In Vitro and in Vivo Regulation of Thyrotropin Receptor mRNA Levels in Dog and Human Thyroid Cells*

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Regulation of thyrotropin (TSH) receptor (TSHr) mRNA accumulation as compared with two other thyroid differentiation markers (thyroglobulin and thyroperoxidase (TPO)) has been investigated by Northern blot. In dogs in vivo, chronic stimulation of the thyroid by treatment with antithyroid drugs did not modify TSHr mRNA although it increased the levels of thyroglobulin and TPO mRNA. In dogs treated with thyroxin, the quiescent thyroids expressed normal levels of TSHr and TPO mRNA but depressed levels of thyroglobulin mRNA. In primary cultures of dog thyrocytes, dedifferentiation of the cells by treatment with epidermal growth factor or 12-O-tetradecanoylphorbol-13-acetate led to decreased TSHr mRNA levels and nearly abolished thyroglobulin and TPO gene expression. However, TSHr mRNA was always present, compatible with the fact that these cells, when treated by TSH, reexpress differentiation. Treatment of the cells with TSH or forskolin transiently increased the TSHr mRNA level after 20 h, an effect inhibited by cycloheximide. This up-regulation was confirmed at the protein level: forskolin-treated cells showed an enhanced cAMP response to TSH and an increased binding of labeled TSH to their membranes. Long term TSH treatment led to a slight down-regulation of TSHr mRNA in dog thyrocytes, but in human thyroid cells no marked down-regulation was observed.

Thyrotropin (TSH) is the main agent regulating the thyroid gland (1-3). Stimulation of thyroid follicular cells by TSH promotes cell proliferation and stimulates the synthesis and secretion of the thyroid hormones, requiring an iodinated glycoprotein precursor, thyroglobulin, and the enzyme thyroperoxidase (TPO), two important markers of the differentiated state of the thyrocyte (4). TSH exerts most of its effects via a receptor positively coupled to adenylate cyclase (1-3).

Previous studies have demonstrated that TSH, via cAMP, increases the mRNA and the transcription of the thyroglobulin and TPO genes (5-9). Although receptors serve as regulators of cellular activities, they are themselves subject to regulation. Stimulation of a receptor is often followed by a desensitization involving negative regulation of the protein level or activity; the former control in general also involves decreased expression of the receptor gene (10-13). Nevertheless, up-regulation of receptors or receptor mRNA after agonist stimulation has also been demonstrated (14-16). In the case of the thyroid, the continuous stimulation of the TSH receptor by autoantibodies in Graves' disease or by excessive secretion of TSH causes hyperthyroidism and goiter, suggesting a very incomplete or nonexistent desensitization (17, 18). The TSH receptor has been shown to be up-regulated in vitro (19) and in vivo (20) whereas other studies on FRTL and human thyroid cells reported down-regulation (21, 22). The recent cloning of the TSH receptor provided us with the opportunity to explore the control of thyrotropin receptor gene expression (23-25). TSHr mRNA levels were investigated in vivo in dogs and in vitro in primary cultures of dog and human thyroid cells subjected to a variety of stimuli and were compared with the amounts of thyroglobulin and TPO mRNA. Our results show that there is little modulation of TSHr mRNA by TSH, in agreement with the clinical observations; TSH through cyclic AMP transiently up-regulates and chronically down-regulates to a small extent TSHr mRNA. On the other hand dedifferentiation of thyroid cells by treatment with epidermal growth factor and phorbol esters greatly decreases TSH receptor gene expression.

MATERIALS AND METHODS

Animal studies were conducted in accordance with the highest standards of care. Dogs were treated for brain studies by oral administration for 4 weeks of methimazole (MMI) (2 x 60 mg/day) and propylthiouracil (PTU) (2 x 150 mg/day) to increase the circulating TSH level, or by thyroxine (T₄) (2 x 600 μg/day Ethyron) to decrease it. Triiodothyronine (T₃) and T₄ concentrations in the serum were followed by radioimmunoassay to ensure that the treatments were effective (T₄, Amerlex; T₃, Gamma- coat, Clinical assays, Travenol Genentech Diagnostics, South San Francisco, CA. For low values, 20 μl instead of 10 μl was used in the assay).

On the day of the experiment, 1 h prior to thyroid resection, the animals received 50 mg/kg bromodeoxyuridine (BrdU) by intravenous injection. Dogs were anesthetized and the thyroid lobes resected. They were dissected free of connective tissue and frozen in small pieces in liquid nitrogen or cut in slices for histological or BrdU labeling analysis. For histology, classical Bouin-fixed, paraffin-embedded thyroid tissues were sectioned at 7 μm and stained by hematoxylin-eosin, green Masson trichrome, or periodic acid-Schiff methods. BrdU labeling analyses were performed as described (26). Two independent series of dogs were handled in this way.

Primary Culture of Dog and Human Thyroid Cells—Dog thyroid...
follicles were obtained as detailed previously (27). The cells were seeded in 100-mm Petri dishes and cultured in a control medium consisting of Dulbecco's minimal essential medium (GIBCO), Ham's F-12 (GIBCO), MCDB 104 (GIBCO) (2:1:1, v/v/v) supplemented with 1 mM sodium pyruvate, 5 μg/ml insulin (Sigma), 40 μg/ml ascorbic acid, 0.3 μg/ml amphotericin B. Fetal calf serum (FCS), 100 units/ml penicillin, 100 μg/ml streptomycin, 1.25 μg/ml proteinase K in 0.3 M, NaCl 0.1% SDS, and phenol/chloroform extractions, the RNA was precipitated and resuspended in water for spectrophotometric quantitation.

Northern Blot Analysis—After denaturation using glyoxal according to the procedure of McMaster and Carmichael (31) equal aliquots (10–15 μg) of total RNA were fractionated on 0.8% agarose gel in 10 mM phosphate buffer, pH 7. Acidine orange staining of independent lanes ascertained that the amounts of RNA were equal in all samples. Denatured RNAs were transferred by diffusion blotting to a nylon membrane (Pall Biodyne A) using SSC 20 × (15 mM NaCl, 0.15 M NaCl, 0.015 M sodium citrate) as described (32). Prehybridization (4 h at 42 °C) and hybridization (overnight at 42 °C) were carried out in 50% formamide, 5 × Denhardt's (0.1% Ficoll, 0.1% polyvinylpyrrolidone), 5 × SSPE (20 × SSPE = 3.6 M NaCl, 0.2 M sodium phosphate, pH 8.3, 20 mM EDTA), 0.3% SDS, 250 μg/ml denatured salmon testes DNA, 200 μg/ml bovine serum albumin. The hybridization solution contained in addition 10% dextran sulfate (w/v) and the heat-denatured probe. cDNA probes (dog and human TSHr cDNA, 2.4-kb BamHI-XhoI insert of a pSVL construct; dog and human TPO cDNA, 2-kb BamHI and 1.9-kb EcoRI insert; dog thyroglobulin cDNA, 5-kb EcorI insert of a pBS construct) were α-32P labeled by random priming extension to a specific activity of approximately 108 cpm/μg in 33. Filters were washed four times for 10 min in 2 × SSC, 0.1% SDS at room temperature and four times for 30 min in 0.1 × SSC, 0.1% SDS at 55 or 65 °C for heterologous or homologous hybridization, respectively. They were then autoradiographed at –70 °C using Hyperfilm (Amersham Corp.) and Siemens intensifying screens.

All experiments were carried out at least twice, with, in each experiment, duplicates of cell Petri dishes (for culture experiments) and samples for each treatment.

RESULTS

**TSHr mRNA Accumulation in Vivo**

Dogs treated with antithyroid drugs and 1-thyroxine were biologically hypo- and hypothyroid at the time of thyroid resection. Serum T3 levels were 3.3 and 6.8 ng/dl in T3-treated dogs, 0.8 and 0.5 mg/dl in MMI/PTU treated dogs, and 1.1, 2.6, 1.0, and 1.2 μg/dl in control dogs for the first and second series, respectively. Serum T3 levels of the second series were < 30 ng/dl for MMI/PTU, 105 ng/dl for T3-treated dogs, and 31 and 42 ng/dl for control animals. Histological examination showed very hyperplastic thyroids in the antithyroid drug-treated animals with almost total disappearance of the colloid, and normal follicles full of colloid in the other thyroids. BrdU labeling analysis of the second series of dogs demonstrated nuclear labeling in 8.1% of the cells in the dog treated with antithyroid drugs and 0.69% and 0.24% of the cells in the control dogs. The T3-treated animals showed virtually absent labeling, less than 0.03% of the cells. There was therefore no doubt that the animals treated with antithyroid drugs were hypothyroid and that their thyroids were chronically hyperstimulated as a consequence. Conversely, thyroxin-treated dogs had higher thyroid hormone levels, and their thyroids were therefore quiescent.

Total RNA was extracted from the dog thyroids and subjected to Northern blot analysis. Fig. 1 shows the Northern blot corresponding to the second series of animals. As described previously (23), a 2.4-kb cDNA probe corresponding to the coding region of the dog TSHr hybridized to a 4.9-kb mRNA transcript. The levels of these transcripts did not differ greatly between normal and MMI/PTU or T3-treated dogs. The in-curved shape of the band is caused by the presence of the 28 S RNA, which has nearly the same size and so displaces the TSHr mRNA. The same blots were then hybridized with a dog TPO cDNA probe which revealed a major transcript around 4.1 kb and a minor one around 3.5 kb. These transcripts have already been reported (36, 37). In the same way, a dog thyroglobulin cDNA probe hybridized to an approximately 8.5-kb mRNA, as described (38, 39). In contrast to the small variation in TSHr mRNA levels the thyroglobulin mRNA level was clearly increased in MMI/PTU and strongly decreased in T3-treated dogs, respectively, reflecting the treatment given. TPO mRNA level was increased in the MMI/PTU-treated dogs, but in T3-treated dogs the situation was not clear: in one experiment it was increased (Fig. 1), and in a second experiment (not shown) it was similar to the control levels.

Thus, modifying in vivo the circulating concentrations of TSH mainly affected thyroglobulin mRNA, to a lesser extent TPO mRNA, but TSHr mRNA was only affected to a small extent.

**TSHr mRNA Accumulation in Vitro**

Chronic Stimulation in Dog and Human Thyroid Cells—Dog thyrocytes were seeded in control medium containing 1% TSH, thyroglobulin (TG), and TPO mRNA levels in in vivo treated dogs. The animals were treated with T3 or MMI and PTU as described under “Materials and Methods” (C, untreated dogs). Total RNA was extracted and subjected to Northern blot analysis. Although it may not appear clearly on the figure, the 3.5-kb TPO transcript is present in all lanes. The right part of the figure shows acidine orange staining of an identical gel.

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**FIG. 1.** TSHr, thyroglobulin (TG), and TPO mRNA levels in in vivo treated dogs. The animals were treated with T3 or MMI and PTU as described under “Materials and Methods” (C, untreated dogs). Total RNA was extracted and subjected to Northern blot analysis. Although it may not appear clearly on the figure, the 3.5-kb TPO transcript is present in all lanes. The right part of the figure shows acidine orange staining of an identical gel.
FCS and then maintained for 5 days in control medium without serum or supplemented with TSH (1 milliunit/ml), forskolin (10^{-6} M), or EGF (25 ng/ml) + 10% FCS. Blot hybridization analyses were then performed to determine the levels of TSHr, thyroglobulin, and TPO mRNA after these incubations. Chronic stimulation by TSH or forskolin, leading to a highly differentiated state (28), resulted in a slight down-regulation of TSHr mRNA and in a high expression of TPO and thyroglobulin mRNA (Fig. 2). On the other hand, differentiating the cells by EGF and FCS (28) reduced TSHr mRNA levels and in a more dramatic way TPO and thyroglobulin mRNA, whose levels became undetectable. The high basal TSHr mRNA level in the control cells is noteworthy by comparison with the very low thyroglobulin or TPO mRNA levels, whose levels became undetectable. The high basal TSHr mRNA level in the control cells is noteworthy by comparison with the very low thyroglobulin or TPO mRNA levels, whose levels became undetectable. The high basal TSHr mRNA level in the control cells is noteworthy by comparison with the very low thyroglobulin or TPO mRNA levels, whose levels became undetectable.

To investigate TSHr mRNA expression in human cells, human thyrocytes were seeded in 1% FCS-containing medium and then incubated (without serum) 2 days in the presence of forskolin (10^{-6} M) or in control medium followed by a 6-h incubation with forskolin (10^{-5} M) or TSH (250 microunits/ml). The 2-day forskolin incubation did not result in TSHr mRNA down-regulation, as the human TSHr mRNA levels were identical in control and forskolin-treated cells. As in dog thyrocytes and in other systems (36, 37, 40, 41), thyroglobulin and TPO mRNA levels were strongly increased following the 2-day forskolin stimulation (Fig. 3). The 6-h treatment with forskolin or TSH did not modify TSHr and thyroglobulin mRNA levels but increased TPO mRNA levels, as expected (5, 7). Contrary to the dog TPO transcripts, the major human TPO mRNA appeared around 3.5 kb. This transcript has been described as generating the protein (42). The longer transcript could be a precursor.

Thus, except for the slight down-regulation of TSHr mRNA, the data obtained in human and dog thyrocytes submitted to chronic activation of the cyclic AMP cascade show little variation in TSHr mRNA levels as opposed to high variations in TPO and thyroglobulin mRNA.

**Accumulation of TSHr mRNA in Response to Short Time Exposures to Various Agents—**Dog thyrocytes were seeded (day 0) and maintained for 1 day in control medium supplemented with 1% FCS. To get enough material, cell proliferation was then stimulated by EGF (25 ng/ml) and 10% FCS (Fig. 1A). After 3 days of such treatment, confluence was achieved while cells lost most of their differentiated characteristics (28, 40) (day 1). Expression of the differentiated functions is restored by washing out the EGF and serum for 2 days followed by addition of an adenylate cyclase activator (at day 6) (28, 40). Fig. 4A shows that TSHr mRNA levels were low but still detectable after a 3-day treatment with EGF and FCS; they were increased again after a further 2 days in control medium. All the experiments described were performed at day 6.

A 20-h incubation with TSH (1 milliunit/ml) or forskolin (10^{-6} M) led to an increase in TSHr, thyroglobulin, and TPO mRNA levels whereas treatment with EGF (25 ng/ml) or TPA (10 ng/ml) markedly reduced thyroglobulin and TSHr mRNA, more so for TPA than EGF for the TSHr mRNA (Fig. 4A). The kinetics of TSHr mRNA induction in the presence of TSH or forskolin showed that the increase culminated at 20 h (Fig. 4B). This up-regulation was short lived; indeed, Fig. 4C shows that the mRNA levels had already decreased after 24 h. Longer exposure times resulted in TSHr mRNA levels equal to or lower than the control levels (Fig. 4, B and C). Similar results have been obtained by in situ hybridization. By contrast and as expected (5, 7), TPO mRNA became apparent after 6 h, and thyroglobulin mRNA content was strongly enhanced after 15 h. A similar time course for TSHr mRNA increase was observed when the cells were seeded and maintained for 4 days in the control medium, without strong proliferative and dedifferentiating pretreatment (not shown).

The increase in TSHr mRNA reflects also an increase in TSH receptors themselves. Indeed, in the same culture conditions as above the capacity of thyroid cells to respond to a TSH (1 milliunit/ml) stimulation by a CAMP elevation became higher when these cells were first pretreated with forskolin. Table I shows that a 15- or 60-min TSH (1 milliunit/ml) incubation of control cells led to a 9- or 23-fold increase in CAMP intracellular concentrations, respectively, but that these increases became 45- or 68-fold, respectively, when the cells were first pretreated 2 days with forskolin (10 or 5 µM) (from day 6). An increase in TSH receptors is also suggested.

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**Fig. 2.** TSHr, thyroglobulin (TG), and TPO mRNA levels after chronic stimulation of dog thyroid cells with TSH (1 milliunit/ml), forskolin (10^{-5} M) or EGF (25 ng/ml) + 10% FCS for 5 days. Total RNA was extracted and subjected to Northern blot analysis. The right part of the figure shows acridine orange staining of an identical gel. C, control; T, TSH; Fo, forskolin; E, EGF + FCS.

**Fig. 3.** TSHr, thyroglobulin (TG), and TPO mRNA levels in human thyrocytes. The cells were stimulated for 2 days with forskolin (10^{-5} M) or for 6 h with forskolin (10^{-5} M) or TSH (0.25 milliunits/ml). Total RNA was extracted and subjected to Northern blot analysis. The right part of the figure shows acridine orange staining of an identical gel. C, control; T, TSH; Fo, forskolin.

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Regulation of Thyrotopin Receptor mRNA

Dog thyrocytes were maintained in control medium or pretreated with forskolin (5 or 10 μM) for 2 days. For cAMP determinations, cells were stimulated by TSH (1 milliunit/ml) for 15 (first experiment) or 60 (second experiment) min. For binding experiments, cells were harvested after the 2 days in control or forskolin-containing medium, membranes were prepared, and TSHr density was determined by binding of 125I-TSH. Data are averages ± S.E. of duplicates (binding values) or triplicates (cAMP values).

### Table I

**cAMP measurements and labeled TSH binding in cultured dog thyroid cells**

| Preincubation | Cell measurements | cpm/20 μg protein | Specific binding |
|---------------|-------------------|------------------|-----------------|
| Control       | TSH binding       | 620 ± 49         | 528             |
| Forskolin (10 μM) | + TSH (30 milliunits/ml) | 92 ± 20         |                 |
| Forskolin (5 μM) | + TSH (30 milliunits/ml) | 1,410 ± 27 | 1,368 |
| Control       | + TSH (30 milliunits/ml) | 42 ± 7          |                 |
| Forskolin (10 μM) | + TSH (30 milliunits/ml) | 536 ± 24        | 328             |
| Forskolin (10 μM) | + TSH (30 milliunits/ml) | 208 ± 56        |                 |
| Forskolin (5 μM) | + TSH (30 milliunits/ml) | 1,196 ± 176 | 976             |

by binding experiments performed on cells submitted to the same protocol with the last 2 days in control medium or in forskolin (10−6 M)-containing medium; a 2.6- and 2.9-fold increase in binding was observed after the forskolin treatment (Table I).

To assess a possible effect of T₃ on TSHr mRNA, dog thyrocytes were incubated for 20 h with various concentrations (10⁻⁷ M, 10⁻⁶ M) of this hormone, with or without forskolin (10⁻⁶ M). No effect of T₃ could be detected on the basal or on the forskolin-enhanced levels (not shown).

**Sensitivity to Cycloheximide**—To determine whether TSH and forskolin exert their actions through newly synthesized protein(s), cells (at day 6) were incubated for 20 h in the presence of cycloheximide (10 μg/ml), alone or with TSH. At this concentration, cycloheximide inhibited more than 90% [35S]methionine incorporation in total proteins (not shown).

Cycloheximide alone markedly reduced TSHr mRNA basal levels, and simultaneous addition of cycloheximide and TSH resulted in an even more pronounced decrease (Fig. 5). This suggests that protein synthesis is involved not only in the TSH stimulation of TSHr but also in the maintenance of the basal levels. As expected (5, 7), the thyroglobulin mRNA increase was also blocked by cycloheximide whereas TPO mRNA remained unaffected under these conditions (not shown). Washing of the cells after cycloheximide treatment is followed by recovery of thyroglobulin mRNA expression (5).

**Role of Insulin**—Insulin, acting presumably through insulin-like growth factor receptors (43), is an important regulatory factor for dog thyroid cells in culture. A role of insulin has clearly been demonstrated in thyroglobulin gene expression but not in TPO gene expression (5, 7, 9, 44-46). To 50 h of incubation with TSH (1 milliunit/ml). d4 and d6, days 4 and 6; C, control; T, TSH; Fo, forskolin; E, EGF.
Regulation of Thyrotropin Receptor mRNA

FIG. 5. TSHr mRNA levels in dog thyrocytes incubated at day 6 with TSH (1 milliunit/ml), cycloheximide (10 μg/ml), or TSH + cycloheximide for 20 h. Total RNA was extracted and subjected to Northern blot analysis. The right part of the figure shows acridine orange staining of an identical gel. C, control; T, TSH; CYC, cycloheximide.

FIG. 6. Effect of insulin on TSHr and thyroglobulin (TG) mRNA levels. Dog thyrocytes were maintained from day 4 with or without insulin (5 μg/ml) and incubated at day 6 with or without TSH (1 milliunit/ml) for 20 h. Total RNA was extracted and subjected to Northern blot analysis. C, control; T, TSH; INS, insulin.

Figure 6 shows the effects of this hormone on TSHr mRNA. Cells were incubated in the presence or absence of insulin (5 μg/ml) and incubated at day 6 with or without TSH (1 milliunit/ml) for 20 h. Total RNA was extracted and subjected to Northern blot analysis. C, control; T, TSH; INS, insulin.

The cell can modulate its response to any factor by controlling the number or the activity of receptors available to this factor. When this results in a decreased response the process is called desensitization. One of the mechanisms involved is the down-regulation, i.e. the decrease in the number of receptors which may result from the regulation of any step involved in receptor gene expression or turnover.

Fig. 7. TSHr mRNA levels in dog thyrocytes incubated with actinomycin D (5 μg/ml) for various periods (two different experiments). Total RNA was extracted and subjected to Northern blot analysis. C, control.

DISCUSSION

In the response of cells to extracellular signals, two elements are involved in the primary event: the signal and the receptor.
ing dogs with MMI/PTU or T₄ resulted in little variation in TSHr mRNA. On the other hand, thyroglobulin and TPO mRNA levels were increased in the MMI/PTU-treated dogs, but only thyroglobulin mRNA levels showed a considerable decrease in the T₄-treated dogs. Lack of precluded the measurement of TSH binding, which still leaves open the possibility of a down-regulation of receptors. However, in guinea pigs no such down-regulation was observed (29). The increase in thyroglobulin mRNA observed in the MMI/PTU-treated dogs was not reported in PTU-treated rats (50, 51) where it was shown that the thyroglobulin gene was maximally transcribed under normal TSH levels. It is probable that this reflects species difference such that, as expected, thyroglobulin gene expression can be up-regulated by TSH, but this phenomenon is not observed in the normally highly active rat thyroid.

In primary cultures of dog and human thyroid cells, TSH through cAMP promotes function, proliferation, and differentiation (3, 28, 40) whereas EGF, TPA, or serum induces mitogenesis but represses differentiation expression (28, 40, 52). Chronic stimulation of dog thyrocytes for 5 days by TSH or forskolin led to a slight down-regulation of TSHr mRNA. The TSH-promoted down-regulation is homologous, and the forskolin-promoted down-regulation is heterologous since the TSH receptor itself is not involved in this process. The latter effect suggests that cyclic AMP is involved in TSH receptor mRNA down-regulation. TPO and thyroglobulin mRNA were on the contrary highly expressed in these cells, which is compatible with a highly differentiated state as also demonstrated by high levels of iodide transport (28). Human thyrocytes cultured 2 days in the presence of forskolin showed similar results except that no down-regulation was observed for the human TSHr mRNA. Thus, in both species, little variation in TSHr mRNA levels was observed by comparison with the high variations in TPO and thyroglobulin mRNA, already described in dog (5, 40), calf (53), and human (36, 41) thyroid cells and in FRTL₅ cells (7, 54, 55). Chronic exposure to EGF and FCS induced a striking fibroblast-like morphology and decreased the differentiation characteristics (iodide trapping and organification, thyroglobulin gene expression) (28, 40). TSHr mRNA levels were strongly repressed, and TPO and thyroglobulin mRNA became undetectable. Thus, despite the fact that the cells became highly dedifferentiated, they still retained detectable amounts of TSHr mRNA. The changes induced by EGF were reversible after its removal; and in the presence of TSH, normal epithelial morphology was then restored. Thyroid cells, even dedifferentiated, never turn the TSHr gene completely off, they always keep expressing the gene which will allow them to return to differentiation upon stimulation by TSH. This is compatible with data obtained on human thyroid tumors, demonstrating that the TSH receptor is the last differentiated characteristic to be lost in increasingly dedifferentiated thyroid neoplasias. A relationship between TSHr mRNA expression and the degree of dedifferentiation was also reported by Ohta et al. (56) in neoplastic human thyroid tissue. This persistence of TSHr mRNA under all these conditions explains why these cells, even when apparently undifferentiated, are still able to respond to TSH in terms of differentiation, proliferation, and function.

Short term incubations of dog thyrocytes with TSH or forskolin resulted in an up-regulation of TSHr mRNA; its levels were maximally increased until 20 h and decreased thereafter to levels equal or lower than the control levels. In the same cells, TPO and thyroglobulin mRNA levels were enhanced after 6 and 10–15 h, respectively, in accordance with other studies on thyroid cells (5, 7). Treatment (20 h) with EGF or TPA markedly reduced TSHr, TPO, and thyroglobulin mRNA. The decrease observed was stronger in the presence of TPA, which could be correlated with the stronger inhibitory effect of this agent on the expression of differentiation in these cells (9).

The observed transient up-regulation of TSHr mRNA contrasts with previous report on the regulatory effects of TSH on its own mRNA; in FRTL₅ cells, Akamizu et al. (21) reported a down-regulation within 8 h of TSHr mRNA in response to agents increasing cAMP levels. This represents another of the many differences in regulation between this cell line and thyroid cells in vivo or in primary culture (57, 58). In transfected Chinese hamster ovary cells, down-regulation of TSHr mRNA was not observed (22). In dog and human thyroid cells, desensitization, i.e. a moderate decrease in cAMP response to TSH, is observed after 2 h until 16 h (59). This phenomenon, impaired by cooling the cells and resistant to protein synthesis inhibition, presumably takes place at the level of the receptor itself (60). In DDT;MF-2 cells it has been demonstrated that cAMP elevation after receptor activation stimulated the β₂-adrenergic receptor gene and also led to receptor phosphorylation (on specific protein kinase A phosphorylation sites) involving receptor desensitization (61). Similarly, a rise in cyclic AMP induces both an increased catabolism of the R and C subunits of cyclic AMP-dependent protein kinase and thus a decrease in enzyme level and an increase in the level of the corresponding mRNA (62). It is tempting to consider a similar autoregulation by cAMP of the TSH receptor. As suggested by Collins et al. (61) this would make physiological sense: the transient increase in TSHr mRNA might allow the cell to maintain receptor number, by compensating an eventual loss of receptors.

Nevertheless, although in DDT;MF-2 cells the number of β₂-adrenergic receptors did not increase with its mRNA level (47). In MA-10 cells, on the contrary, luteinizing hormone/chorionic gonadotropin receptors are increased (48), and in dog thyrocytes the increase in TSHr mRNA is also accompanied by an increase in TSH receptors themselves. Cells treated with forskolin showed an enhanced cAMP response to a TSH stimulation by comparison with untreated cells, and binding experiments revealed higher levels of TSH receptor sites in forskolin-treated cells than in control cells. This suggests that like ACTH, which positively regulates its own receptors and cAMP response in cultured bovine adrenal cells (14), the TSH-induced cyclic AMP accumulation, as mimicked by forskolin, has a positive effect on both TSH receptor number and response. These receptors appear also to be very stable and little desensitized since this response is maintained even after a 2-day forskolin treatment while TSHr mRNA had already declined. Increased content of Gₛₛ, the α subunit of the G protein which activates adenylyl cyclase, might contribute to the higher cyclic AMP response of TSH and forskolin-treated cells (63). The long latency time observed before forskolin or TSH could increase TSHr mRNA levels suggested that prior protein synthesis might be required as in the case of thyroglobulin gene induction (5, 7). To test this hypothesis, experiments were performed in the presence of cycloheximide. Cycloheximide lowered the basal TSHr mRNA levels and completely abolished induction by TSH, suggesting that newly synthesized protein(s) are involved not only in the TSH enhancement of TSHr mRNA level but also in maintaining the basal levels.

In our model of primary culture of dog thyroid cells, insulin markedly potentiated the action of forskolin and TSH on the
rate of DNA synthesis (64). Insulin, acting presumably through insulin-like growth factor I receptors (43), has been shown to be involved in the control of thyroglobulin but not of TPO gene expression (5, 7, 9, 44–46). However, the TSHr mRNA levels were not significantly different with or without insulin. This is in agreement with data demonstrating that the cAMP response to TSH is identical in the presence or absence of insulin. By comparison, rehybridization of the same blots with a thyroglobulin probe clearly demonstrated that the basal and a part of the stimulated levels of thyroglobulin gene expression depended on the presence of insulin in the culture medium, in agreement with the concept of a multihormonal regulation of the thyroglobulin gene (9, 45, 46).

Experiments performed in the presence of actinomycin D showed that the TSHr mRNA in control medium was very stable, since about a 16-h incubation in the presence of this drug was necessary to observe a decline in TSHr mRNA. Half-lives of the same order of magnitude have been observed for the β1-adrenergic receptor mRNA (12 h) (65). Moreover, microinjection of recombinant TSHr mRNA (23) in Y1 cells conferred a TSH-responsive phenotype on them, which was maintained for at least 20 h, suggesting also that receptor and/or mRNA were stable. Taken together with the transient TSH-promoted stimulation of TSHr mRNA (observed at 15 and 20 h and ended by 24 h), these data suggest that an active mechanism is involved in the rapid decline of the raised mRNA level.

In summary, we presented here data on TSHr mRNA regulation which we compared with two other well known thyroid differentiation markers: TPO and thyroglobulin. Regulation of TPO and thyroglobulin mRNA, used to validate our results on TSHr mRNA, is in accordance with previous mRNA level.

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