization*

For *in situ* hybridization, cells were fixed overnight at 4°C in 4% paraformaldehyde in phosphate-buffered saline (PBS) and stored in 70% ethanol at 4°C for up to 4 days. Consequently, cells were rehydrated in 30% ethanol for 5 min and PBS and *in situ* hybridization was undertaken as described previously (8) with some modifications. Cells were treated sequentially with 0.1 *M* glycine–PBS, 0.3% Triton X-100–PBS, acetic anhydride–0.1 *M* triethanolamine, pH 8.0, and 50% formamide, twofold concentration of standard sodium citrate (SSC). Best results were obtained when cells were not allowed to air-dry before *in situ* hybridization was carried out and the proteinase K of the original protocol (8) was omitted. Hybridization was performed in buffer (250 μl/chamber) containing 50% formamide, 2× SSC, 10% dextran sulfate, 0.25% BSA, 0.25% Ficoll, 0.25% polyvinylpyrollidone 360, 250 m*M* Tris (pH 7.5), 0.5% sodium dodecyl sulfate (SDS), 250 μg/ml denatured salmon sperm DNA, and $7.5 \times 10^6$ cpm of the $^{35}$S-labeled antisense RNA probe. Hybridization proceeded at 55°C for 4 h to reduce mRNA diffusion. After removal of the Lab-Tek covers, cells were washed and treated with ribonuclease A and autoradiography and image analysis were undertaken as described before (8). Lab-Teks were exposed for 5 days at 4°C after which autoradiographs were developed in D-19 (Kodak), counterstained with methyl green to assess integrity of cells under brightfield microscopy, and, subsequently, viewed by darkfield microscopy. Specificity of *in situ* hybridization was assessed with the use of a sense RNA probe (not shown). For image analysis autoradiographs were visualized at ×78 under darkfield illumination kept constant with a stabilized power source and captured with a Data Translation (Marlborough, MA) Quick Capture frame grabber board. Analysis was performed with a computerized image analysis system using Image 1.40 (NIH; public domain). Four randomly chosen microscopic fields of every Lab-Tek chamber were analyzed and averaged; the resulting value was considered one observation. Background density points were removed by threshholding the image and integrated optical density values (optical density:area) were measured.

*Northern Blot Analysis*

Total RNA was extracted in guanidinium isothiocyanate using the cesium chloride method (9). RNA samples were electrophoresed and transferred to a nylon membrane using routine procedures (10). The membrane was hybridized with a $^{32}$P-labeled antisense RNA probe as described previously (10). Blots were stripped in 0.01 × SSC/0.5% SDS at 95°C and rehybridized with a hamster actin cDNA probe that was generated using random primer labeling. Blots were exposed for 24–48 h at −80°C in intensifying screens. Analysis of the hybridization signal was carried out with the aid of a computer-assisted image analysis system as for *in situ* hybridization (8). The proTRH mRNA hybridization signal was corrected for the actin mRNA signal to account for minor differences in recovery. A pSP65 antisense rat TRH expression vector containing a 1241-bp *Eco*RI–*Pst*I fragment of the proTRH cDNA was used to generate the RNA probes (10, 11). For the sense RNA probe a pSP64 sense plasmid was used containing the same insert in native orientation downstream of the SP6 promoter. The data shown were obtained in representative experiments that were repeated two to three times with closely agreeing results.

*Stability of mRNA*

Stability of proTRH and actin mRNA in our AP cell culture system was determined according to methods described in a paper by Krane *et al.* (12). In short, cells were treated with actinomycin D (Sigma) at 5 μg/ml in 0.01% dimethyl sulfoxide in the presence or absence of DEX. RNA was extracted 0, 12, 24, and 48 h later and analyzed by Northern blot analysis as described above. The half-life of the mRNA was calculated based on a first-order decay model.

*Statistical Analysis*

All data are represented as means ± SEM and analyzed by analysis of variance followed by the Tukey–Kramer test unless otherwise indicated. The level of significance was set at $P < 0.05$. For statistical analysis undetectable levels of TRH in RIA and proTRH mRNA in measurements of integrated optical density were assigned the value of the detection limit.

## RESULTS

The time course of TRH accumulation in AP cells cultured for up to 4 days (day of plating is Day 0) is depicted in Fig. 1. TRH was first detectable after 3 days in culture (limit of detection, 35 fmol/well) in control cells and rose

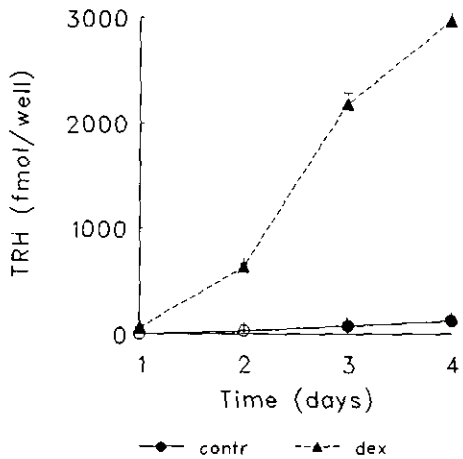


**FIG. 1.** TRH peptide levels (fmol/well/0.2 × $10^6$ cells) in primary cultures of AP cells from Day 1 to Day 4 after dispersion and plating of cells. Cells were cultured in the presence or absence of $10^{-7}$ *M* DEX. Data represent means ± SEM of 6 wells/group. Solid symbols represent detectable, open symbols undetectable (<35 fmol/well) TRH levels. All DEX-treated groups were significantly different at $P < 0.01$ versus time-matched control groups.

significantly to 121 ± 11 fmol/well on Day 4. In DEX-treated cells TRH was already detectable on Day 1 and rose dramatically from 59 ± 3 to 2978 ± 88 fmol/well.

*In situ* hybridization for the visualization of proTRH mRNA revealed that proTRH mRNA was already detectable on Day 1 of culture in DEX-treated cells (Fig. 2). The hybridization intensity increased dramatically toward Day 4 and this was accounted for by an increase in the number of labeled cells rather than density per cell (not quantified) as shown in Fig. 2. In contrast, proTRH mRNA was detectable on Days 2–3 in control cells. Integrated optical density measurements derived from *in situ* hybridization experiments are shown in Fig. 3 which shows that the ontogeny of TRH peptide levels in AP cells correlates very well with that of proTRH mRNA levels.

To test whether DEX acutely increases proTRH gene expression, cells that had been cultured for 12 days were treated with DEX for up to 8 h and proTRH mRNA was examined by Northern blot analysis. A significant threefold stimulation of proTRH mRNA versus actin mRNA accumulation by DEX occurred within 4 h ($P < 0.05$) and mRNA levels rose further after 6 (fourfold; $P < 0.01$) and 8 h (eightfold; $P < 0.01$) (Fig. 4). The apparent size of proTRH mRNA was 1.65 kb and was not influenced by the DEX treatment. The half-life ($t_{\frac{1}{2}}$) of the mRNA was calculated based on a first-order decay model as described previously (12) (Fig. 5). Forty-eight hours after addition of actinomycin D proTRH mRNA was undetectable in both control and treated cells. Therefore, this time point was deleted in determining the $t_{\frac{1}{2}}$ of proTRH mRNA. The calculated $t_{\frac{1}{2}}$ of proTRH mRNA was 13.1 ± 2.8 h in control cells and did not significantly differ from that of DEX-treated cells (12.5 ± 2.8 h). The $t_{\frac{1}{2}}$ of actin mRNA in our cell culture was 6.0 ± 0.5 h (data not shown). Since we could not find a "house-keeping" gene whose RNA is stable for up to 48 h and not influenced by DEX, the data presented in Fig. 5 were not corrected for small differences in recovery of total RNA. However, the ethidium-bromide-stained gel prior to blotting did not indicate major differences in the optical density of the ribosomal bands (data not shown).

## DISCUSSION

Recent studies have demonstrated the presence of hypothalamic hormones and neuropeptides in the AP (1–5). The presence of a hypothalamic releasing hormone such as TRH synthesized in close proximity to its target cells in the AP may point to a specific physiologic role for adenohypophysial TRH in TSH and/or PRL release. Preliminary experiments indeed showed that endogenously synthesized TRH is important for TSH secretion *in vitro* (13). While proTRH mRNA *in vivo* is only detectable by PCR in the human AP (14), the present study demonstrates that proTRH mRNA is readily detectable *in vitro* 1 to 4 days after dispersion and plating of AP cells. Although mRNA levels, as a parameter of prohormone biosynthesis, do not always reflect the levels of the peptide end-product (15), in the present study TRH levels measured by RIA showed an excellent correlation with mRNA levels determined by *in situ* hybridization. We hypothesize that AP proTRH gene expression is inhibited under *in vivo* conditions and that under *in vitro* culture conditions derepression occurs. To understand this phenomenon it is important to investigate the time course and the mechanisms leading to early onset of gene expression. The fact that proTRH gene expression may be derepressed may point to a protective mechanism to maintain hypophysial TSH or PRL release in cases of hypothalamic dysfunction (16). Indeed, in hypothalamic hypothyroidism TSH levels in man may be normal or even elevated (17, 18) and this might account for the clinical observation that tertiary hypothyroidism does not generally cause the severity that may be produced by primary hypothyroidism. Besides the classical hypothalamic–pituitary interaction in which hypothalamic TRH stimulates pituitary TSH release, we propose a paracrine or autocrine role for TRH synthesized within the AP to maintain TSH release in hypothalamic dysfunction.

Recent studies from our group showed that glucocorticoids dramatically increase proTRH gene expression in long-term cultures of AP cells in a dose-dependent manner (5). The present study shows that onset of proTRH gene expression occurs at an early stage in culture within 2 days following plating of AP cells. This process is greatly expedited by DEX treatment. We feel the actin mRNA signal can be used as a control in semiquantitative Northern blot analysis since it is not influenced by glucocorti-

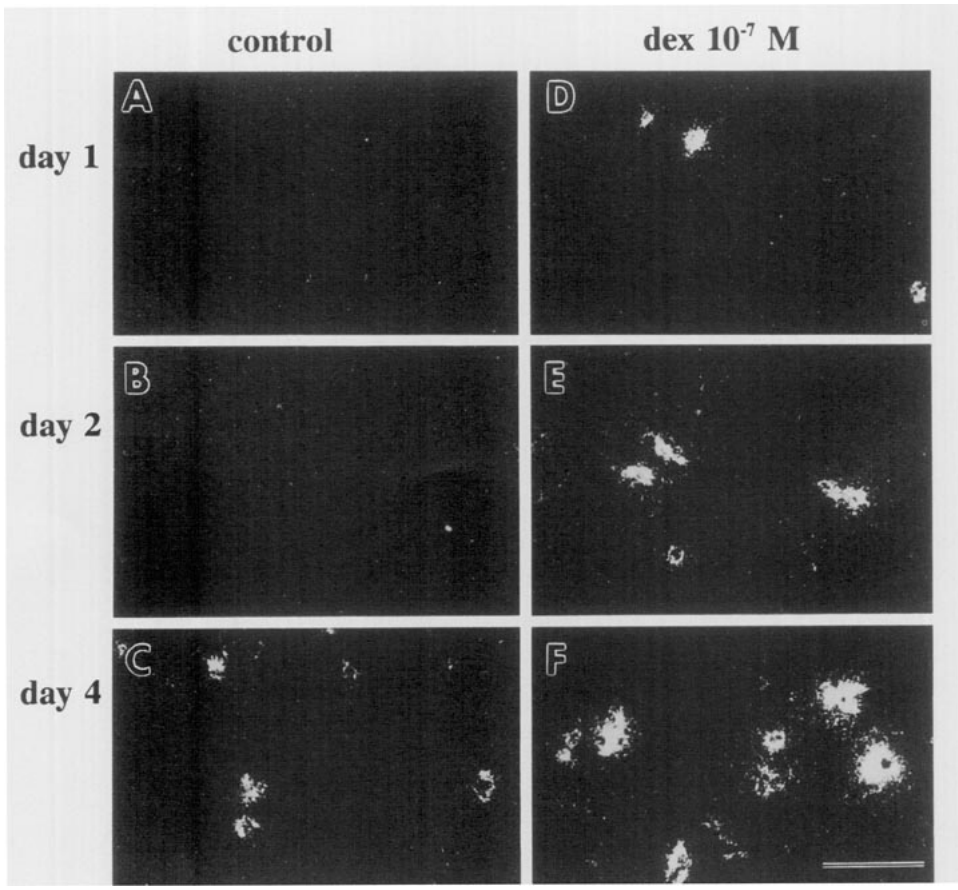


**FIG. 2.** *Darkfield microphotographs of AP cells subjected to in situ hybridization for the visualization of proTRH mRNA after 1 (A, D), 2 (B, E), or 4 days (C, F) in culture. Cells were cultured in the presence (D–F) or absence (A–C) of $10^{-7}$ M DEX. Image analysis data are shown in Fig. 3. Bar = 50 μm.*

coids (personal observation, 19). Moreover, when the glyceraldehyde-3-phosphate dehydrogenase mRNA signal was used instead of the actin mRNA signal to account for differences in recovery the same quantitative increase of proTRH mRNA relative to control was seen after DEX treatment (not shown). The proTRH gene contains a proposed consensus sequence for glucocorticoid receptor binding (11), although transfection studies of proTRH gene constructs with a reporter gene are necessary in order to confirm the importance of this sequence for gene expression. A recent study demonstrated that proTRH gene expression in the hypothalamus *in vivo* is decreased during chronic administration of glucocorticoids to rats (20). It was hypothesized that this decrease in TRH biosynthesis may result in a reduction of TSH levels that had been observed during glucocorticoid treatment (21). The finding that proTRH gene expression in the AP *in vitro* was stimulated by DEX (present study) may suggest cell-specific differential regulation of TRH biosynthesis. However, it should be stressed that the DEX-induced decrease of hypothalamic proTRH gene expression reflects *in vivo* studies and, therefore, might be the result of an indirect effect through other hypothalamic factors. Indeed, preliminary results in our laboratory showed that glucocorticoids increase, not decrease, proTRH gene expression in fetal hypothalamic neurons in primary culture (unpublished data). Similarly, DEX stimulated proTRH mRNA in the CA77 rat medullary thyroid carcinoma cell line (22). The same phenomenon is seen with regard to proCRH gene expression which is inhibited by DEX in the hypothalamic paraventricular nucleus *in vivo* (23), but stimulated in placental cells in culture (24). Regulation of gene expression is the result of complex interplays among receptors, nuclear proteins, response elements, and the promoter complexes (15, 25). The mechanisms through which glucocorticoids can influence proTRH gene expression are currently unknown. We here report that DEX stimulates proTRH gene expression acutely, i.e., within 4 h and that this effect is not mediated by an increase in mRNA stability.

Although splicing and cytoplasmic transport may influence mRNA levels, it is generally believed that mRNA levels are controlled mainly by its synthesis, i.e., gene transcription and its degradation. The stability of mRNA

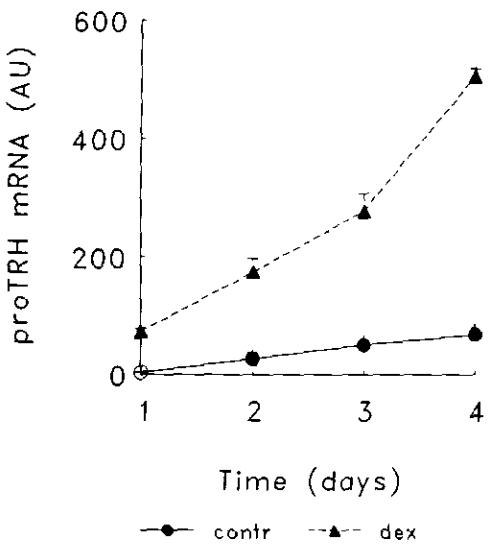


FIG. 3. Integrated optical density measurements (arbitrary units, AU) derived from *in situ* hybridization experiments as shown in Fig. 2. Data represent means ± SEM of 4 wells/group. Images were analyzed without knowing treatment protocols and each determination is the mean of 4 measurements/image. Optical density measurement on Day 1 in control cells (open symbol) was the same as background. All DEX-treated groups were significantly different at $P < 0.001$ versus time-matched control groups.

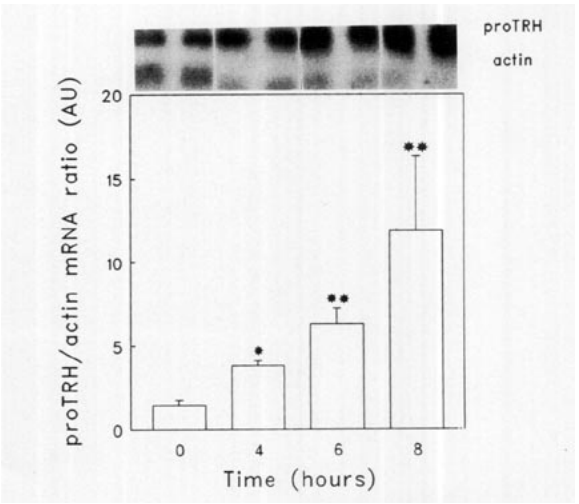


FIG. 4. (Top) Northern blot analysis of proTRH and actin mRNA 0, 4, 6, and 8 h after addition of $10^{-7}$ *M* DEX to AP cells in culture for 12 days. One microgram of total RNA was loaded in each well. (Bottom) Densitometric analysis of a representative experiment. Data represent means ± SEM with $n = 3$ and were analyzed with ANOVA followed by the Dunnet test after logarithmic transformation ($^{*}P < 0.05$, $^{**}P < 0.01$ versus 0 h).

is regulated by cis-elements and their interaction with proteins (26). The rate of mRNA turnover is highly dependent on its poly(A) tail. It is known that several hormones may influence mRNA stability. Estrogen, for example, increases the $t_{\frac{1}{2}}$ of vitellogenin mRNA in Xenopus (27), while DEX has been shown to increase the stability of the human growth hormone mRNA by elongating its poly(A) tail (28). Glucocorticoids may also destabilize mRNA, as has been reported for the interleukin-1$\beta$ mRNA (29). In the present study the size of proTRH mRNA in AP cells was identical to that of the hypothalamus (i.e., 1.65 kb) and was not influenced by DEX treatment. Furthermore, we found that DEX did not increase the $t_{\frac{1}{2}}$ of proTRH mRNA in this system. However, some caution is expressed regarding the effect of actinomycin D in determining mRNA stability since this agent affects the synthesis of multiple cytoplasmic and nuclear proteins that can markedly affect the transcriptional process. With a $t_{\frac{1}{2}}$ of the proTRH mRNA of approximately 12 h one can calculate that an increased stability can maximally account for a 1.5-fold increase of proTRH mRNA 8 h after DEX treatment. Since DEX increases proTRH mRNA 8-fold after 8 h the role of stability is, therefore, minimal. Our present data, however, do not exclude minor effects of stability changes; for a more precise calculation of the role that stability plays in affecting the mRNA signal, extensive statistical analysis increasing the number of observations or more direct pulse–chase studies instead of using actinomycin D are necessary. With these reservations it should be noted that this is the first time the $t_{\frac{1}{2}}$ of proTRH mRNA has been reported. Since most rapidly inducible molecules have unstable mRNAs with half-lives on the order of 10 to 60 min (26), it is of note that proTRH mRNA is very stable.

We conclude that proTRH gene expression is rapidly derepressed after dispersion of AP cells and that glucocorticoids may further enhance the appearance of proTRH mRNA. Since DEX significantly stimulates

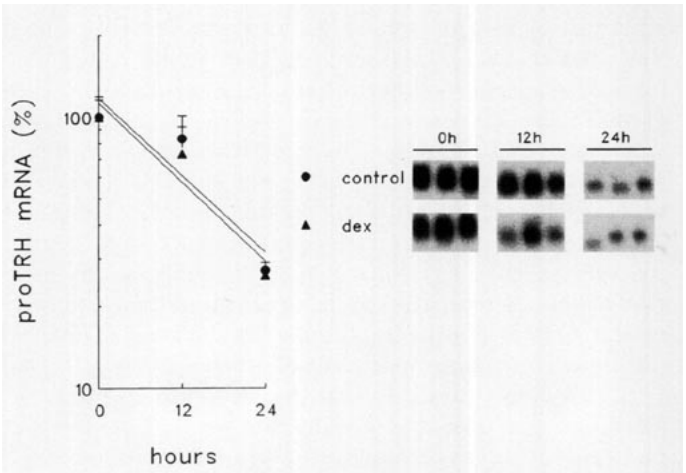


FIG. 5. (Left) Logarithmic presentation of proTRH mRNA degradation after actinomycin D (means ± SEM, $n = 3$). The calculated $t_{\frac{1}{2}}$ of proTRH mRNA in control cells was 13.1 ± 2.8 h (linear fit: $r = -0.86$ with $P = 0.0023$) and in DEX-treated cells 12.5 ± 2.8 h (linear fit: $r = -0.87$ with $P = 0.0031$). (Right) Northern blot analysis of proTRH mRNA 0, 12, and 24 h after addition of 5 μg/ml actinomycin D with or without $10^{-7}$ *M* DEX.

proTRH mRNA levels within 4 h without having major effects on its stability, it seems likely that this effect occurs at the transcriptional level. Our AP culture system provides an *in vitro* model to study tissue-specific regulation of proTRH gene expression.

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