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Antagonistic effects of thyrotropin and epidermal growth factor on thyroglobulin mRNA level in cultured thyroid cells

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Both thyrotropin (TSH) and epidermal growth factor (EGF) are potent mitogenic agents when added to dog thyroid cells in primary culture [Roger, P. P. and Dumont, J. E. (1984) *Mol. Cell. Endocrinol. 36*, 79–93]. The concomitant effect of these agents on the differentiation state of the cells was appreciated using cell morphology, iodide trapping, thyroglobulin synthesis and cytoplasmic thyroglobulin mRNA content as markers. Together with previous results [*Mol. Cell. Endocrinol. 36*, 79–93 (1984)] it is shown that cells cultured in the continuous presence of TSH maintain all the parameters at a near normal level. In the absence of TSH, thyroglobulin mRNA decreased to very low, though still detectable levels. Addition of TSH restored subnormal mRNA levels. Culture of cells in the presence of EGF for 4–6 days affected profoundly their morphology, abolished iodide trapping and decreased thyroglobulin synthesis and cytoplasmic mRNA content to undetectable levels. Addition of TSH to cells previously exposed to EGF reversed the growth factor effect on all four indexes. The redifferentiating effect of TSH was well observed within 3–4 days and was mimicked by the adenylate cyclase activators, forskolin and cholera toxin. When administered simultaneously, TSH and EGF achieved an intermediate situation, EGF antagonizing partially the effect of TSH on the expression of thyroglobulin gene. Another growth factor, fibroblast growth factor, while promoting thyroid cell proliferation also, did not interfere at all with TSH effects on cytoplasmic thyroglobulin mRNA content. Our results make the dog thyroid cell in primary culture an appropriate model to study the mechanisms involved in gene regulation by cyclic AMP and growth factors.

Thyrotropin (TSH) is the main agent regulating the thyroid gland [1]. Stimulation of thyroid follicular cells by TSH promotes cell proliferation and results in the rapid activation of almost every aspect of their metabolism including the synthesis and secretion of thyroid hormones. These hormones are produced by proteolysis of a glycoprotein precursor, thyroglobulin, encoded by an exceptionally large gene ($\approx 250 \times 10^3$ base pairs) [2]. Thyroglobulin is the main product of the thyroid gland, and its synthesis represents 50% of the thyrocyte protein synthesis [3]. The expression of thyroglobulin gene is, therefore, the main differentiation marker of this cell. We have shown that transcription of the thyroglobulin gene in rat *in vivo* requires TSH [4].

The development of a dog thyroid primary culture system [5–7] provides the opportunity to investigate the long-term regulation of thyroglobulin gene expression, in relation to other morphological or biochemical parameters. Furthermore, in this experimental system we have obtained the first indications [7, 8] of a specific negative control by epidermal growth factor (EGF) of the iodide metabolism, a marker of the thyroid differentiation. Similar observations have also been obtained from porcine and sheep thyroid cell cultures [9, 10]. This polypeptide growth factor acquires a hormonal status with regard to its growth-promoting activity and to its ability to modulate specific differentiated functions and specific gene expression in various systems [11–13].

In the present study we have investigated the effects of TSH and EGF on two parameters of thyroid cell differentiation in our dog primary culture system: the trapping of iodide and the expression of thyroglobulin gene as reflected by thyroglobulin synthesis and cytoplasmic thyroglobulin mRNA content.

MATERIALS AND METHODS

Collagenase (150 U/mg) was purchased from Worthington Chemical Co. (Freehold, NJ). Eagle's basal medium (BME), minimum essential medium, Dulbecco's modification of minimum essential medium, Ham's F_{12} medium, MCDB104 medium, glutamine, penicillin-streptomycin, and amphotericin B (fungizone) were obtained from Flow labs (Irvine, UK) and fetal calf serum from Eurobio (Paris). Bovine insulin, transferrin, murine epidermal growth factor (EGF) and pituitary fibroblast growth factor (FGF) were purchased from Collaborative Research (Waltham, MA, USA). Glycylhistidyllysyl acetate and somatostatin were Sigma products (St Louis, MO, USA). Bovine TSH (≈ 1 U/ml) was from Armour Pharmaceutical Co. (Chicago, IL, USA). Cholera toxin was provided by Schwartz-Mann (Division of Becton-Dickinson and Co., Orangeburg, NY, USA). Forskolin was from Hoechst Pharmaceuticals (Bombay, India) and cyclic dibutyryl-AMP from Boehringer Pharmaceutical (Mannheim, FRG). All the other reagents were of the highest purity commercially available.

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Abbreviations. TSH, thyroid-stimulating hormone (thyrotropin); pbTg, plasmid containing bovine thyroglobulin cDNA fragment; EGF, epidermal growth factor; FGF, fibroblast growth factor; SDS, sodium dodecyl sulfate; BME, Eagle's basal medium.

Cell culture

The dog thyroid cells were cultured as described [7]. Briefly, the thyroid tissue was digested by collagenase so that the resulting suspension consisted mainly of fragmented and intact follicles. These follicles were seeded in 50-mm tissue-culture treated plastic petri dishes, and in 1 day adhered to the substratum while a monolayer developed. The seeding was performed so that $5 \times 10^4 - 10^5$ cells/cm^2 attached to the dish after 1 day and one medium change. The cells were cultured in the following mixture, which constitutes the control medium: Dulbecco' minimal essential medium + F12 + MCDB104 (2:1:1, by vol.) with 2 mM glutamine, supplemented by 5 µg insulin/ml, 1.25 µg transferrin/ml, 10 ng glycyl-histidyl-lysyl acetate/ml, 10 ng somatostatin/ml and 40 µg ascorbic acid/ml. Antibiotics, penicillin 100 U/ml, streptomycin 100 µg/ml and amphotericin B 2.5 µg/ml were also added. The Petri dishes were maintained in a water-saturated incubator at 37 °C in an atmosphere of 5% CO_2 in air. The medium was renewed and TSH or other effectors were added as indicated.

Measurement of cell multiplication

Cell multiplication curves were obtained from cell DNA measurements using the increase of fluorescence of ethidium bromide when complexed with nucleic acid [5].

Active transport of iodide

Trapping of iodide was evaluated by the uptake of radioactive iodide at equilibrium. The cells in the petri dishes were incubated for 2 h with $Na^{131}I$ (2 µCi/ml) in 0.01 mM KI in BME at 37 °C. Mercaptomethylimidazole (1 mM) was added to block iodide organification. The cells were then rapidly rinsed with BME three times, scraped and counted in a γ counter. The radioactivity was normalized to the cellular DNA measured from the same dishes. Trapping was also measured as the ratio of radioactive iodide in cells incubated with mercaptomethylimidazole and in cells incubated with mercaptomethylimidazole and 1 mM $NaClO_4$. This ratio provides a good estimate of the commonly used C/M ratio (where C is the radioactivity in the cells and M the radioactivity of a corresponding volume of medium [14, 15]). In the experimental conditions described here, the amount of ^{131}I-DNA (cpm/ng) can be multiplied by 4 to give an estimate of the C/M ratio [15].

Thyroglobulin synthesis measurement

Thyroglobulin synthesis was measured by [^{3}H]leucine incorporation into material immunoprecipitated by anti-thyroglobulin serum. The cells in the petri dishes were incubated for 5 h with [^{3}H]leucine 100 µCi/ml (130 Ci/mmol) in Dulbecco's minimal essential medium without leucine. Cells were then rapidly rinsed twice with 50 mM Tris/HCl/180 mM NaCl pH 7.5, scraped in the presence of 1 ml of the same buffer and transferred to a 1.5-ml microfuge tube; they were pelleted by 1 min centrifugation in a Beckman microfuge B. The pellet was resuspended in 400 µl 50 mM Tris/HCl pH 7.0/150 mM NaCl/1% Triton X-100 and homogenized at 0 °C. The homogenate was centrifuged for 1 h at $100000 \times g$ at 4 °C. Measurement of the radioactivity incorporated into total proteins of the supernatant was carried out on the trichloroacetic acid precipitation of 5 µl of this supernatant supplemented with 5 µg bovine serum albumin [16]. Thyroglobulin in 50 µl was immunoprecipitated in 1 h with 20 µl anti-(bovine thyroglobulin) serum and 5 µg bovine thyroglobulin as carrier in 50 mM Tris/HCl pH 7.5. 150 mM NaCl in a final volume of 180 µl at room temperature. The reaction mixture was layered on 200 µl of a saccharose solution (1 M saccharose/1% sodium deoxycholate/1% Triton X-100/0.1% leucine in 50 mM Tris/HCl/150 mM NaCl, pH 7.5) in 400-µl microfuge tubes. After 1 min centrifugation in the Beckman B microfuge, the pellet was cut and counted. This technique precipitated 65% of ^{125}I-labeled dog thyroglobulin tracer. Thyroglobulin synthesis was expressed as the percentage of total trichloroacetic-acid-precipitable radioactivity.

Measurement of cytoplasmic thyroglobulin mRNA level

Cytoplasmic thyroglobulin mRNA levels were estimated by the cytoplasmic dot-blot hybridization method [17]. Cytoplasmic extracts were prepared, fixed on nitrocellulose filters [17] and hybridized to labeled cloned thyroglobulin cDNA probes [18].

Briefly, filters were prehybridized for 4 h at 42 °C in sealed plastic bags (400 µl solution/cm^2 filter). The prehybridization buffer contained 50% (vol./vol.) formamide, 5 × standard saline citrate (SSC), sonicated denaturated salmon sperm DNA (250 µg/ml), 0.02% ficoll and 0.02% polyvinylpyrrolidone. The hybridization buffer contained 4 parts of the same buffer and 1 part 50% (wt/vol.) dextran sulfate. The probe was a nick-translated bovine thyroglobulin cDNA clone: pbTg 4.7 (specific activity $\approx 5 \times 10^7$ cpm/µg) [19]. Filters were hybridized for 20 h at 42 °C under agitation in sealed plastic bags. After hybridization the filters were washed four times at room temperature in 250 ml 2 × SSC, 0.1% SDS and four or five times for 1 h at 50 °C in 250 ml 0.1 × SSC, 0.1% SDS.

The blots were exposed to X-ray film at −70 °C using a Siemens intensifying screen. The dot-blot hybridization procedure was verified with standards of pure bovine thyroglobulin mRNA and pure rat thyroglobulin mRNA. Other controls were also performed: (a) total liver RNA did not compete for the hybridization of the pbTg probe to thyroglobulin mRNA; (b) the pbTg probe did not recognize any RNA from V79 fibroblasts; (c) the pbTg probe did not bind to thyroid cell cytoplasmic extracts pretreated with ribonuclease.

RESULTS

The dog thyroid cells in culture seem to exist in two distinct states of differentiation depending on the presence of TSH or EGF [7]. TSH stimulates iodide trapping and proliferation and induces or maintains a differentiated epithelial cuboidal morphology. EGF also promotes cells proliferation but, in addition it induces an elongated, fusiform, fibroblast-like morphology, and potently depresses iodide trapping even in the presence of TSH. Our aim was to investigate the expression of the thyroglobulin gene, the main marker of differentiation in the thyroid cells, in these opposite, hormonally dependent states.

Stimulation by TSH of thyroglobulin gene expression and cell differentiation

The cells were seeded at relatively high density ($\approx 10^5$ cells/cm^2) and stimulated to further proliferation by treatment with EGF and 10% serum. Following this treatment the cells reached confluency at day 6 with the previously shown

elongated fusiform morphology (Fig. 3). Iodide trapping, which was high one day after seeding, had declined in culture with EGF and without TSH as previously described [7] and was thus very low (Fig. 1 B) at day 6. Fig. 1 C shows that the synthesis of thyroglobulin had similarly declined and became undetectable at day 6. This decline in thyroglobulin synthesis was correlated with a dramatic decrease in cytoplasmic thyroglobulin mRNA content (Fig. 2). Removal of EGF and serum from the culture medium by washing did not restore iodide trapping (Fig. 1 B), nor thyroglobulin synthesis (Fig. 1 C) and cytoplasmic thyroglobulin mRNA content (Fig. 2) at day 6 and day 9. After removal of EGF and serum, the addition of TSH (1 mU/ml) at day 6 reinduced the differentiated epithelial cuboidal morphology characteristic of dog thyroid cells continuously cultured with TSH [7] (Fig. 3). High iodide trapping levels were also restored by the addition (Fig. 1 B). In these conditions TSH induced thyroglobulin gene expression as seen by the reappearance of thyroglobulin synthesis (Fig. 1 C) and the high cytoplasmic thyroglobulin mRNA content (Fig. 2).

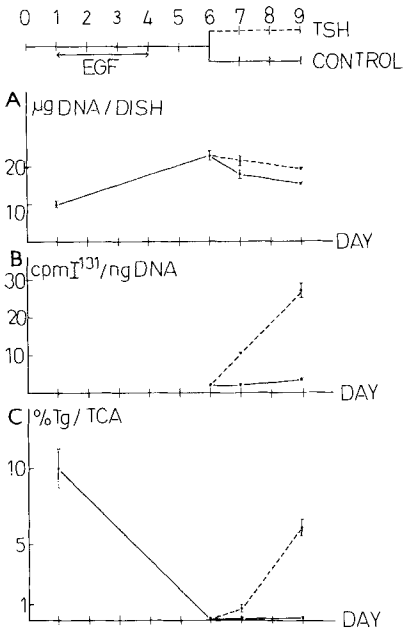

Fig. 1. *Effect of EGF and TSH on cell proliferation and differentiation.* The cells were seeded in control medium at relatively high density (10^5 cells/cm^2) as described in Materials and Methods (day 0). After one day, the culture medium was supplemented with EGF (25 ng/ml) and 10% fetal calf serum and culture was continued for 3 days. After the cells had reached confluency (day 4) they were put back in control medium. Two days later (day 6), the cells were divided in two groups: cultured in control medium (——) or in the presence of TSH (1 mU/ml) (– – –). Cell proliferation (A), iodide trapping (B) and thyroglobulin synthesis (C) were measured as described in Materials and Methods. The thyroglobulin (Tg) synthesis was expressed as a percentage of total trichloroacetic-acid-precipitable radioactivity

Stimulation by cyclic AMP of thyroglobulin gene expression and cell differentiation

It is well established that cyclic AMP is the main intracellular mediator of the effects of TSH in the dog thyroid [20]. TSH activates adenylate cyclase and increases cAMP content in dog thyroid cells in primary culture [5, 21]. Universal adenylate cyclase activators, cholera toxin and forskolin [22] completely reproduced the effects of TSH on iodide transport capacity and cytoplasmic thyroglobulin mRNA levels (Fig. 4). The effect of TSH on cytoplasmic thyroglobulin mRNA level was also completely mimicked by cyclic dibutyryl-AMP (0.1 mM) (not shown).

Inhibition by EGF of thyroglobulin gene expression

When thyroid cells were incubated from the beginning of the culture in the presence of TSH (1 mU/ml), the cytoplasmic thyroglobulin mRNA level remained high (day 8 or 12, Fig. 5). In cells cultured in the absence of TSH, thyroglobulin mRNA decreased to very low though still detectable levels. On the other hand, in cells cultured in the continuous presence

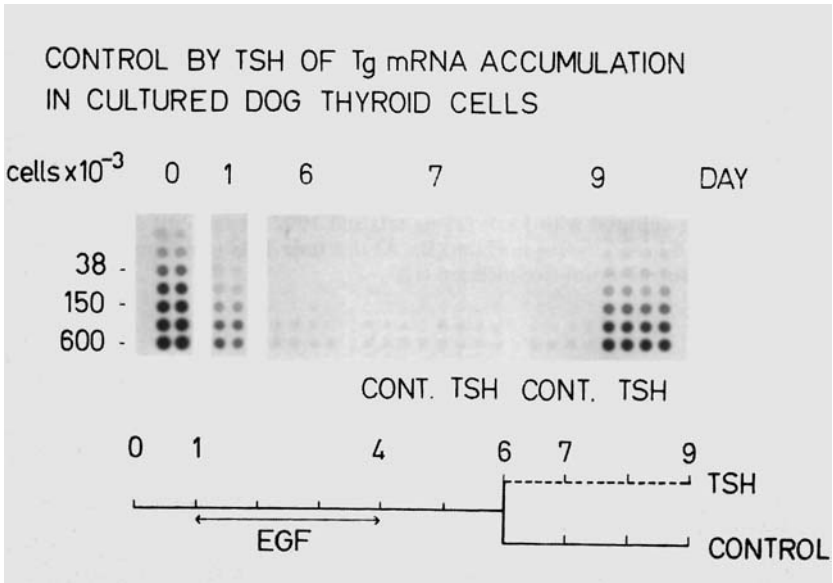

Fig. 2. *Effect of EGF and TSH on cytoplasmic thyroglobulin mRNA content.* The samples were from the same experiment as that illustrated in Fig. 1. Cytoplasmic extracts were prepared from the cells and thyroglobulin (Tg) mRNA content was measured by hybridization to a ^{32}P-labelled bovine thyroglobulin cDNA probe (see Materials and Methods)

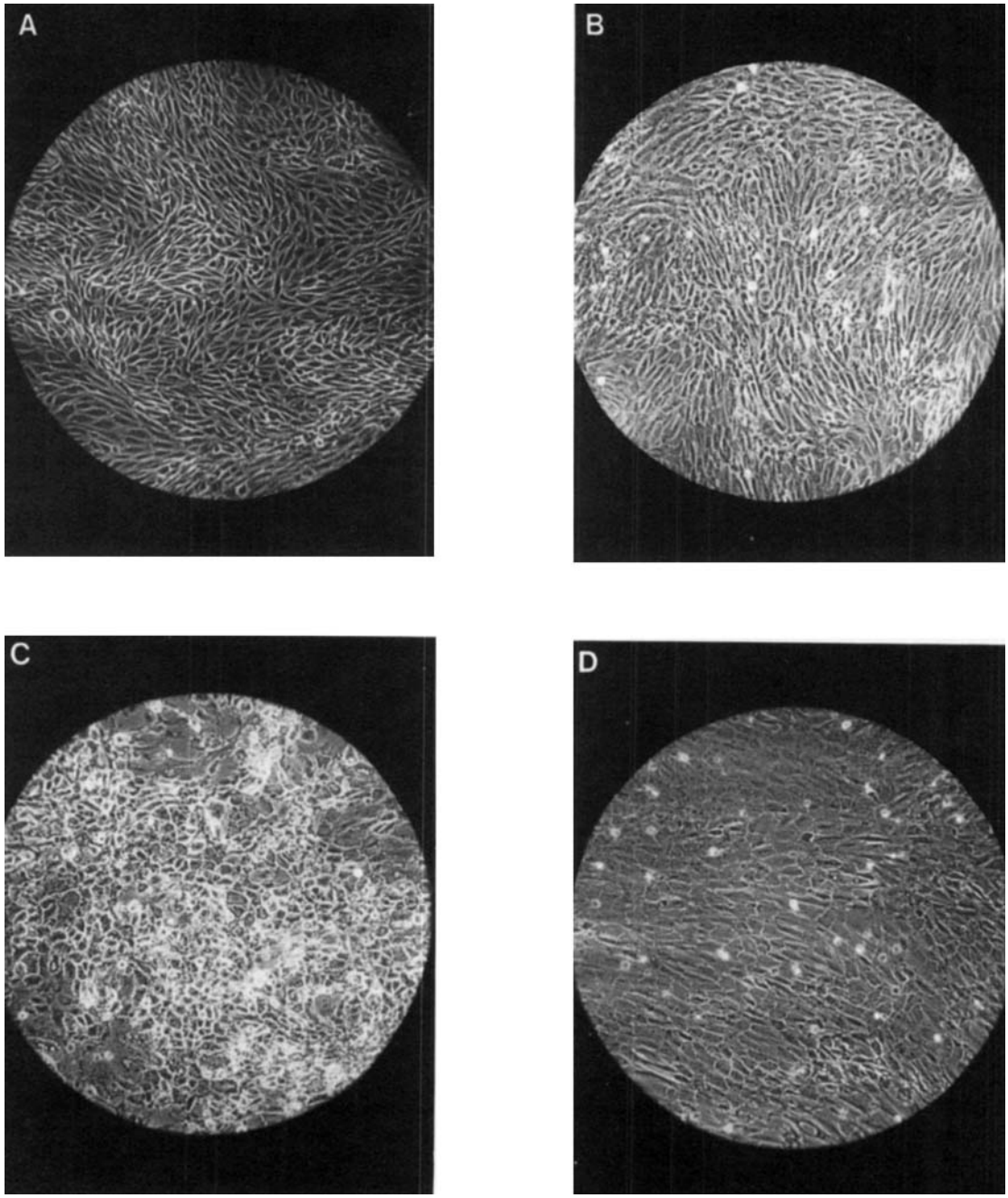

Fig. 3. *Morphology of dog thyroid cells.* Phase-contrast microscopy of living cells (×100) at the different culture stages of the experiment shown in Fig. 4. After day 1 the cells were cultured with EGF (25 ng/ml) and 10% serum until day 6 (A). After elimination of EGF and serum, the cells were maintained until day 8 in serum-free medium (B). At this time TSH (1 mU/ml) was added to some dishes until day 12 (C), while some other dishes remained in control serum-free medium (D)

of EGF (25 ng/ml, 4 nM) thyroglobulin mRNA became undetectable. Moreover, EGF also greatly decreased thyroglobulin mRNA accumulation in TSH-treated cells. This effect was consistent although its importance varied from one experiment to another. It appeared to be specific for EGF, since FGF (fibroblast growth factor), which it also mitogenically active on dog thyroid cells [7], had no such effect (Fig. 5). The addition of EGF together with TSH after 8 days of culture in control medium showed that EGF antagonized partially the TSH effect on thyroglobulin mRNA content (day 12, Fig. 5).

DISCUSSION

Thyroglobulin gene transcription in the rat *in vivo* requires tonic stimulation by TSH. It is greatly decreased in hypophysectomized or triiodo-thyronine-treated animals and it is re-established within 3 h by TSH administration [4]. This effect is mimicked *in vitro* by agents increasing cellular levels of cyclic AMP, such as forskolin, which suggests that it is mediated by cyclic AMP (unpublished). Thus, as with all rapid functional activation types of TSH effects in dog thyroid, the level of expression of the thyroglobulin gene in fully differentiated

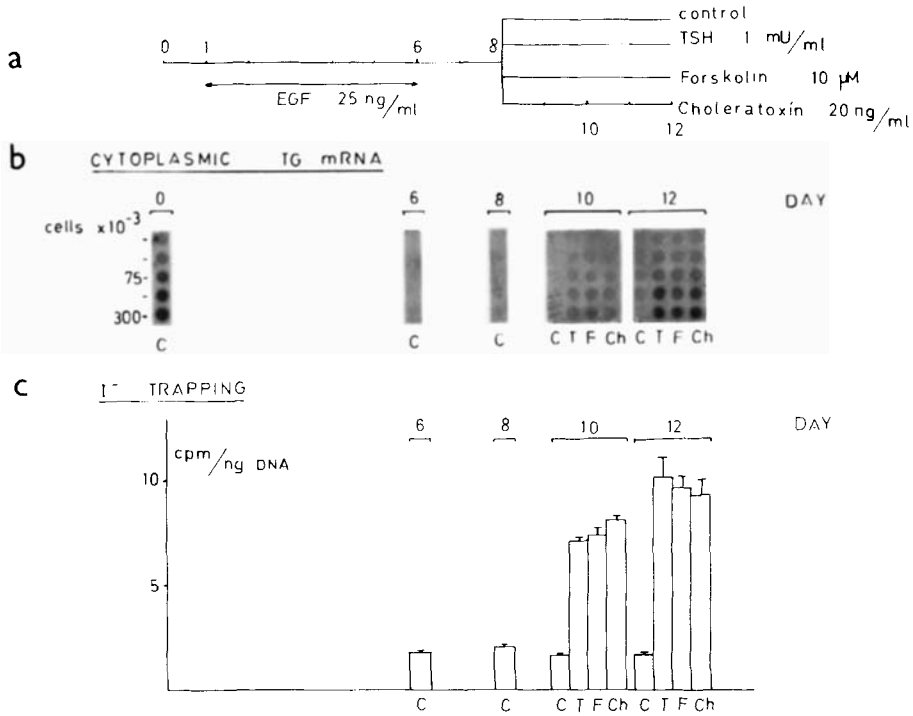

Fig. 4. *Effect of TSH, forskolin and cholera toxin on cell differentiation.* (a) The cells were seeded in control medium (day 0). After one day the culture medium was supplemented with EGF (25 ng/ml) and 10% foetal calf serum and culture was continued until confluency (day 6). At this time the cells were put back in control medium. Two days later (day 8), the cells were divided in four groups and cultured in the following media: control (C), TSH (T), forskolin (F) or cholera toxin (Ch). (b) Cytoplasmic extracts were prepared from the cells and thyroglobulin mRNA content was analyzed as described in the legend to Fig. 1. (c) Iodide trapping was measured as described in Materials and Methods

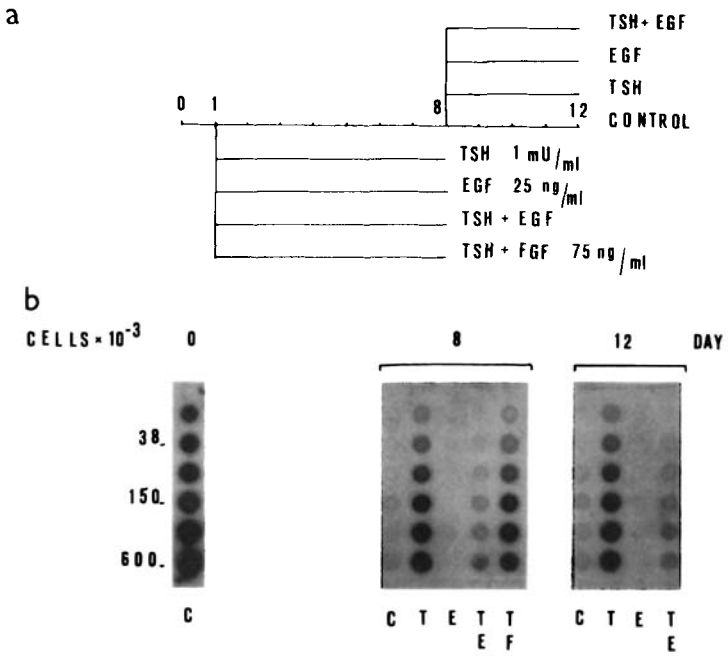

Fig. 5. *Effect of TSH, EGF and FGF on cytoplasmic thyroglobulin mRNA content.* (a) Schematic representation of the experimental schedule. The cells were seeded in control medium (day 0). After one day they were divided in five groups: the first group remained in control medium, the medium of the others were supplemented with TSH (T), EGF (E), TSH + EGF (T−E) or TSH + FGF (T−F). At day 8 the cells cultured in control medium were subdivided into four groups: one remained in control medium, the others were supplemented with TSH (T), EGF (F) or TSH + EGF (T−E). (b) At the time indicated, cytoplasmic extracts were prepared from the cells and thyroglobulin mRNA content was analyzed as described in the legend to Fig. 1

rat thyroid cells is acutely controlled by TSH through cyclic AMP.

The question of the regulation by TSH of thyroglobulin gene expression is more debated when addressed *in vitro* to cell culture systems. From early studies with porcine thyroid cells, it was claimed that the thyroglobulin production is independent of TSH [23, 24]. However, more recently the same authors have observed a stimulation by TSH of thyroglobulin secretion in serum-free culture conditions [25]. The first indications of a stimulation by TSH of thyroglobulin mRNA content were also obtained from porcine thyroid cells in primary culture [26]. However, in these experiments the cells were shown to be poorly responsive to TSH, owing to the lack of accessibility of the receptors [27]. Very recently the partial regulation by TSH of thyroglobulin mRNA content and thyroglobulin synthesis was also reported for the rat FRTL cell line [28]. In this latter system, however, the interpretation of the data is obscured by the fact that TSH is also a survival factor for this line [29]. Indeed a 85% decline in total protein synthesis is observed when the culture medium lacks TSH [28]. What is common to these different studies is that an important part of thyroglobulin gene expression seems to be independent of TSH. Moreover, contrary to what happens *in vivo*, in the culture systems TSH stimulation of thyroglobulin gene expression is a long-term effect observed within days.

In the present study we have investigated the control of the thyroglobulin gene as a differentiation marker of dog thyroid cells. We also describe culture conditions resulting in the loss by the thyroid cell of most of its differentiation characteristics. The cells remain, however, sensitive to TSH as the hormone is able to restore thyroglobulin mRNA accumulation.

In the absence of TSH, dog thyroid cells in primary culture exhibit a progressive disappearance in their differentiation characteristics: iodide trapping and organification [7]. As shown here, thyroglobulin synthesis and thyroglobulin mRNA cell content decrease in parallel. This spontaneous evolution is enhanced by EGF: iodide trapping [7, 8] and thyroglobulin mRNA content (Fig. 5) decrease more than in control cells.

TSH, when added from the beginning of the culture, maintains iodide trapping [7, 8] and thyroglobulin mRNA content (Fig. 5). When added after a proliferation period induced by EGF and serum, it restores both indexes almost to their initial levels. EGF acts in an opposite manner to TSH when it is added from the beginning of the culture or after 8 days. The reinduction by TSH of iodide trapping and thyroglobulin gene expression occurs parallel to dramatic morphological changes from the elongated fusiform EGF-dependent shape to the epithelial cuboidal morphology, which is the characteristic of TSH-treated cells.

There was, therefore, a good parallel between various biochemical parameters of differentiation, such as iodide trapping and thyroglobulin gene expression, and morphological changes in response to TSH, drugs enhancing cyclic AMP and growth factors. Moreover both of these responses were slow (Fig. 1) when compared with the rapid *in vivo* effects of TSH on functional activation [20] and on the level of thyroglobulin gene transcription [4]. This suggests the existence of a slow, qualitative modulation of gene expression, i.e. a modulation of the expression of differentiation characteristics. However, in these experiments the commitment of the cells to thyroid differentiation remains established: fusiform, fibroblast-like cells with no iodide trapping and thyroglobulin mRNA can be reset by TSH and by non-specific cyclic AMP enhancers, such as forskolin and cholera toxin, to express again these characteristics and to return to an epithelial morphology.

With regard to the thyroglobulin gene it is, therefore, possible to distinguish three levels of control: a qualitative control leading to differentiation of the thyrocytes during embryogenesis, a slow modulation of the expression of differentiation characteristics (as shown in this study) and a rapid quantitative control of gene transcription [4]. The first level has not yet been explored. Our primary cell cultures provide an experimental model for the second and the rat, *in vivo*, for the third. It is possible that different mechanisms operate for these three levels of control. In the case of regulation of the metallothionein and vitellogenin genes, different segments of promotor sequences or different chromatin configurations have been shown to correspond to different types of control [30, 31].

In the dog thyroid cells, TSH and EGF both stimulate proliferation but play opposite roles on differentiation expression. Such an opposite role on differentiation expression has also been observed in mouse mammary epithelial cells [13]. The level at which EGF antagonizes the action of TSH in dog thyroid cells is presently not known. However, the effects of TSH and EGF on differentiation expression are not a direct consequence of the action of these agents on proliferation:

a) Both TSH and EGF enhance proliferation while having antagonistic effects on differentiation expression; FGF increases proliferation while not affecting iodide trapping and thyroglobulin mRNA nor the effect of TSH on these parameters.

b) The induction of thyroglobulin gene expression and iodide trapping by TSH were observed on the late phase of the culture, during which only little proliferation or cell renewing occur.

However, the possibility is not completely excluded that a limited stimulation of proliferation might be an important step of action of TSH or EGF on differentiation.

In dog thyrocytes the effects of TSH on differentiation expression, including thyroglobulin mRNA accumulation, were mimicked by non-specific activators of adenylate cyclase, such as forskolin and cholera toxin, and by a cyclic AMP analogue cyclic dibutyryl-AMP. This suggests that this action of TSH is mediated by cyclic AMP. Moreover, this constitutes the first evidence that the thyroglobulin gene may be sorted in the category of eukaryotic genes regulated by cyclic AMP. Only a few examples of these genes are known, namely the genes coding for tyrosine aminotransferase [32], phospho-*enol*pyruvate carboxykinase [33, 34], prolactin [35], lactate dehydrogenase [36], α-lactalbumin and casein [37]. By increasing cyclic AMP, TSH is thus able to stimulate acutely thyroid functions, to enhance the level of thyroglobulin gene expression and to induce both cell proliferation and differentiation. The range of hormonal actions mediated by this cyclic nucleotide appears unique to the dog thyroid cell.

It is assumed that in the thyrocytes, as in most experimental systems, the level of thyroglobulin mRNA reflects the level of transcription of its gene. However, as we have only studied the cytoplasmic amounts of the messenger we do not exclude the possibility of complementary posttranscriptional effects on thyroglobulin mRNA stability or on the processing of nuclear precursor transcripts as recently shown in porcine thyroid cells [38]. These results provide us with a useful experimental model to study the control of thyroglobulin gene expression by TSH, cyclic AMP and growth factors. Together

with the availability of cloned thyroglobulin gene promoters [39, 40], this system and a derived bovine cell system [41] will now be used to study the molecular mechanisms involved in gene regulation by cyclic AMP and EGF.

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