Thyroid cell culture models and the dog thyroid primary culture system

Provided adequate culture conditions are found, specialized cells may keep in vitro some differentiated functions they are known to exert in the whole organism. These functions are responsive in vitro to purified hormones, neurotransmitters or growth factors normally secreted by other cell types. These factors are, therefore, assumed, but frequently not proved, to produce analogous effects in vivo, thus participating in the homeostasis of the whole organism. In this way, much of the modern understanding of the regulation of a defined cell within the organism relies on a mental reconstitution, which combines bits of experimental information obtained from various artificial culture systems, within a framework of 'rules' dictated by well admitted clinical observations and decades of in vivo animal experimentation. In vitro experimental observations that fit these rules are, therefore, called 'mechanisms': the others are disregarded as 'culture artefacts'. This view is of course too simplistic. Transgenic and knockout animals remind us that in vitro and in vivo mechanisms may differ. On the other hand, previously unsuspected functions or regulations demonstrated in culture systems may eventually prove to have important physiological or pathological relevance. However, the correlation between in vivo and in vitro observations requires an in depth knowledge and understanding of the in vitro systems.

For the study of thyroid cell differentiated functions and proliferation, various culture systems have been developed, using either primary cultures or immortal cell lines of different species. Both have advantages and disadvantages. The rat FRTL5 cell line is by far the most frequently used system. It retains most of the features of differentiated follicular thyroid cells, such as thyroid-stimulating hormone (TSH)-dependence of growth (but also survival), iodide uptake, thyroglobulin (Tg) and thyroperoxidase (TPO) gene expression. However, the immortality of this cell line is sufficient evidence that it has lost some of the basic mechanisms of cell cycle control. In nude mice, FRTL5 cells develop TSH-dependent tumors (1). Moreover this system suffers from instability (2) and clonal variability (3, 4) which explains the opposite results sometimes obtained in different laboratories. The FRTL5 cell line has lost the epidermal growth factor (EGF) control of growth (5), and β-adrenergic cAMP regulation (6), which is re-acquired in one of its variants (4). Because of its simplicity and accessibility, because it allows permanent transfections and genetic experiments and also because of the increasing difficulty in obtaining animals for experimentation, FRTL5 is now the preferred and often only used system in the majority of in vitro studies of thyroid biology (over 600 studies, mainly from the United States, Italy and Japan).

Perhaps because of a personal bias, we consider that cells that are not selected by long-term in vitro propagation are less remote from physiology. We have thus always been surprised to observe that the relevance for the thyroid gland of observations from thyrocyte primary cultures is often definitively accepted only once they have been confirmed from FRTL5 cells, rather than conversely (we are all guilty of regarding what exists in our own model system as the only truth!). The confrontation of the different thyroid primary culture systems has pointed out the importance of possible species differences, but also the influence of culture conditions, and the fact that cells have a 'memory', which means that their characteristics are not fully stabilized and may evolve depending on their previous in vivo and in vitro story. In the last decade, the amount of information collected from the dog thyroid primary culture system has justified it emerging as the 'challenger' of the FRTL5 cell line as an in vitro model of thyroid cell biology. The aim of this review is to summarize the characteristics of this system and its contribution to the regulation of thyroid function, cell proliferation and differentiation. Whereas differences between systems are fully recognized (7), this review is not aimed at drawing up their catalog. We hope that the information on the properties of dog thyrocytes in culture assembled here may help other thyroidologists...
Dog thyroid glands were first studied as short-term in vitro incubations of tissue slices in our laboratory because after extensive investigation they had been shown to be the only large thyroids responding reproducibly and markedly to TSH with regard to cAMP accumulation and functional effects (8). Inspired by Kerkof and Fayet’s methodologies developed for sheep and pig thyroid cells, Winand and Kohn (9) and Rapoport (10) established dog thyroid primary cultures for the first time two decades ago using conventional serum-supplemented media. These cells were proved to retain an excellent responsiveness to TSH (adenyl cyclase activation and cAMP production (10), typical morphological responses (10)) and a low but significant iodide uptake capacity when cultured in the presence of TSH (11). In addition they were shown to grow for a limited number of culture passages (10). First aiming to analyze the regulation of cell multiplication by TSH, we slightly adapted the Rapoport method of dog thyrocyte preparation (12) which has the advantage of allowing a purification of follicular cells (initially about 70% of cells in the gland). The originality of this method is the use of collagenase instead of trypsin, which releases fragmented and intact thyroid follicles with a high yield rather than isolated cells. Follicles are separated from isolated cells and cell debris by several low speed centrifugations, and seeded in polystyrene tissue culture Petri dishes (10, 12). Follicular cell aggregates rapidly attach and cells migrate from the follicle remnants and develop as colonies of 30–200 monolayer cells with an epitheloid cuboidal appearance. During this process, which takes about 2 days and is independent of cell multiplication, cells remain closely associated by tight junctions. The cell spreading process is associated with the development of an abundant network of actin microfilament stress fibers, and the appearance of high molecular weight tropomyosin isoforms (Tm 1, 2, 3) which have a higher affinity for binding to actin (13).

We first used a high serum culture medium (12, 14), but soon developed a serum-free culture medium (15, 16) based on a mixture of DMEM+F12+MCDB104 (2:1:1) (+ascorbic acid and antibiotics), which quantitatively differs from the Coon’s modification of F12 medium used for the FRTL5 cell line. This medium has also been recognized by others as the best basis to support the growth of human thyroid cells (17). This basal medium supports the attachment, spreading and survival of dog thyrocytes for at least 10 days and superior TSH responsiveness (differentiated functions) than in serum-supplemented media. In some experiments, this basal medium was supplemented with only 1% fetal calf serum. This allows a better maintenance of the cell responsiveness and survival after the first week of culture and increases the proliferation rates (18). With a supplement of 5 μg/ml insulin in the absence of serum (our usual control medium), cells are quiescent but DNA synthesis and cell division can be induced in all the cell population by an adequate combination of mitogens (15, 16). This constituted the first demonstration that all the initially differentiated cells from thyroid follicles (rather than a small population of stem cells) have conserved the capacity to proliferate (15). However, even in response to maximal growth stimulation, the proliferation capacity is limited to four to seven population doublings in different experiments (both in the presence of serum or purified hormones and growth factors) independently of cell density (19). All the attempts to derive continuous cell lines from dog thyroid primary cultures have failed (P P Roger and M Baptist; G Fayet, unpublished observations). Very few cells have the capacity to grow beyond this limit, but finally stop proliferating. This limitation could result from a deficiency of the culture medium, but most likely corresponds to an intrinsically limited division capacity (life span) (7). Once this limit has been reached, or if the cells are deprived of mitogenic factors, the proliferation abruptly stops, but cells can survive for at least 2 months, with regular renewals of the culture medium. A residual DNA synthetic and mitotic activity is compensated for by a low rate of apoptotic cell death (20).

In the absence of serum or with 1% serum, the cultures are almost exclusively constituted of epithelial cells characterized by cytokeratin intermediate filaments network (cytokeratin 8, 18 and possibly 19) (21) and junctional proteins such as E-cadherin (22). Electron microscopy showed that the monolayer cells are polarized (P Ketelbant-Balasse, P Servais and P P Roger, unpublished observations). The apical membrane, facing the culture medium, is decorated by microvilli. Cells are associated at the apical pole by tight junctions. The basal membrane is underlined by an extracellular matrix secreted by the cells, but which remains to be characterized. At cell confluence density, the monolayer presents a high electric resistivity (E Raspé, unpublished observations), which demonstrates its tightness. It also presents some signs of functional polarity, such as, after a continuous TSH treatment in the presence of 1% serum, the development of domes (blisters), which denotes a transepithelial transport of water and electrolytes (18). Nevertheless, at variance with pig thyrocyte monolayers, which present a strict functional polarity with most receptors and iodide uptake at the baso-lateral pole and Tg secretion and iodide efflux at the apical pole, even in very dense dog thyrocyte monolayers these functions seem to be largely redistributed on both cell sides.
Though this represents an obvious difference with the situation in the thyroid follicle in situ, this apparent redistribution has the practical advantage that it allows an unrestricted access of iodide, hormones, growth factors and neurotransmitters to their transporters and receptors, which in pig thyrocyte monolayers are only fully accessible in bicameral culture devices.

Appropriately stimulated (TSH) dog thyrocyte monolayers have been demonstrated to perform all the thyroid specific functions required for the synthesis and secretion of thyroid hormones, including Tg (23) and TPO (24, 25) gene expression, iodide uptake (11, 14, 18), H$_2$O$_2$ generation (26), iodide efflux (27) and macropinocytosis (28). However, they are not expected to produce high amounts of thyroid hormones, since like FRTL5 they poorly reform follicle lumina, and thus release Tg. H$_2$O$_2$ and iodide in the culture medium where their dilution prevents an efficient iodination of Tg. Indeed the protein iodination capacity, which is high in freshly isolated follicles (14, 29), disappears during the cell monolayer formation (14), and is only restimulated by TSH in highly confluent monolayers (18) where some intracellular or oligocellular microfollicular lumina are reformed. By autoradiography, those microfollicles were seen to concentrate fixable (i.e. protein bound) radioiodine, but these structures concern at best 10% of cells (J Coclet and P Nève, unpublished observations).

**Factors acting on cultured dog thyrocytes and the signaling cascades they control**

Many of the factors that modulate dog thyroid function, and thyroid hormone secretion have been characterized or demonstrated thanks to a system of short-term incubations of thyroid tissue slices (8, 30–32). At least qualitatively, most of the results on thyroid signaling obtained with this system have been confirmed and extended from dog thyroid primary cultures. The adenyl cyclase and cAMP production are stimulated by TSH (10, 33, 34), other TSH receptor agonists (thyroid stimulating immunoglobulins (TSI) (35) and prostaglandin E (PGE). After TSH stimulation, the cAMP levels rise within a few minutes, reach a maximum after 30 min and then the TSH response is subjected to a partial desensitization (by 4 to 8 h), the mechanism of which is not elucidated (36), but might depend on protein neosynthesis (37, 38). One day after TSH stimulation, high cAMP levels are restored, which denotes an escape from this refractoriness, possibly as a consequence of the stimulation by TSH of TSH receptor expression (39, 40). By contrast, the cAMP response to PGE is rapidly and totally desensitized after 1 h. As in dog thyroid slices, the TSH response is inhibited in part by norepinephrine (29) and by iodide (to be observed in culture after more than 1 day, this last effect requires an exogenous enzymatic H$_2$O$_2$-generating system (41)). cAMP is expected to activate cAMP-dependent protein kinases (PKA) (I and II; both are equally present in dog thyrocytes before stimulation (42)). The released catalytic C subunit of PKAs migrates to the nucleus (43) where it phosphorolysates transcription factors including cAMP-response element binding protein (CREB) (S Dremier, unpublished observations) and presumably CAMP-response element modulators. Probably because C and the RI regulatory subunit, more than RII, are rapidly degraded once they are freed by PKA activation, cells chronically stimulated by TSH or other adenylyl cyclase activators such as forskolin lose type I PKA isozyme (42, 44).

Several hormones and neurotransmitters activate the signaling pathways triggered by phospholipase (PL) C $\beta$ activation (i.e. inositol triphosphate (IP$_3$)/Ca$^{2+}$ and diacylglycerol/protein kinase C (PKC)) in dog thyrocyte cultures, including carbachol (Cch) through muscarinic receptors, ATP, PGE2α, thyrotropin-releasing hormone (TRH) and bradykinin (BK) (26, 45–48). Cch is by far the most potent activator. It increases the generation of inositol phosphates and mobilizes Ca$^{2+}$ from intracellular stores within seconds (26, 46, 47). Afterwards the concentration of Ca$^{2+}$ declines to a plateau higher than the basal level. This plateau level is dependent on extracellular Ca$^{2+}$ and on the continuous activation of muscarinic receptors (26, 46, 47). The responses to the other factors are more rapidly desensitized (26). TSH has been repeatedly reported (47, 49) as increasing IP$_3$ generation and intracellular Ca$^{2+}$ concentration in dog thyrocytes. However, these effects should be appreciated cautiously, as they are very weak, obtained with huge concentrations of TSH (50), and could reflect an increased labeling of IP$_3$ precursors (49) but not a real increase of the IP$_3$ concentration (51). Phorbol ester (TPA) tumor promoters have been used as PKC activators (29, 52) (in dog thyroid, only alpha and zeta kinase C isozymes are found (53)). They mimic the effects of Cch (E Raspé and F Lamy, unpublished observations) and a membrane permeant diacylglycerol on the phosphorylation of various proteins separated by two-dimensional gel electrophoresis (54).

Dog thyrocytes in primary culture are responsive to several hormones and local growth factors acting through tyrosine kinase membrane receptors, including EGF (18, 55), hepatocyte growth factor (HGF) (56), fibroblast growth factor (FGF) (18), insulin, insulin-like growth factor-I (IGF-I) and IGF-II (15, 16, 57) (but not to other factors such as platelet-derived growth factor, keratinocyte growth factor, bombesin). The analysis of the phosphorylation of intact cell proteins separated by two-dimensional gel electrophoresis, has given a high-resolution picture of the activation state of protein kinase-dependent regulatory networks (54). EGF rapidly induces the phosphorylation of five proteins, including the phosphorylation on serine of the 28 kDa heat shock proteins and the activatory phosphorylations of the two related 42 kDa and 44 kDa MAP kinases on tyrosine and threonine residues (54, 58). HGF also
activates MAP kinases (56) and like EGF, it induces the nuclear translocation of MAP kinases (58, 59). Cch and TPA via PKC activation also cause MAP kinase phosphorylation and nuclear translocation, which thus represents an important early convergence point of tyrosine kinase and PKC signaling cascades (54, 58, 59). In addition they induce the phosphorylation/dephosphorylation of another set of proteins that could be related to the acute functional and morphological effects specific to PKC activation (54). By contrast, TSH via cAMP induces the phosphorylation of a completely different set of proteins that includes neither the MAP kinases, the 28 kDa heat shock proteins, nor any phosphorylation on tyrosine residues (54, 58).

The related insulin and IGF-I receptors have been characterized in dog thyrocytes (57). They also activate a kinase cascade culminating in MAP kinase phosphorylation and nuclear translocation (through receptor β subunit autophosphorylation, and insulin receptor substrate-like protein tyrosine phosphorylation) (57). Importantly, whereas IGF-I receptors appear to be constitutively expressed, the presence of insulin receptors is strikingly dependent on TSH (cAMP) (57). By inducing insulin receptors, TSH thus exerts a delayed (16 h) facilitative action on the signaling cascade triggered by insulin (and by IGF-II and even IGF-I which bind weakly to insulin receptors) (57).

EGF and more markedly TPA (36, 44) and Cch (60) partly inhibit the cAMP (adenyl cyclase) response to TSH. The long-lasting feature of this effect is probably mediated by the inhibition of TSH receptor expression (39, 40). In addition, Cch exerts a rapid inhibition on TSH-dependent cAMP levels (60). This is due to a stimulation of phosphodiesterase through a Ca²⁺-dependent mechanism, which may be partially complemented by a PKC-dependent inhibition of adenylyl cyclase (31, 60).

Transforming growth factor β1 (TGFβ1) represents another class of factors which act in dog thyrocytes (61). Its receptor harbors a serine/threonine kinase activity but the signaling cascade it controls is still poorly characterized.

Iodide has long been known to have various regulatory effects on thyrocytes. These effects are at least in part mediated by 2-iodohexadecanoyl (IHDA), a compound which is produced in dog thyrocytes incubated with iodide (in culture, this generation requires an exogenous H₂O₂ generating system; see above) (62) and mimics the inhibitory effects of iodide on adenylyl cyclase (63) and cell function (64).

Regulation of dog thyrocyte function

The regulation of thyroid function, i.e. of events directly involved in the synthesis of thyroid hormones (iodination of Tg) and their secretion (cell phagocytic activity) is regulated at two major levels. Some stimulations are acute, bear on enzyme activity and are independent of protein synthesis, whereas others involve the stimulation of the expression of thyroid specific genes encoding key proteins of thyroid hormone synthesis. This enhances the functional responsiveness of cells to acute stimuli, and in fact reflects the expression of thyroid differentiation.

Acute functional regulation

These effects are better studied in dog thyroid slices which retain the in vivo structure and closely reproduce the in vivo situation (8, 32). As explained above, the protein iodinating capacity is low in dog thyrocyte primary cultures compared with the levels reached in dog thyroid tissue slices or freshly isolated follicles (14). In the different experimental systems, it is stimulated by TSH through cAMP (18, 29). This function is also stimulated by Cch and TPA in tissue slices and follicles, but acutely inhibited by these factors in primary cultures (29). This last phenomenon should be interpreted as a culture artefact. Indeed, because of the monolayer organization of these cultures, the iodination of proteins is exceedingly dependent on the iodide transport capacity of the cells, which is acutely inhibited by Cch and TPA (29). It has been shown in dog thyroid slices that in the presence of high iodide concentrations the rate limiting substrate of Tg iodination is H₂O₂ (32). Protein iodination independently of iodide transport is thus regulated by H₂O₂ generation. In primary cultures as in tissue slices (32), H₂O₂ is generated in response to the acute stimulation by both the cAMP signaling cascade elicited by TSH, and the PLC/Ca²⁺ cascade triggered by Cch, PGF2α, BK, ATP, TRH (26, 65). The Cch effect is mimicked by both Ca²⁺ ionophores and TPA, indicating that the diacylglycerol/PKC and IP₃/Ca²⁺ branches of the PLC/Ca²⁺ cascade are involved (26). It is inhibited by IHDA as a possible mediator of the Wolf–Chaikoff inhibitory effect of iodide (64). The oxidation of glucose is stimulated by TSH (cAMP), Cch or TPA (66) in parallel with H₂O₂ generation as a consequence of NADPH₂ oxidation by the H₂O₂ generating and reducing systems (32). The transport of glucose is also stimulated by these agents (66) in order to sustain the availability of the required glucose.

The efflux of I⁻ from dog thyrocytes is better studied in cultures than in slices where it is masked by the trapping mechanism. It is activated by the same panel of agents activating either the PLC/Ca²⁺ or the cAMP cascades (27). It involves an I⁻ transport system with pharmacological and regulatory properties different from those of the baso-lateral Na⁺/I⁻ symporter, which is likely the apical I⁻ channel whose activity would be required for I⁻ availability in the follicular lumina and Tg iodination (27, 67).

TSH through cAMP, but not TPA, rapidly induces the phagocytic activity of dog thyrocytes in primary culture (28). This process, involving the formation of apical pseudopodes, is probably the in vitro reflection of the
macropinocytosis of the luminal iodinated Tg, which is the first step in stimulated thyroid hormone secretion (8).

Therefore, despite the loss of the follicular architecture, which impedes an efficient hormonogenesis, the key mechanisms involved in this process and their regulation are remarkably well preserved in dog thyrocyte primary cultures.

**Regulation of differentiation expression** In dog thyrocytes cultured in the absence of TSH, the expression of several differentiated functions progressively declines to a low but significant basal level. This applies to iodide transport capacity (Na⁺/I⁻ symporter) (11, 14, 18), synthesis (23), mRNA accumulation (23, 39, 68) and gene transcription (69) of Tg, activity (70), mRNA accumulation (24, 39, 71) and gene transcription (25, 69) of TPO, and the capacity of cells to generate H₂O₂ (65) and release iodide (27) in response to an acute stimulation by Cch. All these functions are maintained at a high level in the presence of TSH or any factors increasing cAMP. They are inhibited, both in the presence or absence of TSH or cAMP analogs, by EGF and TPA (18, 23, 27, 39, 52, 55, 65, 68, 71). The inhibitory effect of EGF on the expression of these differentiation markers is independent of its mitogenic activity (see below). It is specific since EGF does not inhibit the TSH effects on the synthesis of a variety of proteins separated by two-dimensional gel electrophoresis (72). The proliferation of dog thyrocytes in the presence of EGF or TPA seems to abolish the expression of differentiation (18, 23, 52). However, EGF-treated cells remain committed to re-express the thyroid differentiation, i.e. in the terminology of embryologists they remain determined. Indeed, after elimination of EGF and addition of TSH, high iodide trapping capacity and Tg and TPO mRNA are restored (18, 23, 68). In the other thyroid primary culture systems this redifferentiation activity of TSH has not been demonstrated and the maintenance of differentiated functions requires the continuous presence of TSH. Other mitogenic factors including FGF (18, 23), HGF (56) and serum more weakly affect the differentiation expression, and inhibit the iodide transport capacity mainly during the proliferation phase of the culture (14, 18, 56). At variance with other thyroid culture systems, TGFβ does not inhibit the TSH-stimulated iodide transport of dog thyrocytes (61).

The expression of the various differentiation markers, though apparently closely coordinated, is subjected to partly separated regulations (see also later section on transcriptional regulation of thyroid-specific genes). The basal expression of Tg gene (but not its stimulation by TSH) is strictly dependent on the presence of insulin in the culture medium (68, 69), but insulin does not affect TPO gene expression (69, 71) and iodide transport (72). Hydrocortisone potentiates the TSH-stimulation of iodide transport capacity (14) but does not modify Tg mRNA accumulation (the opposite is found in calf thyroid primary cultures (73)). The iodide transport capacity responds to lower TSH concentrations than the other functions (18) and may thus be more sensitive to physiological TSH concentrations in vivo. TPO mRNA accumulation differs from Tg mRNA and iodide transport by the rapidity of its control by cAMP (see section on transcriptional regulation of thyroid-specific genes), and its rapid disappearance upon cessation of stimulation (71). The regulation of TPO synthesis rather than its activity may thus play a key role in the acute control of thyroid hormone synthesis by TSH.

TSH receptor mRNA expression also constitutes a differentiation marker of thyroid cells. TSH through cAMP transiently up-regulates and chronically down-regulates to a small extent TSH receptor mRNA (39). It is independent of insulin, but inhibited by EGF and TPA (39, 40). However, in all the circumstances the TSH receptor remains expressed to allow further TSH responses (39, 40), which explains that such cells are still able to respond to TSH and re-express differentiation.

The synthesis of another protein (23 kDa) is stimulated by TSH, but inhibited by EGF and TPA in dog thyroid primary cultures (72), and thus may appear as a possible differentiation marker, though it is not specifically expressed in thyroid cells. Interestingly, this protein had previously been shown to be acutely phosphorylated in response to TSH and cAMP enhancers, which suggests a role in functional activation. The molecular cloning of its cDNA has revealed a striking analogy with calcymophosine. This protein has been shown to bind Ca²⁺ (74) and therefore has been called calcymophosine.

**Regulation of dog thyrocyte proliferation**

A stimulation of cell proliferation by a huge concentration (100 mU/ml) of a crude TSH preparation was reported from dog thyroid primary cultures by Winand and Kohn (9) in 1975. However, this effect was not mimicked by dibutyryl cAMP, and is thus most likely explained by the contamination of the TSH preparation by pituitary growth factors. We have shown that pure preparations of TSH enhance the proliferation of dog thyroid cells in primary culture in the presence of serum (12, 18), and trigger DNA synthesis (16, 66), cell cycle progression (75) and cell proliferation, when administered to quiescent cells in serum-free medium. We have demonstrated these proliferation effects of TSH using various methodologies including cell counting (12), DNA measurement (12, 18), incorporation of [³H]thymidine (16), counting of thyrmidine (16) or bromodeoxyuridine-labeled nuclei, and cell cycle analysis using the proliferating cell nuclear antigen (PCNA) as a marker (75). The stimulation is detectable with 0.03 mU TSH/ml, half-maximum with 0.05–0.1 mU/ml and maximum at 0.5–1 mU/ml (16, 18). Unlike what has been claimed on several occasions from the
FRTL5 system, the induction of dog thyrocyte DNA synthesis by TSH is independent of the PLC/Ca^{2+} cascade (50), or of the phospholipase A2/arachidonic cascade and cyclooxygenase metabolites since it is not affected by indomethacin (76). Moreover it is not affected by pertussis toxin, and is thus not mediated by Gi proteins that are also coupled to TSH receptors in dog thyrocytes (77). The stimulation of DNA synthesis and proliferation by TSH is fully mediated by the increase of cellular cAMP concentrations (12, 16, 18, 78), since it is totally mimicked by adenyl cyclase activators such as cholera toxin and forskolin, by dibutyryl cAMP and other cAMP analogs, and by the microinjection of the mRNA of receptors constitutively activating adenyl cyclase (such as the A2a adenosine receptor (79) or TSH receptors activated by mutation (43)). DNA synthesis, as well as function and differentiation expression, present the same synergistic dependence on pairs of specific cAMP analogs as does the activation of PKA (78). This suggested that PKA indeed mediates the cAMP action on these processes. Nevertheless, in contrast to stimulation of function and differentiation expression, DNA replication seems more sensitive to PKAI activation (78), and the specific desensitization of the cAMP-dependent growth response is accompanied by the disappearance of PKAI but not PKAII after several days of culture with TSH or forskolin (42). On the other hand, whereas the microinjection of the heat stable PKA protein inhibitor partly inhibited the cAMP dependent DNA synthesis (43), all the attempts to induce DNA synthesis by microinjecting the active catalytic subunit of PKA have failed (43). The possibility thus remains that the action of the catalytic PKA subunit is not sufficient for mitogenesis and that additional effects of a free PKA regulatory subunit or even other cAMP binding proteins may be required.

Independently of cAMP and PKA, several factors have been found to induce the proliferation and DNA synthesis of dog thyrocytes. They include serum (15, 18), purified growth factors including EGF (15, 18, 55), FGF (18), the very potent HGF (56), and tumor promoting TPA (52). However, physiological activators of the PLC/Ca^{2+} cascade (Cch, BK, ATP) have no or only a very marginal mitogenic activity (50). High insulin concentrations, which activate both insulin and IGF-I receptors, and are present in our usual control culture conditions, have only a very weak mitogenic effect per se. Insulin (>10^{-7} mol/l), IGF-I and more weakly IGF-II are required for, or markedly potentiate the mitogenic effects exerted either by TSH through cAMP, or by the cAMP-independent factors including EGF and TPA (15, 16, 57). The action of HGF is partly dependent on insulin/IGF in some experiments, but not in others. In some experiments, TSH also weakly stimulates DNA synthesis in the absence of insulin or exogenous IGFs (15, 57). This effect partly depends on an autocrine production of IGF-I or -II, since it is inhibited by antibodies that neutralize IGFs or IGF-I receptors (57). Noteworthy, whereas the stimulation of DNA synthesis by cAMP-independent mitogens absolutely requires high supraphysiological concentrations of insulin, 100- to 1000-fold lower concentrations of insulin are sufficient to support some stimulation of DNA synthesis by TSH (15, 57). This permissive effect of physiological concentrations of insulin in the presence of TSH is mediated by insulin receptors (since it is blocked by the MA-10 insulin receptor blocking antibody) and is completely independent of IGF-I receptors (57). This action is allowed by the delayed induction by TSH via cAMP of the expression of insulin receptor gene (57). Especially in the presence of low insulin concentrations, TSH via cAMP may thus very potently promote dog thyrocyte proliferation by directly controlling cell cycle progression through a MAP kinase independent mechanism (see later section on dog thyroid cell cycle biochemistry), and by indirectly stimulating (with a delay) the MAP kinase-dependent insulin permissive pathway, at least in part by inducing insulin receptors (57). Both mechanisms obviously cooperate.

For a cell to divide, or at least for a sustained multiplication, it must double its mass as well as its DNA content before each division. Strikingly, while potently stimulating DNA synthesis in the presence of insulin, TSH unlike cAMP-independent mitogens, does not increase the overall protein synthesis (80) and protein accumulation (81, 82). Insulin and IGF-I, on the contrary, while being not sufficient mitogens, stimulate protein accumulation leading to the hypertrophy of cells (81, 82). This has led to the proposal that this insulin-independent increase of cell mass could be a prerequisite for the mitogenic effect of TSH (81). Nevertheless, the hypertrophic effects of insulin and IGF-I are not sufficient to explain their permissive effects on DNA synthesis.

In the presence of insulin, EGF synergizes with cAMP enhancers to induce DNA synthesis (15) (in some experiments, this is obscured because the TSH stimulation is close to the maximum DNA synthesis rate), but its effects are not additive to those of TPA (52). This was a first indication that EGF and TPA act through a common cascade that is different from the cAMP pathway. Mitogenic effects of TSH and TPA are less than additive (52), probably because TPA unlike EGF inhibits the effects of TSH and cAMP on mitogenesis (44). In dog thyrocytes, TGFβ potently inhibits DNA synthesis induced by TSH, adenyl cyclase activators and cAMP analogs, but poorly affects the mitogenic response to serum, EGF, TPA or HGF (61). This also differentiates the mitogenic stimulations by cAMP or growth factors.

**Relationship between differentiation expression and cell proliferation** According to an old but still current view, growth and differentiation are often considered mutually exclusive processes in cell life. The apparent paradox between this antagonism of
growth and differentiation and the stimulation of both processes by cAMP has been addressed in dog thyrocyte primary cultures. While EGF and TPA stimulate proliferation and inhibit differentiation, studies of the induction of Tg and TPO mRNA accumulations by in situ hybridization demonstrated that they are fully compatible with cell cycle progression when they are promoted by cAMP (68, 71). In fact, both the differentiation induced by TSH and the dedifferentiation induced by EGF and TPA are independent of the effects of these agents on proliferation (68, 71). Suppression of differentiation expression in thyrocytes is not a consequence of cell cycling itself, but could be related to the activation of some mitogenic signaling cascades (68, 91) (see later section on regulation of early immediate genes).

**Morphological and cytoskeletal responses of dog thyrocytes**

Cell morphology as well as the composition and the organization of the cytoskeleton are considered to have major influences on cell growth, differentiation and gene expression. Using human thyrocytes, we have ourselves suggested that many effects of TSH on the synthesis of proteins are mediated by the morphological changes this hormone induces (83). Like the regulation of function, the control of cell morphology and the underlining cytoskeletal changes are subjected to both acute and long-term modulations involving (de)phosphorylations of cytoskeleton proteins and changes in the synthesis of cytoskeleton components. When cells are well spread in monolayer, the addition of TSH produces a dramatic morphological change, first detected after 15 min of stimulation, which involves the thickening and retraction of the cell border, then the formation of a network of dendrite-like cytoplasmic projections resulting from both cell retraction and active cytoplasmic extension (this process is often called ‘cell arborization’) and an increase in the number of surface microvilli (13, 18, 34, 84). This process is associated with the stimulation of cell phagocytic activity (28), and depends on a rapid reorganisation of actin and myosin microfilament stress fibers in a thin ‘microtrabecular’ network (13, 28, 34, 85) (Fig. 1). This process is mediated by cAMP (18) and mimicked by the microinjection of the catalytic subunit of PKA (86). PKC activators including TPA and Cch, but not EGF, induce another dramatic morphological modification, which also depends on actin stress fiber disorganisation, but involves a redistribution of actin in very prominent lamellar membrane structures accompanied by blebbing and ruffling of cell periphery membranes (13, 28) (Fig. 1). This process precedes a distortion of cell shape, partial disruption of cell contacts and an increased cell motility (13, 52). TPA unlike TSH stimulates the phosphorylation of cytokeratins (87), and disrupts the cytokeratin tonofilaments associated with the nucleus, cytoplasm and desmosomes (87).
The disorganization of actin/myosin microfilaments in response to both TSH and TPA possibly results from the observed dephosphorylation of myosin light chains (28, 54, 88), likely because the myosin light chain kinase is inhibited by its phosphorylation by PKC and PKA. In addition, TSH, more rapidly than TPA, causes the dephosphorylation of two related actin-binding proteins, destrin and cofilin (85). In the case of TSH, we have postulated that this event may be associated with the stimulated macropinocytic activity (85). In response to TSH and TPA but not EGF, the synthesis of actin (89, 90) and high molecular weight tropomyosin isoforms is inhibited (13). This may result from and prolong the effect of the acute disruption of actin bundles. Noteworthy, these high molecular weight tropomyosin isoforms have since been ascribed a role of tumor and growth suppressor.

Dog thyrocytes chronically treated with TSH display a characteristic cuboidal epitheloid morphology (18) (Fig. 2) associated with a redistribution of the cytokeratin network and a marked cytokeratin and actin immunoreactivity at the cell junctions (21). TSH chronically repress the synthesis of vimentin (the ‘mesenchymal’ intermediate filament protein often considered as associated with dedifferentiation of cultured epithelial cells, but found in 40% of thyrocytes in freshly isolated follicles) (21), and stimulates the synthesis of the junctional protein E-cadherin (22). These changes may be related to the differentiation state of the cells and suggest that TSH induces the formation of junctional complexes, which remain to be characterized but might increase the tightness of the epithelium and affect the intercellular communication between adjacent cells. By contrast, chronic cell treatment with EGF or TPA (but not FGF) inhibits the long-term morphological effects of TSH and cAMP, and induces the cells to adopt a fusiform, fibroblast-like pattern (18, 21, 52) (Fig. 2). This suggested that these agents induce profound changes in the differentiation program. The opposite effects of TSH or EGF on differentiation expression and morphology are likely to be interdependent, at least in part. The EGF effect is associated with a generalization of the presence of vimentin (21), but does not involve an inhibition of cytokeratin (21) or E-cadherin synthesis (22). It is reversible, and EGF-treated cells thereafter cultured with TSH regain an epitheloid morphology (18, 23) and differentiation in spite of the persistency of a complete network of vimentin (21).

Intercellular heterogeneity of morphology, differentiation and proliferation responses

In biochemical studies, cultured cells are generally investigated as a whole presumably homogeneous population. However, qualitative or quantitative intercellular heterogeneities have been found at each level of our ‘in situ’ investigations of morphological, differentiation
and proliferative responses of unselected dog thyrocytes in primary culture (92). Examples of such heterogeneities include the presence or absence of a vimentin intermediate filament network in adjacent cells (21), the fact that quantitative in situ hybridization demonstrated that the induction of Tg mRNA, which was observed in more than 90% of TSH or forskolin treated cells, is very heterogeneous (68) (positive cells range from 10 to 180 autoradiographic silver grains; after EGF treatment, Tg mRNA becomes detectable 16 h after TSH addition in some cells, but the response of other cells requires a very long (48 h) lag), and the fact that at a given moment only part of a stimulated cell population is progressing through the cell cycle (15, 19). These different heterogeneities are not obviously related at the individual cell level and should depend on at least partly distinct mechanisms (92), which is not in favor of the claimed (93) coexistence in thyroid of distinct subpopulations of cells stably expressing special properties.

The heterogeneity of proliferation responses was peculiarly intriguing. When initially quiescent thyrocytes are stimulated by TSH (cAMP), TPA, EGF, or serum, the limited increase in the thymidine-labeling index after 48 h suggested that the proliferative fraction could be markedly inferior to 1 (15). Nevertheless, the great majority of the thyrocyte population is capable of progressing through the cell cycle when stimulated by a combination of TSH, EGF and serum (15), or if the stimulation by EGF or TSH is prolonged for several days (19). Using a double-labeling methodology (autoradiography of [3H]thymidine and immunofluorescent detection of bromodeoxyuridine) that allows us to trace the proliferative behavior of cells at different culture times, we observed that in the cases of stimulation by either TSH or EGF, cells that divide at day 4 may not divide at day 8, and vice versa, which demonstrated that the heterogeneity observed at one moment does not reflect a permanent situation (19). As especially well seen in the case of stimulation with suboptimal TSH concentrations, proliferating cells are frequently gathered in large clusters, which, strikingly, poorly overlap clusters of cells having proliferated a few days earlier (19). This indicated a local synchrony of proliferating cells which implies a communication between neighboring cells. Therefore, a clustered distribution of cells exhibiting similar properties, as observed in histological sections of thyroid gland (93), does not necessarily imply that these cells are members of a same progeny with special inherited properties.

However, this dynamic heterogeneity resulting from temporary characteristics of cells or groups of cells can produce a qualitative change in the mitogen responsiveness, thus generating a more stable heterogeneity. The progeny of the fraction of cells having proliferated during a limited treatment with cAMP-independent mitogens (EGF, serum) retains a reduced but marked responsiveness to EGF, but these cells quite unexpectedly lose the mitogenic sensitivity to TSH or forskolin (19). The neighboring cells that have not replicated DNA during the momentary EGF stimulation are not affected in their responsiveness to TSH (which thus generates two distinct subpopulations). By contrast, the mitogenic responses to TSH/cAMP or EGF are not abolished in cells that have divided in response to TSH. Further characterization of this extinction phenomenon, including analysis of dikaryons formed by the fusion of thymidine-labeled and unlabeled cells, suggested that the suppression of the TSH/cAMP-dependent mitogenic pathway is due to the delayed induction of a dominant intracellular inhibitory factor during the cAMP-independent cell cycle progression (19). This quite well illustrated the cell ‘memory’ (and exemplified how the TSH (cAMP) inducibility of growth could have been easily lost in other systems). Other responses to TSH and cAMP including morphological changes with cytokeratin redistribution (21) and Tg and TPO mRNA expression are not affected (68). Again, the heterogeneities of proliferative responses, differentiation expression and morphology are unrelated.

Towards an understanding of molecular mechanisms of dog thyrocyte growth and differentiation

Regulation of early immediate genes

Contrary to growth factors, TSH and cAMP can trigger both proliferation and differentiation programs in the same cells at the same time (68). The expression of the early immediate genes, protooncogenes encoding nuclear transcription factors might explain such differences.

The expression of c-fos has been studied by Northern and Western analysis and by indirect immunofluorescence in dog thyrocytes (59, 83). As in other cell types, the tyrosine kinase cascades of EGF and HGF and the PKC cascade of TPA and Cch enhance c-fos expression, likely as a consequence of the activation of p42 and p44 MAP kinases (59, 91). c-Fos protein accumulation (and MAP kinase nuclear translocation) are induced by EGF and HGF in variable fractions of the cell population, which could suggest that the heterogeneity of proliferative responses to these factors could be due to the lack of c-Fos and MAP kinase responses in many cells (59). By contrast in the case of stimulations by TPA, c-Fos accumulation and MAP kinase translocation occur within the whole cell population, which does not explain the heterogeneity of the growth response to this factor (59). Cch and insulin (used alone) activate MAP kinases and induce c-Fos, but do not stimulate DNA synthesis (50, 57, 82). Thus, these ‘early mitogenic events’ might be necessary but not sufficient for the induction of DNA synthesis by cAMP-independent factors (50, 59). On the other hand, TSH or forskolin have a smaller inductive effect on this protooncogene
expression (59). This response was observed in only a minority of cells. This means that in response to these factors many cells progress through the cell cycle without any detectable increase of c-Fos protein content, suggesting that a high c-Fos expression is not necessary in the cAMP mitogenic pathway. Nevertheless, cAMP potentiates and generalizes the c-Fos (but not MAP kinase) responses to EGF, which could contribute to explaining the synergy of EGF and cAMP effects on DNA synthesis (59, 91).

In contrast fos B is essentially stimulated by the TSH/ cAMP cascade, to a small extent by TPA and HGF, and not at all by EGF or IGF-I (82). Fra-1 is little induced by TPA, EGF or HGF, and Fra-2 by insulin and cAMP but less extensively than the other protooncogenes of the family (82). The functional entity in the cell is the transcription factor AP-1, composed of various dimers of Jun and Fos proteins, which may differentially affect the pattern of gene transcription and the cellular behavior. The main fos-partner to the AP-1 complex could thus be c-Fos in the tyrosine kinase and PKC cascades and Fos B in the cAMP-dependent cascade.

With regard to the Jun protooncogenes family, the mRNA expression of c-Jun, jun B and to a lesser extent jun D is stimulated in fibroblasts by serum, TPA, EGF or other growth factors. In the same cells, in which it inhibits growth, cAMP represses c-Jun expression while it induces jun B and jun D mRNA accumulation (94). In dog thyrocytes, in response to EGF or HGF, jun D is not induced but its mRNA levels are transiently increased in response to cAMP (95). After EGF, insulin, IGF-I, TPA or HGF treatments, jun B expression is increased and sustained. An important induction also arose after cAMP cascade stimulation but the kinetics were very sharp and transient (94). In our system, c-Jun is induced by TPA, EGF, HGF or insulin, but interestingly its expression is inhibited after stimulation of the cAMP-dependent cascade (95). Contrary to the dogma, there is therefore no parallel between c-Jun expression and proliferation in the dog thyroid cells, but it is possible that c-Jun could be a factor involved in the negative control of differentiation expression. Among the Jun genes, jun B correlates best to the proliferative state of the thyroid cells (96). The Jun-partners to the AP-1 complex could thus be c-Jun or Jun B in the tyrosine kinase and PKC cascades and Jun B or Jun D in the cAMP-dependent cascade.

The activity of AP-1 dimers as transcription factors depends on the phosphorylation of the Jun protein by MAP kinases that are activated by the cAMP-independent cascades but not by the mitogenic cascade of cAMP. Moreover, in contrast to c-Jun, Jun B and Jun D have often been found to be repressors of AP-1 activity and even to inhibit cell proliferation and transformation (97, 98). Thus, the role of AP-1 transcriptional activity appears well established in the tyrosine kinase and PKC cascades, but whether and how it is also involved in the cAMP cascade is less clear.

A sustained increase of c-myc expression is considered to be required for G1 phase progression and DNA synthesis initiation. A rapid and dramatic decrease in c-myc mRNA by antisense myc sequences prevents DNA synthesis but induces differentiation of a variety of cell types (99, 100). In several systems where cAMP inhibits growth, it also inhibits c-myc expression, e.g. in HL60, B-precursor cells or fibroblasts. In systems in which cAMP is mitogenic, it enhances c-myc expression (see (101) for a review). In the case of dog thyrocytes in primary culture as in other systems, EGF, HGF, insulin or IGF-I, TPA and Cch enhance c-myc mRNA and protein accumulation for several hours (82, 83, 91, 102, 103). After activation of the cAMP cascade, the kinetics of the c-myc gene appear as for jun B tightly controlled. After a first phase of 1 h of higher level of c-myc mRNA (102), c-myc expression is decreased below the control levels (91, 103). At 1 h, the effects of EGF and cAMP are additive, but at 3 h cAMP markedly inhibits the stimulation of c-myc expression by EGF (91). Thus, in this second phase cAMP decreases c-myc mRNA as it does in cells in which it inhibits the proliferation. This tight control results from a complex transcriptional and posttranscriptional regulation (103). As c-myc is downregulated upon induction of differentiation in a variety of cell types, it is tempting to relate the later down-regulation of c-myc by TSH, compared with the sustained expression caused by EGF and TPA, to the fact that TSH induces both proliferation and differentiation, whereas the other agents induce mitogenesis and dedifferentiation. However, c-myc expression is clearly not sufficient for mitogenesis, since it is induced by a non-mitogenic factor (Cch) (50), or by insulin or growth factors used alone (i.e. in conditions where they little, or not at all, induce DNA synthesis) (82). The involvement of c-myc in the cAMP-dependent mitogenic pathway is unclear. In response to TSH or forskolin, c-myc expression is too transient to explain the continuous requirement for high cAMP during the progression into G1 phase (104). Moreover, c-Myc activity as a transcription factor also has been reported to require its phosphorylation by MAP kinases.

Max: the direct partner of c-Myc, is generally expressed at relatively constant levels that are independent of proliferative status (105). In dog thyrocytes, Max mRNA accumulation is subject to regulation with delayed early-genes kinetics: TPA and EGF stimulate mRNA induction in a protein synthesis dependent manner; cAMP inhibits Max mRNA accumulation in a way which is independent of new protein synthesis (106). The Max mRNA level is thus, as for its partner c-Myc, inversely related to the establishment by the cell of a differentiated phenotype.

The early transient expression of NGFI-B/nur 77 gene leads to various cellular events, such as proliferation, differentiation and apoptosis. It is an early induced nuclear receptor transcription factor implicated in the cellular response to growth factors. In dog thyrocytes,
NGFI-B mRNA is barely detectable in the absence of any stimulation (107). The mitogenic agents TSH, TPA and EGF all induce a transient expression of NGFI-B mRNA but at very different levels and with different kinetics. TSH and forskolin induce a high production of NGFI-B mRNA, whereas much lower stimulations are elicited by TPA and especially by EGF (107). Forskolin also induces a high capacity of NGFI-B transactivation in dog thyrocytes (107). Its role is presently being investigated.

All together, these observations demonstrate the convergence of tyrosine kinase and PKC cascades, and the partial divergence of the cAMP cascade, at the level of immediate early genes encoding important transcription factors.

**Regulation of dog thyroid cell cycle**

**Cell cycle kinetics**

Dog thyrocytes cultivated without serum but with high insulin concentrations are essentially quiescent (16). DNA synthesis can be induced in a significant part of the cell population by TSH, EGF, HGF, TPA or serum, after a prereplicative lag phase (G0–S) of 18–20 h (15). Using these cells, we have found that the presence of the DNA polymerase δ associated PCNA and its subcellular localizations allow identification of each phase of the cell cycle (75). When the cAMP-dependent stimulation (TSH, forskolin) is compared with the various cAMP-independent mitogenic stimulations (EGF+serum, EGF, serum, TPA, HGF), a lengthening of the cell cycle is observed in S phase (TSH: 9–10 h, EGF+serum: 6–7 h) and above all in G2 phase (TSH: >5 h, EGF+serum: 3 h) (75). In cells stimulated by the adenylyl cyclase activator forskolin, the removal of this drug at late stages of the prereplicative phase prevented cells from initiating DNA synthesis (104), while a similar forskolin removal once cells have reached S phase, hastened their entry into mitosis (75). Thus in dog thyrocytes cAMP positively controls a late restriction point (104) (the no-return point in late G1 beyond which the cell cycle can proceed independently of exogenous factors) just before DNA synthesis, but negatively influences the G2/mitosis transition (75) as observed in a large variety of systems (101). The cooperation of cAMP-dependent (TSH) and cAMP-independent (EGF+serum) mitogenic pathways results in an increase of the fraction of cycling cells (15), and in a 3–4 h reduction of the prereplicative phase (compensated for by a lengthening of S phase) (75). This illustrated the fact that TSH cooperates with cAMP-independent mitogens by stimulating steps that are rate limiting for DNA synthesis initiation. Addition of forskolin 24 h after EGF, or EGF 24 h after forskolin also resulted in the amplification of the DNA synthesis response, but with a lag after the addition of the second factor equal to the total prereplicative phase observed with both factors added simultaneously (15). This result was especially amazing because both cAMP and EGF can independently control the overall progression into prereplicative phase (104). It suggested that mitogenic events induced by cAMP can no longer be integrated in the sequence of events induced by EGF, when the two stimulations are not applied simultaneously (15). Moreover, the pre-treatment of dog thyrocytes for 10 h with forskolin (a treatment too short to induce DNA synthesis by itself) did not reduce the lag time for initiation of DNA synthesis after stimulation by EGF. Thus a 10 h progression into prereplicative phase induced by cAMP is not helpful for the progression into cell cycle induced by EGF. These results suggested that distinct ordered sequences of mitogenic events stimulated by either EGF or cAMP should be self-sufficient and, in parallel, lead to commitment for DNA replication (15, 101).

**Dog thyroid cell cycle biochemistry**

The absence of MAP kinase activation, the low or absent c-fos and c-myc expressions and transient c-myc expression in the TSH/cAMP cascade imply that the pattern of late gene expression could be different in the cAMP-dependent and -independent mitogenic pathways. Indeed the patterns of protein synthesis (as analyzed by twodimensional gel electrophoresis) induced by activation of dog thyrocytes by TSH and cAMP, on the one hand, and EGF, TPA, and serum, on the other hand, are different during G1 phase progression (72, 80). The transient induction of an 80 kDa protein in mid G1 constitutes a marker of G1 phase progression stimulated by cAMP-independent mechanisms (EGF, serum, TPA), but not of the activation by cAMP (72). The synthesis and nuclear accumulation of PCNA are already stimulated by TSH via cAMP in mid G1, but they are detected only a few hours before the onset of S phase in response to EGF+serum (72, 75). Kinetic experiments have suggested that the accumulation of PCNA to threshold levels could be a rate-limiting event for the initiation of DNA replication in the case of cAMP-independent mitogenic stimulations, but not in the case of TSH (75).

It is now admitted that the different transitions of the cell cycle require the sequential activation and deactivation of a family of cyclin-dependent protein kinases (CDK) whose activity depends on their association with cyclins but is restrained by various inhibitory proteins (CDK inhibitors, CK1). As expected, the cAMP-dependent (TSH), and cAMP-independent (EGF+serum) mitogenic pathways converge before G1–S transition at the late stage of these proteins that control the cell cycle machinery (108). The common events include the phosphorylation and nuclear translocation of CDK2 in late G1, the induction of cyclin A and the accumulation and phosphorylation of p34<sup>cdk2</sup> (CDK1) during S and G2 phases, and the degradation of cyclin A at metaphase (108). These events depend on the presence of insulin in the culture medium, and their stimulation by TSH is inhibited by TGFβ (61). Differences in the kinetics of these events in cells stimulated by TSH or EGF+serum...
correlate with the differences of cell cycle kinetics (108). The lengthening of G2 phase in the cAMP-dependent cell cycle is associated with a stabilization of the inhibitory tyrosine phosphorylation of p34\(^{\text{ck2}}\) and the ‘clamping’ of the nuclear accumulation of cyclin A and CDK2 (108).

The activation of CDK4, which phosphorylates and inactivates the growth and tumor suppressing proteins of the Rb family, is now considered a molecular link at the ‘restriction point’ between the mitogenic signaling cascades and the largely self-regulated cell cycle machinery. In different systems, growth factors activate CDK4 by inducing cyclins D and inhibiting the expression of the CKi p27\(^{\text{kip1}}\) (109). By contrast, cAMP inhibits the synthesis of cyclins D and/or stimulates the expression of p27\(^{\text{kip1}}\) in macrophages and fibroblasts, which has been claimed to explain its growth inhibitory effects in these cells. In dog thyrocytes, unlike EGF+serum and TPA, TSH via cAMP stimulates the accumulation of p27\(^{\text{kip1}}\), including in cells progressing in G1 and S phases (110), and does not stimulate the expression of cyclin D1, cyclin D2 or cyclin D3 (111, 112). During the prereplicative phase, TSH even partially inhibits the basal expression of the most abundant cyclin D3 (112). These effects are thus paradoxically similar to those observed in cells blocked in G1 by cAMP. These opposite effects of TSH and growth factors on major G1 phase regulators are likely to result from the analogous differential effects observed at the level of earliest events of the mitogenic cascades, such as MAP kinase activation, c-myc, c-jun and c-fos expression, but not from the common responses to TSH and growth factors on related genes such as jun D and jun B.

However, TSH enhances the nuclear immunofluorescent detection of cyclin D3 (111), apparently by unmasking an epitope close to its domain of interaction with CDK4 (112). Like the stimulation of DNA synthesis, this effect is dependent on insulin and inhibited by TGFB\(^\alpha\). Moreover, the microinjection of a neutralizing cyclin D3 antibody blocked the stimulation of DNA synthesis by TSH in the presence of insulin, but not the stimulation by HGF (112). In dog thyrocytes, contrary to cell types characterized by a G1 cell cycle block by cAMP, TSH via cAMP induces in late G1, like growth factors, the phosphorylation of Rb and related p107 and p15\(^{\text{ink4a}}\) (113). Again this TSH effect is dependent on insulin (113) and inhibited by TGFB\(^\alpha\). TSH and EGF+serum also induce a not previously described nuclear translocation of CDK4, the assembly of immunoprecipitable cyclin D3–CDK4 complexes and the Rb kinase activity of these complexes (112). The CDK4 activity is essential in the cAMP-dependent mitogenic pathway, since the microinjection of the CDK4 inhibitor p16\(^{\text{ink4a}}\) blocked the stimulation of DNA synthesis by TSH, exactly as in growth factor stimulated fibroblasts with a normal Rb function (112, 114). These results thus identified the first convergence point in late G1 of the parallel cAMP-dependent and -independent mitogenic pathways. It involves the activation of cyclin D3 through a new mechanism which remains to be explored, CDK4 nuclear translocation, and assembly of active cyclin D3–CDK4 complexes, despite opposite effects of these pathways on the expression of the known growth factor-dependent modulators of CDK4 activity.

**Transcriptional regulation of thyroid-specific genes**

Like other specialized cells, the thyroid follicular cell distinguishes itself by expressing a specific subset of the genome. Among those genes that are specifically expressed in the thyrocyte, the ones coding for the synthesis of Tg and TPO have been most studied so far. Considering the very restricted pattern of expression of these genes and the major role their products play in the physiology of the thyrocyte, the main questions that have been dealt with concern the nature of the mechanisms involved in their cell type specific activation and how their transcriptional activity is modulated in response to physiological stimulation of the cell. The dog thyrocytes maintained in primary culture proved to be an extremely useful experimental system regarding this last question as the controls exerted on the transcription of both Tg and TPO genes in the dog cells are very comparable to the ones that have been shown to exist in the intact tissue.

Experiments conducted in the rat had shown that a normal level of circulating TSH is required to sustain transcription of the Tg gene at a detectable level in the intact animal (115). This effect of TSH on the transcription of the Tg gene had also been shown to be mediated by cAMP as it could be reproduced by forskolin in the intact rat tissue incubated *in vitro* (116). This cAMP-dependent control on the transcription rate of the Tg gene is conserved in the dog thyrocyte, with the exception that the increase in transcriptional activity resulting from cAMP (TSH/forskolin) stimulation is observed after a longer time in the cultured cells as compared with the intact tissue (23, 69). Interestingly, whereas in the animal (rat) and in thyroid tissue slices (rat, dog), ongoing transcription of Tg gene is modulated by TSH or forskolin within 1 h (69), the Tg gene response is delayed in dog thyrocyte primary cultures (23, 69) by a time lag that depends on both culture conditions and local cell organization (68). The response is often more rapid in dense cell aggregates; cells that have not proliferated *in vitro* present shorter lag times (8 h) for Tg mRNA reinduction than cells that have proliferated in the presence of EGF (about 20 h) (68). 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 might be akin to a cell differentiation process.

The effect of cAMP (TSH/forskolin) stimulation on transcription of the TPO gene in the primary cultured
thyrocytes is identical to that observed in dog thyroid tissue slices incubated in vitro. In both cases, transcription is detectable at 1 h after stimulation (25, 69). This indicates that the pathways used to regulate the transcriptional activity of Tg and TPO genes differ at some step(s) downstream from the generation of the cAMP signal. Several other differences regarding the nature of the control mechanisms regulating Tg and TPO genes transcription have been noted. As mentioned above, insulin exerts a positive control on Tg gene transcription, independently of the effect of cAMP, whereas it has no detectable effect on transcription of the TPO gene (69). Also, experiments in which the protein synthesis inhibitor cycloheximide was used, both in cultured dog cells and in tissue slices, showed that Tg gene transcription requires on-going protein synthesis whereas TPO transcription does not (69). Clearly, although both genes are positively controlled at the transcriptional level by a cAMP-dependent mechanism, the molecular events involved in both cases must be partially different.

Chromatin structure of the Tg gene Expressed genes distinguish themselves by an altered chromatin structure, as compared with bulk chromatin, that renders their DNA more accessible to non-chromosomal proteins. This ‘open’ chromatin state can be evidenced by the increased sensitivity of the corresponding DNA to the action of nucleases. Within these nuclease-sensitive regions, which encompass entire genes, restricted areas may exhibit a still greater sensitivity. These hypersensitive sites have been shown in many cases to correspond to regulatory elements participating in the control of transcriptional activity. The study of the chromatin structure of the 5’ end of the Tg gene in cattle had identified the presence of two hypersensitive sites in thyroid chromatin, whereas none was detected at this level in liver chromatin (117). The first hypersensitive site was found to correspond to the DNA sequences surrounding the transcription start of the gene; the other one was located more upstream (about 2 kb from transcription start). To investigate whether the presence of the hypersensitive sites could be related to the actual transcriptional status of the gene, primary cultured dog cells with regard to the expression of differentiation (73). When these cells were maintained in the presence of TSH or forskolin, both hypersensitive sites were detectable in the chromatin corresponding to the 5’ end of the Tg gene. If the cells were maintained in dedifferentiating conditions, by substituting a high serum concentration to the agents mentioned above, only the upstream hypersensitive site could still be detected. Returning the same cells to stimulated conditions (TSH or forskolin in the absence of serum) resulted in the reappearance of the hypersensitive site encompassing the transcription start (117). Thus, the hypersensitive character of the chromatin at the transcription start is dependent on actual transcription of the gene, whereas hypersensitivity at the upstream position is not directly related to transcription but may be considered as reflecting the ‘committed’ state of the Tg gene gained upon thyrocyte differentiation.

Promoter regions of Tg and TPO genes The DNA sequences preceding the transcribed parts of Tg and TPO genes were expected to contain promoter elements directing transcription of these genes. The identification of these promoter regions required the use of a functional assay allowing investigation of their ability to initiate transcription in response to cAMP stimulation. As both the cloned bovine Tg promoter sequences and primary cultures of calf thyrocytes were available, attempts were made to obtain a detectable transcriptional activity of the transfected promoter in this homologous experimental system. Despite numerous efforts, this approach failed. It was then proposed to use the primary cultured dog thyrocytes as recipients for the transfected promoter sequences. For still unknown reasons, this proved to be the right choice. Transfection of DNA constructs containing the chloramphenicol acetyltransferase reporter gene under the control of the sequences located 5’ to the transcribed part of the Tg gene demonstrated that these DNA sequences are able to control transcription of the reporter gene in response to cAMP stimulation in the dog cells (118–120). The minimal sequences required for proper promoter activity were narrowed down by a deletion approach to about the first 200 bp preceding the transcription start of the gene. Most of these sequences are part of the proximal hypersensitive site identified previously in Tg gene chromatin in thyroid cells (see earlier section on chromatin structure of the Tg gene). Tg promoters from different mammalian species, including dog, cattle, man and rat, are equally active when transfected into dog thyrocytes, which suggests that some basic molecular mechanisms involved in the control of Tg gene transcription have been conserved among these species (119). Mutagenesis experiments revealed that thyroid transcription factor-1 (TTF-1) (121) plays a major role in the transcriptional activity of the Tg promoter (122). However, whether TTF-1 is only required for basal promoter activity or whether it mediates the effect of cAMP remained an open question (123, 124), as the very low level of transcriptional activity of the promoters when transfected in unstimulated cells precluded a clear demonstration of the role of TTF-1 in this basal activity. The DNA sequences corresponding to the upstream hypersensitive site in bovine Tg gene chromatin (see earlier section on chromatin structure of the Tg gene) were shown to exhibit enhancer activity when assayed in transfected dog thyrocytes (125). This enhancer element is composed of three contiguous binding sites for TTF-1 as determined by footprinting experiments. A similar region has also been identified.
recently in man (126). In addition to TTF-1 binding sites, the human Tg enhancer also contains a CAMP-response element (CRE) that is recognized by a complex involving CREB (127).

The promoter sequences of the human TPO gene were also characterized by transient transfection in the cultured dog thyrocytes. As expected, the TPO promoter is able to direct the expression of reporter genes in response to cAMP (TSH/forskolin) stimulation of the transfected cells (128). Footprinting experiments using nuclear extracts from the primary cultured thyrocytes identified a cAMP-modulated binding activity (129) that is likely to correspond to thyroid transcription factor-2 (TTF-2) (130).

Both cloned Tg and TPO promoter regions thus proved to be functional when transfected in the primary cultured dog thyrocytes and to exhibit the expected cAMP-dependence. In the case of the TPO promoter, TTF-2 appears as a likely candidate mediating the effect of cAMP on promoter activity. However, no such clear picture has emerged for the Tg promoter.

**Role of DNA methylation in Tg promoter activity**

Previous data obtained in man had shown that Tg gene sequences have a reduced content in methylated cytosine residues in thyroid cell DNA as compared with DNA extracted from other cell types (131). Transfection of *in vitro* methylated, or unmethylated, DNA reporter constructs in the dog thyrocytes indicated that cytosine methylation may repress Tg promoter activity in unstimulated cells, but does not do so when the transfected cells are maintained in differentiating culture conditions (i.e. in the presence of forskolin or TSH) (132). This suggested that DNA methylation may help to maintain the Tg gene inactive in non-thyroid and undifferentiated thyroid cells and that the presence of modified cytosine residues does not impair transcriptional activation of the gene as soon as the complete set of adequate transcriptional activators accumulates in the stimulated, fully differentiated, thyrocyte. Thus, the demethylation event that accompanies Tg gene expression does not seem to be a prerequisite for transcriptional activity.

**Expression of thyroid-specific transcription factors**

The transcription factors TTF-1 and Pax-8 appear as critical regulatory molecules controlling thyroid-specific gene expression (123, 133). Their expression was studied at RNA and protein levels in the primary cultured dog thyrocytes maintained in dedifferentiating (EGF+serum), control and cAMP-stimulated (forskolin) conditions (124, 134). Both factors are less abundant in dedifferentiated cells (EGF+serum conditions) than in the control cells. The expression of TTF-1 is only slightly increased in the cAMP-stimulated cells as compared with control conditions, but Pax-8 accumulates significantly in the cells following cAMP stimulation. Pax-8 factor thus appears as a likely candidate for mediating a delayed effect of cAMP on gene expression (134). This effect would also require on-going protein synthesis. Although these features are reminiscent of some known characteristics of the cAMP-dependent Tg gene transcription (delayed, cycloheximide-sensitive) as determined in the same cells (see above), no experimental data support the existence of a causal link between the increase in Pax-8 and the stimulation of Tg promoter activity at the present time.

In the same studies (124, 134), both TTF-1 and Pax-8 were shown to be phosphorylated nuclear proteins in control cells. No dramatic change in their phosphorylation could be detected following cAMP stimulation, which at first argues against the possibility that cAMP-dependent Tg gene transcription relies essentially on PKA-mediated phosphorylation of one, or both, of these factors. Our present interest focuses on TTF-2 expression in dog thyrocytes. TTF-2 mRNA concentration was recently shown to be dramatically increased following cAMP stimulation in these cells, whereas TPA and EGF had no significant effect. Whether the cAMP pathway also acts at the level of TTF-2 protein itself will be the subject of future investigations.

**Concluding remarks and perspectives**

The dog thyrocyte primary culture system was demonstrated to keep most of the known in vivo characteristics of thyroid follicular cells. As illustrated in this review, the in-depth analysis of this model system has provided coherent answers to questions concerning the physiological or pathological regulation of the thyroid gland. Several initial observations from this system led to predictions that are now well verified, or provided incentives to investigations of new physiological regulations. In dog thyrocytes, three classes of factors controlling three major signaling cascades were found to exert well-distinct end effects.

(i) The PLC/Ca^{2+} signaling pathway, first evidenced from the dog thyroid tissue slice system and abundantly characterized in dog thyrocyte primary cultures, is mainly activated by various neurotransmitters. It has little influence on cell proliferation, but acutely stimulates (and might coordinate) the simultaneous availability at the luminal pole of cells of the two rate-limiting substrates for Tg iodination: H_{2}O_{2} and iodide. It could thus play a major role in the regulation of iodide storage in Tg and thyroid hormone synthesis. The physiological meaning of this control should be clarified. It will require understanding of the role of thyroid innervation. The fact that almost all thyroid follicles are innervated by at least one nerve fiber (especially cholinergic nerves in the dog thyroid) suggests that this role may be important (135).

(ii) TSH, by activating the cAMP signaling pathway, activates function, differentiation expression (the transcription of thyroid-specific genes) and growth (cell
cycle progression). The last features correspond to known chronic in vivo effects of the hormone, which contribute to enhance the capacity of the gland to respond to acute functional stimuli, and may play a role in thyroid development. The transcriptional effect of cAMP on Tg and TPO genes has been well characterized in the dog thyrocytes. The promoter elements involved have been delineated functionally and, in the case of the Tg gene, they correspond perfectly with sequences exhibiting an altered chromatin structure in the thyroid cells. The expression of some of the factors implicated in the transcriptional activation of Tg and TPO genes has been investigated in detail with the aim of uncovering a possible link with the effect of cAMP. It is noteworthy that the experimental system described here appears as a reference regarding this aspect (133). The cAMP pathway has been shown to regulate the expression of some transcription factors (Pax 8, NGFI-B, TTF-2) in the thyrocytes in a specific way, but the information gathered to date still does not explain how cAMP controls the transcription of Tg and TPO genes at the molecular level. One may thus hope that in the near future additional elements will be characterized in this privileged experimental system.

We have confirmed in primary cultures of human thyrocytes our demonstration that cAMP mediates the proliferation effects of TSH (136) responsible for goiter formation in vivo. This conclusion was also relevant to the action of hypersecreted TSH in thyrotrph adenomas and of TSH receptor stimulating antibodies in the autoimmune Graves’ disease. Since cAMP does stimulate human thyrocyte proliferation, TSHs that activate adenyl cyclase must be thyroid growth stimulating immunoglobulins (TGI). Conversely, the claimed existence of TGIs stimulating growth through TSH receptors but other signaling cascades (PLC/Ca²⁺, PL2) is as yet not well supported (see reference (137) for an opposite opinion). Whether the known promoting influence of TSH and TSI on follicular carcinomas also requires the activation of the cAMP-mitogenic pathway has not been clarified. Our observations of dual stimulatory effects of cAMP on both cell proliferation and thyroid-specific gene expression led us to postulate that overactivity of the cAMP cascade could provoke hyperfunctioning tumors in thyroid and other tissues (138). This hypothesis was confirmed in vivo by experiments in transgenic mice where the expression in thyroid of a constitutively active A2a receptor coupled to adenyl cyclase elicits goiter and hyperthyroidism (139), and if the cell cycle inhibitory function of Rb has been inhibited, follicular carcinomas (140). The finding of adenyl cyclase activating mutations of Gsα (141) and quite more frequently of the TSH receptor (142) in thyroid hyperfunctional adenomas and familial hyperthyroidism (143), provided a definitive demonstration from human pathology of the dual proliferative and functional effects of cAMP. The demonstration of the mitogenic activity of cAMP in primary cultures is still now crucial for the interpretation of these somatic and germline mutations as oncogenic mechanisms.

(iii) First demonstrations of TSH-independent regulations of thyroid growth and differentiation expression were also obtained from the dog thyrocyte primary culture system. EGF (55), FGF (18) and HGF (56), through tyrosine kinase receptors, are potent mitogens for these cells. In addition, EGF strongly inhibits the expression of differentiation. These growth factors were found to produce quite similar effects in some animals in vivo. In mice, the injection of EGF promotes DNA synthesis in thyroid and inhibits iodide uptake (144). By contrast, the injection of FGF induces a colloid goiter with no inhibition of iodide metabolism or Tg and TPO mRNA accumulation (145). EGF and FGF have since been found to be locally synthesized in the thyroid gland, as a possible response to thyroxine (146) and TSH (147) respectively. Their exact role as autocrine and/or paracrine agents in the development and function of the thyroid gland of different species has yet to be clarified. It has been proposed that EGF could locally participate to a short feedback loop by which thyroxine might inhibit thyroid gland function and responsiveness to TSH, and thus regulate its own production (18, 146, 148). On the other hand, the subversion of tyrosine kinase pathways similar to those normally operated by local growth factors (i.e. the activation of ret/PTC and TRK, the overexpression of MET/HGF receptor or erbB2/EGF receptor) was causally associated with TSH-independent papillary carcinomas.

IGF-I and TGFβ are other local factors in the thyroid gland, the action of which has been characterized in dog thyrocyte primary cultures. They modulate positively (IGF-I) or negatively (TGFβ) the mitogenic effects of TSH. TGFβ may also inhibit the proliferation of rat thyrocytes in vivo, as recently suggested by the injection of neutralizing TGFβ antibodies (149), and contribute to a mechanism limiting undue proliferation in the thyroid. Whereas IGF-I has been ascribed a role in thyroid growth in other species as an autocrine or paracrine factor regulated by TSH, in dog thyrocyte primary cultures TSH induces the accumulation of insulin receptors that mediate the permissive effects of low physiological concentrations of insulin on the action of TSH. This raised the intriguing possibility of TSH/insulin interactions in vivo, which could allow circulating insulin, besides locally produced IGF-I, to support the mitogenic action of TSH.

The dog thyrocyte primary culture system has allowed a systematic comparison of biochemical events involved in the mitogenic pathways activated by growth factors or TSH through cAMP. This in depth analysis led us to conclude that these pathways are essentially distinct and parallel during most of the G1 phase. Unlike the rapidly converging tyrosine kinase and PKC growth-signaling pathways, the cAMP-mitogenic cascade of dog thyrocytes does not utilize the phosphorylation of proteins on tyrosine and the
activation of MAP kinases, it poorly induces c-Fos protein accumulation, it down-regulates c-jun mRNA and, after a short initial induction c-myc mRNA and protein, and it does not stimulate the accumulation of cyclins D but paradoxically increases the expression of the CDK inhibitor p27kip1. By contrast, TSH activates cyclin D3 by a new mechanism that allows the assembly of active cyclin D3–CDK4 complexes. In a given cell, different strategies utilizing different genes and sequences of regulatory events may thus coexist, separately leading to the cell commitment for DNA replication at the G1 phase ‘restriction point’.

The TSH-controlled cAMP-mediated growth pathway, which is compatible with cAMP-dependent thyroid-specific gene transcription may appear itself as a differentiated characteristic (19, 101). Whether it could depend, like thyroid-specific differentiation genes, on thyroid-specific transcription factors, would be an interesting area of research. As a specialized regulation, the elements of the cAMP-dependent mitogenic pathway appear to be adjunctive to the more general mechanisms operated by growth factors. This could explain its various odd characteristics, which have systematically contradicted generalizations on the necessary biochemical steps in mitogenesis. We are now using dog thyrocyte primary cultures as a unique model of the possible diversity of the mechanisms of cell cycle control by distinct intracellular cascades. To what extent some features of this model could be generalized to the growth stimulatory effects of cAMP frequently observed in other specialized cell types (101, 138) still awaits further studies.

Unlike immortal cell lines, dog thyrocyte primary cultures cannot be considered ‘easy experimental objects’. However, they have proved to be especially fascinating as they have demonstrated an extreme variety of physiologically or pathologically relevant actions of cAMP, which concern many basic aspects of cell biology including cytoskeleton organization, cell cycle, hormone synthesis and secretion, and the transcription of different categories of genes.

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