A Mechanism Generating Heterogeneity in Thyroid Epithelial Cells: Suppression of the Thyrotropin/cAMP-dependent Mitogenic Pathway after Cell Division Induced by cAMP-independent Factors

Pierre P. Roger, Mireille Baptist, and Jacques E. Dumont

Institute of Interdisciplinary Research, Université Libre de Bruxelles, Campus Erasme, 1070 Brussels, Belgium

Abstract. The mechanisms that generate the intercellular heterogeneity of functional and proliferation responses in a tissue are generally unknown. In the thyroid gland, this heterogeneity is peculiarly marked and it has been proposed that it could result from the coexistence of genetically different subpopulations of thyrocytes. To evaluate the heterogeneity of proliferative responses in primary culture of dog thyrocytes, we asked whether the progeny of cells having incorporated ^{3}H thymidine in a first period of the culture could have a distinct proliferative fate during a second labeling period (incorporation of bromodeoxyuridine revealed by immunofluorescence staining combined with autoradiography of ^{3}H thymidine). No growth-prone subpopulations were detected and the great majority of cells were found to response to either EGF or thyrotropin (TSH) through cAMP. However, only a fraction of cells replicated DNA at one given period and a clustered distribution of labeled cells within the monolayer, which was different for thymidine- or bromodeoxyuridine-labeled cells, indicates some local and temporal synchrony of neighboring cells.

The TSH/cAMP-dependent division of thyrocytes preserved their responsiveness to both TSH and EGF mitogenic pathways. By contrast, cells that had divided during a momentary treatment with EGF lost the mitogenic sensitivity to TSH and cAMP (forskolin) but retained the sensitivity to EGF. Since cells that had not divided kept responsiveness to both TSH and EGF, this generated two subpopulations differing in mitogen responsiveness. The extinction of the TSH/cAMP-dependent mitogenic pathway was delayed (1–2 d) but stable. Cell fusion experiments suggest it was due to the induction of a diffusible intracellular inhibitor of the cAMP-dependent growth pathway.

These findings provide a useful model of the generation of a qualitative heterogeneity in the cell sensitivity to various mitogens, which presents analogies with other epigenetic processes, such as differentiation and senescence. They shed a new light on the significance of the coexistence of different modes of cell cycle controls in thyroid epithelial cells.

INDIVIDUAL cells in a tissue may display different properties, in relation to their differentiation stage (coexistence of stem cells, transit cells, and terminally differentiated cells of a given lineage (Hall and Watt, 1989; Potten and Loeffler, 1990)), or because of a different microenvironment (influenced by microvascularization, innervation, cellular interactions through cell adhesion molecules, substrate adhesion molecules, extracellular matrix or paracrine and autocrine factors). A striking heterogeneity may arise even among cells derived from a same clone in tissue culture. Thus, marked differences in multiplication rates or growth factor sensitivity are observed at high frequency even between sister cells in normal human fibroblasts or in immortal 3T3 cell lines (Smith and Whitney, 1980; Brooks et al., 1984; Grundel and Rubin, 1988). Heterogeneity increases during aging in vivo and during senescence in tissue culture as exemplified by a progressive decrease of the proliferative fraction in cells with finite life span (Hayflick, 1977; Smith and Whitney, 1980; Matsumura, 1984). Intercellular heterogeneity is also one of the striking features of tissue pathology, mostly in diseases involving cell multiplication processes. This is true even when a single cause is involved. An interesting example of such tissue heterogeneity in disease is provided by thyroid goiter, whether due to a single genetic defect (e.g., iodide oxidation), nutritional deficiency (iodine), or abnormal stimulation (thyroid stimulating antibodies) (Ingbar and Braverman, 1986).

A central question is thus whether heterogeneity of cellular responses results from stochastic intracellular factors, changes in cell microenvironment, coexistence of genetically different subpopulations, or on the acquisition of stable shifts in phenotypic expression. Primary cultures of unselected cells allow the study of cells representative of in vivo situations under controlled conditions. However, in biochemical studies, these cells are generally investigated as a whole presumably homogeneous population. The great majority of unselected cells that constitute the primary cultures of epithelial dog thyroid cells (thyrocytes), share both

the capacity to proliferate (Roger et al., 1987*a*) and to express thyroid differentiation (Pohl et al., 1990). However, the rates of differentiation expression (stimulated by thyrotropin [TSH]) present a high intercellular variability (Pohl et al., 1990) and the general proliferative response is dependent on a very strong stimulation by a combination of mitogens acting through quite different mechanisms. When thyrocytes are stimulated by their physiological signal, TSH acting through cAMP, or by other cAMP-independent mitogens such as EGF, phorbol esters, and serum, each added separately, the limited increase in the thymidine-labeling index (Roger et al., 1987*a*) suggests that the proliferative fraction could be markedly inferior to 1, as shown in 3T3 fibroblasts stimulated by suboptimal serum concentrations (Brooks et al., 1984; Brooks and Riddle, 1988). These data, together with a qualitative intercellular difference in cytoskeleton composition (presence or not of vimentin beside cytokeratin) (Coclet et al., 1991), are indicative of a considerable intercellular heterogeneity, which might reflect the well-known in vivo heterogeneity of growth and function responses in thyroid gland (Dumont, 1971; Studer et al., 1989).

In this study, using a method of double labeling to demonstrate DNA synthesis at different periods of the culture, we assess the heterogeneity of proliferative responses of these cultured dog thyrocytes. We show that the great majority of these cells share the capacity of proliferating in response to either TSH or EGF, though the mitogenic responses need several days before concerning the whole cell population. On the other hand, we report evidence that a specific inhibitor(s) of the TSH/cAMP-dependent mitogenic pathway is lately induced in the fraction of cells that have divided during a momentary treatment with cAMP-independent mitogens (EGF, serum). This inhibitory factor(s) is trans-dominant in cell fusion experiments. By contrast, the cAMP-dependent division of thyrocytes preserves their susceptibility to both cAMP-dependent and independent mitogenic pathways. These unexpected findings provide a useful model of the generation of a stable qualitative heterogeneity, presumably epigenetic, in the cell sensitivity to various mitogens.

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; Worthington Biochemical Corp., Freehold, NJ) so that the resulting suspension consisted mainly of fragmented and intact follicles. These follicles were seeded ($\pm 2.10^4$ cells/cm^2) in 35-mm tissue culture-treated petri dishes; the follicles attached with a high yield (±75%) and developed in 1–2 d as a monolayer. The cells were cultured in the following mixture that constitutes the control medium (Roger and Dumont, 1984; Roger et al., 1987*a*): DMEM + Ham's F12 medium + MCDB104 medium (Gibco Laboratories, Paisley, UK) (2:1:1 by vol) with 1 mM sodium pyruvate, supplemented with ascorbic acid (40 μg/ml), insulin (5 μg/ml) (Sigma Chemical Co, St Louis, MO), 1% FBS (Sera-Lab, Sussex, UK), antibiotics (100 U penicillin/ml, 100 μg streptomycin/ml, 2.5 μg amphotericin B/ml). The cells were kept in a water-saturated incubator at 37°C in an atmosphere of 5% CO_2 in air. The medium was renewed every other day or every day when cells approached confluency.

As indicated in the Results, thyrocytes in primary culture were submitted to various additions: bovine TSH (Sigma Chemical Co.), murine EGF (Sigma Chemical Co.), forskolin (Calbiochem-Behring Corp., La Jolla, CA) and 10% FBS.

1. *Abbreviations used in this paper*: BUDR, 8-bromo-deoxyuridine; TSH, thyrotropin.

As discussed earlier (Roger and Dumont, 1984) and assessed recently by thyroglobulin mRNA in situ hybridization (Pohl et al., 1990) and cytokeratin immunofluorescence (Coclet et al., 1991), the cultures consist of >98% thyrocytes.

The cell DNA assay described previously (Roger et al., 1982) uses the increase of fluorescence of ethidium bromide when complexed with nucleic acids.

Double Labeling of DNA Synthesis

As indicated in the Results, cells were incubated for a first 24-h labeling period in the complete medium containing 3×10^{-5} M thymidine, 10^{-4} M deoxycytidine, and 10 μCi/ml ^{3}H thymidine (Amersham International, Amersham, UK). It was verified that these conditions of ^{3}H thymidine incubation did not affect the subsequent proliferation of thyrocytes, in growth curves obtained with or without ^{3}H thymidine (not shown). After two washings of the cells, cultures were continued as indicated and the cells were then incubated for a second 24-h labeling period in the complete medium containing 10^{-4} M 8-bromo-deoxyuridine (BUDR) (Sigma Chemical Co.) and 2×10^{-6} M fluorodeoxycytidine. Cells in the petri dishes were fixed in methanol for 10 min at −20°C, and kept in pH 7.5, PBS.

Cells were permeabilized with Triton X100 (0.15%) for 10 min at room temperature, incubated with 2 N HCl for 30 min (room temperature), then rinsed and incubated for 10 min in a borax buffer (0.1 M $Na_2B_4O_7$, pH 8.5). Subsequent rinses were followed by addition, for 30 min, of normal sheep serum (1/20 in PBS with 0.1% BSA). The cells were then incubated overnight at 4°C in PBS/BSA with the antibromodeoxyuridine mAb (1/50, Becton Dickinson), together with a polyclonal rabbit broad-spectrum anticytokeratin antibody (1/50, Dakopatts Z622). Then cells were washed and successively incubated for 1 h at room temperature with biotinylated sheep anti-mouse Igs (1/50 RPN1001 from Amersham International) together with Texas red-conjugated donkey anti-rabbit Igs (1/50, N2034 from Amersham International) and then with fluorescein-streptavidin (1/30, RPN1232 from Amersham International).

The cells were finally processed for autoradiography (one week exposure at 4°C using 1:1 diluted Ilford K5 emulsion); counterstained with propidium iodide (0.5 μg/ml for 3 min); and mounted in Gelvatol (polyvinylalcohol, Monsanto) solution (20 g Gelvatol + 80 ml PBS + 40 ml glycerol) containing 100 mg/ml 1,4 diazobicyclo(2,2,2) octane to delay the fluorescence fading.

Cells were viewed with a Leitz epifluorescence microscope (50× oil immersion lens; L2, I2, and N3 fluorescence filter blocks), and the proportion of cytokeratin-positive cells that are labeled with thymidine, BUDR, or both was determined by counting 500–1,000 cells per dish. Microphotographs were taken using 400 iso Fujichrome films.

Cell Fusion Experiments

Thyroid follicles were seeded in control medium in 90-mm tissue culture-treated petri dishes and submitted 1 d later to EGF (25 ng/ml) or TSH (1 mU/ml) for 3 d, with ^{3}H thymidine during the last 24 h. After rinsing, the cells were cultured for two additional days in control medium and detached from the dishes with a trypsin/EDTA solution. Cells from two dishes were pooled in a 12-ml polystyrene conical centrifuge tube. After centrifugation, the cell pellet was rinsed with culture medium with 1% serum and one more time with serum-free medium. The last pellet was obtained by centrifuging cells for 1 min at 1,000 rpm. It was then gently resuspended in 0.7 ml of a sterile (0.22 μm filtered) solution of polyethylene glycol 4000 (Hipure grade, Koch-Light, Haverhill, UK) made 50% wt/vol in serum-free medium, by rolling the tube for 90 s. The cell suspension was progressively diluted by successively adding at 60-s intervals 0.7, 1.4, 2.8, and 5.6 ml of serum-free medium. Between each addition, the cell suspension was made homogeneous by gently rolling the centrifuge tube. Cells were centrifuged, resuspended in 10 ml control medium, and seeded in nine 35-mm culture dishes. One day after, fused cells were treated by TSH, EGF or control medium for 40 h with BUDR for the last 24 h, and fixed with methanol. The cells were then processed for double labeling of DNA synthesis as above. The cell survival was ~50% and the yield of cell fusion, ~20%. BUDR incorporation was analyzed in each sort of dikaryons (with two thymidine-labeled nuclei, one thymidine-labeled nucleus, or two unlabeled nuclei) and compared to unfused cells.

DNA Synthesis in Dog Thyroid In Vivo

Dogs (male, 12-mo-old) received, by oral administration for 4 wk,

methimazole (2 × 60 mg/d) and propylthiouracil (2 × 150 mg/d) to increase the circulating TSH level. 1 h before thyroid resection, treated and control animals received 50 mg/kg BUDR by intravenous injection. Immunohistochemistry of the BUDR labeling was performed as described (Coclet et al., 1989).

Results

Proliferative Capacity of Dog Thyroid Cells

The proliferative curves obtained in dog thyroid cell primary cultures initiated from a high yield preparation of thyroid follicles are shown on Fig. 1. As previously shown (Roger and Dumont, 1984), a weak proliferation occurred in control condition only during the first 4 d. It is due to the presence of insulin and 1% FCS (Roger et al., 1987*a*). TSH and EGF at optimal concentrations potently stimulated the proliferation. The fraction of DNA replicating cells (1-h incubation with ^{3}H thymidine) was 10% at day 5 in primary culture of dog thyrocytes continuously stimulated with TSH (1 mU/ml). This figure was quite similar to the rate of DNA synthesis obtained in vivo in the follicular cells of the thyroid gland of dogs treated with methimazole and propylthiouracil in order to chronically elevate the endogenous TSH levels: the BUDR-labeling index of thyrocytes was 8.1% in treated dogs, versus 0.17% in control animals.

Even in the presence of the potent mitogenic combination of TSH and EGF, proliferation (and DNA synthetic activity, data not shown) of cultured thyrocytes stopped after the first subcultivation, well before confluency was reached (Fig. 1). A maximum of four to six population doublings was obtained in different experiments. Whether this limitation was due to the exhaustion of an intrinsically limited proliferation capacity or to a deficiency of the culture system is not known. However, it corresponds to the calculated number of cell population renewals in the thyroid gland during adult life in several species, including dogs (Coclet et al., 1989).

The Proliferative Response of Thyroid Cells to TSH and EGF Is General but Spread over Several Days

Previous studies based on labeling of cells during 24–48 h incubations with ^{3}H thymidine have suggested that only a fraction of cells participate to a proliferation response to TSH or EGF, even when used at optimal concentrations (Roger et al., 1987*a*). Our aim has thus been to define whether all the cells could participate to the growth responses shown in Fig. 1, but at different stages of the culture, or whether the proliferation curves could be due to successive divisions in a rapidly proliferating subpopulation. The second possibility would imply the existence in the thyroid of fractions of cells with a higher propensity to proliferate or to respond to the mitogenic stimuli provided by TSH or EGF and that these properties would be retained in the progeny of such cells.

To discriminate between both possibilities, we asked whether the progeny of cells that had incorporated ^{3}H thymidine in a first period of the culture, had more chance to synthesize DNA in a second period. This second labeling was achieved by the incubation of cells with the thymidine analogue BUDR, the nuclear incorporation of which was revealed by indirect immunofluorescence staining with fluores-

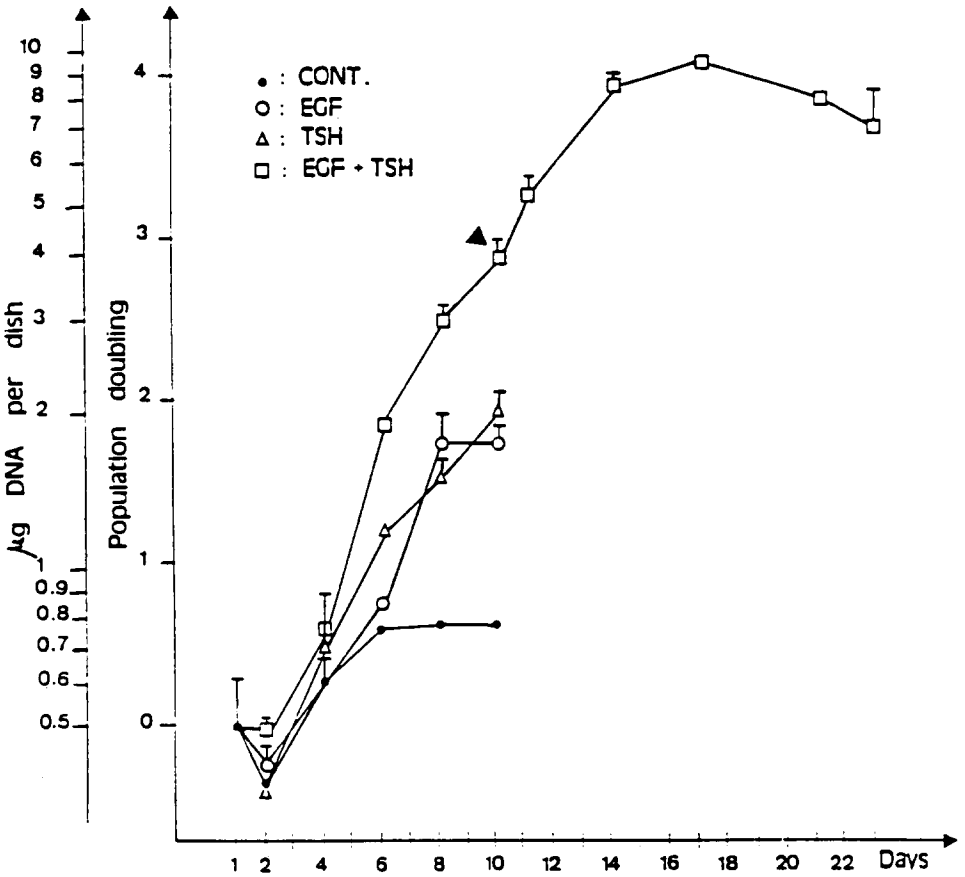

Figure 1. Proliferation of dog thyrocytes. Cells were seeded in control medium. At day 1, TSH (1 mU/ml), EGF (25 ng/ml), or both were added to some culture dishes and their presence was maintained for the rest of the culture. At day 10 (*arrowhead*), some EGF + TSH-treated cells were detached by trypsin/EDTA and re-seeded at 10-fold dilution. DNA content of trypsinized cells was multiplied by 10.

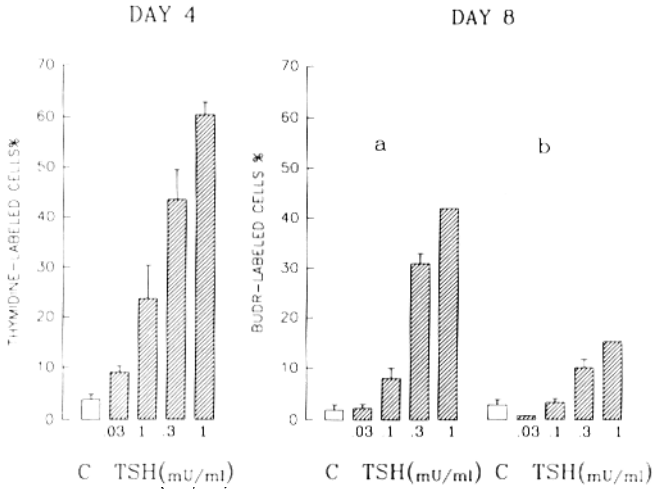

Figure 2. Effect of various TSH concentrations on the fraction of dog thyrocytes that replicated DNA at day 4 or at day 8 of the culture. Cells were seeded in control medium (*C*) and continuously cultured in the presence of different TSH concentrations after day 1. They were incubated with ^{3}H thymidine between day 3 and 4 (some cells were fixed and autoradiographed at this stage), and with BUDR between day 7 and 8. Comparison at day 8 of the incorporation of BUDR in: (*a*) cells which had not incorporated ^{3}H thymidine between day 3 and 4. (*b*) progeny of cells which had incorporated ^{3}H thymidine between day 3 and 4.

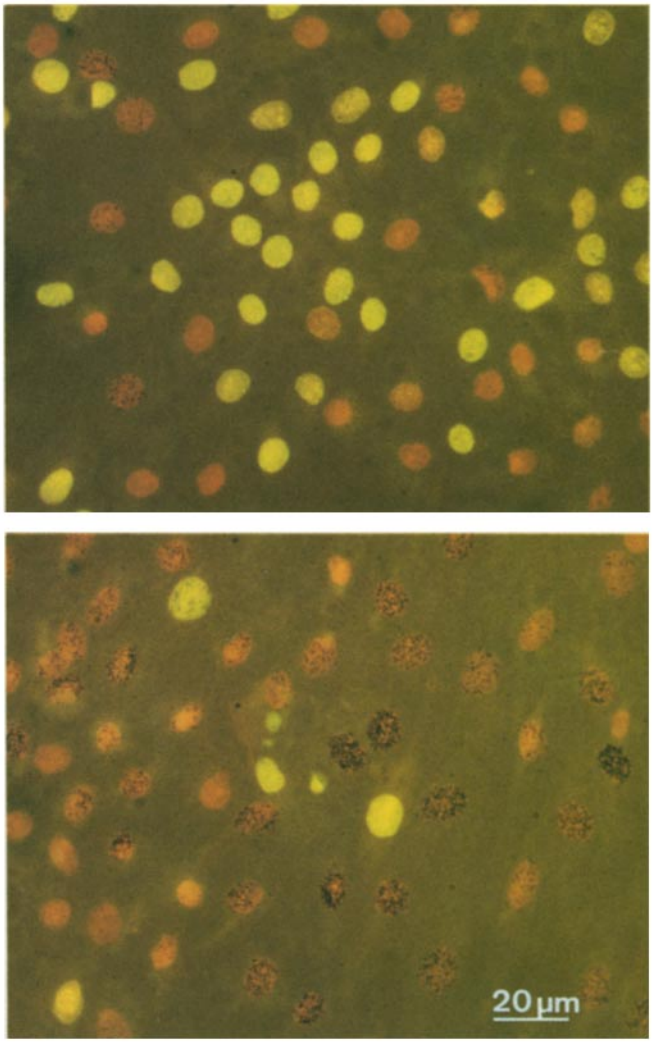

Figure 3. Distribution of cells that had replicated DNA at day 4 (24-h ^{3}H thymidine labeling) or at day 8 (24-h BUDR labeling) in response to a continuous treatment with a suboptimal TSH concentration (0.3 mU/ml) applied at day 1. Both epifluorescence micrographs were taken from two adjacent fields of the same culture dish with cells fixed at day 8 and then treated for BUDR immunofluorescence staining (*yellow-green* fluorescence of nuclei) and autoradiography (*silver* grains on ^{3}H thymidine labeled nuclei). Propidium iodide counterstaining (*red* fluorescence). BUDR-labeled and thymidine-labeled cells occurred in clusters that poorly overlapped.

cein. The procedure thus utilizes the frequently ignored possibility of simultaneous observation of immunofluorescence staining and autoradiographic labeling. The indirect immunofluorescence staining of cytoplasmic cytokeratins with Texas red was joined as a third label in order to visualize morphology and ascertain the epithelial nature of the cells. Nuclear DNA was counterstained with propidium iodide (red fluorescence).

Primary cultured thyroid cells were maintained for 8 d either in the control conditions, or in the continuous presence of different TSH concentrations. They were incubated for 24 h with ^{3}H thymidine between day 3 and 4 (some cells were fixed and subjected to autoradiography at this stage) and for 24 h with BUDR between day 7 and 8. Increasing TSH concentrations produced a gradual increase in the fraction of cells synthesizing DNA at a given time of the culture (Fig. 2). It has been claimed that this reflects the presence in the normal thyroid follicular epithelium of subpopulations of cells with different hereditary growth propensities (Studer et al., 1989). On the contrary, Fig. 2 shows that cells that had proliferated in response to low concentrations of TSH (and thus could be more sensitive to TSH) were not able to pass this characteristic to their progeny. Indeed, during continuous stimulation with suboptimal TSH concentrations, the proliferative activity (at days 7–8) in the progeny of cells that had replicated DNA at days 3–4 was always lower than in their parent cells or than in cells that had not replicated DNA in the first labeling period (Fig. 2). This shows no indication of the existence of growth-prone subpopulations, but on the contrary a relative desensitization of the growth response.

Especially in the case of stimulations with suboptimal TSH concentrations, cells that proliferated at a given time were unevenly dispersed in the cell monolayer. In a same culture dish, zones with high proliferative activity were observed neighboring zones with very few DNA synthesizing cells. As exemplified in Fig. 3, the zones with high proliferative activity at days 7–8 were frequently different from the zones with high proliferative activity at days 3–4. This indicates that some previously quiescent groups of cells later became highly responsive to low mitogenic concentrations of TSH, while other groups with high DNA replicating activity became proliferatively silent. These alternating groups of resting and dividing cells, which formed a striking patchy distribution within the cell monolayer, suggest some spatial and temporal coordination of neighboring cells. However, a greater overlap of cells replicating DNA at day 4 and at day 8 was observed in cultures subjected to maximal concentrations of mitogens, which induce DNA replication in larger fractions of the cells population (Fig. 2).

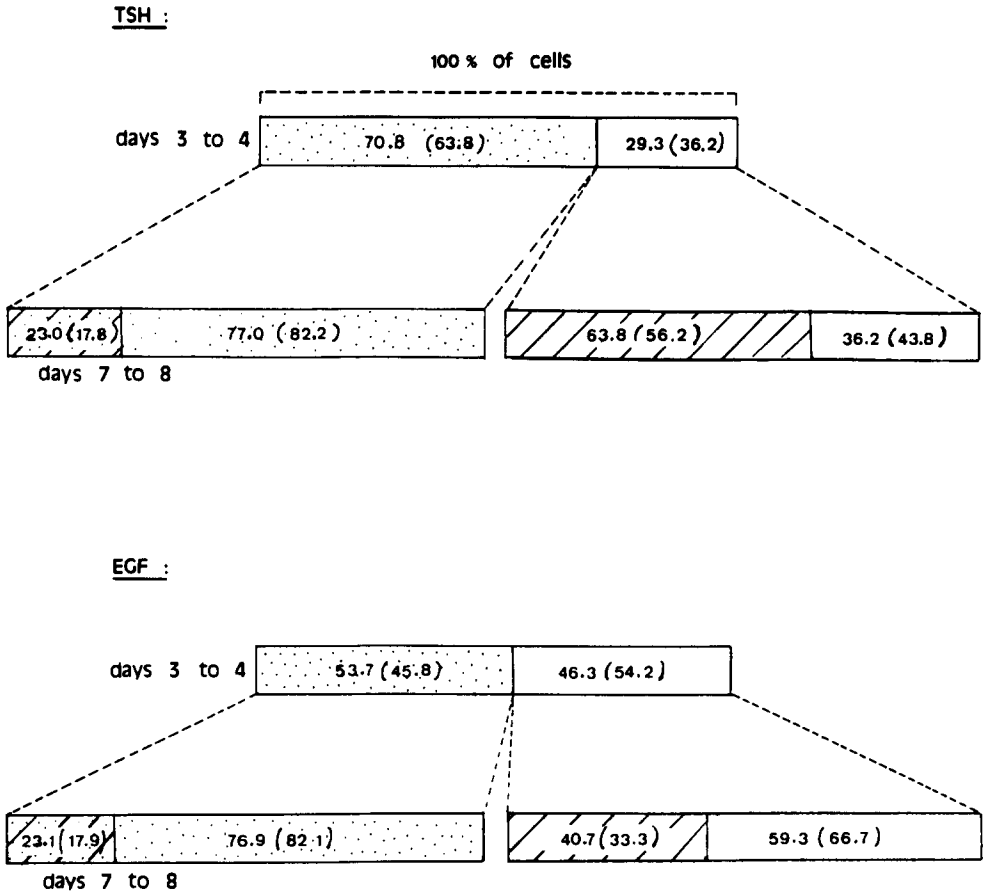

Figure 4. Combining DNA synthesis labeling indices of thyrocytes from two different labeling periods reveals that the majority of thyrocytes can proliferate in response to maximal concentrations of either TSH (1 mU/ml) or EGF (25 ng/ml). After day 1, cells were continuously stimulated with either TSH or EGF. They were incubated with ^{3}H thymidine between day 3 and 4, and with BUDR between day 7 and 8. Cells were fixed at day 4 or 8 and processed for double labeling of DNA synthesis as described in Materials and Methods. Values are the fraction (%) of labeled cells (n^*) as they were counted. Values in brackets are the fraction (%) of cells undergoing DNA synthesis (n_S) during the 24-h labeling period. n_S is calculated by the formula

$$n_S = \frac{n^*}{1 + f(1 - n^*)}$$

Where f is evaluated to be 9/24 = 0.375 considering that, on the average, only the cells having started DNA synthesis during the first 9 h of the 24-h labeling period could have divided at time of fixation (on the average, the duration of S phase + G2 + mitose is 15 h in dog thyrocytes treated with TSH or EGF; our unpublished observations). Percentages are the mean from five independent experiments (TSH) or seven experiments (EGF).

In other experiments, thyrocytes were continuously stimulated with maximal concentrations of either TSH or EGF (Fig. 4). The 36% of TSH-treated cells that had not replicated DNA in the first labeling period were able to respond later. Indeed, many (56%) of them synthesized DNA during the second labeling period (Fig. 4). Thus, by combining the labeling indices obtained for both culture periods, it is concluded that at least 84% of cells (64 + 36 * 0.56) are able to proliferate in the presence of TSH (Fig. 4). Similarly, a total of 64% of EGF-treated cells (46 + 54 * 0.33) (Fig. 4) were seen to proliferate either at days 3–4 or at days 7–8. By comparison, only 17% of control cells replicated DNA, mostly during the first labeling period. The values of Fig. 4 should represent only a minimal evaluation of the fraction of cells that can proliferate in response to TSH or EGF. Many cells also proliferate during the three days between both labeling periods (see Fig. 8 for other choices of labeling windows). In fact, when EGF-labeled cells were continuously incubated with ^{3}H thymidine between day 3 and day 6, 93% of cells were labeled, and 49% of the very few thymidine-unlabeled cells still incorporated BUDR during an additional 24-h labeling period. Therefore, the great majority of dog thyrocytes were able to proliferate in response to either TSH or EGF.

Again, in the case of growth stimulation by either TSH or EGF, the progeny of cells that had replicated DNA during the first labeling period presented a weaker DNA replication response at the end of the culture, as compared to cells that had not incorporated thymidine (Fig. 4). Clearly, the fraction of cells that proliferated during the first labeling period in response to TSH or EGF did not give rise to a progeny with higher susceptibility to these mitogens. On the contrary, their reduced proliferation at late stages of the culture might reflect a relative desensitization of the growth response or, as suggested by Fig. 1, a partial exhaustion of an intrinsically limited potential of divisions.

Suppression of cAMP-dependent Mitogenesis after Division in Response to cAMP-independent Stimuli

In a second set of experiments, we assessed whether the sensitivity of a cell progeny to various mitogenic stimuli could have been modified depending on which mitogenic treatment had triggered the division of its parent cell. As shown in Fig. 5, cells that did not incorporate thymidine during the first part of the culture, either in control conditions or in response to EGF or TSH (or forskolin, the general adenylate cyclase activator that mimics the TSH mitogenic action [Roger et al., 1987*b*]), were however quite responsive to TSH or EGF, which produced additive mitogenic effects during the second part of the culture. Similarly, the progeny of cells that had incorporated thymidine in response to TSH (or forskolin, not shown) during the first part of the culture, retained the capacity to respond to TSH (forskolin) and EGF applied at day 6

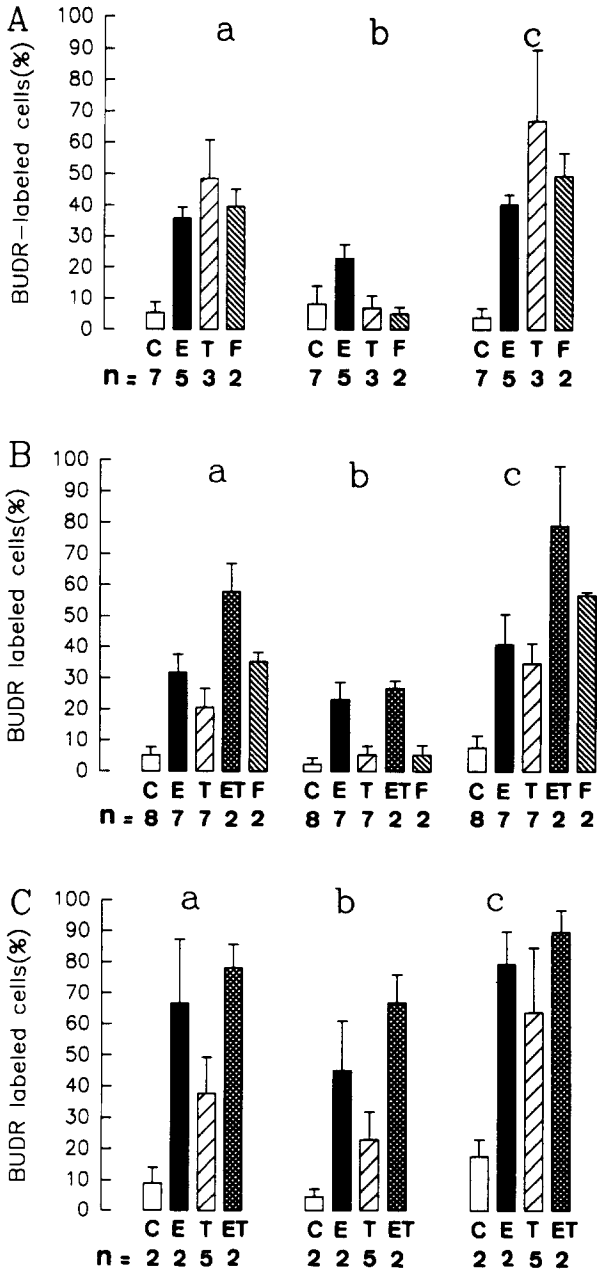

Figure 5. DNA synthesis responses of thyrocytes that had divided in the presence of different mitogenic pretreatments. After day 1, cells were cultured until day 6, with a 24-h incubation with ^{3}H thymidine between day 3 and 4, in (*A*) control medium; (*B*) EGF (25 ng/ml); or (*C*) TSH (1 mU/ml). At day 6, cells were rinsed and stimulated for 2 additional days as indicated on the figure with BUDR for the last 24 h. *C*, control; *E*, EGF (25 ng/ml); *T*, TSH (1 mU/ml); *ET*, EGF + TSH; *F*, forskolin (10^{-5} M). The fraction of cells incorporating BUDR was determined at day 8 in the total cell population (*a*); in the progeny of cells that had incorporated ^{3}H thymidine at day 4 (*b*); and in the cells that had not incorporated ^{3}H thymidine at day 4 (*c*). Results are the mean + SD of data from independent experiments (*n* is the number of experiments for each condition).

of the culture (Fig. 5 *C*). By contrast, the progeny of cells that had incorporated thymidine at days 3–4 in control conditions (Fig. 5 *A*) or in response to EGF (Fig. 5 *B*) (or 10% serum, data not shown) presented a quite altered pattern of mitogenic responses. A marked, though somewhat reduced (as shown in Fig. 4), proliferative response to EGF was retained, but the mitogenic response to TSH or forskolin (alone, or in addition to EGF) was abolished (Fig. 5). Since it has been shown that originally the great majority of cells do have the capacity to respond to either EGF or TSH (Fig. 4), this implies that the mitogenic sensitivity to TSH and cAMP has been specifically suppressed in response to cell division supported by cAMP-independent mitogenic treatments (control [insulin + 1% serum], EGF or serum; but not TSH). This quite unexpected result was consistently reproduced in all the experiments performed (Fig. 5). The inhibition appeared stable: even a 4-d treatment with forskolin applied at day 6 did not restore the cAMP-dependent proliferation response in the progeny of cells that had divided in response to EGF (data not shown).

This phenomenon thus generates two subpopulations in dog thyroid cell cultures. As illustrated in Fig. 6, after mitogenic pretreatment with EGF in the presence of ^{3}H thymidine, thymidine-unlabeled cells frequently incorporated the BUDR label after TSH stimulation, whereas thymidine-labeled cells failed to respond to the mitogenic stimulus of TSH. The thymidine-labeled cells were not completely insensitive to TSH, which induced, in these cells as in thymidine-unlabeled cells, the same morphological response associated with a cytokeratin redistribution (Fig. 6 *C*). By comparison, in the converse situation, after a mitogenic pretreatment with TSH in the presence of ^{3}H thymidine, cells frequently incorporated BUDR after EGF stimulation, regardless of whether they were labeled with thymidine or not (Fig. 7).

The Suppression of the cAMP-dependent Mitogenesis Is a Delayed Phenomenon Which Does Not Depend on a Continuous Stimulation with cAMP-independent Mitogens

In the previous experiments, the suppression of the cAMP-dependent mitogenic stimulation was observed in the progeny of cells that had incorporated thymidine at day 4 in the presence of EGF which was maintained for two additional days before the addition of TSH. During this 2-d period, both ^{3}H thymidine-labeled and -unlabeled cells could have proliferated, and it was not clear why they were so different in their mitogenic sensitivity to TSH. The apparent paradox is explained by the experiment illustrated in Fig. 8. After incorporation of thymidine in the presence of EGF, cells were either immediately stimulated by TSH (or EGF), or placed in control medium (which strongly reduces the proliferative activity) for one to three days before addition of TSH (or EGF). As shown in Fig. 8, thymidine-labeled cells were insensitive to the mitogenic action of TSH when TSH was added 2 or 3 d after the ^{3}H thymidine incubation. However, when TSH was added immediately after the thymidine incubation, an important mitogenic response was still observed in these thymidine-labeled cells (Fig. 8 *A*). This experiment suggests that the suppression of the TSH-dependent mitogenesis after proliferation of cells in the presence of EGF concerns only those cells that have replicated DNA at least 1–2 d before TSH addition. The 2-d period between ^{3}H thymidine exposure and TSH addition (as in the protocol of Figs. 5 and 6) is thus necessary to observe the full inhibition of the TSH-

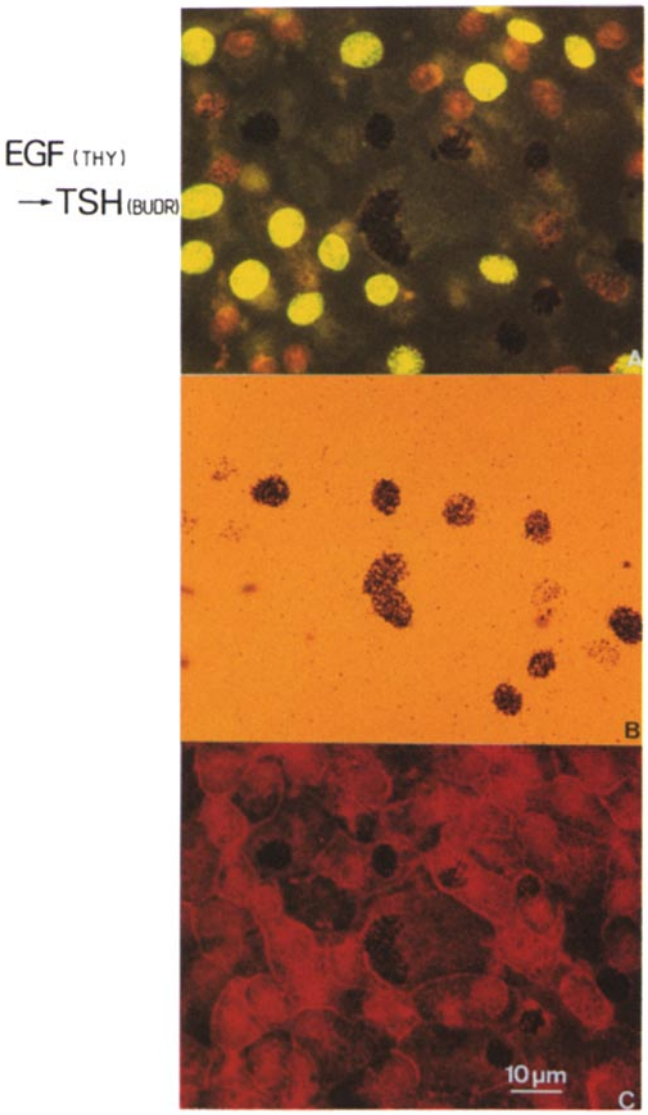

Figure 6. Suppression of the mitogenic response to TSH in the fraction of thyrocytes that had divided in the presence of EGF. After day 1, cells were cultured until day 6 in the presence of EGF (25 ng/ml) (with ^{3}H thymidine between day 3 and 4), and then stimulated by TSH (1 mU/ml) until day 8 (with BUDR for the last 24 h). *A–C* were taken from the same microscope field. (*A*) BUDR immunostaining with fluorescein (*yellow* nuclei). Red counterstaining of DNA with propidium iodide. (*B*) Bright field micrographs showing the silver grains of autoradiography on ^{3}H thymidine-labeled nuclei. (*C*) Cytokeratin immunostaining with Texas red. Red staining of nuclei with propidium iodide. Note that the BUDR incorporation in response to TSH was exclusively restricted to ^{3}H thymidine-unlabeled cells (*A* and *B*). By contrast, note the homogeneous morphological response of cells to TSH (*C*).

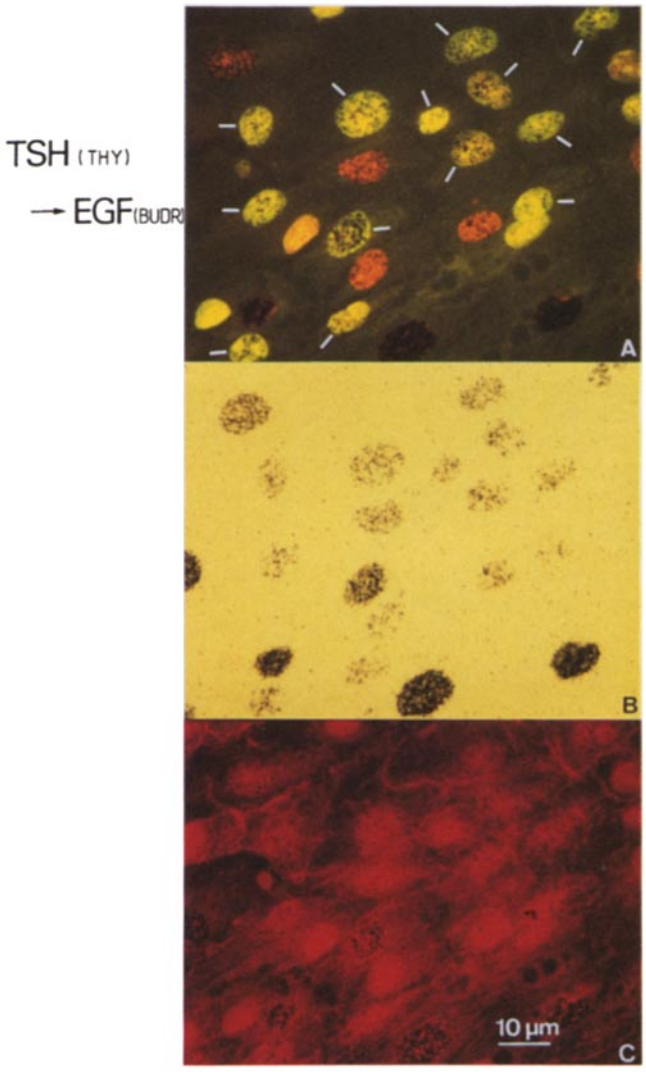

Figure 7. Successive mitogenic responses of thyrocytes to TSH and EGF. After day 1, cells were cultured until day 6 in the presence of TSH (1 mU/ml) (with ^{3}H thymidine between day 3 and 4), and then stimulated by EGF (25 ng/ml) until day 8 (with BUDR for the last 24-h). *A–C* were taken from the same microscope field. (*A*) BUDR immunostaining with fluorescein. Propidium iodide counterstaining. (*B*) Autoradiographical silver grains of ^{3}H thymidine-labeled nuclei. (*C*) Cytokeratin immunostaining with Texas red. Propidium iodide staining of nuclei. Note the high frequency of BUDR/^{3}H thymidine double-labeled cells (shown by white marks in *A*) demonstrating the mitogenic response to EGF in cells that had divided in response to TSH. Also note the morphological response to EGF (*C*) with elongated cells.

dependent proliferation in ^{3}H thymidine-labeled cells. However, the continuous presence of EGF and thus proliferation of cells during this 2-d period is not necessary. This experiment shows that the suppression of the cAMP-dependent mitogenic stimulation is a delayed phenomenon, the completion of which requires 1 or 2 d after its commitment during the execution of cell cycle in the presence of EGF.

By contrast, the DNA synthesis response to EGF remained elevated throughout the experiment (Fig. 8). Noteworthy, its partial desensitization in ^{3}H thymidine cells (which is in accordance with Figs. 4 and 5) also was delayed.

The Suppression of the cAMP-dependent Mitogenesis Is a Trans-dominant Phenomenon in Cell Fusion Experiments

The suppression of the cAMP-dependent mitogenesis could conceivably be caused either by the induction of intracellular factor(s) that inhibit the cAMP-dependent mitogenic pathway (but not the cAMP-independent one), or by the disappearance of factor(s) that would be specifically necessary for

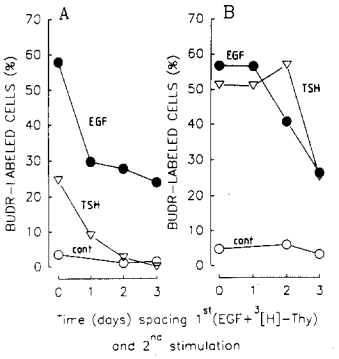

Figure 8. Kinetics of disappearance of the responsiveness to the mitogenic action of TSH after division of thyrocytes in the presence of EGF. After day 1, cells were cultured in the presence of EGF (25 ng/ml) until day 4, with ^{3}H thymidine between day 3 and 4. After day 4, cells were rinsed and placed in control medium for the time (*0, 1, 2,* or *3* d) indicated on the abscissa, before being treated for two additional days, with BUDR for the last 24 h, with control medium (○), EGF (25 ng/ml) (●), or TSH (1 mU/ml) (▽). The fraction of cells incorporating BUDR was determined in the progeny of cells that had incorporated ^{3}H thymidine at day 4 (*A*), and in cells that had not incorporated ^{3}H thymidine at day 4 (*B*).

the cAMP-dependent mitogenic pathway. In the first possibility, it is expected that the inhibition of the cAMP-dependent mitogenesis in cells having replicated DNA in the presence of EGF (thymidine-labeled cells) would be dominant upon fusion with a competent cell for cAMP-dependent mitogenesis (i.e., a cell from the same culture dish that had not incorporated thymidine in response to EGF). In the second hypothesis, the sensitivity to the cAMP-dependent mitogenic stimulation would be restored in the dikaryons resulting from a fusion with a cAMP-dependent growth competent cell. Thyroid cells were stimulated by EGF in the presence of ^{3}H thymidine, and then maintained for 2 d in control medium to allow full expression of the inhibition of the cAMP-dependent mitogenic stimulation. They were detached by trypsin, fused by polyethyleneglycol treatment, and the DNA replication activity (incorporation of BUDR) was studied in dikaryons after stimulation by TSH or EGF. Autoradiography was performed to identify the informative dikaryons, i.e., those with only one thymidine-labeled nucleus.

In almost all dikaryons (99%), both nuclei behaved similarly, i.e., both incorporated BUDR or not. As shown in Table I *A*, homodikaryons containing two thymidine-labeled nuclei or two thymidine-unlabeled nuclei responded as unfused cells labeled or not with thymidine. Some thymidine-unlabeled homodikaryons replicated DNA in response to either TSH or EGF, while thymidine-labeled homodikaryons were insensitive to the mitogenic action of TSH. Heterodikaryons with only one thymidine-labeled nucleus behaved exactly as thymidine-labeled homodikaryons. They still responded to EGF, but the mitogenic response to TSH was abolished. Thus, the suppression of the mitogenic stimulation by cAMP and TSH behaves as a dominant factor. As a control, the same experiment was performed with cells stimulated by TSH in the presence of ^{3}H thymidine (Table I *B*). In that case, a mitogenic response to either EGF or TSH was retained in each sort of dikaryons. Altogether, the results suggest that there is a commitment during cAMP-independent cell cycle progression for the delayed induction of a diffusible factor that specifically represses the cAMP-dependent mitogenic pathway.

Noteworthy, the partial homologous desensitizations (as shown in Figs. 4 and 5) of EGF (Table I *A*) or TSH (Table I *B*) growth responses also were dominant in heterodikaryons with only one thymidine-labeled nucleus.

Table I. DNA Synthesis in Dikaryons of Thyrocytes

	BUDR-labeled dikaryons %		
	○○	●●	○●
A			
Control	4.7 ± 3.3	0.9 ± 0.7	1.7 ± 2.5
EGF (25 ng/ml)	33.1 ± 6.4	15.5 ± 3.0	13.4 ± 6.9
TSH (1 mU/ml)	21.0 ± 5.3	1.3 ± 0.3	1.0 ± 0.8
B			
Control	9.7	2.6	0.0
EGF (25 ng/ml)	32.1	12.4	28.6
TSH (1 mU/ml)	38.7	12.2	16.7

Cells were cultured for 3 d in the presence of EGF (25 ng/ml) (*A*) or TSH (1 mU/ml) (*B*), with ^{3}H thymidine for the last 24 h. After rinsing, the cells were cultured for two additional days in control medium, detached from the dishes, fused with polyethylene glycol, and seeded in control medium. One day after, fused cells were stimulated for 40 h as indicated with BUDR for the last 24 h. For each treatment, BUDR incorporation was analyzed in each sort of dikaryons (with two thymidine-labeled nuclei, ●●; two thymidine-unlabeled nuclei, ○○; or one thymidine-labeled nucleus, ○●) by counting a total of ~300 dikaryons. Values in *A* are the mean ± SD of results from three independent experiments.

Discussion

The present results show that the great majority of dog thyroid cells in primary culture have initially the capacity to proliferate in response to either TSH or EGF. However, the mitogenic response of the whole population is spread over a considerable time. The apparent heterogeneity in the cell sensitivity to mitogens, as it is observed at one time point, is not due to the coexistence of different subpopulations with different hereditary growth propensities. The latter hypothesis was based on the demonstration in histological sections of clustered patterns of heterogeneous cell responses (Studer et al., 1989) and on the situation found in the immortal but unstable FRTL-5 thyroid cell line in which each cell seems endowed with its own growth pattern and degree of TSH dependency which are inherited in the majority of its progeny (Huber et al., 1990). In sharp contrast, using primary cultures of thyroid cells with a limited division potential that could correspond to a finite life span in vivo, we observe here that the progeny of cells that have proliferated at a given period have a rather lower probability to divide at a second period than cells that have remained quiescent during the first period. Thus in the case of stimulation by either TSH (even at low concentrations) or EGF, cells that divide at day 4 may not divide at day 8 and vice versa, which demonstrates that the heterogeneity of growth observed at one given moment is not constant in time. This has been similarly shown for thyroid function in mice, since follicles that are not trapping iodide at a given time have the same iodide content as highly iodide trapping follicles (Mestdagh et al., 1990).

Why some cells do proliferate at the beginning of the culture and others several days later is unclear. As also reported by Derwahl et al. (1990) in FRTL-5 cells, dog thyroid cells that divide at a given time are not randomly scattered in the monolayer. As best observed in the case of stimulations with suboptimal TSH concentrations, proliferating cells are frequently gathered in large clusters. Cell clusters that had proliferated at the beginning of the culture often poorly overlap cell clusters proliferating a few days later. This indicates a local synchrony of proliferating cells, which implies a communication between neighboring cells, through diffusion of

locally produced autocrine factors, or through gap junctions that exist in thyroid cells (Munari-Silem et al., 1990). Therefore, clustered distribution of cells exhibiting similar properties, as observed in histological sections in thyroid gland, may reflect local cell communication (Yap et al., 1987; Munari-Silem et al., 1990). It does not necessarily imply that these cells are members of a same progeny with special inherited properties (as argued by Studer et al., 1989).

Generation of Stable Heterogeneity

The heterogeneity of growth responses described above appears essentially to result from temporary characteristics of individual cells or groups of cells. However, this "dynamic" heterogeneity can produce a qualitative change in the mitogen responsiveness, thus, generating a more stable heterogeneity with two clearly distinct subpopulations. The progeny of the fraction of cells having proliferated in control conditions or during a limited treatment with EGF or serum retain a reduced but marked responsiveness to EGF, but these cells lose the mitogenic sensitivity to TSH and forskolin. The latter phenomenon differs from the inhibitory effects of EGF on functional differentiation, which are reversible and independent of cell cycle progression (Roger et al., 1985; Pohl et al., 1990). As summarized in Table II, it is specific in multiple respects. (*a*) The fraction of cells that have not replicated DNA during the limited EGF mitogenic treatment are not affected in their responsiveness to TSH, which thus generates two subpopulations qualitatively differing in their growth responsiveness to TSH and cAMP. (*b*) Other responses to TSH and cAMP such as cytokeratin redistribution (Fig. 6; Coclet et al., 1991) and thyroglobulin mRNA expression (Pohl et al., 1990) are not affected in cells having proliferated with EGF, which indicates that the TSH/cAMP/protein kinase A cascade is not grossly altered. (*c*) The cAMP-dependent but not the EGF mitogenic stimulation is abolished; and (*d*) the TSH/cAMP mitogenic response is reduced but not abolished in cells that have divided in response to TSH. Further characterization, including analysis of heterodikaryons formed by fusion of thymidine-labeled and unlabeled cells, suggests that the suppression of the cAMP-dependent mitogenic pathway is due to the delayed induction of a dominant inhibitory factor(s). To our knowledge, a similar phenomenon has not yet been described in the growth control of other systems. It has several implications and warrants parallels with other epigenetic processes such as differentiation and senescence.

Implications for the Two-pathways Hypothesis of Growth Control

Our previous studies have indicated that the cAMP-dependent mitogenic pathway (TSH) and the rapidly converging cAMP-independent ones (EGF, phorbol esters, and serum) remain partly separated until late commitment of DNA synthesis (Roger et al., 1986, 1987*a*,*b*; Maenhaut et al., 1991). Differences include divergent patterns of protein phosphorylations and thus utilization of different protein kinases (Contor et al., 1988), partly divergent expression of protooncogenes (Reuse et al., 1990, 1991), and synthesis of different proteins during G1 phase (Lamy et al., 1989). Not only the cell signalling cascade, but even the biochemical nature of the G1 phase might be specific to the mitogenic stimulus (Dumont et al., 1989). The TSH controlled cAMP-mediated growth pathway may appear as an adjunctive differentiated trait. Its unique characteristics might explain how it can be compatible with induction of differentiation expression (Pohl et al., 1990; Reuse et al., 1990; Maenhaut et al., 1991). The present data demonstrate directly that the EGF tyrosine protein kinase and the cAMP mitogenic pathways coexist in parallel in the same cells. The responsiveness of proliferation to either TSH or EGF is spread over the majority of the cell population. Cells that have divided with TSH can subsequently divide with EGF and vice versa (in the EGF then TSH sequence, this is restricted to the lag before suppression of the cAMP-dependent growth pathway).

Table II. Summary of Observations

Mitogenic treatment (days 1–4)	Thymidine incorporation at day 4	Mitogenic response (BUDR) (days 6–8)		Morphology/ differentiation* responses (days 6–8)
		EGF	TSH/forskolin	TSH/forskolin
Control/EGF/ FCS (10%)	Yes	+	−	++
	No	++	++	++
TSH/forskolin	Yes	+	+	++
	No	++	++	++

* Pohl et al. (1990).

The fact that the cAMP-dependent mitogenic pathway was inhibited, but much less the EGF-stimulated one, constitutes an additional difference between the pathways. Also in rat thyroid in vivo, an irreversible desensitization mechanism specifically affects the TSH-dependent proliferation, but not the proliferation induced by tissue wounding (Wynford-Thomas et al., 1983; Smith et al., 1987). The nature and target of the inhibitory factor(s) remain to be determined. Our fusion experiments suggest it should be a diffusible intracellular factor that is stable or continuously produced. It should interact with event(s) that are rate limiting in the cAMP-dependent mitogenic pathway but not in the cAMP-independent one, with no overall alteration of the cAMP/protein kinase A cascade. The concept of such intracellular negative regulators of cell cycle progression has been derived from the analysis of heterokaryons of dividing and nondividing cells (Stein and Yanishevsky, 1981; Adlakha et al., 1983; reviewed by Zelenin and Prudovsky, 1989) and more recently from the identification of tumor suppressor genes (for reviews see Harris, 1990; Levine and Mormand, 1990; Weinberg, 1990). However, the proposed factors have not been shown to specifically inhibit one particular mitogenic pathway.

Another new dimension of the two mitogenic pathways hypothesis is provided by the observation that the suppression of the cAMP-dependent growth pathway is induced during cell cycle performance in response to cAMP-independent factors (EGF, 10% serum or even with 1% serum + insulin [control conditions]), but not during the TSH-stimulated cAMP-dependent cell cycling. This implies that the cAMP-dependent and independent cell cycles are not equivalent in terms of the maintenance of one important feature of the thyroid phenotype.

Parallels with Differentiation and Senescence

The concept of two kinds of cell cycle – either preserving or

changing differentiation phenotypes—is well admitted in various models of cell differentiation (Lajtha, 1979; Yamada, 1989). Moreover, differentiation processes generally include modifications of growth characteristics. As pointed out above, the TSH/cAMP-dependent mitogenic pathway, which is repressed in cell fusion experiments, could be analyzed as a differentiated trait of thyrocytes. The TSH/cAMP growth pathway is specifically lost in somatic hybrids of FRTL-5 thyroid cells and BRL rat liver cells (Veneziani et al., 1990), or in hybrids of FRTL-5 and undifferentiated FRT thyroid cells (Zurzolo et al., 1991). Differentiated functions are also generally suppressed in heterokaryons or somatic hybrids formed by the fusion of a cell expressing this function with a nonexpressing cell (Harris, 1990). In some cases, it has been demonstrated that the extinction is due to a trans-dominant inhibitory gene locus which is normally expressed in the cell that does not express the differentiated function (Killary and Fournier, 1984; Chin and Fournier, 1987; Boshart et al., 1990; Thayer and Weintraub, 1990).

In the present case, the trans-dominant activity that suppresses the cAMP-dependent mitogenic pathway is not initially present in differentiated thyrocytes. Paradoxically, in several respects its appearance also resembles a differentiated process. As adipose conversion of fibroblasts, for instance (Kuri-Harcuch and Marsch-Moreno, 1983), it is a stable but delayed phenomenon, the induction of which requires the performance of one mitotic cell cycle. Its inheritance after further division is unfortunately not testable, since likely it would be restored at each cAMP-independent division.

The suppression of cAMP-dependent mitogenic pathway after division of dog thyrocytes induced by cAMP-independent factors also resembles a senescence process. Cellular senescence involves irreversible changes in proliferation capacity and mitogen responsiveness occurring during successive cell cycles, which lead to a gradual increase in the heterogeneity of individual growth responses (Bell et al., 1978; Smith and Whitney, 1980; Brooks and Riddle, 1988*b*), until complete cessation of proliferation. The finite life span phenotype is also dominant and senescent human fibroblasts express one or more dominant inhibitors of proliferation (Stein and Yanishevsky, 1981; Pereira-Smith and Smith, 1988). The quite abrupt extinction of the cAMP-dependent proliferation might thus be considered as some kind of conditional (heterologous) pathway-specific senescence. It should be distinguished from the more progressive and relative reduction of mitogen responsiveness observed in dog thyrocytes continuously stimulated with TSH or EGF, which is also dominant in cell fusion experiments, but probably reflects the very limited division potential of these cells (Fig. 1; Coclet et al., 1989).

Implications for Thyroid Goitrogenesis

The goiter, which results from thyroid hyperplasia, is diffuse in a first stage, but becomes heterogeneous, then multinodular later. Although its initial goitrogenic cause may be unique (congenital defect, goitrogen, iodide lack), it is characterized by a very important regional heterogeneity of function and growth of thyroid epithelial cells. The cause of this heterogeneity is much debated (Peter et al., 1985; Studer et al., 1989; Many et al., 1986; Denef et al., 1989). It might depend on environmental factors, or on intrinsic properties of follicular thyroid cells. The nodules in a multinodular goiter are polyclonal (Namba et al., 1990) unlike the solitary nodules which represent true adenomas (Thomas et al., 1989; Namba et al., 1990). Therefore, they do not result as suggested (Peter et al., 1985; Studer et al., 1989) from an amplification during growth of genetically different subpopulations. Here, we have shown that heterogeneity may arise from the competition between two different modes of cell cycle progression, either preserving or transforming growth responses to external endocrine (e.g., TSH) or paracrine (e.g., EGF) factors. Since these responses seem to occur in clusters of communicating cells, this could explain the regional, patchy pattern of the resulting stable heterogeneity, as it is observed in multinodular goiter.

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