Differentiation Expression during Proliferative Activity Induced through Different Pathways: In Situ Hybridization Study of Thyroglobulin Gene Expression in Thyroid Epithelial Cells

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Abstract. In canine thyrocytes in primary culture, our previous studies have identified three mitogenic agents and pathways: thyrotropin (TSH) acting through cyclic AMP (cAMP), EGF and its receptor tyrosine protein kinase, and the phorbol esters that stimulate protein kinase C. TSH enhances, while EGF and phorbol esters inhibit, the expression of differentiation. Given that growth and differentiation expression are often considered as mutually exclusive activities of the cells, it was conceivable that the differentiating action of TSH was restricted to noncycling (Go) cells, while the inhibition of the differentiation expression by EGF and phorbol esters only concerned proliferating cells.

Therefore, the capacity to express the thyroglobulin (Tg) gene, the most prominent marker of differentiation in thyrocytes, was studied in proliferative cells (with insulin) and in quiescent cells (without insulin). Using cRNA in situ hybridization, we observed that TSH (and, to a lesser extent, insulin and insulin-like growth factor I) restored or maintained the expression of the Tg gene. Without these hormones, the Tg mRNA content became undetectable in most of the cells. EGF and 12-0-tetradecanoyl phorbol-13-acetate (TPA) inhibited the Tg mRNA accumulation induced by TSH (and/or insulin). Most of the cells (up to 90%) responded to both TSH and EGF. Nevertheless, the range of individual response was quite variable. The effects of TSH and EGF on differentiation expression were not dependent on insulin and can therefore be dissociated from their mitogenic effects.

Cell cycling did not affect the induction of Tg gene. Indeed, the same cell distribution of Tg mRNA content was observed in quiescent cells stimulated by TSH alone, or in cells ~50% of which had performed one mitotic cycle in response to TSH + insulin. Moreover, after proliferation in "dedifferentiating" conditions (EGF + serum + insulin), thyrocytes had acquired a fusiform fibroblast-like morphology, and responded to TSH by regaining a characteristic epithelial shape and high Tg mRNA content. 32 h after the replacement of EGF by TSH, cells in mitosis presented the same distribution of the Tg mRNA content as the rest of the cell population. This implies that cell cycling (at least 27 h, as previously shown) did not affect the induction of the Tg gene which is clearly detectable after a time lag of at least 24 h.

The data unequivocally show that the reexpression of differentiation and proliferative activity are separate but fully compatible processes when induced by cAMP in thyrocytes. The inhibition of differentiation could be a side effect of the activation of the EGF and phorbol ester pathways, rather than a consequence of cell cycling itself.

ADULT thyroid epithelial follicular cells (thyrocytes) in primary culture keep many thyroid functions and responsiveness to various hormones, neurotransmitters, and growth factors (Lissitzky et al., 1971; Westermark et al., 1983; Roger and Dumont, 1984; Roger et al., 1982, 1985; Errick et al., 1985; Raspé et al., 1986; Gérard et al., 1988, 1989*a*). Moreover, they can proliferate in defined culture medium in response to different growth factors and hormones (Roger et al., 1987*a*). Therefore, these primary cultures are good experimental models for the study of how growth and differentiation expression are regulated, how they interact, and what molecular mechanisms are involved. We have demonstrated different proliferation controls in dog thyrocytes in primary culture: thyrotropin (TSH)[1] acting through a continuous cAMP rise induces DNA synthesis af-

1. *Abbreviations used in this paper*: Tg, thyroglobulin; TPA, 12-0-tetradecanoyl-13-phorbol acetate; TSH, thyrotropin.

ter a 16–20 h prereplicative phase (Roger et al., 1983, 1987*b*). EGF and the tumor promoter 12-0-tetradecanoyl-13-phorbol acetate (TPA), acting respectively through the receptor protein tyrosine kinase and through protein kinase C, cause DNA synthesis with the same kinetics but independently of cAMP (Roger et al., 1986, 1987*a*; Contor et al., 1988; Lamy et al., 1989). Low concentrations of insulin are permissive for the mitogenic effect of TSH, whereas higher concentrations are necessary to obtain the mitogenic effect of EGF (Roger et al., 1987*a*). The cAMP-dependent mitogenic pathway induced by TSH appears essentially distinct from the rapidly converging EGF and TPA cascade (Dumont et al., 1989), as judged from the patterns of protein phosphorylation and synthesis (Contor et al., 1988; Lamy et al., 1989).

These distinct mitogenic pathways are associated with opposite effects on the expression of thyroid differentiation (Roger and Dumont, 1984). TSH acting through cAMP induces thyroglobulin (Tg) mRNA accumulation and iodide transport; EGF and TPA antagonize these effects (Roger et al., 1985, 1986). Tg, the precursor of thyroid hormone, is a specific thyroid protein.

Proliferation and differentiation are often considered as mutually exclusive genetic programs of the cell. The induction of both by TSH through cAMP raises the question as to whether an individual cell could express both simultaneously or not. To answer this question, we have used Tg mRNA, as demonstrated by in situ hybridization with antisense canine Tg cRNA probes. We have characterized, in response to the above-mentioned mitogenic agents, the differentiation expression (Tg mRNA) in individual dog thyrocytes in primary culture versus their capacity to enter into mitosis.

Glossary

Factors Controlling Tg Gene Expression: Abbreviations and Concentrations

α	absence of hormone (insulin) and serum
I	insulin (8.3 10^{-7} M, 5 μg/ml)
i	insulin (1.6 10^{-9} M, 10 ng/ml)
T	TSH (thyrotropin, 10^{-9} M, 1 m U/ml)
E	EGF (4.1 10^{-9} M, 25 ng/ml)
TPA	12-0-tetradecanoylphorbol-13-acetate (1.6 10^{-8} M, 10 ng/ml)
IGF-1	insulin-like growth factor-1 (2.7 10^{-9} M, 20 ng/ml)
F	forskolin (10^{-5} M)
S	1% FCS

Materials and Methods

Primary Cultures of Dog Thyroid Follicular Cells

The cells were obtained from dog thyroid as detailed previously (Roger et al., 1982). Briefly, the tissue was digested by collagenase (type I, 150 U/ml; Worthington Biochemical Corp., Freehold, NJ) so that the resulting suspension consisted mainly of fragmented as well as intact follicles. These follicles were seeded ($\pm$ 2.10^4 cells/cm^2) on glass coverslips (Corning Glass Works, Corning, NY; n°2, 22 × 22 mm); the attached follicles developed in 1–2 d as a monolayer.

The cells were cultured in the following mixture that constituted the control medium (Roger et al., 1987*a*): DMEM + Ham's F12 medium (Flow Laboratories, Irvine, UK) + MCDB 104 medium (Gibco Laboratories, Pailey, UK) (2:1:1 by vol.) supplemented with 2 mM glutamine (Flow Laboratories), ascorbic acid (40 μg/ml), antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml and 2.5 μg amphotericin B/ml, Flow Laboratories).

The cells were kept in a water-saturated incubator at 37°C in an atmosphere of 5% CO_2 in air. Thyrocytes in primary culture were submitted to various additions: bovine TSH (Armour Pharmaceutical Co., Chicago, IL); forskolin (Hoechst Pharmaceutical, Bombay, India); bovine insulin (Sigma Chemical Co., St. Louis, MO); murine EGF (Sigma Chemical Co.); TPA (Sigma Chemical Co.), FCS (Gibco Laboratories); insulin-like growth factor-1 (Kabi Vitrum, Stockholm, Sweden).

RNA Probes

Plasmid pBSdTg 11 was prepared by ligating Pst I–cleaved dog thyroid cDNA to Pst I–linearized pBS M13+ vector (Stratagene, La Jolla, CA). Recombinant plasmids containing the dog Tg sequence were identified by hybridization with cloned bovine Tg cDNA fragments (Christophe et al., 1980). They were characterized by partial sequencing and compared with the sequence of bovine Tg (Mercken et al., 1985). Clone pBSdTg 11 contained an 1,133-bp long insert corresponding to positions 6,543 to 7,676 of bovine Tg cDNA. Sense (negative) and antisense (hybridizing) probes ($\pm$ 250 bp) were obtained after in vitro transcription of Pvu II cleaved pBSdTg 11 DNA using T3 and T7 RNA polymerases (Stratagene) with 80 μM tritiated uridine triphosphate nucleotide (40–50 Ci/mmol; Amersham Corp., Arlington Heights, IL).

In Situ Hybridization

The following criteria for in situ hybridization were met to allow a quantitative analysis of the results: absence of labeling with "sense" cRNA probes in thyrocytes treated with TSH; absence of labeling with "antisense" cRNA probes in nonthyroid cells (L929 cultured cells); significant labeling with "antisense" probe in thyroid TSH-treated cells; and absence of labeling in cells that had been treated with ribonuclease before hybridization and postfixed with paraformaldehyde.

Cultured thyroid cells on coverslips were fixed with 4% paraformaldehyde in 0.1 M PBS (pH 7.4) + 5 mM $MgCl_2$ + 15 mM vanadyl sulfate (Berger and Birkenmeier, 1979) for 12 min at room temperature. They were rinsed twice (5 min) in PBS; dehydrated in increasing ethanol concentrations; air dried; attached with mounting medium on ribonuclease-free (sulfochromic-treated) glass slides; kept frozen (−20°C); and desiccated.

Before hybridization, slides were brought to room temperature and dipped in PBS for 10 min; treated 30 min with 0.01% saponin (Sigma Chemical Co.); rinsed twice (5 min) in PBS; digested 5 min with pronase (Calbiochem-Behring Corp., San Diego, CA; 50 μg/ml in 50 mM Tris [pH 7.5], 5 mM EDTA); rinsed 30 s in glycine (Aldrich Chemical Co., Milwaukee, WI, gold label, 2 mg/ml in PBS); 30 s in PBS; treated with 0.25% (vol/vol) acetic anhydride according to the procedure of Hayashi et al. (1978); rinsed twice (3 min) in 2 × SSC; dehydrated in increasing ethanol concentrations; and air dried.

"Antisense" and "sense" Tg RNA probes were lyophilized before use (stock: $\pm$ 1.10^8 dpm/μg kept at −80°C as precipitate in 72% ethanol + 1/10th vol 3 M sodium acetate; final concentration: 1–2 μg/ml in hybridization mixture [Cox et al., 1984]). Other components were added to give a final concentration of 50% formamide (recrystallized at least twice at 0°C); 2 × SSC, 1 × Denhardt (0.02% BSA; 0.02% Ficoll, and 0.02% polyvinylpyrollidone); 1 U/μl ribonuclease inhibitor (Boehringer Mannheim Diagnostics, Houston, TX); 5 mM DTT; 10% dextran sulfate (Sigma Chemical Co.) (Wahl et al., 1979); 100 μg/ml *Escherichia coli* tRNA (Boehringer Mannheim Diagnostics, RNAase negative); 25 μg/ml sonicated DNA from salmon (Sigma Chemical Co.); 10^{-5} M uridine 5'triphosphate (Pharmacia Fine Chemicals, Piscataway, NJ). Hybridization mixture was applied (18 μl/coverslip) and cells were overlaid for protection with coverslips (Thermanox) (13-mm diam); sealed with rubber cement; and placed in boxes humidified with 50% formamide in 2 × SSC. They were left at 60–65°C overnight.

Slides were then rinsed, for 15 min, twice in 2 × SSC; ribonuclease-treated (ribonuclease A; Sigma Chemical Co., 10 μg/ml in 10 mM Tris [pH 8.0], 0.5 M NaCl) for 30 min at 37°C; rinsed at 37°C for 30 min in 10 mM Tris (pH 8.0), 0.5 M NaCl + 100 μg/ml brewer's yeast tRNA (Boehringer Mannheim Diagnostics) + uridine triphosphate nucleotide 10^{-5} M (Aldrich Chemical Co.). After that, they were washed in 50% formamide; 0.25 SSC (2 × 15 min at 45°C, 4 × 30 min at 20°C); and rinsed 30 min in 0.5 ×

SSC without formamide. Slides were finally dehydrated in 70 and 94% ethanol + 300 mM ammonium acetate and air dried. For autoradiography, they were exposed for 2–3 wk at 4°C to Ilford K2 emulsion diluted 1:1 with distilled water + 2% glycerin + 300 mM ammonium acetate; developed with D19 (Kodak); fixed with Hypam (Ilford); and stained with 5% giemsa (E. Merck, Darmstadt, FRG).

Autoradiographic silver grains were counted "double blind" under a light microscope in duplicate samples of 200 random spread out cells; counts made by independent investigators were similar. Cellular background, when present, was deduced in the numerical data reported. The results were quite similar for different sets of experiments, and the mean value of the labeling was quite reproducible for duplicate slides in a given experiment. Each figure represents one typical experiment that was representative of at least three separate experiments.

Results

Tg Gene Expression in Thyrocytes Cultured with Different Mitogenic Agents (Figs. 1–3)

Freshly isolated dog thyroid follicles adhering to glass coverslips presented a high Tg mRNA content as judged by intense labeling with the Tg cRNA probe (not shown). High Tg mRNA contents were maintained in cells proliferating as a monolayer in the presence of TSH + insulin + 1% serum (Fig. 1 *b*), but declined to a low basal level in the absence of TSH (Fig. 1 *a*) (mean value: 15 grains/cell versus 181 grains/cell with TSH). Although most of these TSH-treated cells (>90%) contained Tg mRNA (Fig. 1 *b*), a marked heterogeneity was observed from cell to cell (Fig. 1 *d*). This variability in Tg mRNA content was not due to gross differences in metabolism rates or viability within the cell population since all the cells presented similar levels of protein synthesis, as judged by ^{3}H leucine incorporation revealed by autoradiography (not shown). Neither was the heterogeneity due to differences in TSH receptor density or accessibility, since exactly the same cell distribution of Tg mRNA (data not shown) was obtained in cells cultured with TSH or forskolin, the direct adenylate cyclase activator (Seamon and Daly, 1986) that perfectly mimics all the effects of TSH in dog thyrocytes (Roger et al., 1985; 1987*b*), but freely diffuses through membranes. On the other hand, highest mRNA contents were not especially associated with a peculiar cell morphology (Fig. 1 *d*); nevertheless, they were more often observed in less spread cells, originating from the remnants of the seeded follicles (not shown). The biochemical basis for this variability is unknown but a highly heterogeneous response to TSH from follicle to follicle and even cell to cell has also been observed for various functions of the thyroid in vivo (Dumont et al., 1971; Bergé-Lefranc et al., 1983; Studer et al., 1989).

These cultures of cells continuously stimulated by TSH are in fact a mixture of asynchronously cycling cells with some nonproliferating cells. It was therefore plausible that lowest amounts of Tg mRNA corresponded to most actively cycling cells. TSH could maintain Tg gene expression and support cell proliferation, but not in all the cells at the same time (Roger and Dumont, 1983). When the proliferating fraction of cells was increased as a result of the additive effects of TSH and EGF (Roger and Dumont, 1984; Roger et al., 1987*a*), the Tg mRNA accumulation was inhibited in most of the TSH + EGF-treated cells (mean value, 68 grains/cell) (Fig. 1 *c*) with only a few cells still presenting high Tg mRNA levels, as compared to the amounts induced by TSH alone (181 grains/cell). Cells that proliferated for 5 d in the presence of EGF + insulin + 1% serum contained only very low amounts of Tg mRNA (mean value, 7 grains/cell). The inhibitory effect of EGF was not because of a decrease of Tg mRNA stability. EGF did not accelerate the spontaneous disappearance of Tg mRNA labeling (half-life, ~24 h; data not shown) after the removal of TSH or forskolin.

From these preliminary experiments, it was conceivable that the differentiation action of TSH was restricted at any time to noncycling (Go) cells, while the inhibition of the differentiation by EGF only concerned proliferating cells. Drawing firm conclusions as to whether or not an individual cell could simultaneously express differentiation and cycle in response to TSH would require double labeling experiments (e.g., thymidine incorporation versus Tg mRNA in situ hybridization). However, they would not be conclusive in these asynchroneous cell populations, since Tg mRNA is too stable and would not completely disappear during cell cycle progression (27 h minimum; Roger et al., 1987*a*). Therefore, preliminary experiments were needed to select the experimental culture conditions able to suppress the basal expression of the Tg gene in the thyrocytes; indeed such a requirement would be essential to observe if a given cell were able to reexpress the Tg gene and to enter into mitosis simultaneously. It was also necessary to define precisely the time course of Tg mRNA accumulation in response to TSH to compare it with the previously published kinetics of cell cycle progression (Roger et al., 1987*a*). In addition, we were interested in examining whether the growth effects of TSH or EGF could be dissociated from their effects on differentiation.

Reinduction of Tg Gene Expression in Proliferative and Unproliferative Thyrocyte Populations

After a 4-d culture period in α medium (medium lacking insulin and serum), no proliferation at all was observed; cells acquired an undifferentiated spread-out morphology. Very few cells (<20%) still had a very low content in Tg mRNA (mean value in the total population, 3 grains/cell); the rest of the cell population was unlabeled (Fig. 2 *a*). Kinetics of Tg mRNA accumulation from 0 to 72 h in response to TSH after α pretreatment (Fig. 2 *a*), showed that ~20% of thyrocytes had started to accumulate Tg mRNA within 8 h. This proportion reached 80% at only 16 h after TSH addition. Tg mRNA then progressively accumulated within the whole cell population, but here also the individual responses were quite heterogeneous (Fig. 2 *a*; 72 h, 5–100 grains/cell).

The induction of DNA replication in dog thyrocytes by TSH requires insulin as a comitogenic factor (Roger et al., 1987*a*) and is usually studied starting with cells that are quiescent after 4 d in culture with a high insulin concentration (5 μg/ml) (Roger et al., 1987*a*) that could saturate IGF-1, receptors (Straus, 1981). We have compared the rate of expression of the Tg gene in 4-d-old α medium–pretreated cell culture when submitted to the action of various mitogenic agents for 72 h (Fig. 3). The reexpression of the Tg gene induced by TSH was almost inhibited by EGF ($\pm$ insulin and serum) or TPA, in the whole thyrocyte population. However, as shown in Fig. 3, insulin itself stimulated Tg mRNA accumulation. The comitogenic effect of the high insulin supplement can be partly replaced by either low insulin concen-

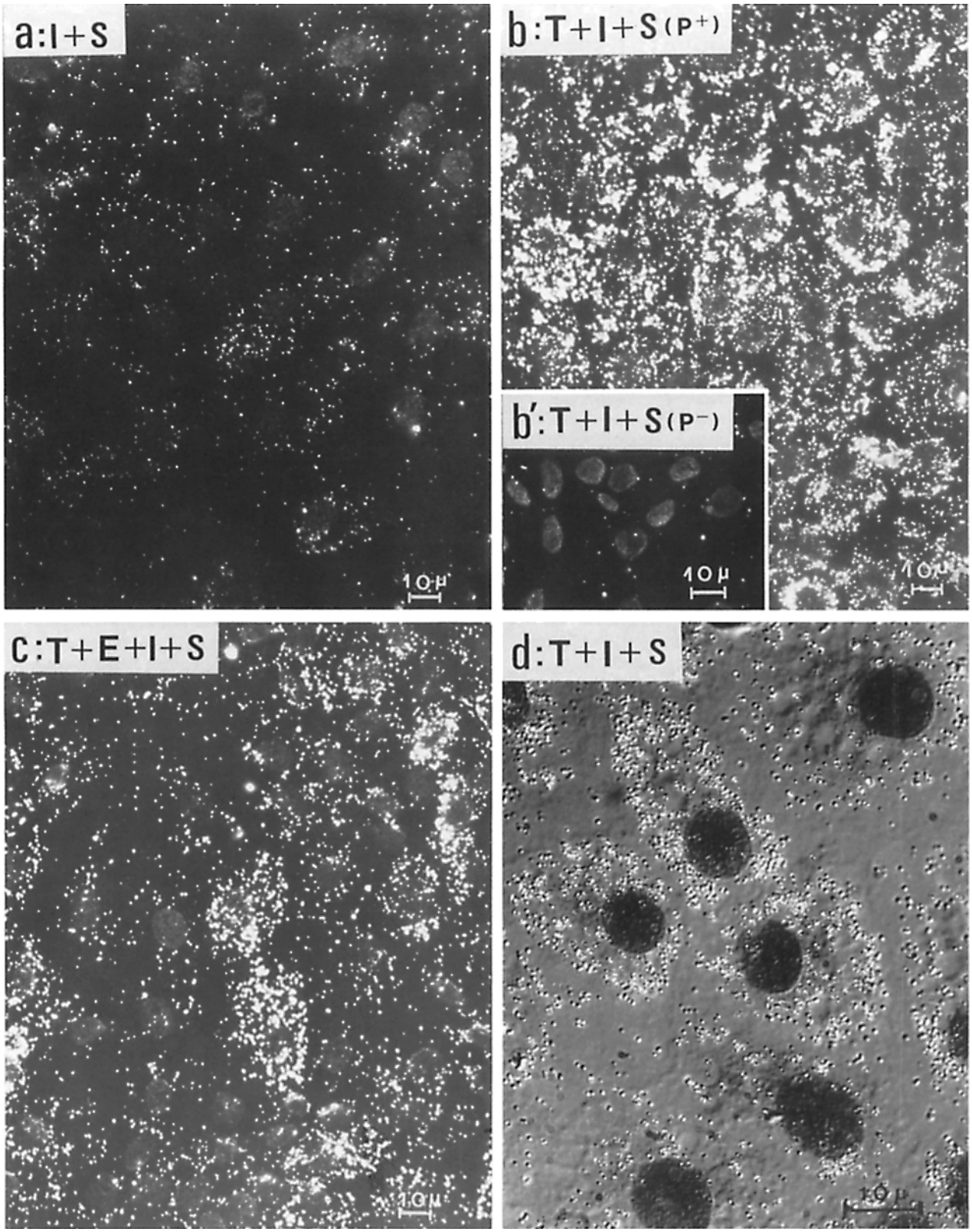

Figure 1. Autoradiography of dog thyrocytes in primary culture when hybridized with Tg cRNA probes. Thyrocytes were seeded in insulin (*I*) + 1% serum (S) medium. 24 h later, they were cultured for 5 d either in the same medium (*a*); plus TSH (T + I + S) (*b* and *d*); or plus TSH + EGF (T + E + I + S) (*c*). The hybridization was performed with antisense (hybridizing) Tg cRNA probe P^+ (*a*, *b*, *c*, and *d*) or with sense (negative) probe P^-. *b′*, inserted in *b*; *a*, *b*–*b′*; *c*, dark field. *d* (Nomarski differential interference contrast) illustrates the heterogeneousness of the Tg mRNA content in TSH-treated thyrocytes (same treatment as in *b*). The cells were fixed and processed for hybridization in situ and autoradiography, as described in Materials and Methods except that K5 emulsion was used in the place of K2 to get a better illustration of the labeling.

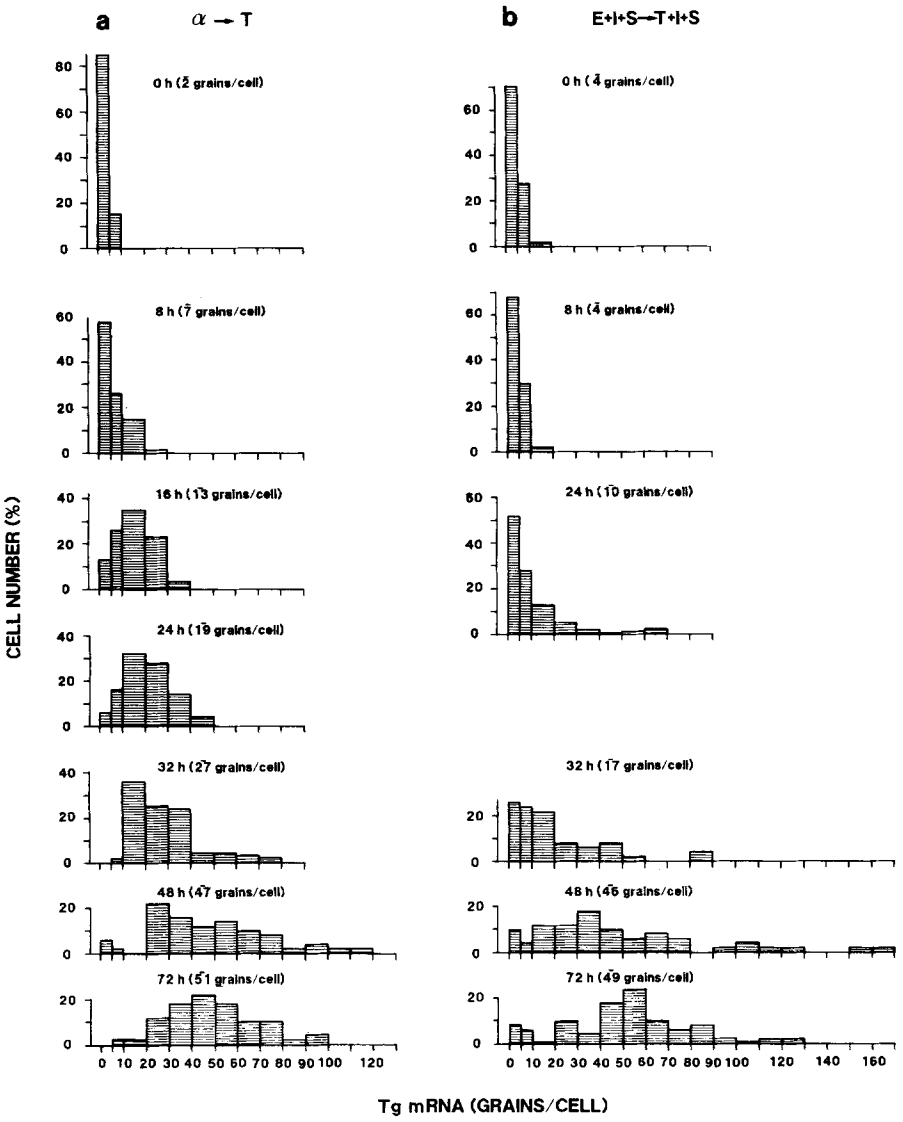

Figure 2. Evolution of Tg mRNA content distribution in quiescent (*a*) and proliferative (*b*) thyrocyte populations after stimulation by TSH. (*a*) Thyrocytes were seeded and cultured for 4 d in α medium; they were then transferred to TSH (*T*) for the indicated times (0–72 h). (*b*) Thyrocytes were maintained for proliferation during 5 d in medium containing EGF(*E*) + insulin (*I*) + 1% serum (*S*), then transferred for the indicated times (0–72 h) into TSH (*T*) in the presence of insulin (*I*) and 1% serum (*S*). The Tg mRNA content was determined after in situ hybridization and autoradiography as detailed in Materials and Methods.

trations (Roger et al., 1987*a*) or by a low IGF-1 concentration (our unpublished observations). But both a low "physiological" insulin concentration (10 ng/ml) and, more weakly, a low IGF-1 concentration also induced Tg mRNA accumulation (Fig. 3). Thus, these characteristics did not allow to dissociate the comitogenic action of insulin in the presence of TSH from its effect on Tg gene expression, and to fulfill the requirement of inducing proliferation in cells devoid of Tg mRNA. This insulin effect on Tg gene expression was unexpected: insulin does not induce other thyroid differentiation markers such as iodide transport (Lamy et al., 1989) and thyroperoxydase gene (Gérard et al., 1989*b*).

Nevertheless these results (Fig. 3) provide conditions that allow a comparison of the distribution of Tg mRNA induced by TSH (72 h) in proliferative and unproliferative thyrocyte populations (Fig. 4). DNA replication was evaluated by the thymidine labeling index obtained in the continuous presence (72 h) of tritiated thymidine (data not shown) (Roger et al., 1987*a*). Interestingly, very similar patterns were observed whether the cells had been precultured, during 4 d, in the presence of insulin (Fig. 4 *c*) or not (Fig. 4 *d*). Under these conditions, ~50% of insulin-treated cells underwent DNA replication after TSH addition while cells not exposed to insulin did not. The superimposable patterns demonstrate that all the cells in both populations accumulated Tg mRNA. This strongly suggested that TSH is able to promote, as compatible programs in the individual cell, both cell division and expression of the differentiated phenotype. Nevertheless, low amounts of Tg mRNA might be present in some cycling cells, because of the action of insulin during the 4-d pretreatment (Fig. 4, *a* versus *b*), or because it had been relatively rapidly induced by TSH (Fig. 2) before cells entered the cell cycle.

Reinduction of Tg Gene Expression after Multiplication in Dedifferentiation Conditions (EGF + Insulin + 1% Serum)

Cells were kept for 5 d in E + I + S medium after seeding in I + S (1 d). Active proliferation affecting 80–90% of the cell population occurred during this pretreatment (Roger and Dumont, 1984; our unpublished observations). The cells

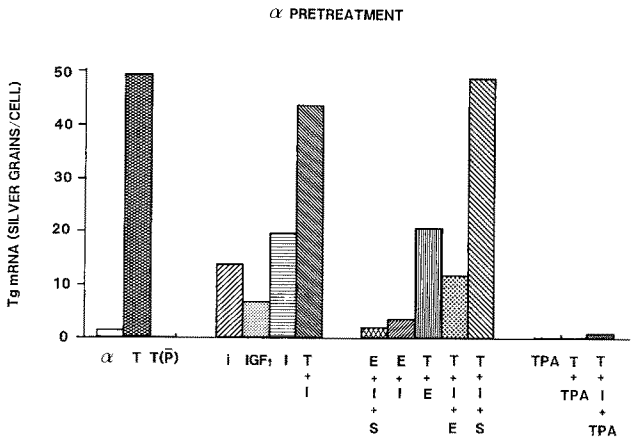

Figure 3. Mean TgmRNA content of quiescent thyrocytes subjected to various mitogenic agents for 3 d. Dog thyrocytes were seeded and cultured for 4 d in α medium. They were then subjected, for an additional 3 d, to various agents as indicated: *T*, TSH; *E*, EGF; *i*, insulin (10 ng/ml); *I*, insulin (5 μg/ml); *TPA*, 12-0-tetradecanoyl-phorbol-13-acetate. Tg mRNA content was determined after in situ hybridization with hybridizing (antisense) cRNA probes in every case, except for T(P^-) where a negative (sense) probe P^- was used, as a control, on TSH-treated cells (*T*). After that, the cells were processed for autoradiography (see Materials and Methods).

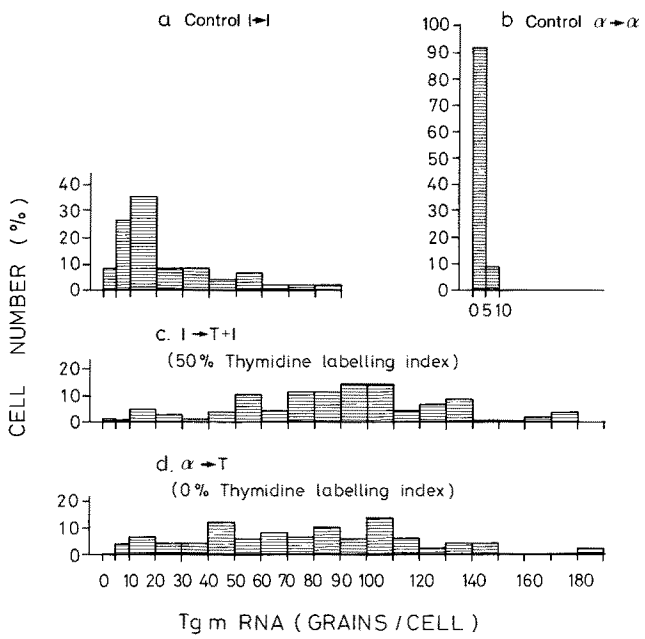

Figure 4. Differentiation expression versus proliferative activity. Comparative distribution of TSH-induced Tg mRNA content in proliferating and unproliferating thyrocytes. Proliferative conditions were realized as maintaining the cells for 4 d in insulin followed by 72 h in TSH + insulin (*T* + *I*). Tg mRNA distribution induced by *T* + *I* is shown in 4 *c* with its control in insulin alone (4 *a*). Unproliferative conditions were realized as detailed in Fig. 2 *a* (4 d in α medium followed by 72 h in the presence of TSH alone). Tg mRNA distribution induced by TSH (*T*) is shown in Fig. 4 *d* with its control in α conditions (Fig. 4 *b*). After that, thyrocytes were processed for Tg cRNA hybridization and autoradiography as detailed in Materials and Methods.

adopted an elongated fusiform morphology (Roger and Dumont, 1984) with few cells still containing Tg mRNA (mean value, 4 grains/cell). The inhibitory action of EGF on Tg gene expression was essentially reversible since, 72 h after the removal of EGF, TSH had reinduced Tg mRNA accumulation in ~90% of the cells (Fig. 2 *b*). The cuboidal, differentiated morphology characteristic of TSH-treated cells was also restored (Roger et al., 1985).

The kinetics (0 to 72 h) of Tg mRNA accumulation in response to TSH (Fig. 2 *b*), in the presence of insulin + 1% serum, showed a shift from almost unlabeled cells (0 h) to cells with high Tg mRNA content (72 h). The shift did not present a steady progression: up to 24 h after TSH addition, very few cells increased their Tg mRNA content. After 32 h, 75% of the cells had started to accumulate Tg mRNA, occasionally at high levels (5 to 80–90 grains/cell), but part of the cell population (25%) remained essentially unlabeled (0–4 grains/cell). Later (48–72 h), a generalized but heterogenous response to TSH (90% of the cells) was achieved with a progressive increase in the Tg mRNA content (72 h, 0–5 to 130 grains/cell). Thus, as compared to Tg mRNA reinduction after an α pretreatment (Fig. 2 *a*), the response was delayed (24 h at least) and asynchronous, some cells requiring a very long lag (48 h) before accumulating Tg mRNA (Fig. 2 *b*). This long lag time (24 h at least) was merely because of the presence of EGF during the pretreatment as shown in experiments where thyrocytes were pretreated by I + S alone without EGF (time lag, <16 h, data not shown).

After EGF pretreatment, TSH can still induce a weak proliferative activity in the presence of insulin and 1% serum. 32 h after TSH addition, some mitoses were observed, merely dependent on the presence of TSH (1.5% in TSH-treated cells versus 0.3% in control cells). As shown in Fig. 5, identical cellular distribution of Tg mRNA, with occasionally very high contents, were observed in these cells in mitosis (Fig. 5 *c*) and in the rest of cells (Fig. 5 *b*) 32 h after TSH addition. Since the minimum time of the cell cycle in dog thyrocytes in primary culture is 27 h (Roger et al., 1987*a*) and since the reinduction of the Tg gene expression required a 24-h time lag at least (Fig. 2 *b*), this demonstrates that TSH was able to induce simultaneously both proliferative activity and differentiation expression in the same thyrocytes (Fig. 6, *a*, *b*, and *c*), as already suggested in Fig. 4.

Inhibition by EGF of Tg-gene Expression in Nonproliferating Thyrocytes

The mitogenic effects of EGF are dependent on high insulin concentration as a comitogenic factor (Roger et al., 1987*a*; Lamy et al., 1989). In α pretreated cells (4 d in medium lacking insulin and serum), TSH enhanced Tg mRNA accumulation. This effect was inhibited by EGF in the presence or absence of insulin (Fig. 3). Even more potently than EGF, TPA also inhibited Tg mRNA accumulation in the absence of insulin (Fig. 3). Thus, the inhibiting effects of EGF and TPA are independent of insulin.

Discussion

In dog thyrocytes, the TSH-cyclic AMP mitogenic pathway induces differentiation expression as measured by Tg mRNA content and iodide trapping. On the other hand, the cAMP-independent mitogenic pathways activated by EGF and TPA

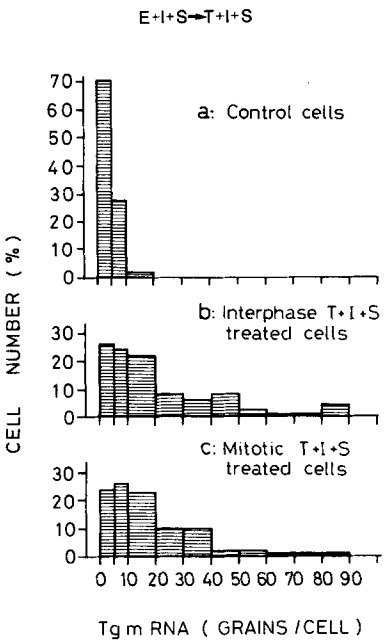

Figure 5. Differentiation expression versus proliferative activity. Comparative distribution of TSH-induced Tg mRNA in mitotic and interphase thyrocytes. Thyrocytes were pretreated by EGF (*E*) + insulin (*I*) + 1% serum (*S*) as detailed for Fig. 2 *b*. Tg mRNA distribution was compared, 32 h after the transfer in TSH (*T*) + I + S, in interphase (*b*) and in mitotic cells (1.5% of the population) (*c*). The distribution of Tg mRNA, before the transfer to T + I + S, is illustrated (*a*). The cells were processed for Tg cRNA hybridization and autoradiography as described in Materials and Methods.

decrease this expression. These observations raise questions about the old but still current concept that differentiation expression and proliferation are antagonistic processes in cell life. Rather than a consequence of cell cycle progression, a loss of differentiation expression might be dependent on the activation of only some mitogenic pathways. Otherwise, the apparent converse relation between growth and differentiation could be merely coincidental, reflecting independent effects of hormones and growth factors. The present study aims to distinguish between these alternatives in our experimental model.

Thyroglobulin Gene Expression as Differentiation Marker

Extensive in situ hybridization experiments were performed to quantitate the induction of Tg mRNA as a differentiation marker in individual dog thyrocytes in primary culture, in response to different mitogenic agents. When proliferating in the presence of TSH and insulin, these cells retained high levels of Tg mRNA. In the absence of any hormone or in dedifferentiating conditions (EGF + insulin + serum), the Tg gene expression became undetectable, but it could be reinduced by TSH. Such a tight hormonal dependence of Tg gene expression has not been observed in other thyroid systems where a constitutive basal expression has been reported (Chebath et al., 1979; Fayet and Hovsepian, 1979; Fayet et al., 1982; Aouani et al., 1987; Chambard et al., 1987; Santisteban et al., 1987). The induction of Tg mRNA accumulation by low physiological concentrations of insulin, and the lack of insulin influence on the TSH control of Tg gene, are

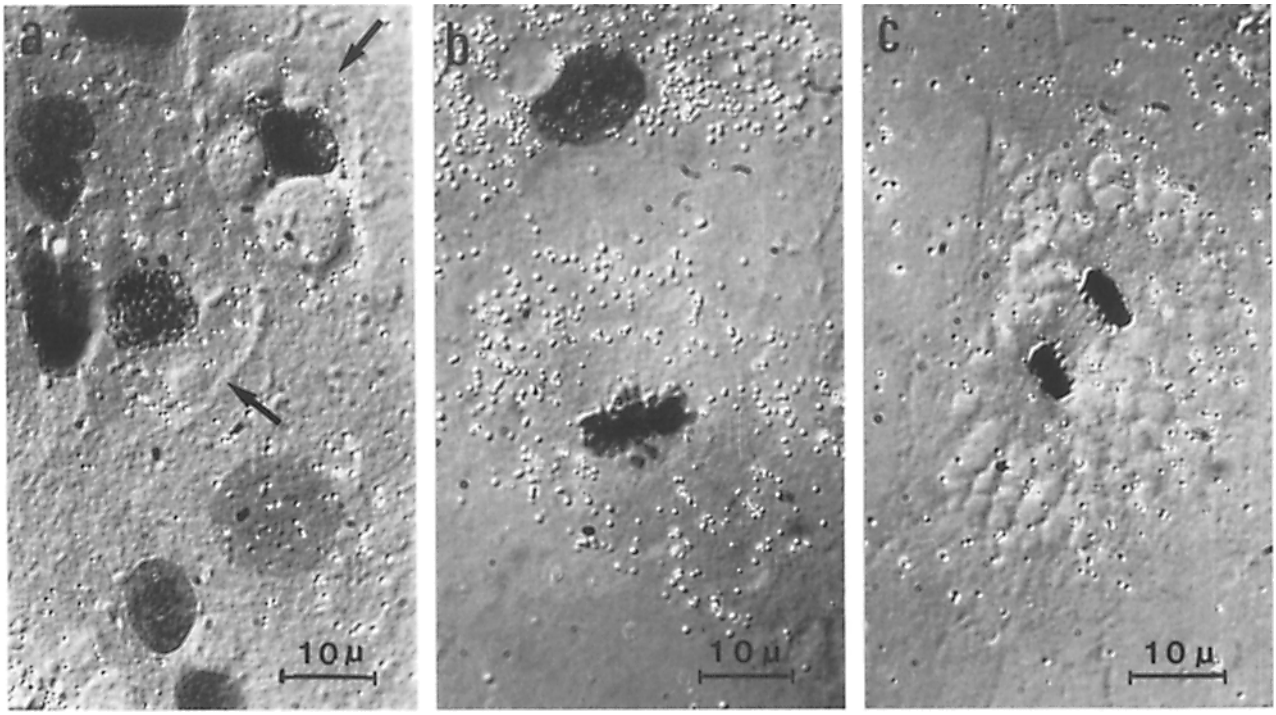

Figure 6. Expression of differentiation (Tg mRNA) in dog thyrocytes in mitosis (autoradiography). Treatment of TSH-treated dog thyrocytes was realized as detailed for Fig. 5. In situ labeling of Tg mRNA in thyrocytes is shown at different stages of mitosis: prophase (*a*), metaphase (*b*), and telophase (*c*). Nomarski differential interference contrast was used. The cells were processed for Tg cRNA hybridization and autoradiography as described in Materials and Methods.

novel observations. Unlike TSH, insulin does not increase the levels of cAMP in dog thyrocytes (Van Sande and Roger, unpublished observations); this shows that the Tg gene expression can be induced separately by cAMP-dependent and independent mechanisms. To our knowledge, this constitutes the first evidence of an effect of insulin at physiological concentration on a thyroid-specific function, which might contribute to explain the impairment of thyroid function reported in insulin-dependent diabetes in animal and in man (Jolin et al., 1970; Gray et al., 1980; Bagchi et al., 1981).

Although the Tg gene transcription is inducible through cAMP, its promoter lacks CRE and AP-2 consensus sequences (de Martynoff et al., 1987). TSH induction of Tg gene transcription requires ongoing protein synthesis (Gérard et al., 1989*b*) and involves changes in chromatin accessibility in culture (Hansen et al., 1988). While in vivo and in dog thyroid tissue incubations, ongoing transcription of Tg gene is quickly modulated by TSH (Van Heuverswyn et al., 1984; Gérard et al., 1989*b*), the Tg gene response was delayed in primary cultures by a time lag that depended on both culture conditions and local cell organization. The response was often more rapid in dense cell aggregates; cells that had not proliferated in vitro (either with or without insulin) presented shorter lag times for Tg mRNA reinduction than cells that had proliferated actively in the presence of EGF, which inhibits differentiation expression. The kinetics of Tg gene induction by TSH seem, therefore, quite dependent on the differentiation state of the cells before stimulation. In cultured dog thyrocytes, they are akin to a cell differentiation process rather than to a rapid functional control.

The Tg mRNA accumulation, either stimulated by TSH or insulin, was inhibited (as iodide trapping and the maintenance of an epithelial morphology) by the cAMP independent mitogenes EGF and TPA. EGF has probably an effect on gene transcription, as it did not modify the disappearance of Tg mRNA in the absence of TSH.

Mitogenesis and Differentiation Expression Are Separate Processes That Can Be Compatible

Mitogenesis and differentiation are often considered mutually exclusive activities of the cells; this obviously applies to terminal differentiation processes necessarily accompanied by an irreversible cessation of growth. In addition, several examples of initially highly differentiated cell types present a temporal loss of differentiation expression during the proliferative phase; this was reported very early for iris pigmented cells (Doljanski, 1930), for retinal pigment cells (Rodesch, 1973), for hepatocytes (Leffert et al., 1978; Nakamura et al., 1984*a*, 1985), hepatoma cells (Nakamura et al., 1984*b*), thyroid medullary carcinoma cells (Berger et al., 1984; de Buostros et al., 1986), ovarian granulosa cells (Epstein-Almog, 1985), and for prostatic epithelial cells (Chevalier et al., 1981). Similar results were obtained with dog thyroid cells in the presence of 15% serum (Roger and Dumont, 1983; Magnusson and Rapoport, 1985). In such systems, the long prereplicative phase was interpreted as including the necessary time for the shift of the genetic activity from a specialized program to a more general growth-related one (Boynton and Whitfield, 1983). By contrast, in embryonic rat hepatocytes, differentiation process (accumulation of carbamoylphosphate synthetase) was found to occur in each phase of the cell cycle (van Roon et al., 1989).

That mitogenesis and Tg mRNA expression are compatible is already suggested by the effects of insulin treatment that induces Tg mRNA synthesis and promotes the TSH stimulation of growth. Moreover, in serum-free conditions, we observed the same cell distribution of Tg mRNA without any unresponsive cells, in quiescent cells (in the presence of TSH alone; Fig. 4 *d*), and in cells ~50% of which had performed at least one mitotic cycle (in the presence of TSH + insulin; Fig. 4 *c*).

At a given time, the different mitogenic agents stimulate cell proliferation into a fraction of the thyrocyte population: after 2 d of treatment, ~50, 30, and 20% of the cells undergo DNA replication in response to TSH, EGF, and TPA (Roger et al., 1986, 1987*a*). The present results clearly showed that nearly the whole cell population (up to 90%) was responsive to both types of agents: TSH induced the accumulation of Tg mRNA, and EGF and TPA counteracted this action of TSH in virtually all the cells. The fact that Tg mRNA content was modulated in the same way in all the cells submitted to differentiating (TSH, forskolin) and dedifferentiating treatments (EGF and TPA) shows that differentiation expression was similar qualitatively, if not quantitatively, in all cells submitted to a given treatment whether they were proceeding to mitosis.

Still, considering the quantitative heterogeneity of cell responses, it was conceivable that the differentiating action of TSH occurred predominantly in the nonproliferating cells of the stimulated population. However, after culture in dedifferentiating conditions (EGF + serum + insulin), in situ hybridization revealed that, 32 h after TSH addition, cells in mitosis (Fig. 5 *c*) presented a distribution of Tg mRNA content similar to that of the rest of the cell population (Fig. 5 *b*). This showed that the mitotic cycle (minimum 27 h) stimulated by TSH did not antagonize the long TSH-dependent reinduction of Tg mRNA accumulation in these conditions. These data unequivocally demonstrate that TSH, through cAMP, could simultaneously reinduce proliferative activity and differentiation expression in the same cell (Fig. 6). Independent factors should be responsible for the intercellular heterogeneity of either differentiation or growth responses.

Our previous studies have indicated that the cAMP-dependent mitogenic pathway (TSH) and the cAMP-independent ones (EGF and TPA) remain partly separated until late commitment of DNA synthesis (Roger et al., 1987*a*,*b*). They could involve different biochemical events in addition to common ones, which are necessary, but not sufficient, for cell cycle progression (Contor et al., 1988; Lamy et al., 1989; Reuse et al., 1986, 1990). Not only the cell signaling cascade, but even the biochemical nature of the G1 phase, might be specific to the mitogenic stimulus (Dumont et al., 1989). Therefore, the cAMP-dependent mitogenic pathway, by opposition to cAMP-independent ones, might have unique characteristics that make it compatible with differentiation expression. A clue to the mechanism involved is that the c-myc mRNA response to EGF and TPA in dog thyrocytes is relatively stable while its response to TSH and cAMP is transient, due to a specific cAMP-dependent inhibitory mechanism (Reuse et al., 1986, 1990). In several systems, c-myc expression not only appears as a prerequisite for growth stimulation, but also it prevents differentiation (Heikkila et al.,

1987; Yokoyama and Imamoto, 1987; Prochownik et al., 1988; Griep and Westphal, 1988). Perhaps, the rapid termination of c-myc induction in TSH-stimulated thyrocytes is necessary to prevent interference with the simultaneous induction of differentiation expression by TSH. The apparent growth-related dedifferentiation induced by EGF or TPA could thus be a side effect of the activation of these mitogenic pathways rather than a necessary event for proliferation itself in dog thyrocytes. Nevertheless, the inhibition by EGF of differentiation expression (iodide transport [Lamy et al., 1989] and Tg mRNA accumulation [this study]) can be dissociated from its stimulation of DNA synthesis; indeed, in the absence of insulin, EGF induces dedifferentiation, but not DNA synthesis and proliferation.

The subversion of the two types of mitogenic pathways, which are coupled with different patterns of differentiation, should lead to the generation of tumors with quite different phenotypes and prognoses.

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