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## Chapter 1 – The Phylogeny, Ontogeny, Anatomy and Regulation of the Iodine Metabolizing Thyroid.

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## PHYLOGENY

The primary event in the phylogeny of the thyroid was the development in living forms of the capability of collecting iodide ion and binding it to protein. These activities have been observed widely among plants and in the invertebrate members of the animal kingdom. Brown algal kelps are the most efficient accumulators of iodide identified with enrichment factors for iodine of up to $10^6$ (1). In *Laminaria digitata*, for example, the iodine content can reach up to 5% of the dry weight. However, only a minor fraction of iodine is stored in the form of iodinated amino acid residues including monoiodotyrosine (MIT) and diiodotyrosine (1) (2) (3). The biochemical pathways involved in iodine uptake, accumulation and metabolism in these algae have still not been fully elucidated. Recent studies suggest that iodide is oxidized by a vanadium-dependent iodoperoxidase (4) yielding more lipophilic iodine species which diffuse across cell membranes and are subsequently sequestered as labile iodine species in the apoplastic compartment (1). Thus, the iodide uptake mechanism utilized by these algae appears very different from that of vertebrate thyroid follicular cells and the organification of iodine is still considered a by-product of the reactive environment.

In invertebrates, endogenous synthesis of iodothyronines including thyroxine (T4) and triiodothyronine (T3) has been clearly demonstrated for urochordates and cephalochordates (5), whereas evidence for endogenous iodothyronine synthesis outside the chordates is very limited (6;7) (8) (9). Nevertheless, invertebrates deserve attention when analyzing the evolution of the hormonal signalling function of iodothyronines. Already in 1896, Drechsel (10) recognized that sponges and corals contain large quantities of iodine as iodotyrosines. Iodohistidine and bromotyrosine have also been detected. Monoiodotyrosine (MIT) and diiodotyrosine (DIT) have been found in starfish, mollusks, annelids, crustaceae, and insects (9) (11) (12) In insects, several organs and tissues can concentrate radioiodide but there is no evidence that this results in TH (TH) formation (11). One process that is likely to yield iodinated compounds is cuticle formation(9). It has been suggested that iodinated substances may be by-products of the process of "quinone tanning". The formation of

benzoquinone cross-linkages in the molecular structure of scleroproteins is probably responsible for hardening of the cuticle, and it is known that, in the presence of inorganic iodide, benzoquinones can bring about the iodination of proteins in vitro(13). Thus, the iodination of tyrosine may be mediated quite accidentally by quinones that are involved in the general tanning reaction of the exoskeleton. However, recent studies by Heyland et al. (6) (7) suggest that at least some echinoderms and molluscs might produce T4 and T3 which was detected by thin layer chromatography and confirmed by ELISA measurements. Interestingly, iodothyronine synthesis in these organisms was prevented by thiourea but not by perchlorate treatment.

Even though most invertebrates might not be able to endogenously synthesize TH, a wealth of data indicate that organic iodine species are taken up from the environment (e.g., via the food) and function as signalling molecules with pleiotropic effects on various aspects of invertebrate physiology (9). In analogy to vitamins, Eales coined the term " vitamones" to describe this ancient function of iodinated compounds as external morphogenic signals governing larval development in some invertebrates. This provocative model has been supported by experimental studies (8;11;12). Recently, putative orthologs of vertebrate TH receptors (TRs) have been identified in various invertebrates (14;15) (16). Functional characterizations are still sparse for these proteins. On the other hand, in the case of the urochordate TR, binding studies failed to demonstrate T3 binding to the receptor (16). However, T4 and T3 might not necessarily represent the biologically active iodothyronines in invertebrates. For example, experimental data in insects suggest that iodothyronines such as T2 may be more potent signalling molecules than T4 or T3 to induce specific responses (8;11).

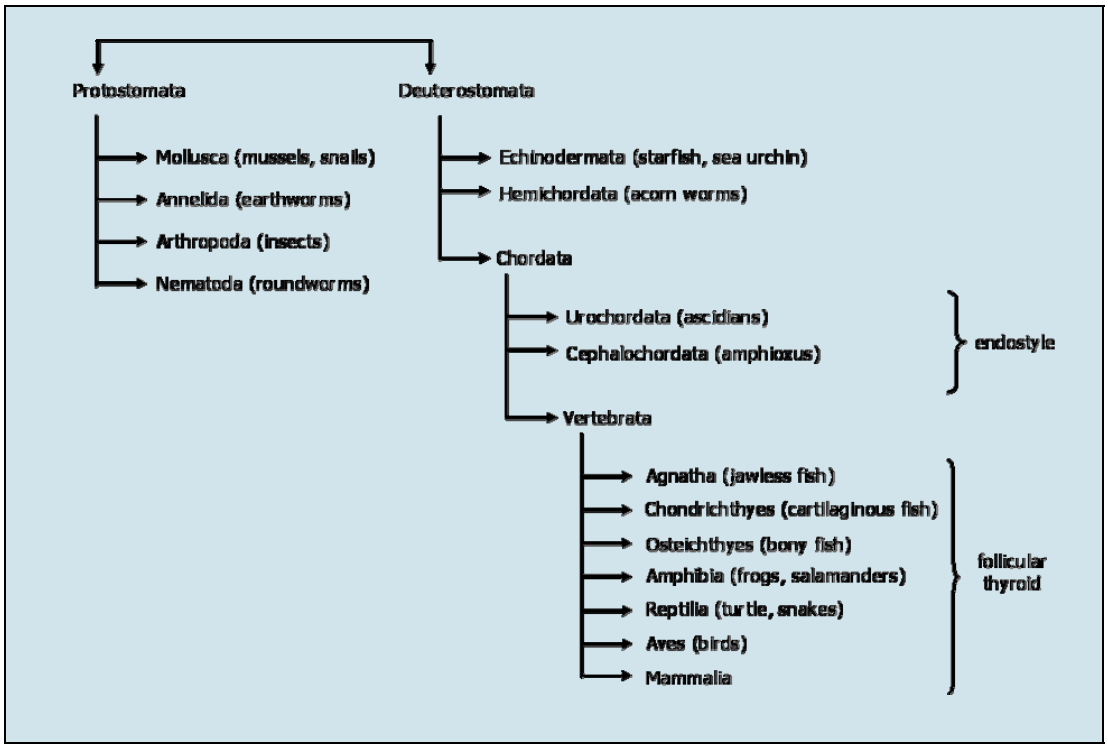
Gorbman(17) has hypothesized that during evolution, organisms became accustomed to a supply of iodotyrosines and iodothyronines derived from external sources, and eventually developed a requirement for the iodinated amino acids. The first evidence of an organ capable of providing iodothyronines and thus, related to the vertebrate thyroid, is found in the protochordates, comprising the subphyla Urochordata (ascidians), and Cephalochordata (amphioxus) (Fig. 1-1). In the origin and evolution of the thyroid gland, the protochordates occupy key positions in phylogeny, because ascidians are the most basal in the phylum Chordata and amphioxus are the closest living relatives of vertebrates (5). In ascidians and amphioxus, an organ known as the endostyle lies on the floor of the pharynx and connects with the pharynx by a duct. Notably, an endostyle is still present in the basalmost vertebrates, the lamprey larvae (ammocoete).

From the present point of view, the significant evolutionary event was the development of iodination centers within the endostyle. The differentiated endostyles in protochordates and lamprey larvae are histologically divided in "zones" containing different cell types (18). In the

ascidian *Ciona intestinalis*, these iodination centers are present in zones 7, 8 and 9 at the tip of the endostyle (19). The amphioxus endostyle contains seven zones and iodide organification has been observed in zones 5a, 5b, and 6 which are also located at the tip of the endostyle (20). In amphioxus, Barrington (21) has shown that an iodinated glycoprotein is formed in these iodination centers, probably on the surface of the cell. The endostyle secretes a mucus that passes down the duct into the pharynx and thence is moved along into the alimentary canal, presumably carrying iodinated protein along with it. Although early accounts were sometimes conflicting (22), accumulation of radioiodide, peroxidase activity as well as endogenous synthesis of T4 and T3 has been demonstrated in the endostyle of several protochordate species (23-25) (20;26).

In the ascidian *Ciona intestinales*, the proposed organ homology between the endostyle and the vertebrate thyroid was strengthened in recent molecular studies. For *Ciona* orthologs of vertebrate thyroid-specific marker genes, including the transcription factors Pax2/5/8, TTF-1 and TTF-2, expression was demonstrated in several zones of the endostyle in adult *Ciona*(18;27). In addition, *in situ* hybridization revealed expression of *Ciona* orthologs of TPO and Duox in the iodide-concentrating zone 7 of the endostyle (28). An interesting observation was that Pax2/5/8 and TTF-2 expression domains overlapped with those of TPO and Duox whereas TTF-1 expression was not detectable in zone 7. In amphioxus, however, all three transcription factors, Pax2/5/8, TTF-1 and TTF-2, were expressed together with TPO in the iodide-concentrating zones (18). The recently published sequence of the *Ciona intestinales* genome provided a first overview of the repertoire of ascidian genes encoding for components of the thyroid system (29). Orthologous sequences were identified encoding for a NIS-like iodide transporter, a pair of closely related iodothyronine deiodinases and a TR. The *Ciona* genome apparently does not contain a sequence homologous to thyroglobulin (TG) suggesting that other protein substrates are used by ascidians for iodotyrosine synthesis (30). In amphioxus, a TG-like protein has been described by Monaco et al. (31), but a molecular characterization of this protein is not yet available. Further, no clear homologs of thyrotropin-releasing hormone (TRH) or the two subunits of thyroid-stimulating hormone (TSH) have been detected in the *Ciona* genome, which is in accordance with the current view that ascidians do not have a pituitary gland(32). Based on a comparison of *Ciona* and vertebrate genomes, Campbell (30) concluded that some features of the vertebrate thyroid system appear well represented in urochordates but that critical genes involved in the neuroendocrine control of thyroid function are lacking.

**Figure 1. Phylogeny of the development of the thyroid gland.**




The most primitive vertebrates in which a follicular thyroid gland can be definitely demonstrated are the jawless fishes (agnathans). Concerning the origin and the evolution of the thyroid gland, lampreys are of particular interest because they are the only known vertebrates that possess a larval endostyle that directly transforms into a follicular thyroid during metamorphosis (33) (34). Although the endostyle of the lamprey larvae (ammocoete) has a different structure and organization compared to the endostyle of protochordates, physiological and molecular characteristics are very similar. The lamprey endostyle shows iodide uptake and organification, the latter involving a protein that is apparently related to TG (34) (35;36) (37). The TG-like protein undergoes proteolytic digestion in the intestinal tract to liberate T4 and T3 which are probably taken up directly from the gut lumen to enter blood circulation (38;39). Given this pathway of TH synthesis and release, it is of particular interest that high 5'-deiodinase activities were determined in the larval intestine (40). Comparative analyses of thyroid-related genes confirmed the expression of Pax2/5/8 (41) and TTF-1 orthologs (33) (27) in the lamprey endostyle. Interestingly, similar to ascidians, the expression domains of TTF-1 were not clearly overlapping with domains of iodide concentration, Tg synthesis or T4 synthesis (18;33).

Very high plasma concentrations of TH have been determined in lamprey larvae (42). A unique feature of lamprey developmental endocrinology is a dramatic decrease of circulating TH levels concomitantly with the onset of metamorphosis. This developmental TH profile is in sharp contrast to metamorphosis in amphibians and various fishes where high TH titers are associated with metamorphosis. Moreover, metamorphosis in lamprey larvae can be induced by anti-thyroidal compounds such as perchlorate or methimazole suggesting that the abrupt decline of plasma TH levels might trigger the onset of metamorphosis (43).

During metamorphosis of the ammocoete into the adult lamprey, the endostyle loses its connection with the pharynx and becomes a thyroid composed of scattered follicles (34) These follicles are not encapsulated, but they have the typical biosynthetic functions associated with hormone formation in the adult vertebrates. In the lamprey, the biosynthesis of Tg in larval forms has the same characteristics as that formed in thyroid follicles of the adult form, with a 12S as the precursor of the 18-19S protein. Total I in Tg is very low (0.002%) and about 5% is present in the form of T3 and T4 (44). Curiously, the thyroid appears to play no role in the metamorphosis of the ammocoete, although the gland itself undergoes a remarkable change.

The striking relationship of the thyroid to the gastrointestinal tract is apparent in all phylogenetic studies. Dunn (45) has actually found ciliated thyroid cells in the mouse and shark, a reminder of the origin of the gland from endoderm. In mammals the gastric mucosa and the salivary glands retain a functional relationship to the thyroid in that they too can concentrate iodide (46), and the salivary gland contains a peroxidase.

Thus, a thyroid capable of forming iodotyrosines and iodothyronines is present in all vertebrates. Its level of function varies widely from species to species and season to season. With the exceptions noted below, thyroid activity in the poikilotherms is very low. Seasonal changes in thyroid activity have been found in both warm- and cold-blooded animals. Certain morphologic changes occur after the biochemical evolution of the thyroid has ceased. In the adult lamprey and in most bony fishes, the gland is not encapsulated. The follicles may be widely scattered, either singly or in small clusters, especially along the course of the ventral aorta and in the kidneys (47). In cartilaginous fish, the thyroid is encapsulated. In the higher vertebrate forms, the thyroid is a one- or two-lobed encapsulated structure.

## Function of the Thyroid in Lower Forms

A functioning thyroid is evident in forms as primitive as lampreys and hagfishes. TH are critically important for the regulation of diverse biological processes associated with development, growth and metabolism in non-mammalian vertebrates (48) (49;50) (50). In particular, the vital importance of TH for the regulation of early developmental processes is

not limited to human or mammalian species (51), but is well conserved throughout the vertebrate kingdom (49) (52) (53). In avian species, for example, TH are required for nervous system and skeleton development (49) and TH action has also been demonstrated to regulate both direct larval and metamorphic development in fish (50;54). In amphibians, TH are the primary morphogen regulating postembryonic development (metamorphosis) (52).

Most fish species undergo similar developmental phases as described for amphibians including a larval stage, juveniles and adults (53) with a larvae-to-juvenile transition often associated with metamorphic changes (50). Experimental manipulation of the thyroid status as well as the recent cloning of fish TRs and the characterization of TR developmental expression profiles clearly demonstrated the important role of THs for early fish development (50) At later life stages, TH have been shown to assist in the control of various physiological functions in fish including osmoregulation, metabolism, somatic growth, and behaviour (50) (53). In salmonids, for example, TH are important for migration from fresh water to salt water (smoltification), and the high T4 plasma levels during smoltification represent some of the highest circulating T4 levels in fish (55;56).

Initially, it was believed that TH had little or no stimulatory effect on the oxidative metabolism of cold-blooded species. Now it is known that the effect of the thyroid on metabolic activity in cold-blooded species is strongly dependent on environmental temperature. For example, T4 causes stimulation of metabolism in lizards at 32°C, but not when they are acclimated to low temperatures (57). The thyroid gland is also more active at higher temperatures (23°-32°C) than at 10°-15°C in snakes, fish, amphibians, turtles, and lizards (58).

TH levels are also influenced by the nutritional status in both endothermic (birds, mammals) and ectothermic (fish) vertebrates with a decreased T3 and T4 to T3 conversion in caloric deficient states (59).

A most striking effect of TH is the induction of metamorphosis in certain amphibia, first reported by Gundernatsch in 1912 (60). The physiological role of TH during anuran metamorphosis is best exemplified by the fact that surgical removal of the thyroid gland or chemical blockage of TH synthesis leads to complete cessation of metamorphic development (61) On the other hand, addition of minute amounts of T4 (1 nM) to the rearing water of tadpoles during premetamorphosis leads to a precocious induction of metamorphosis (52). Particularly the metamorphosis of the South African clawed frog *Xenopus laevis* has been used for years as a highly successful animal model to understand TH function in a developmental context (52) (62) (63;64) (65). The relationship between the functional state of the thyroid system and the progress of postembryonic development is well documented in this species (52) (66). The premetamorphic period is characterized by rapid growth of tadpoles with only minor morphological changes (67) and TH levels are very low (66) Growth and differentiation of the hind limbs are the earliest TH induced modifications marking the

onset of the prometamorphic period during which levels of circulating TH steadily increase (26) stimulating further morphogenesis and differentiation of the limbs. The emergence of fore limbs marks the beginning of metamorphic climax characterized by rapid and dramatic changes in morphology (e.g., intestinal remodelling, complete resorption of gill and tail tissue) under the influence of peaking TH levels (52) (62). Towards the end of metamorphosis, plasma TH decline and low levels are present in juvenile and adult frogs (66).

A fascinating aspect of anuran metamorphosis is that a single type of hormone, TH, induces different tissues and organs to undergo remodelling in a highly coordinated spatio-temporal fashion (52) (62) (68). Similar to mammals, TH synthesis is regulated by thyroid-stimulating hormone (TSH) (64). T4 is the major hormone secreted by the thyroid gland while the secretion of T3 is low in X. laevis tadpoles (69). Expression of glycoprotein □-subunit and TSH □-subunit (TSH□) mRNAs in the pituitary of metamorphosing X. laevis tadpoles increase from low premetamorphic levels to maximum levels at early climax stages and decrease towards the end of metamorphosis (70) (71). Thus, there is a concurrent increase of TSH expression, thyroid activity and circulating T4 levels from premetamorphosis to early climax stages. Different hypotheses have been put forward to explain this condition. Some authors stressed the importance of climax stage induction of type II iodothyronine deiodinase (D2) expression in pituitary thyrotrophs as a molecular switch to establish a negative feedback control of TSH synthesis (72). In contrast, other studies could demonstrate negative feedback control of TSH expression by T4 at much earlier stages suggesting that the developmental TSH expression profile is the net result of negative feedback action of circulating TH and a concomitant increase in pituitary stimulation by hypothalamic factors (73) (65). Of relevance for the increased hypothalamic stimulation of pituitary thyrotrophs might be the TH-dependent maturation of the median eminence (74) as well as increased synthesis and release of hypothalamic peptide hormones (73).

The extent to which different plasma proteins such as thyroxine-binding globulin (TBG), transthyretin (TTR) and albumin account for TH binding in the blood varies among different groups of vertebrates (75). In humans and rodents, TBG and TTR are the main THBP, respectively, showing a higher affinity for T4 than T3 (76). TTR is assumed to be the main TH-binding plasma protein in metamorphosing tadpoles and many teleost fish. One characteristic property of amphibian and teleost TTRs is that they display a several-fold higher affinity for T3 than for T4(76).

In their target cells, the biological action of TH is mediated by activation of nuclear TH receptors (TRs). Two TR subtypes (TR$\alpha$, TR$\beta$) which are encoded by separate genes have been described in X. laevis (82). Due to pseudo-tetraploidy, there are two TR$\alpha$ (A and B) and

two TRβ (A and B) in X. laevis(77). Similar to mammals, TRs can bind to TH response elements (TREs) weakly as homodimers whereas heterodimers of TR and 9 cis retinoic acid receptors (RXR) strongly bind with TREs (63). Extensive investigations on the gene expression profiles induced by T3 made X. laevis one of the leading resources for understanding TH action during vertebrate development (63) (78). Gene transcription in X. laevis tadpoles can be either up- or down-regulated by TH(79;80). For the category of genes that are up-regulated by TH, it has been shown that unliganded heterodimeric TR-RXR complexes can bind to TRE but repress transcription through recruitment of corepressor (63). In X. laevis, low mRNA expression has been demonstrated for TRα and TRβ in oocytes and embryos but the putative functions are still poorly defined (81) (63). During postembryonic development, the expression profiles of TRα and TRβ show striking differences (52) (63) (68;77). TRα is expressed at high levels during early premetamorphic stages and expression is maintained at an elevated level throughout metamorphic development(63) (82). TRβ shows a more complex developmental expression profile, characterized by low expression during premetamorphosis and a dramatic up-regulation in parallel with the increasing TH plasma levels during prometamorphosis (63). When analyzed in individual tissues, TRβ was found to be up-regulated particularly during periods of active tissue remodelling (63) (77). Another important aspect of TH action in X. laevis tadpoles is that exogenous T3 regulates the expression of its cognate receptors (63). Among the most rapid changes in gene expression induced by T3, a dramatic up regulation of TRβ gene expression has been observed in all organs and tissues analyzed (63) (77). In addition, recently developed transgenic models carrying dominant negative and constitutively activated TR mutants could clearly demonstrate the important role of TR in mediating the developmental effects of TH during X. laevis metamorphosis (63).

Three types of iodothyronine deiodinases (D1, D2, D3) have been identified in vertebrates which differ in tissue distribution, substrate specificity and sensitivity to inhibiting compounds (83). D1 and D2 catalyze primarily the removal of one iodide from the outer tyrosine ring of T4 to produce T3. D3 catalyzes the cleaving of one iodide from the inner tyrosine rings of T4 and T3 generating inactive iodothyronine derivatives (e.g. reverse T3 and diiodothyronines), respectively. In amphibian tadpoles, the coordinated progression of metamorphic development requires a high degree of local control of T3 production which apparently dominates over the general supply of T3, at least during metamorphosis (84). Only recently, a putative X. laevis homologue of mammalian D1 has been identified (85) but neither the expression profile nor the putative regulatory role of D1 during metamorphic development have been characterized so far. However, several studies have investigated the role of D2 and D3 in controlling TH action during metamorphosis of anuran tadpoles (84). The data

derived from these studies support the view that both D2 and D3 play a central role in modulating the tissue responsiveness to TH by either increasing intracellular concentrations of biologically active T3 (e.g., D2 in hind limbs) or by preventing TH action via rapid inactivation of T4 and T3 (e.g., D3 in tadpole tail).

Central to the understanding of TH function in lower vertebrates are the manyfold interactions of TH with other hormones (e.g., corticosterone, prolactin, and growth hormone) contributing to a fine-tuning of developmental TH action. For example, several studies have shown that high concentrations of corticosterone can provoke both inhibitory or accelerating effects on amphibian metamorphic development, depending on whether treatment is initiated at early (inhibition) or at late developmental stages (acceleration) (86). Concerning the accelerating effects of corticosterone on the development of tadpoles at late stages, two molecular mechanisms have been proposed including corticosterone -induced increases in T3 receptor binding capacities and corticosterone -induced increases in peripheral deiodination of T4 to T3 (52). Similarly, corticosterone is known to affect peripheral deiodination of T4 to T3 in avian species, including inhibitory effects on D3 and D1 activities in chicken liver (87).

Another hormone that has received a great deal of attention with regard to modulation of TH dependent metamorphic development is prolactin. Early studies using mammalian prolactin preparations could demonstrate antagonistic effects of prolactin on TH action in various peripheral tissues (88). Inhibitory effects on TR autoinduction by TH have been suggested as a primary mechanism of prolactin action to antagonize TH action in peripheral tissues. It should be noted, however, that in a transgenic frog model of prolactin overexpression, no retardation of tadpole development was detectable, with the exception of blocked tail resorption in a limited number of transgene animals(89).

In chicken embryos, GH appears to be a potent inhibitor of hepatic D3 activity resulting in increased T3 availability (87). This GH action on TH metabolism probably represents a physiologically relevant hormonal response linking the nutritional state with the activity of the thyroid system. Given the great diversity and heterogeneity in fish physiology and ecology, it is not surprising that a multitude of hormonal interferences have been described in various model species. The reader is referred to several comprehensive reviews about this hormonal interplay in fish (90-92).

## Hypothalamic and Pituitary Control

Hypothalamic-pituitary control of thyroid function in the most primitive vertebrates represented by hagfishes and lampreys is still poorly understood (93) (94). Although the pituitary of these primitive fishes might contain thyrotrophic factors (93), their nature and their

hypothalamic-releasing factors are unknown. TRH and TSH were largely ineffective in stimulating thyroid activity as assessed by different experimental protocols (93). Instead, numerous studies support the concept that peripheral deiodination of TH might provide the major regulatory mechanism for the control of thyroid status (92). The intestinal tract seems to be a major site of this TH metabolism in larval lamprey (40).

Neuroendocrine control of thyroid activity has been established for teleost fishes, amphibians, birds and reptiles (95). TRH is regarded as the main regulator of TSH secretion in mammals, and TSH-releasing activity of this peptide hormone has also been observed in various non-mammalian vertebrates. In avian species, injection of TRH has been shown to preferentially increase circulating T3 instead of T4, an effect that was later related to a GH-dependent inhibition of hepatic D3 activity subsequent to a stimulation of somatotrophs by TRH(96). In fact, preferential binding of TRH was demonstrated for somatotrophs in chicken. In recent years, it became clear that another hypothalamic factor, corticotropin-releasing hormone (CRH), is a more potent stimulator of TSH release in non-mammalian vertebrates. In chicken, CRH was found to increase both T3 and T4 plasma levels via stimulation of TSH release (97). Studies by de Groef (98) could show a preferential expression of CRH receptors type 1 (CRH-R1) and type 2 (CRH-R2) in chicken corticotrophs and thyrotrophs, respectively. The role of CRH R2 in mediating the CRH effect on TSH release in chicken was corroborated in further studies using CRH R2-specific agonists and antagonists.

In amphibians, the regulation of TSH release by TRH and CRF appears to be dependent on the life stage. While TRH was able to increase TSH secretion in adult frogs but not in tadpoles (99;100), the finding that TRH stimulates TSH release at least in adult amphibians argues against the hypothesis that the TSH-stimulating activity of TRH has only recently been coopted in association with the development of endothermy. Similar to the studies with chicken, CRF proved to be the most potent releasing factor for TSH in the tadpole pituitary in vitro (100;101). In vivo, injection of CRH increased plasma T4 levels in Xenopus tadpoles and accelerated metamorphic development in ranids. In addition, TSH synthesis and release by the amphibian pituitary is under the control of a negative TH feedback (73;102). Few studies have addressed CRH effects on thyrotrophs in fish but the available data indicate that CRH but not TRH stimulates TSH release in salmonids (103).

## ONTOGENY

The main anlage of the thyroid gland develops as a median endodermal downgrowth from the tongue. It can be seen in the human embryo at the end of the third week (104). It is located near the primordium of the heart, and as the heart is pulled caudally, the thyroid

anlage follows. At the 5th week the thyroglossal duct starts to breakdown. At about the 30th day it has developed into a hollow bilobed structure, and by the 40th day, the original hollow stalk connecting it to the pharyngeal floor atrophies and then breaks. Shortly thereafter the lateral extensions of the median anlage make contact with the ultimobranchial bodies developing from the 4th pharyngeal pouches, the so-called lateral anlage of the thyroid. The ultimobranchial cells or neural cells accompanying them are the origin of calcitonin-secreting C-cells in the thyroid gland and may contribute to the formation of follicular cells as well (105). By the 8th week the cells have a tubular arrangement, and cell clusters are apparent. Two weeks later, when the embryo is approximately 80 mm long, follicles are present. Shortly after this time the follicles contain colloid, and the thyroid accumulates and binds iodide by the 11th-12th week (Fig. 2). Secondary follicles arise by budding from the primary follicles; they increase in number until the embryo reaches a length of about 160 mm. After this time the follicles increase in size, but the number remains the same. Under intense stimulation, the adult thyroid can form new follicles.

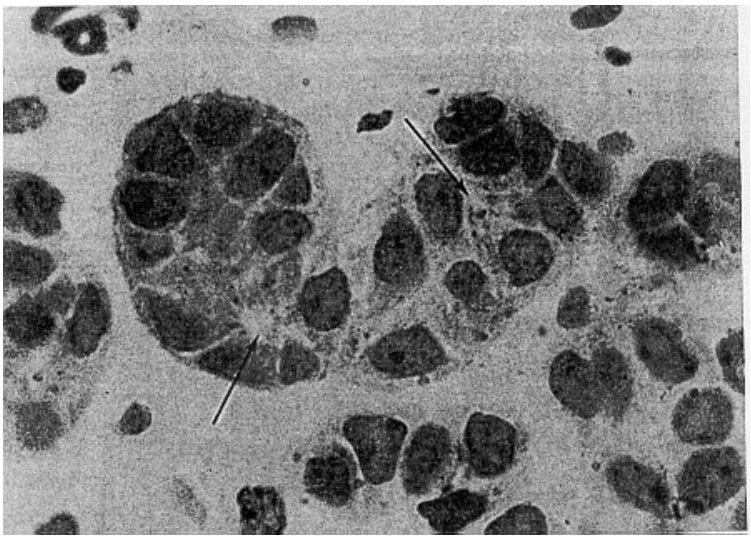


***Fig. 1-2.*** *Photograph of thyroid tissue from a fetus with a 50-mm crown-rump length, estimated gestational age 64 days. The arrows indicate two intracellular canaliculi. During incubation of the tissue in organ culture in vitro, there was no uptake or fixation of iodide. The figure shows the earliest stage of formation of colloid spaces. The tissue was fixed in ozmium, embedded in Epon epoxy resin, and sectioned at 1-mm thickness (x2,400). (From T.H. Shephard, J. Clin. Endocrinol. Metab., 27:945,1967, with permission).*

Fujita and Machino (106) have studied the origins of the follicular lumen in the chick embryo. They found that colloid droplets, 1-5μm in diameter and enclosed by a limiting membrane, first appear within the cytoplasm of parenchymal cells. As the droplets enlarge, they

approach the cell membrane and come in contact with the droplets of an adjoining cell. The limiting cell membrane disappears, and the droplets fuse. By an extension of this process to cells close to the original droplet, an acinar structure containing colloid and enclosed by a ring of parenchymal cells is formed. A similar process can be demonstrated in aggregates of isolated thyroid cells in vitro (107).

## GROSS ANATOMY

### Physical Appearance and Anatomic Location

The Germans call the thyroid the "shield gland" (Schilddrüse), and the English name, derived from the Greek, means the same thing. Such a term gives a most erroneous impression of its shape. It is interesting, however, that in the Minoan culture, a shield was used that had a shape somewhat like that of the mammalian thyroid gland. The gland as seen from the front is more nearly the shape of a butterfly. It wraps itself about and becomes firmly fixed by fibrous tissue to the anterior and lateral parts of the larynx and trachea. Anteriorly, its surface is convex; posteriorly, it is concave. The isthmus lies across the trachea anteriorly just below the level of the cricoid cartilage. The lateral lobes extend along either side of the larynx as roughly conical projections reaching the level of the middle of the thyroid cartilage. Their upper extremities are known as the upper poles of the gland. Similarly, the lower extremities of the lateral lobes are spoken of as the lower poles, although they make no such prominent projections as do the upper (Fig. 1-3).

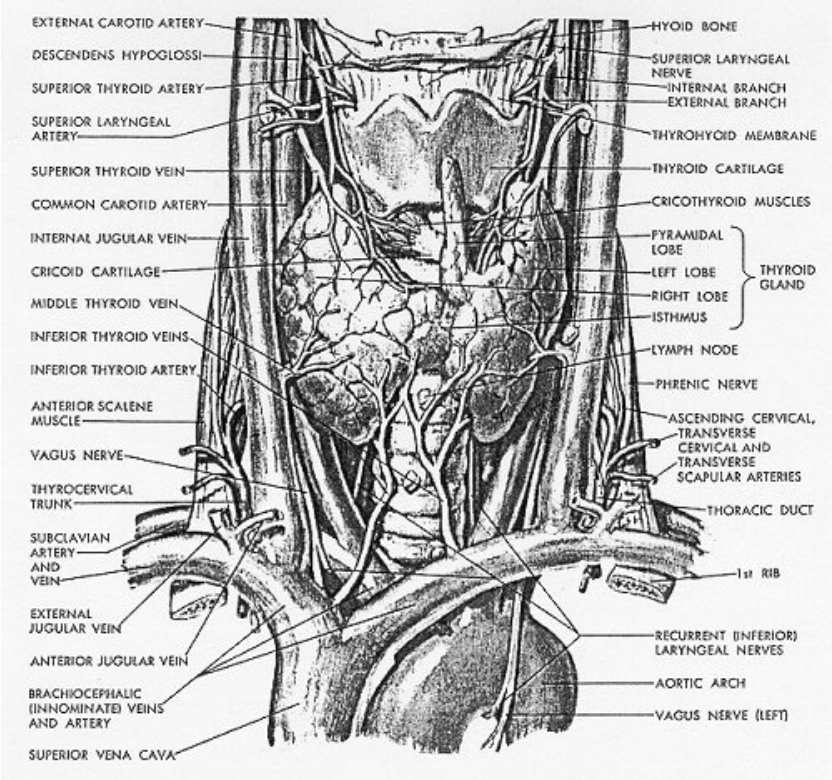



***Fig. 1-3**. Gross Anatomy of the thyroid and surroundings (from: Netter FH, The Ciba Collection of Medical Illustrations, vol. 4, Endocrine system and selected metabolic diseases, Ciba, 1965, with permission).*

The weight of the thyroid of the normal nongoitrous adult is 6-20 g depending on body size and iodine supply. The width and length of the isthmus average 20 mm, and its thickness is 2-6 mm. The lateral lobes from superior to inferior poles usually measure 4 cm. Their breadth is 15-20 mm, and their thickness is 20-39 mm.

The thyroid is enveloped by a thin, fibrous, nonstripping capsule that sends septa into the gland substance to produce an irregular, incomplete lobulation. No true lobulation or lobation exists. In fact, the gland is throughout a uniform agglomeration of follicles packed like berries into a bag. It has no true subdivisions. The lateral lobes lie in a bed between the trachea and the larynx medially and between the carotid sheath and the sternomastoid muscles laterally. The deep cervical fascia, dividing into an anterior and a posterior plane, lines this bed and makes a loosely applied false or surgical capsule for the lateral portions of the gland. In front are the thin, ribbon-like infrahyoid muscles. The thyroid is molded perfectly to fit the space available between the neighboring structures, and is superficially placed. It can usually be

outlined by careful palpation in normal humans, but if the neck is thick and short or the sternomastoid muscles heavily developed, it may be impossible to feel the gland.

The shape and attachments of the organ are important in examination and diagnosis. The relation of the thyroid gland to the parathyroids, which are usually situated on the posterior surface of the lateral lobes of the gland within the surgical capsule, and to the recurrent laryngeal nerves, which run in the cleft between the trachea and esophagus just medial to the lateral lobes, are most important to the surgeon. The relationship to the trachea is important from the point of view of pressure symptoms.

The pyramidal lobe is a narrow projection of thyroid tissue extending upward from the isthmus and lying on the surface of the thyroid cartilage, to the right or left of the prominence of that structure. It is a vestige of the embryonic thyroglossal tract. The importance of the pyramidal lobe is in its relation to developmental anomalies and also in its propensity to undergo hypertrophy when the rest of the thyroid has been removed. Any pathologic process that is diffuse may involve the pyramidal lobe, for example, Graves' disease or Hashimoto's thyroiditis. It becomes thus an item of some importance diagnostically and in thyroid surgery. A pyramidal lobe is found by the surgeon in about 80% of patients.

## Blood Supply

The thyroid gland has an abundant blood supply. It has been estimated that the normal flow rate is about 5 ml/g of thyroid tissue each minute. The blood volume of normal humans is about 5 liters and total blood flow 5 liters/min. This mass moves through the lungs about once a minute, through the kidneys once in five minutes, and through the thyroid approximately once an hour. Although the thyroid represents about 0.4% of body weight it accounts for 2% of total blood flow. In disease the flow through the gland may be increased up to 100-fold.

This abundant blood supply is provided from the four major thyroid arteries. The superior pair arise from the external carotid and descend several centimeters through the neck to reach the upper poles of the thyroid, where they break into a number of branches and enter the substance of the gland. The inferior pair spring from the thyrocervical trunk of the subclavian arteries and enter the lower poles from behind. Frequently, a fifth artery, the thyreoidea ima, from the arch of the aorta, enters the thyroid in the midline. There are free anastomoses between all of these vessels. In addition, a large number of smaller arteriolar vessels derived from collaterals of the esophagus and larynx supply the posterior aspect of the thyroid. The

branching of the large arteries takes place on the surface of the gland, where they form a network. Only after much branching are small arteries sent deep into the gland. These penetrating vessels arborize among the follicles, finally sending a follicular artery to each follicle. This, in turn, breaks up into the rich capillary basket like network surrounding the follicle.

The veins emerge from the interior of the gland and form a plexus of vessels under the capsule. These drain into the internal jugular, the brachiocephalic, and occasionally the anterior jugular veins.

The adult thyroid is composed of follicles, or acini. These have lost all luminal connection with other parts of the body and may be considered, from both the structural an functional points of view, as the primary, or secretory, units of the organ. The cells of the follicles are the makers of hormone; the lumina are the storage depots. In the normal adult gland the follicles are roughly spherical and vary considerably in size. The average diameter is 300 microns. The walls consist of a continuous epithelium one cell deep, the parenchyma of the thyroid. The epithelium of the normal gland is usually described as cuboidal, the cell height being of the order of 15 μm. In the resting gland the cells may become flatter. Under chronic TSH stimulation such as occurs with iodide deficiency, the height

## Lymphatics

A rich plexus of lymph vessels is in close approximation to the individual follicles, but no unique role in thyroid function has been assigned to this system. The major normal, if not only, secretory pathway for thyroid hormone is through the venous drainage of the thyroid rather than through the lymphatics but thyroglobulin appears in the lymph.

## Innervation

The gland receives fibers from both sympathetic and parasympathetic divisions of the autonomic nervous system. The sympathetic fibers are derived from the cervical ganglia and enter the gland along the blood vessels. The parasympathetic fibers are derived from the vagus and reach the gland by branches of the laryngeal nerves. Both myelinated and nonmyelinated fibers are found in the thyroid, and occasionally in the ganglion cells as well. The nerve supply does not appear to be simply a secretory system. The major neurogenic modifications of thyroid physiology have to do with blood flow and are reviewed in Chapter 4. However neurotransmitters have direct effects on thyroid follicular cells, which vary from one species to another. The physiological relevance of these effects remains to be proved.

## The Secretory Unit - The Follicle

The adult thyroid is composed of follicles, or acini. These have lost all luminal connection with other parts of the body and may be considered, from both the structural an functional points of view, as the primary, or secretory, units of the organ. The cells of the follicles are the makers of hormone; the lumina are the storage depots. In the normal adult gland the follicles are roughly spherical and vary considerably in size. The average diameter is 300 microns. The walls consist of a continuous epithelium one cell deep, the parenchyma of the thyroid. The epithelium of the normal gland is usually described as cuboidal, the cell height being of the order of 15 μm. In the resting gland the cells may become flatter. Under chronic TSH stimulation such as occurs with iodide deficiency, the height increases, and the term columnar is applied. Such stimulation, which increases colloid resorption, also leads to a reduction in size the follicular lumen. As a result, the height of the epithelium is often inversely proportional to the diameter of the lumen of the follicle.

Within the follicle and filling its lumen is the homogeneous colloid. This is a mixture of proteins, principally thyroglobulin, but there are other lightweight iodoproteins and serum proteins and albumin, originating from the serum, as well.

In addition to the acinar cells, there are individual cells or small groups of cells that are seen not to extend to the follicular lumen and which may appear as clusters between follicles (Fig. 1-4a,b). These light cells, or C-cells, are a distinct category probably derived from the neural crest via the ultimobranchial body, as shown by studies in quail chicks by Le Douarin and Le Lièvre (108). The C-cells secrete calcitonin (or "thyrocalcitonin") in response to an increase in serum calcium (109). This hormone is important in the regulation of bone resorption and to a lesser extent influences the concentration of serum calcium. Calcitonin acts primarily by suppressing resorption of calcium from bone and therefore lowers plasma free $Ca^{++}$ levels. C-cells also contain somatostatin, calcitonin gene related peptide, gastrin-releasing peptide, katacalcin and helodermin that could have either a stimulatory or inhibitory activity on thyroid hormone secretion. Their physiological relevance is doubtful (see "Other Regulatory Factors" in this chapter). The C-cells are also the origin of the "medullary" thyroid cancers. In adult human they represent 1% of the cell population.

Outside the follicles two other types of cells populate the thyroid the endothelial cells and fibroblasts. In normal dog thyroids the relative proportions of follicular, endothelial cells and fibroblasts are 70%, 6% and 24% (110).

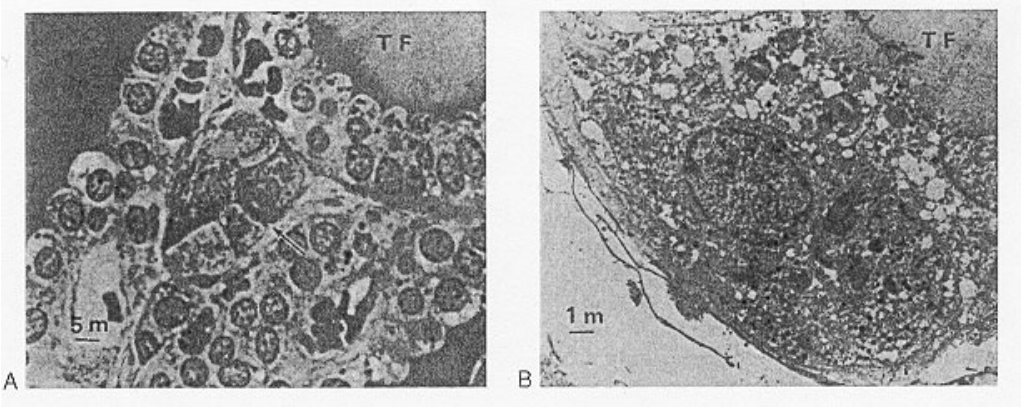



***Fig. 1-4.*** *(A) Light microscopy of a parafollicular cluster (arrow) in relationship to thyroid follicle (TF) (x900). (B) Parafollicular cell in characteristic position between follicular cells and follicular basement membrane, not abutting on colloid (TF) (x4,200). Tissue was obtained from normal thyroid tissue of a 26-year-old woman operated on because of a solitary thyroid adenomatous nodule. Specimens were fixed in glutaraldehyde and embedded in Araldite-502. (From Teitelbaum et al. Nature, 230,1971, with permission).*

## THE FINE STRUCTURE OF THE THYROID CELLS

The follicular organization and the polarity of the thyrocytes are essential to the specialized metabolism of the organ : with the vectorial transport of thyroglobulin and iodide at the apex, the synthesis of thyroid hormones at the apical membrane, the storage of iodine and thyroid hormone within thyroglobulin in the lumen and endocytosis of thyroglobulin also at the apex. The onset of thyroid function in embryo coincides with the appearance of this structure.

The acinar surface of thyroid parenchymal cells appears to be smooth in the light microscope, but the electron microscope shows that it is covered with tiny villi and some pseudopods. Each cell displays a cilium in the follicular lumen. The base of the cell abuts on a capillary and is separated from it by a two-layer basement membrane visible under the electron microscope. In the usual hematoxylin and eosin stain, the cell cytoplasm is neutrophilic, and colloid droplets may be present. The nucleus is at the base of the cell.

The colloid is variable in tinctorial response but tends to be strongly eosinophilic in resting follicles and pale-staining or even slightly basophilic when the gland is stimulated. In hyperactive follicles the margin of the colloid is scalloped by resorption vacuoles. These vacuoles may represent the "negatives" of the resorption process.

The villi are extensions of cytoplasm which increase cell secretory surface. In acutely stimulated thyroids, pseudopods extend out into the colloid and surround and ingest it by macropinocytosis. Over the course of several hours, the ingested droplets move toward the base of the cell (111). These droplets of resorbed colloid are processed for secretion as hormone by the gland (112).

The resolving power of the electron microscope has been turned upon the thyroid acinar cell by several investigators, among them Wissig (113), Dempsey and Peterson (114), Ekholm and Sjöstrand (115) and Herman (116). Wissig's and Ekholm's findings are presented here in detail and can be taken as typical of the cytologic picture of most species. The entire follicular cell is covered by an uninterrupted plasma membrane (Fig. 1-5 and Fig. 1-6). The apical surface of the cell is dome-shaped and is provided with numerous microvilli that are approximately 0.35 mm tall and 0.07 mm broad. This membrane is composed of two dark layers separated by a single pale layer and is 70 Å thick. Terminal bars join opposing cells at the apical margin, and desmosomes often occur on contacting cell surfaces. Vesicular structures, approximately 60 mm broad, appear in the microvilli and contain material that has the same density as colloid. Beneath the apical border there is a band of cytoplasm that is approximately 0.5 mm wide and devoid of organelles, although microtubular and microfilamentous structures are seen in this area. Beneath this band, a few apical vesicles of 400-15,000 Å are seen, and beneath this area and extending to the base of the cell are the channels of the endoplasmic reticulum, also known as ergoplasmic vesicles. These vesicles, or channels, are limited by a single membrane (the a cytomembrane) approximately 60-70 Å thick, and their outer surface is studded with ribosomes approximately 130-150 Å in diameter. In some areas the membrane covering the cytomembrane is devoid of ribonucleoprotein particles, and in between the vesicles the ribonucleoprotein granules may be seen to lie free. The endoplasmic vesicles are very pleiomorphic. Small vesicles are seen near the apical surface, 50 nm to several microns in diameter, and closed by a single-layer membrane 5 nm in thickness. These droplets appear especially in the apex of the cell and are thought to be secretion droplets. The material within them is frequently quite dense. Large vesicles of up to 1 micron appear especially in stimulated thyroids. These are called colloid droplets because the material within the vesicles is homogeneous and has the density of colloid and results from colloid endocytosis.

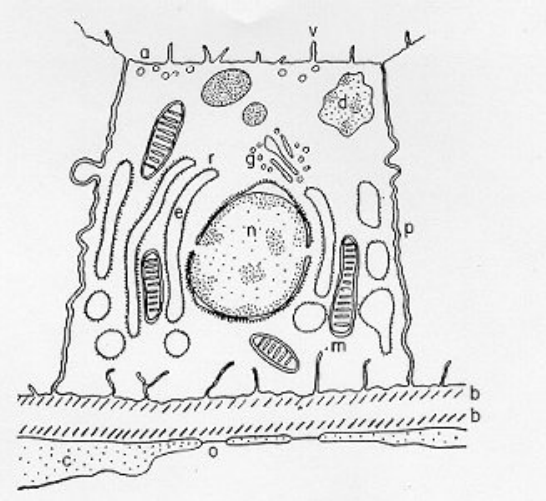



***Fig. 1-5**. A thyroid follicular cell, including: (a) apical vessel of cell; (e) endoplasmic reticulum; (d) colloid droplets; (v) microvilli; (r) ribosomes on endoplasmic reticulum; (g) Golgi apparatus; (m) mitochondrion; (p) plasma membrane; (c) capillary cells; (n) nucleus; (b) basement membrane; (o) open "pore" endothelial cells ; (c) cilium. (Reproduced by permission of the Journal of Ultrastructural Research).*

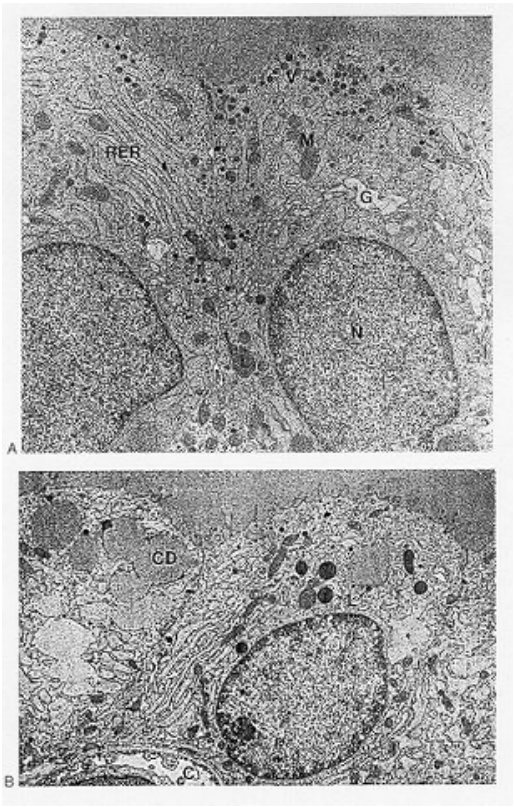



***Fig. 1-6.*** *Electron micrographs of rat thyroid. (a) Appearance after inactivation of the gland by two daily doses of T4. The micrograph shows two cell nuclei (N), well-developed rough-surfaced endoplasmic reticulum (RER), Golgi apparatus (G), mitochondria (M), lyosomes (L), and numerous dense, apical (exocytocic) vesicles (V). Because of the T4-induced TSH suppression, no colloid droplets are present (no hormone release); TG synthesis, however, is still going on, as indicated by the dense apical vesicle. (b) Appearance 20 minutes after intravenous administration of TSH (100 mU) to a rat treated with T4 for two days. The most characteristic features of these cells are the large number of colloid droplets (CD) and the almost complete disappearance of dense apical vesicles; TSH has induced an emptying of these vesicles into the follicle lumen. Other organelles are similar to those in Fig. 1-2. Note the close relation relation between colloid droplets and lysosomes (L). At the base of the follicle cells part of a blood capillary (C) is seen. (Micrographs kindly supplied by Professor Ragnar Ekholm, Goteborg, Sweden).*
*The Golgi apparatus is located near the nucleus and consists of small vacuoles and vesicles 400-800 Å in diameter. No nucleoprotein granules are found on the surface of these vesicles. The content of the Golgi vesicles has a density similar to that of secretion droplets.*

Numerous rod-shaped or irregular mitochondria are present. Their average diameter is 0.2 mm. They are bordered by a triple-layered membrane 160 Å in width consisting of two opaque layers and a less opaque interposed layer. The inner opaque layer is thrown up into folds, or cristae, which run irregularly, either in the long or the short axis of the mitochondrion.

The nucleus is enclosed within a double-walled envelope whose layers are separated by a less dense area approximately 200 Å thick. The outer nuclear membrane is continuous with the membranes forming the endoplasmic reticulum. The nuclear envelope has characteristic pores 400 Å in diameter.

The abutting plasma membranes of adjacent cells parallel one another and are about 70 Å thick. They are separated by a space 150 Å wide, which contains a material of the same density as the basement membrane. The membrane at the base of the cell is covered on the outer surface by a basement membrane that is approximately 400 Å in width. A thin layer of fibers about 400 Å in diameter may occur at the outer surface of the basement membrane. The basement membrane of the follicular cell is separated by a clear area from the basement membrane of the opposing capillary endothelium. At frequent intervals, the wall of the endothelial cell is interrupted by a pore approximately 450 Å in diameter. Here the lumen of the capillary appears to be in direct contact with the basement membrane of the endothelial cell. The thyroid follicle cells are separated by two layers of basement membrane from the capillaries, but the pores in the endothelial lining of the capillaries may allow, some plasma to come in direct contact with basement membrane. This arrangement should allow free diffusion of materials into and out of the acinar cell.

Ribosomes of the ergastoplasm synthesize thyroglobulin which is processed in the smooth reticulum and Golgi apparatus.

The colloid lumen is sealed by various cell-cell junctions: 1) the tight junctions of the zonula occludens, close to the apical border and which separate the basal from the apical membrane (main protein constituents : occludins); 2) further from the apex are the tight junctions (main protein components : cadherins) and 3) further the desmosome junctions (main protein components : desmogleins and desmocollins). All these junctions are linked to the cytoskeleton. Gap junctions (main protein components : connexins) provide joint channels allowing the passage of small molecules between the cells.

## GENERAL METABOLISM OF THE FOLLICULAR THYROID CELL

The metabolism of the thyroid as related to hormone synthesis and secretion is discussed in Chapter 2. In this section, a review of some general aspects of metabolism of the thyroid acinar cell is provided. The metabolism of the thyroid has been studied by all the usual techniques - in vivo in men and mice, in situ, in in vitro perfusion, in slices, cells, homogenates, or subcellular fractions. Several species, including humans, have been investigated, often with obvious and consistent species-related differences. Conditions of tissue preparation and assays have varied widely.

### Energy metabolism

Energy supply in the human thyroid cell is necessary for many activities like synthesis of nucleotides, proteins, nuclear acids, lipids, transport functions and other activities like phagocytosis, lysosome movement etc. It is mainly produced by mitochondrial oxidative phosphorylation (about 85%) and to a minor extent by cytosolic aerobic glycolysis (117). Energy metabolism in the human thyroid cell resembles in many aspects that in the dog thyroid. However, the absolute values of oxygen uptake, glucose uptake and lactate formation are significantly less in the human thyroid slices. Adenosine triphosphate (ATP) concentration in the thyroid cell is in the millimolar range and about 90% of ribonucleotides are in the form of triphosphates (118). Free fatty acids are probably the main source of energy in thyroid cells as respiration is maintained for long periods in vitro in the absence of exogenous substrate. As there is hardly any glycogen present in these conditions, most probably free fatty acids are the endogenous substrates. Compartmentation may make glycolytic ATP the main energy source for some membrane functions such as endocytosis of colloid. Indeed inhibition of glycolysis inhibits colloid endocytosis much more than total energy metabolism and addition of glucose counteracts this effect (118).

Mitochondrial inhibitors abolish the stimulatory effect of TSH on thyroid cell respiration (119). Cyclic AMP does not influence mitochondrial respiration in a direct manner. It is therefore assumed that the stimulatory effect of TSH on respiration is secondary to its enhancing effect on energy (i.e. ATP) consuming cellular processes.

### Carbohydrate metabolism

The main source of energy delivery for metabolic processes in the thyroid cell are free fatty acids. However, glucose metabolism has an important function in the thyroid for several reasons. About 70% of the glucose taken up by dog or human thyroid slices is transformed to

lactate, a further 5% is catabolized through the Embden-Meyerhof pathway and the Krebs cycle (120). Another 6% of glucose carbon is incorporated into protein and less than 1% into lipids and glycogen. The remaining part (about 10%) is oxidized through the hexose monophosphate pathway (HMP). Most of the enzymes participating in the Embden-Meyerhof pathway, HMP and Krebs cycle have been demonstrated in the human thyroid (121) (122) (123). As hexokinase instead of glucokinase is present in the thyroid, the rate of phosphorylation of glucose is probably independent of its concentration because of the low Km of hexokinase for glucose.

Glucose metabolism serves several purposes. The incorporation of glucose carbon into proteins is related to the function of the thyroid cell in protein synthesis i.e. synthesis of thyrogobulin which contains 10% carbohydrate. The metabolism of glucose along the HMP is related to the generation of NADPH and pentoses in this pathway. The production of pentoses is obviously necessary for generation of nucleotides. NADPH production is necessary in several respects (Fig. 7). It is needed for generation of H2O2 for oxidation, organification of iodide and thyroid hormone synthesis. NADPH is also an important cofactor in iodotyrosines deiodination. It is also needed to reduce oxidized glutathione after its generation by GSH peroxidase in the detoxification of the H2O2 leaking in the cell (124).

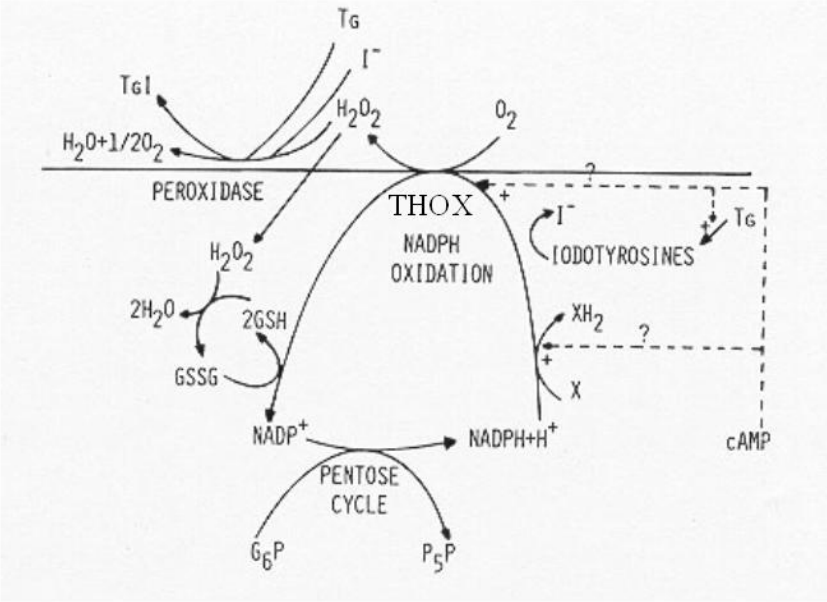



***Fig. 1-7.*** *Postulated NADP oxidation-reduction cycle in thyroid. Four*

*mechanisms of NADPH oxidation are outlined: the reduction of any intermediate X by an NADPH-linked dehydrogenase, the deiodination of iodotyrosines released by thyroglobulinolysis, the generation of H2O2 by THOX (thyroid H2O2 generating enzyme), and the reduction of H2O2 through GSH peroxidase. TG and TGI: uniodinated and iodinated thyroglobulin. +, activation. (From: Dumont JE (124).*

H2O2 generation is stimulated by TSH through cAMP in dog thyroid and by $Ca^{++}$ and diacylglycerol in all investigated species, including humans and dogs.

TSH enhances carbohydrate metabolism in the dog thyroid (120). During the stimulation there is selective increase in the activity of the HMP whereas incorporation of glucose into proteins and lipids decreases (120). The activity of TSH in this metabolism is probably mediated by cAMP, since this nucleotide can reproduce the TSH effects on glucose uptake, catabolism, incorporation in protein and lipids and on the HMP pathway. TSH also causes an increase in NADPH and NADP+ concentration through increased NAD+ kinase activity (124).

The increased metabolic activity induced by TSH mainly reflects increased consumption by NADPH dependent processes stimulated via cAMP. For instance, the activity of the HMP pathway is predominantly dependent on the availability of the substrate NADP+ generated during oxidation of NADPH (124).

## Mitochondrial Respiration

The mitochondrion has appropriately been termed the "powerhouse" of the cell. It provides about 85% of generated ATP in the thyroid cell, only 15% coming from glycolysis. The thyroids of different animal species contain mitochondria with their typical morphology, having electron transport chain, Krebs cycle enzymes, coupled oxidative phosphorylation and good respiratory control (119). The activity of mitochondria is controlled by adenosine diphosphate (ADP) levels. Also respiration linked Ca++ accumulation plays a general and fundamental role in vertebrate cell physiology (125). Free fatty acids are the preferred substrate of oxidation in the unstimulated thyroid, presumably through mitochondrial pathways (126). In thyroids of patients operated for hyperthyroid Graves' disease all enzyme activities studied were increased suggesting an increase in the mitochondrial population in chronically stimulated thyroid cells (118). TSH increases oxygen consumption in thyroid slices by 20 - 30% within a few minutes, independently of exogenous substrates. The increased respiration is oligomycin and antimycin sensitive. Thus, respiration is of largely mitochondrial origin and probably represents the effect of TSH in increasing metabolic

activities and consequently ATP consumption (see section on Energy Metabolism) (124). TSH augments oxidation of pyruvate and acetate by thyroid slices. Compounds such as perchlorate, methimazole, iodide, thiocyanate and T4 have no significant direct action on thyroid mitochondria (119). In isolated thyroid mitochodria protein synthesis is dependent on intact electron transport and oxidative phosphorylation. It is inhibited by chloramphenicol but not by cycloheximide (127).

## RNA and DNA Metabolism

Chronic TSH stimulation produces cell hypertrophy, and proliferation with a greater increase of RNA than of DNA. Since RNA and DNA synthesis are required for cell growth and division, it is not surprising that TSH stimulation causes rapid and continued increases in synthetic activities. When given in vivo, TSH stimulates uptake and incorporation of RNA precursors within one hour and net RNA increases in about 12 hours (128) (129). TSH stimulates cell uptake and synthesis of purine and pyrimidine precursors (130) (131) and purine and pyrimidine synthesis. Synthesis of both messenger RNA (mRNA) and ribosomal RNA (rRNA) is stimulated by TSH (132). The population of mRNA preferentially synthesized in response to TSH and cyclic AMP is important and includes specific thyroid gene expression such as thyroperoxidase (TPO) Na+/I- cotransporters (NIS) (133), thyroglobulin etc.. RNA degradation is not known to be influenced by TSH.

Formation of polyamines is closely linked to cell growth, although the mechanism is not known. TSH and cAMP enhance ornithine decarboxylase activity, the rate-limiting enzyme in polyamine synthesis (134).

## Protein Metabolism

Thyroid tissue is composed of cells and a storage protein, and the kinetic behavior of each compartment varies enormously with the conditions. Thus, in the colloid especially, protein storage and degradation go on concurrently, and content at any time reflects a balance between these activities.

TSH enhances uptake of amino acids by isolated thyroid cells, and stimulates protein synthesis within 30 minutes to 4 hours in some preparations. Because of effects on thyroglobulin (TG) degradation, and dilution of amino acid precursor pools, stimulation of synthesis is more difficult to demonstrate in whole tissues (135) (136) (137). However, if thyroid slices are incubated in high concentration of leucine to obliterate any separate effect

of TSH on cell uptake of amino acid, a clear stimulation of protein synthesis by TSH can be demonstrated in vitro in dog thyroid slices (137), and also in isolated thyroid cells but not in primary cultures of dog thyroid cells. Within 12-24 hours of chronic TSH stimulation in vivo, net protein content may be decreased by active TG hydrolysis, but after this, protein content is increased (138). This response remains nearly linear over four to five weeks as thyroid size in animals quintuples. The response is primarily due to production of new cells, since DNA and protein change in parallel.

Huge polysomes (40 to 80 ribosomal units) connected by mRNA have been demonstrated in the thyroid (139) (140) and were shown to incorporate precursors into TG-related peptides. (Fig.1-8). In dog thyroid slices, TSH also shifts thyroid monosomes to polysomes, and this is stimulated by cAMP. This action suggests a direct effect on translation (141).

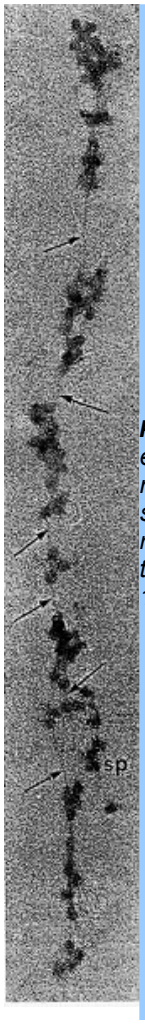



***Fig. 1-8***. *Electron microscopic photograph of an enormous polysome containing 60 or 70 monosomes, the presumed source of TG synthesis. The arrows point to a thread possibly representing mRNA holding the polysome together. (From Keyhani et al., J. Microscop., 10:269,1971, with permission).*

## Lipid metabolism

Free fatty acids are the main fuel of the thyroid cell and they may be completely oxidized. Sufficient endogenous substrate is present to sustain respiration for several hours during in vitro incubation of thyroid slices (142) (143) (144). Studies on localization of lipids in human thyroids have shown that small amounts are only present in goitres from thyrotoxic patients, but that appreciable amounts are present in the normal human thyroid i.e. phospholipids, cholesterol and gangliosides: 5.2, 4.3 and 0.12 mmol/kg fresh tissue. C-cells contain most abundantly phospholipids. The human thyroid contains phospholipids in the proportion: phosphatidylcholine (41.8%), phosphathidylethanolamine (26.9%), phosphathidylserine (10.4%), phosphathidylinositol (4.4%), cardiolipin (3.4%), sphyngomyelin (12.4%) (145) (146) (147). TSH enhances the incorporation of precursors into most phospholipids. The effect is believed to reflect a direct stimulation of synthesis of phospholipids. However, as TSH also stimulates phospholipid degradation, increased phospholipids synthesis under the influence of TSH could correspond in part to this accelerated turnover rather than to an accumulation {123}. TSH also stimulates incorporation of inositol into phosphoinositides in a glucose free system. TSH specifically enhances synthesis of phosphatidic acid from glycerophosphate after in vivo administration (148) (149).

## Electrolyte Transport and metabolism

The mean resting transmembrane potential as studied in rat, rabbit and guinea-pigs thyroid cells varies between -60 and -70 mV. The magnitude of the membrane potential was found te be dependent mainly upon the gradient for K+ across the membrane. A high intracellular K+ and low Na+ concentration is maintained by ouabain sensitive Na+ - K+ ATPase. The activity of this ATPase varies in direct relation to chronic TSH stimulation, probably corresponding to cell hypertrophy and hyperplasia. There is no evidence for direct action of TSH on this enzyme. Acute stimulation of thyroid cells induces a depolarization of the cell, which is accompanied by a decrease in membrane resistance. The depolarization may correspond to increased permeability to predominantly extracellular cations, such as Na+ or to a decreased permeability to predominantly intracellular cations, such as K+. Administration of TSH or veratridine, a sodium channel agonist, depolarized cultured thyroid cells and increased the secretion of radioiodine from the organically bound pool. Depolarization of the cells by increasing the potassium concentration in the medium failed to promote secretion of radioactive iodine indicating that the sodium influx rather than the depolarization itself, may mediate the secretory response (124).

# THYROID REGULATORY FACTORS

## In Physiology

Four major biologic variables are regulated in the thyrocyte as in any other cell type: function, cell size, cell number, and differentiation. The first three variables are quantitative and the latter is qualitative. In this chapter we consider the factors involved in these controls in physiology and in pathology, the main regulatory cascades through which these factors exert their effects, and the regulated processes, which are function, proliferation and cell death, gene expression, and differentiation. Whenever possible, we describe what is known in humans.

The two main factors that control the physiology of the thyroid after embryogenesis are the requirement for thyroid hormones and the supply of its main and specialized substrate iodide (Table 1-1). Thyroid hormone plasma levels and action are monitored by the hypothalamic supraoptic nuclei and by the thyrotrophs of the anterior lobe of the pituitary, where they exert a negative feedback. The corresponding homeostatic control is expressed by thyroid-stimulating hormone (TSH, thyrotropin). The hypophysis adjusts its secretion of TSH to the sensitivity of the thyroid, increasing TSH levels when thyrocyte sensitivity decreases (e.g. because of reduced TSH receptor expression) (150) . The TSH receptor is also stimulated by a new different natural hormone cloned by homology, thyrostimuline. The physiological role of this protein is unknown but its level is not controlled by a thyroid hormone feedback and it does not participate in the homeostatic control of the thyroid (151). Iodide supply is monitored in part through its effects on the plasma level of thyroid hormones, but mainly in the thyroid itself, where it depresses various aspects of thyroid function and the response of the thyrocyte to TSH. These two major physiologic regulators control the function and size of the thyroid - TSH positively, iodide negatively (124;152-154). These are the specific controls exerted at the level of the thyrocyte itself. The follicular cells themselves probably regulate the other thyroid cells, fibroblasts and endothelial cells through local extracellular signals such as NO, prostaglandins, growth factors etc.

**TABLE 1-1 THYROID REGULATORY FACTORS**

| | FUNCTION | DIFFERENTIATION | PROLIFERATION |
|---|---|---|---|
| Specific | | | |
| *Physiologic* | | | |
| TSH | ↗ | ↗ | ↗ |
| Thyrostimuline | ↗ | ↗ | ↗ |
| LH, hCG (high levels) | ↗ | ↗ | ↗ |
| $I^-$ | ↘ | ? | ↘ |
| $T_3$, $T_4$ | ? | ? | ↘? |
| *Pathologic* | | | |
| TSAb | ↗ | ↗ | ↗ |
| TSBAb | ↘ | ? | ↘ |
| Non Specific | | | |
| *Physiologic* | | | |
| Hydrocortisone | 0 | ↗ | 0 |
| $IGF_1$ | ? | ↗ | ↗ |
| EGF | ↘/0 | ↘ | ↗ |
| FGF | ? | ↘/0 | ↗ |
| TGFβ | ? | ↘ | ↘ |
| Norepinephrine | ↗ | 0 | 0 |
| PGE | ↗ | 0 | 0 |
| ATP,Bradykinin, TRH | ↗/↘ | ? | ? |
| *Pathologic* | | | |
| IL1 | ↘ | ↘ | ↘ |
| TNF | ↘ | ↘ | ↘ |
| IFNγ | ↘ | ↘ | ↘ |

↗, stimulation ; ↘, inhibition ; 0, no effect;EGF, epidermal growth factor; FGF, fibroblast growth factor; hCG, human chorionic gonadotropin; IFN, interferon; IGF, insulin-like growth factor; IL, interleukin; LH, luteinizing hormone ; PGF, prostaglandin F; T3, triiodothyronine; T4, thyroxine; TSBAb, thyroid-blocking Antibody; TGF, transforming growth factor; TRH, thyrotropin-releasing hormone ; TSAb, thyroid-stimulating antibodies.

In mice embryo, other unknown factors control differentiation and organ growth which takes place normally in the absence of TSH receptor (155;156). However, homozygous inactivating mutations of the TSH receptor in familial congenital hypothyroidism were found to be associated with a very hypoplastic thyroid gland (157). Although the thyroid contains receptors for thyroid hormones and a direct effect of these hormones on thyrocytes would make sense (158), as yet little evidence has indicated that such control plays a role in physiology (159). However expression of dominant negative thyroid hormone receptors in mice represses PPARγ expression and induces thyroid tumors in thyroid (160). Luteinizing hormone (LH) and human chorionic gonadotropin (hCG) at high levels directly stimulate the thyroid, and this effect accounts for the depression of TSH levels and sometimes elevated thyroid activity at the beginning of pregnancy (161-163).

The thyroid gland is also influenced by various other nonspecific hormones (164). Hydrocortisone exerts a differentiating action in vitro (165). Estrogens affect the thyroid by unknown mechanisms, directly or indirectly, as exemplified clinically in the menstrual cycle and in pregnancy. Growth hormone induces thyroid growth, but its effects are thought to be mediated by locally produced somatomedins (IGF-I). Nevertheless the presence of basal TSH levels might be a prerequisite for the growth promoting action of IGF-I, because a GH replacement therapy did not increase thyroid size in patients deficient for both GH and TSH (166). The anomalously low endemic goiter prevalence among pygmies living in iodine deficient areas (167), who are genetically resistant to IGF-I, is also compatible with an in vivo

permissive effect of IGF-1 and IGF-1 receptor on TSH mitogenic action. Indeed thyroids of transgenic mice overexpressing IGF1 and IGF1 receptor develop hyperplasia and a degree of autonomy vs TSH: their serum TSH is lower and thyroid hormones level normal which shows that they require less TSH to maintain normal thyroid hormone levels (168;169). In dog and human thyroid primary cultures, the presence of insulin receptors strictly depends on TSH, suggesting that thyroid might be a more specific target of insulin than generally considered (170;171). It is permissive for TSH mitogenic action in vitro.

Effects of locally secreted neurotransmitters and growth factors on thyrocytes have been demonstrated in vitro and sometimes in vivo, and the presence of some of these agents in the thyroid has been ascertained. The set of neurotransmitters acting on the thyrocyte and their effects vary from species to species (152;172). In human cells, well-defined direct, but short-lived responses to norepinephrine, ATP, adenosine, bradykinin, and thyrotropin-releasing hormone (TRH) have been observed (152;173;174). In rat, as evidenced by superior cervical ganglion decentralization, sympathetic activity positively modulates function and size of the thyroid (175).

Growth factor signaling cascades demonstrated in vitro can exert similar effects in vivo. In nude mice, the injection of EGF promotes DNA synthesis in thyroid and inhibits iodide uptake in xenotransplanted rat (176) and human thyroid tissues (177). By contrast, the injection of FGF induces a colloid goiter in mice with no inhibition of iodide metabolism or thyroglobulin and thyroperoxidase mRNA accumulation (178). These effects are the exact replica of initial observations from the dog thyroid primary culture system (179) and other thyroid primary culture systems (180-183). EGF and FGF have since been reported to be locally synthesized in the thyroid gland, as a possible response to thyroxine and TSH (184) respectively. Their exact role as autocrine and/or paracrine agents in the development, function and pathology of the thyroid gland of different species has yet to be clarified (185;186). HGF does not activate mitogenesis in normal human thyrocyte. The Transforming Growth Factors □(TGF)β constitute another category of cytokines that are produced locally by thyrocytes and influence their proliferation and the action of mitogenic factors (185;187). TGFβ inhibits proliferation and prevents most of the effects of TSH and cAMP in human thyrocytes in vitro (188;189). TGFβ is synthesized as an inactive precursor which can be activated by different proteases produced by thyrocytes. TGFβ expression is upregulated during TSH-induced thyroid hyperplasia in rats, suggesting an autocrine mechanism limiting goiter size (190). Activin A and the bone morphogenetic peptide (BMP), which are related to TGFβ, □□are also present in thyroid and inhibit thyrocyte proliferation in vitro (191). Unlike TGFβ, they are directly synthesized as an active form. Elements of a Wnt/β catenin signaling pathway (Wnt factors, Frizzled receptors and disheveled isoforms) have been identified in human thyroid and

thyroid cancer cell lines (192). The eventual role in vivo in humans of most of these factors remains to be proved and clarified.

Thyroglobulin has been reported as a negative feedback inhibitor repressing the expression of specific thyroid transcription factors TTF1, TTF2, Pax8 and acting through a putative receptor at the apical membrane (193). However, as previous claims by the same group (the exophtalmic producing factor, ganglioside as the TSH receptor, etc) this one is neither substantiated nor supported by others.

Human thyroid cells contain androgen and estrogen receptors (194). Estrogens promote the growth of these cells (195) which may explain the higher prevalence of thyroid tumors and diseases in women, particularly between puberty and menopause.

## In Pathology

Mutated constitutively active TSH receptors and Gs proteins cause thyroid autonomous adenomas (196;197). Mutations conferring higher sensitivity of the TSH receptor to LH/HCG cause hyperthyroidism in pregnancy (198) (199). Pathologic extracellular signals play an important role in autoimmune thyroid disease. Thyroid-stimulating antibodies (TSAbs), which bind to the TSH receptor and activate it, reproduce the stimulatory effects of TSH on the function and growth of the tissue. Their abnormal generation is responsible for the hyperthyroidism and goiter of Graves' disease. The kinetic characteristics of TSH and TSAbs differ: TSH effects on camp accumulation are rapid and disappear rapidly in the absence of the hormone (minutes) while TSAbs effects are slow and persistent (hours) (200). Thyroid-blocking antibodies (TBAbs) also bind to the TSH receptor but do not activate it and hence behave as competitive inhibitors of the hormone. Such antibodies are responsible for some cases of hypothyroidism in thyroiditis. Both stimulating and inhibitory antibodies induce transient hyperthyroidism or hypothyroidism in newborns of mothers with positive sera (153). The existence of thyroid growth immunoglobulins has been hypothesized to explain the existence of Graves' disease with weak hyperthyroidism and prominent goiter (201). The thyroid specificity of such immunoglobulins would imply that they recognize thyroid-specific targets. This hypothesis is now abandoned (202-204). Discrepancies between growth and functional stimulation may instead reflect cell intrinsic factors. Local cytokines have been shown to influence, mostly negatively, the function, growth, and differentiation of thyrocytes in vitro and thyroid function in vivo. Because they are presumably secreted in loco in autoimmune thyroid diseases, these effects might play a role in the pathology of these diseases, but this notion has not yet been proved (152) (164). Moreover in selenium

deficiency secretion of TGFβ by macrophages has been implicated in the generation of thyroid fibrosis (205) (206) and the pathogenesis of thyroid failure in endemic cretinism. The overexpression of both FGF and FGF receptor 1 in thyrocytes from human multinodular goiter might explain their relative TSH-independence (207). 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 (208) and TRK (209), the overexpression of Met/HGF receptor sometimes in association with HGF (210), or erbB/EGF receptor in association with its ligand TGFα (211) can be causally associated with TSH-independent thyroid papillary carcinomas. An autocrine loop involving IGF-II and the insulin receptor isoform-A is also proposed to stimulate growth of some thyroid cancers (212). Thyroid cancer cells often escape growth inhibition by TGFβ (213).

## REGULATORY CASCADES

The great number of extracellular signals acting through specific receptors on cells in fact control a very limited number of regulatory cascades. We first outline these cascades, along with the signals that control them, and then describe in more detail the specific thyroid cell features: controls by iodide and the TSH receptor.

### The Cyclic Adenosine Monophosphate Cascade

The cyclic adenosine monophosphate (cAMP) cascade in the thyroid corresponds, as far as it has been studied, to the canonic model of the β-adrenergic receptor cascade (153) (Fig. 1-9). It is activated in the human thyrocyte by the TSH and the β-adrenergic and prostaglandin E receptors. These receptors are classic seven-transmembrane receptors controlling transducing guanosine triphosphate (GTP)-binding proteins. Activated G proteins belong to the $G_s$ class and activate adenylyl cyclase; they are composed of a distinct $\alpha_s$ subunit and nonspecific β and γ monomers. Activation of a G protein corresponds to its release of guanosine diphosphate (GDP) and binding of GTP and to its dissociation into $\alpha_{GTP}$ and βγ dimers. $\alpha_{sGTP}$ directly binds to and activates adenylyl cyclase. Inactivation of the G protein follows the spontaneous, more or less rapid hydrolysis of GTP to GDP by $\alpha_s$ GTPase activity and the reassociation of $\alpha_{GDP}$ with βγ. The effect of stimulation of the receptor by agonist binding is to increase the rate of GDP release and GTP binding, thus shifting the equilibrium of the cycle toward the $\alpha_{GTP}$ active form. One receptor can consecutively activate several G proteins (hit-and-run model). The thyroid contains mainly three isoforms of adenylyl cyclase : III, VI and IX (214). A similar system negatively controls adenylyl cyclase through $G_i$. It is stimulated in the human thyroid by norepinephrine through $\alpha_2$-receptors. Adenosine at high concentrations directly inhibits adenylyl cyclase. The cAMP generated by adenylyl cyclase

binds to the regulatory subunit of protein kinase A (PKA) that is blocking the catalytic subunit and releases this now-active unit. The activated, released catalytic unit of protein kinase phosphorylates serines and threonines in the set of proteins containing accessible specific peptides that it recognizes. These phosphorylations, through more or less complicated cascades, lead to the observed effects of the cascade. cAMP-dependent kinases have two isoenzymes (I, II), the first of which is more sensitive to cAMP, but as yet no clear specificity of action of these kinases has been demonstrated. In the case of the thyroid, this cascade is activated through specific receptors, by TSH in all species, and by norepinephrine receptors and prostaglandin E in humans, with widely different kinetics: prolonged for TSH and short lived (minutes) for norepinephrine and prostaglandins (215). Other neurotransmitters have been reported to activate the cascade in thyroid tissue, but not necessarily in the thyrocytes of the tissue (174). Besides PKA, in the thyroid cAMP activates EPAC (Exchange Proteins directly Activated by cAMP) or Rap guanosine nucleotide exchange factor-1 (GEF-1) and the less abundant GEF-2, which activate the small G protein Rap (216). However, despite high expression of EPAC1 in thyrocytes and its further increase in response to TSH, all the physiologically relevant cAMP-dependent functions of TSH studied in dog thyroid cells, including acute regulation of cell functions (including thyroid hormone secretion) and delayed stimulation of differentiation expression and mitogenesis, are mediated only by PKA activation (217). The role of the cAMP/EPAC/Rap cascade in thyroid thus remains largely unknown. Activation of PKA inactivates small G proteins of the Rho family (RhoA, Rac1 and Cdc42), which reorganizes the actin cytoskeleton and could play an important role in stimulation of thyroid hormone secretion and induction of thyroid differentiation genes (218). Of the other known possible effectors of camp, cyclic guanosine monophosphate (cGMP-dependent protein kinases are present in the thyroid but cyclic nucleotide-activated channels have not been looked for. For several effects of cyclic AMP (eg NIS and thyroglobulin induction, DNA synthesis) protein kinase A is required.

The cAMP cascade is also controlled by several negative feedbacks. The most important is the activation and induction by PKA of PDE4 D3 and other phosphodiesterases (219) (133)

The thyrocyte is very sensitive to internal cAMP: a mere doubling of its concentration is sufficient to elicit near maximal thyroglobulin phagocytosis (220).

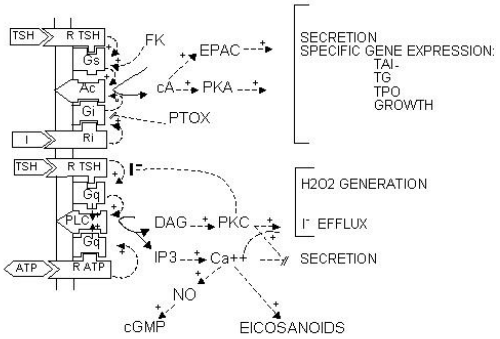



***Fig .1.9.*** *Regulatory cascades activated by thyroid-stimulating hormone (TSH) in human thyrocytes. In the human thyrocyte, $H_2O_2$ (H2O2) generation is activated only by the phosphatidylinositol 4,5-bisphosphate ($PIP_2$) cascade, that is, by the $Ca^{2+}$ (Ca++) and diacylglycerol (DAG) internal signals. In dog thyrocytes, it is activated also by the cyclic adenosine monophosphate (cAMP) cascade. In dog thyrocytes and FRTL-5 cells, TSH does not activate the $PIP_2$ cascade at concentrations 100 times higher than those required to elicit its other effects. Ac, adenylate cyclase; cA, 3'-5'-cAMP, cGMP, 3'-5'-cyclic guanosine monophosphate; FK, forskolin; Gi, guanosine triphosphate (GTP) binding transducing protein inhibiting adenylate cyclase; Gq, GTP-binding transducing protein activating $PIP_2$ phospholipase C; Gs, GTP-binding transducing protein activating adenylate cyclase; I, putative extracellular signal inhibiting adenylate cyclase (e.g., adenosine through $A_1$ receptors); $IP_3$, myoinositol 1,4,5-triphosphate; EPAC: cAMP dependent Rap guanyl nucleotide exchange factor; PKA, cAMP-dependent protein kinases; PKC, protein kinase C; PLC, phospholipase C; PTOX, pertussis toxin; R ATP, ATP purinergic $P_2$ receptor; R TSH, TSH receptor; Ri, receptor for extracellular inhibitory signal I; TAI, active transport of iodide; TG, thyroglobuline; TPO, thyroperoxidase.*

**Comment [U1]:** Modifier figure pour rôle Epac

**The $Ca2^{+}$– Inositol 1,4,5-Triphosphate Cascade**

The $Ca^{2+}$–inositol 1,4,5-triphosphate ($IP_3$) cascade in the thyroid also corresponds, as far as has been studied, to the canonic model of the muscarinic or $\alpha_1$-adrenergic receptor–activated cascades. It is activated in the human thyrocyte by TSH, through the same receptors that stimulate adenylyl cyclase, and by ATP, bradykinin, and TRH—through specific receptors. In this cascade, as in the cAMP pathway, the activated receptor causes the release of GDP and the binding of GTP by the GTP-binding transducing protein ($G_q$) and its dissociation into $\alpha_q$ and βγ. $\alpha_{GTP}$ then stimulates phospholipase C. Gs and Gq compete for the same TSH receptor, with a higher affinity for Gs (221-223). Phospholipase C hydrolyzes membrane phosphatidylinositol 4,5-bisphosphate ($PIP_2$) into diacylglycerol and $IP_3$. $IP_3$ enhances calcium release from its intracellular stores, followed by an influx from the extracellular

medium. The rise in free ionized intracellular $Ca^{2+}$ leads to the activation of several proteins, including calmodulin. The latter protein in turn binds to target proteins and thus stimulates them: cyclic nucleotide phosphodiesterase and, most importantly, calmodulin-dependent protein kinases. These kinases phosphorylate a whole set of proteins exhibiting serines and threonines on their specific peptides and thus modulate them and cause many observable effects of this arm of the cascade. Calmodulin also activates constitutive nitric oxide (NO) synthase in thyrocytes. The generated NO itself enhances soluble guanylyl cyclase activity in thyrocytes and perhaps in other thyroid cells and thus increases cGMP accumulation (224). Nothing is yet known about the role of cGMP in the thyroid cell but NO causes vasodilatation. Diacylglycerol released from $PIP_2$ activates protein kinase C, or rather the family of protein kinases C, which by phosphorylating serines or threonines in specific accessible peptides in target proteins causes the effects of the second arm of the cascade (225). It inhibits phospholipase C or its $G_q$, thus creating a negative feedback loop. In the human thyroid, the $PIP_2$ cascade is stimulated through specific receptors by ATP, bradykinin, TRH and by TSH (174) (226) (227). The effects of bradykinin and TRH are very short lived. Acetylcholine, which is the main activator of this cascade in the dog thyrocyte (228), is inactive on the human cell, although it activates nonfollicular (presumably endothelial) cells in this tissue (174).

## Other Phospholipid-Linked Cascades

In dog thyroid cells and in a functional rat thyroid cell line (FRTL5), TSH activates $PIP_2$ hydrolysis weakly and at concentrations several orders of magnitude higher than those required to enhance cAMP accumulation. Of course, these effects have little biologic significance. However, in dog cells, at lower concentrations TSH increases the incorporation of labeled inositol and phosphate into phosphatidylinositol. Similar effects may exist in human cells, but they would be masked by stimulation of the $PIP_2$ cascade. They may reflect increased synthesis -perhaps coupled to and necessary for cell growth (229).

Diacylglycerol can be generated by other cascades than the classic $Ca^{2+}$-$IP_3$ pathway. Activation of phosphatidylcholine phospholipase D takes place in dog thyroid cells stimulated by carbamylcholine. Because it is reproduced by phorbol esters, that is, by stable analogues of diacylglycerol, it has been ascribed to phosphorylation of the enzyme by protein kinase C, which would represent a positive feedback loop (230). Although such mechanisms operate in many types of cells, their existence in human thyroid cells has not been demonstrated (231).

Release of arachidonate from phosphatidylinositol by phospholipase $A_2$ and the consequent generation of prostaglandins by a substrate-driven process are enhanced in various cell types through G protein–coupled receptors, by intracellular calcium, or by phosphorylation by

protein kinase C. In dog thyroid cells all agents enhancing intracellular calcium concentration, including acetylcholine, also enhance the release of arachidonate and the generation of prostaglandins. In this species, stimulation of the cAMP cascade by TSH inhibits this pathway. In pig thyrocytes, TSH has been reported to enhance arachidonate release. In human thyroid, TSH, by stimulating $PIP_2$ hydrolysis and intracellular calcium accumulation, might be expected to enhance arachidonate release and prostaglandin generation, but such effects have not yet been proved.

## Regulatory Cascades Controlled by Receptor Tyrosine Kinases

Many growth factors and hormones act on their target cells by receptors that contain one transmembrane segment. They interact with the extracellular domain and activate the intracellular domain, which phosphorylates proteins on their tyrosines. Receptor activation involves in some cases a dimerization and in others a conformational change. The first step in activation is interprotein tyrosine phosphorylation, followed by binding of various protein substrates on tyrosine phosphates containing segments of the receptor. Such binding through src homology domains (SH2) leads to direct activation or to phosphorylation of these proteins on their tyrosines and to membrane localization. In turn, these cause sequential activation of the *ras* and *raf* proto-oncogenes, mitogen-activated protein (MAP) kinase kinase, MAP kinase, and so on, on the one hand, and phosphatidylinositol-3-kinase (PI-3-kinase), protein kinase B (PKB), and TOR (target of rapamycin) on the other hand. The set of proteins phosphorylated by a receptor defines the pattern of action of this receptor. In thyroids of various species, insulin, IGF-I, EGF, FGF, HGF, but not platelet-derived growth factor activate such cascades (232;233). In the human thyroid, effects of insulin, IGF-I, EGF, FGF, but not HGF have been demonstrated (152;171;207;234-236). Transforming growth factor-β, acting through the serine threonine kinase activity of its receptors and intermediate proteins (Smad), inhibits proliferation and specific gene expression in human thyroid cells (188;189;237). TSH and cAMP do not activate either the MAPK-ERK nor the JUK and p38 phosphorylation pathways in dog or human thyroids (233).

## Cross-Signaling between the Cascades

Calcium, the intracellular signal generated by the $PIP_2$ cascade, activates calmodulin-dependent cyclic nucleotide phosphodiesterases and thus inhibits cAMP accumulation and its cascade (238). This activity represents a negative cross-control between the $PIP_2$ and the cAMP cascades. Activation of protein kinase C enhances the cAMP response to TSH and inhibits the prostaglandin E response, which suggests opposite effects on the TSH and

prostaglandin receptors (239). No important effect of cAMP on the $PIP_2$ cascade has been detected. On the other hand, stimulation of protein kinase C by phorbol esters inhibits EGF action.

Cross-signaling between the cyclic AMP pathway and growth factor activated cascades have been observed in various cell types (240;241). In ovarian granulosa cells, FSH through cAMP activates MAP kinases and the PI3 kinase pathway (242). In FRTL5, but not in WRT cell lines, TSH through cAMP activates MAP kinases. In WRT cells but not in PCCl3 cells, TSH and cAMP activate PKB (243;244). Such cross signallings have not been observed in human or dog thyroid cells. Ras, MAPK, p38, Jun kinase and ERK5, as well as PI3 kinase and PKB, are not modulated by cAMP (232;233) (245) (246).

Other growth activating cascades have been little investigated in the thyroid. In dog and human cells TSH or cAMP have no effect on STAT phosphorylations i.e. on the JAK-STAT pathways. The NFKβ pathway has not yet been investigated in thyroid cells.

## SPECIFIC CONTROL BY IODIDE

Iodide, the main substrate of the specific metabolism of the thyrocyte, is known to control the thyroid. Its main effects in vivo and in vitro are to decrease the response of the thyroid to TSH, to acutely inhibit its own oxidation (Wolff-Chaikoff effect), to reduce its trapping after a delay (adaptation to the Wolff-Chaikoff effect), and at high concentrations to inhibit thyroid hormone secretion (Fig. 1-10). The first effect is very sensitive in as much as small changes in iodine intake are sufficient to reset the thyroid system at different serum TSH levels without any other changes (e.g., thyroid hormone levels), which suggests that in physiologic conditions, modulation of the thyroid response to TSH by iodide plays a major role in the negative feedback loop (154;247). Iodide in vitro has also been reported to inhibit a number of metabolic steps in thyroid cells (248) (249). These actions might be direct or indirect as a result of an effect on an initial step of a regulatory cascade. Certainly, iodide inhibits the cAMP cascade at the level of $G_s$ or cyclase and the $Ca^{2+}$-$PIP_2$ cascade at the level of $G_q$ or phospholipase C; such effects can account for the inhibition of many metabolic steps controlled by these cascades (250) (251). In one case in which this process has been studied in detail, the control of $H_2O_2$ generation, that is, the limiting factor of iodide oxidation and thyroid hormone formation, iodide inhibited both the cAMP and the $Ca^{2+}$-$PIP_2$ cascades at their first step but also the downstream effects of the generated intracellular signals cAMP, $Ca^{2+}$, and diacylglycerol on $H_2O_2$ generation (252). This effect account for the inhibition by iodide of its oxidation i.e. the Wolff-Chaikoff effects (253).

The mechanism of action of iodide on all the metabolic steps besides secretion fits the "XI" paradigm of Van Sande (254). These inhibitions are relieved by agents that block the

trapping of iodide (e.g., perchlorate) or its oxidation (e.g., methimazole)—the Van Sande criteria. The effects are therefore ascribed to one or several postulated intracellular iodinated inhibitors called XI. The identity of such signals is still unproved. At various times several candidates have been proposed for this role, such as thyroxine, iodinated eicosanoids (iodolactone) (255), and iodohexadecanal (256). The latter, the predominant iodinated lipid in the thyroid, can certainly account for the inhibition of adenylyl cyclase and of $H_2O_2$ generation (256) (257) (258). It should be emphasized that iodination of the various enzymes, as well as a catalytic role of iodide in the generation of $O_2$ radicals (shown to be involved in the toxic effects of iodide), could account for the Van Sande criteria with no need for the XI paradigm (254) (259). Besides, an inhibition of thyroid secretion by iodide in antithyroid drugs treated hyperthyroid patients suggests a direct Xi independent effect.

Distinct from its inhibitory effects, iodide also activates H2O2 generation and therefore protein iodination in the thyroid of some species including humans. This effect is also inhibited by inhibitors of thyroperoxidase and NIS. It would link the generation of H2O2 to the availability of its cosubstrate iodide (260).

Iodide in vivo, at moderate doses in dog, decreases cell proliferation and the expression of TPO and NIS mRNA but not the synthesis or secretion of thyroid hormones. The downregulation of NIS explains the well known delayed decrease of iodide transport in response to iodide, i.e. the adaptation to the Wolff-Chaikoff effect (261).

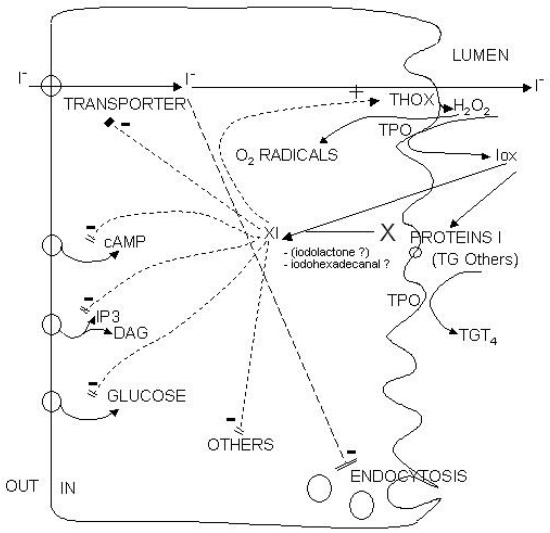



***Fig 1-10.*** *Effects of iodide on thyroid metabolism. All inhibitory effects of iodide, except in part the inhibition of secretion, are relieved by drugs that inhibit iodide trapping (e.g., perchlorate) or iodide oxidation (e.g., methimazole). All effects are direct inhibitions except the effect on iodide transport which bears on the transcription of Na/I- symporter gene. Three possible mechanisms corrresponding to this paradigm are outlined: generation of $O_2$*

*radicals, iodination of target proteins and synthesis of an XI compound. Any of these mechanisms could account for the various steps ascribed to XI inhibition by $I^-$ (indicated by slashes).*

## THE THYROID-STIMULATING HORMONE RECEPTOR

As the sensor for the main regulatory agent acting on the thyroid and the target of autoimmune reactions, the TSH receptor had been extensively studied before it was cloned. Studies from affinity-labeling experiments described the receptor quite accurately as an integral membrane protein inserted in the basolateral plasma membrane and consisting of a relatively large extracellular domain responsible for TSH binding linked to a membrane-spanning domain (262). The number of receptors was estimated to be around 1000/cell. Because of its coupling to the generation of cAMP, it was classified as a member of the large family of G protein-coupled receptors interacting with Gs (see earlier). It was its belonging to this large receptor family, and the expectation that it would display significant sequence similarity with other members of the family (in particular with the FSH and LH receptors), that allowed the cloning of the TSH receptor cDNA (263) {1199} (264) (265) (266) (267)).

### Structure of the Protein.

The beta subunits of glycoprotein hormones, to which TSH belongs, are encoded by paralogous genes displaying substantial sequence similarity (Fig. 1-11). The corresponding receptors, FSHr, LH/CGr and TSHr, are members of the rhodopsin-like G protein-coupled receptor family. As such, TSHr contains a "serpentine" portion containing seven transmembrane helices with many (but not all) of the sequence signatures typical of this receptor family. In addition, and a hallmark of the subfamily of glycoprotein hormone receptors, it contains a large (about 400 residues) aminoterminal ectodomain responsible for the high affinity and selective binding of TSH (268) (269) (270). The higher sequence identity of the serpentine portions of glycoprotein hormone receptors (about 70%) when compared with the ectodomains (about 40%, see Fig. 1-11) suggested early that the former are interchangeable modules capable of activating the G proteins (mainly $G\alpha_s$ ?) after specific binding of the individual hormones to the latter (153). Contrary to other rhodopsin-like GPCRs, binding of the hormones to their ectodomains can be observed with high affinity in the absence of the serpentine (271). The intramolecular transduction of the signal between these two portions of the receptors involves mechanisms specific to the glycoprotein hormone receptor family (see below). The relatively high sequence identity between the

hormone-binding domains of the TSH and LH/CG receptors opens the possibility of spillover phenomena during normal or, even more so, molar or twin pregnancies, when hCG concentrations are several orders of magnitude higher than TSH. This provides an explanation to cases of pregnancy hyperthyroidism (163).

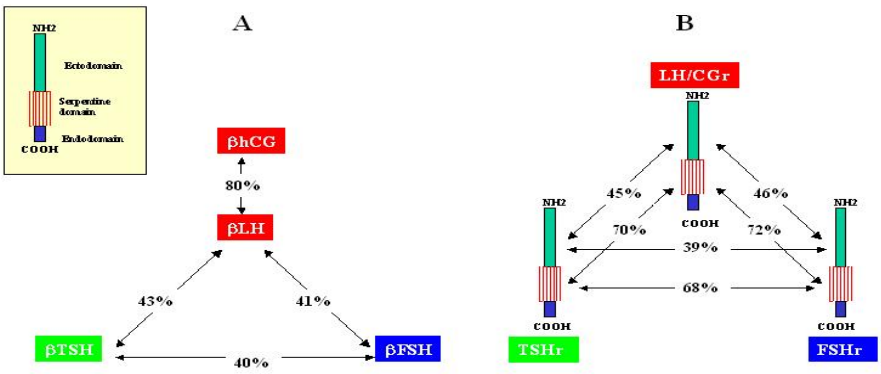



***Fig. 1-11.*** *Both the glycoprotein hormone receptors (panel A) and the beta subunits of glycoprotein hormones (panel B) are encoded by paralogous genes. Sequence identities are indicated, separately for the ectodmains and serpentine domains of the three receptors, and for the beta subunits of the four hormones. The pattern of shared similarities suggests coevolution of the hormones and the ectodomain of their receptors, resulting in generation of specificity barriers. The high similarity displayed by the serpentine portions of the receptors is compatible with a conserved mechanism of intramolecular signal transduction (272).*

## Activation by TSH

TSH is a glycoprotein, consisting of two subunits, an $\alpha$ and a $\beta$ chain . TSH action requires binding to a TSH-specific membrane receptor which activates adenylate cyclase through a G-protein coupled system. A recent report suggests that the $\beta$-unit of human TSH is necessary for recognizing the TSH receptor in FRTL-5 cells while the $\alpha$-subunit is required for signal transduction.

Binding to the receptor of TSH, its natural ligand, results mainly in the activation of Gs causing the stimulation of adenylyl cyclase. The EC50 for adenylyl cyclase activation by bovine TSH in human thyrocytes and in CHO cells transfected with the human cDNA is around 0.3 to 0.4 mU/ml (226). The dissociation constant for TSH binding (bovine TSH) expressed in biological units is close to the same value (1.5 to 1.8 mU/ml) (273) (274). In the absence of a reliable knowledge of the bioactivity of pure intact human TSH (275), it is difficult to translate this value in molar terms. The consensus is that it must be in the

nanomolar range. The bioactivity of TSH is highly dependent on the glycosylation state of the hormone. In this respect it is interesting that artificially deglycosylated TSH behaves as a potent antagonist (276). The bioactivity of human TSH (and of all glycoprotein hormones, including hCG) is lower than that of bovine TSH and of other non primate mammals. This is due to positive selection in higher primates of alpha subunits in which several key basic aminoacids have been substituted (277). The observation that this phenomenon paralels the evolution of chorionic gonadotropin suggests that it may be related to protection against promiscuous stimulation of the TSH receptor by hCG during pregnancy.

In humans, but not in the dog, TSH is also able to activate phospholipase C, resulting in the accumulation of IP3 and diacylglycerol (see earlier) (226) (227). The EC50 for the activation of this pathway is about one order of magnitude higher (about 5 mU/ml) than for the cAMP cascade. The ability to activate adenylyl cyclase and phospholipase C is an intrinsic property of the receptor, since the cloned molecule expressed in Chinese hamster ovary (CHO) cells displays both activities (226). Interestingly, the cloned canine TSH receptor exhibits the same dual potential when expressed in CHO cells, even though it activates only cAMP accumulation in the dog thyrocyte. It is likely that it is the G protein and receptor localization or a different set of still unknown accessory proteins that determines whether the receptor couples to one cascade or the other or both. In dog and human thyroid membranes and cells, TSH also activates Gi but no role has yet been ascribed to this effect.

The thyroid adjusts rapidly to the current level of TSH (278) and cyclic AMP generation and intracellular concentration is instantaneously regulated by the level of receptor occupancy (200).

Expression of the TSH receptor gene is largely thyroid specific. Constructs made of a chloramphenicol acetyltransferase reporter gene under control of the 5' flanking region of the rat gene show expression when transfected into FRTL5 cells and FRT cells but not into nonthyroid HeLa or a rat liver cell line (BRL) cells (279). However, TSH receptor mRNA has been clearly demonstrated in fat tissue of the guinea pig (280), and following differentiation of preadipocytes into adipocytes (281;282). Functional significance in man of reports showing its presence in lymphocytes, extraocular tissue and, recently, in cartilage and bone, requires additional studies. In particular the reported effect of TSH on bone density (283) remains controversial (284). Expression of the TSH receptor in thyroid cells is extremely robust. It is moderately upregulated and downregulated by TSH in vitro (285) and downregulated by iodide in vivo (261).

Recognition of the receptor by TSH

The three-dimensional structures are available for hCG and FSH (236-238) which allows accurate modelization of TSH on these templates. The crystal structure of the human FSHr-FSH complex (286) has confirmed that the ectodomain of glycoprotein hormone receptors belongs to the family of proteins with leucine-rich repeats (LRRs) as was previously suggested by sequence analysis and homology modeling (287). The concave inner surface of the receptor (Fig. 1A) is an untwisted, non-inclined b-sheet formed by ten LRRs. Whereas the N-terminal portion of the beta-sheet (LRR1–7) is nearly flat, the C-terminal portion (LRR7–10) has the horseshoe-like curvature of LRR proteins. The crystal structure of part of the TSHr ectodomain in complex with a thyroid-stimulating autoantibody has recently been obtained (288). Notably, both the structure of the ectodomain of TSHr and the receptor binding arrangements of the autoantibody are very similar to those reported for the FSHr-FSH complex. The ectodomain of glycoprotein hormone receptors also contains, downstream of the LRR region, a cysteine cluster domain of unknown structure (the hinge region), involved in receptor inhibition/activation and containing sites for tyrosine sulfation important for hormone binding (see below).

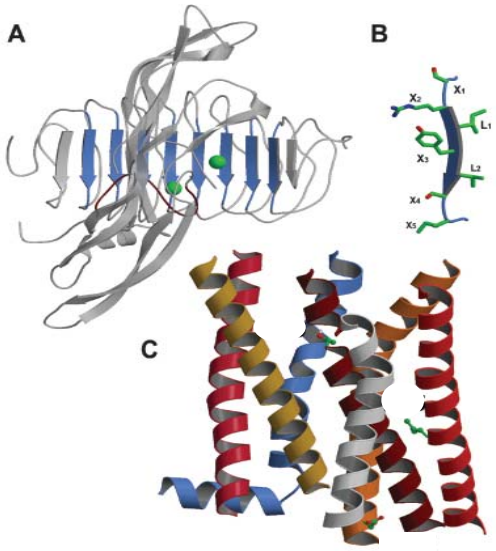



***Fig. 1-12***. *(A) General view of the follicle-stimulating hormone receptor (FSHr)-FSH crystal structure as a template to model the interaction between TSH and the TSH receptor (288). The concave inner surface of the receptor, formed by ten leucine rich repeats (LRR2–9, shown in blue), contact the middle section of the hormone molecule, both the C-terminal segment of the a subunit and the "seat-belt" segment of the b-subunit (shown in red). (B) EachLRR is composed of theX1-X2-L-X3-L-X4-X5 residues (where X is any amino acid, and L usually is Leu, Ile, or Val), forming the central X2-L-X3-L-X4 a typical beta-strand, while X1 and X5 are parts of the adjacent loops. (C) Molecular model of the transmembrane domain of the TSH receptor, constructed from the crystal structure of bovine rhodopsin (289). The color code of the a-carbon ribbons is: transmembrane helix 1 (crimson), 2 (goldenrod), 3 (dark red), 4 (gray), 5 (red), 6 (orange), and 7 (blue), and Helix8 (blue). Recently the crystal*

*structure of the beta2-adrenergic receptor bound to the partial inverse agonist carazolol has been published (290;291). The structure of both rhodopsin and the beta2 receptor are similar at the transmembrane domain.*
*Adapted from Caltabiano et al (292).*

Extensive amino acid substitutions by site-directed mutagenesis of the $X_i$ residues in the LRR portion of the TSHr for their counterparts in the LH/CGr have been preformed (293). Exchanging eight amino acids of the TSHr for the corresponding LH/CGr residues resulted in a mutant displaying a sensitivity to hCG matching that of the wild type LH/CGr. Surprisingly, while gaining sensitivity to hCG, the mutant kept a normal sensitivity to TSH, making it a dual specificity receptor. It is necessary to exchange twelve additional residues to fully transform it into a bona fide LH/CGr (293). From an evolutionary point of view, these observations indicate that nature has built recognition specificity of hormone-receptor couples on both attractive and repulsive residues, and that residues at different homologous positions have been selected to this result in the different receptors.

Inspection of electrostatic surface maps of models of the wild type TSH and LH/CG receptors and some of the mutants is revealing in this respect (292;293). The LH/CGr displays an acidic groove in the middle of its horseshoe, extending to the lower part of it (corresponding to the C-terminal ends of the beta strands). Generation of a similar distribution of charges in the dual-specificity and reverse-specificity TSHr mutants suggests that this is important for hCG recognition. A detailed modelization of the interactions between TSH and the ectodomain of its receptor has been realized (292). In addition to the hormone-specific interactions genetically encoded in the primary structure of glycoprotein hormones receptors and their ligands, the importance of non-hormone specific ionic interactions has been demonstrated, involving sulphated tyrosine residues present in the ectodomains of all three receptors (294). Sulfation in the TSHr takes place on both tyrosine residues of a conserved Tyr-Asp-Tyr motif located close to the border between the ectodomain and the first transmembrane helix (Fig. 1-13). However, only sulfation of the first tyrosine of the motif seems to be functionally important (294), contributing crucially to the binding affinity, without interfering with specificity. The functional role of this postranslational modification of the TSH receptor has been confirmed by demonstration of profound hypothyroidism due to resistance to TSH in mice with inactivation of Tpst2, one of the enzymes responsible for tyrosine sulfation (295)(296).

Activation of the serpentine portion of the TSH receptor

As it belongs to the rhodopsin-like GPCR family and displays many of the cognate signatures in primary structure, the serpentine portion of the TSH receptor is likely to share with rhodopsin common mechanisms of activation (289;297).

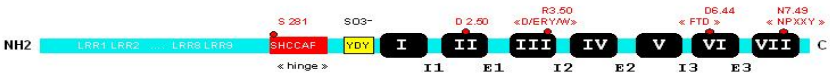



***Fig. 1-13.*** *Linear representation of the TSH receptor. Sequence signatures common to all rhodopsin-like GPCRs and sequence signatures specific to the glycoprotein hormone receptor gene family are both implicated in activation of GPHRs. Key residues are indicated (red dots) as well as conserved motifs: $SO3^{--}$ stands for postranslational sulfation of the indicated tyrosine residues. The black boxes stand for transmembrane helices and I1-I3, E1-E3, for intracellular and extracellular loops, respectively (272).*

However, sequence signatures characteristic of the serpentine portion of the glycoprotein hormone receptors suggest the existence of idiosyncrasies associated with a specific mechanism of activation (Fig. 1-13). In addition, over the past ten years, the TSHr has been found to be activated by a wide spectrum of gain of function mutations (298-300). Compilation of available data identifies more than 30 residues, the mutation of which causes constitutive activation (for a complete list of TSHr mutants, see (301) and http://gris.ulb.ac.be. As many somatic mutations affecting a given residue have been found repeatedly, it is likely that we are close to a saturation map for spontaneous gain of function mutations. Attempts to translate this map into mechanisms of transition between inactive and active conformations of the receptor have been made, in the light of rhodopsin structural data. Three sequence patterns affected by gain of function mutations deserve special mention and may help understanding how the TSH receptor is activated.

***The first***, is centered on an aspartate in position 6.44 (Asp633), at the cytoplasmic side of transmembrane helix VI (TM-VI). When mutated to a variety of aminoacids the result is constitutive activation (169;298;302). This suggested that the gain of function resulted from the breakage of (a) bond(s), rather than the creation of novel interaction(s) by the mutated residue, and the main partner of Asp6.44 was identified as Asn7.49, in transmembrane helix

VII. From a series of site-directed mutagenesis studies (169;303), it was tentatively concluded that in the inactive conformation of the TSH receptor, the side chain of Asp7.49 is normally “sequestered” by both Thr6.43 and Asp6.44, and that the active conformation(s) require(s) establishment of novel interaction(s) of N7.49 involving most probably Asp in position 2.50.

***The second***, glutamate 3.49 and arginine 3.50 of the highly conserved “D/ERY/W” motif at the bottom of TM-III form an ionic lock with aspartate 6.30 at the cytoplasmic end of TM-VI. Disruption of this ionic lock, e.g. by mutations affecting Asp6.30, leads to constitutively active mutant receptors (303). Thus, the movements of TM-III and TM-VI at the cytoplasmic side of the membrane, i.e. presumably an opening of the receptor, is necessary for receptor activation (304). The existence of this ionic lock has however been questioned recently, as it is not found in the determined structure of the beta2 adrenergic receptor (291).

***The third***, serine 281 belongs to a GPHR-specific “YPSHCCAF” sequence signature located at the carboxyl terminal end of the LRR portion in the ectodomain of the receptors (see Fig. 96-5). After mutation of this serine residue had been shown to activate the TSH receptor constitutively (305), this segment, sometimes referred to as the “hinge” motif, was shown to play an important role in activation of all three glycoprotein hormone receptors (151). The functional effect of substitutions of S281 in the TSHr likely results of a “loss-of-structure”, locally, since the more de-structuring the substitutions, the strongest the activation (151;306). This observation, together with results showing that mutation of specific residues in the extracellular loops of the TSHr cause constitutive activation (197), led to the hypothesis that activation of the receptor could involve the rupture of an inhibitory interaction between the ectodomain and the serpentine domain (see below) (305).

<u>Interaction between the ectodomain and the serpentine domain</u>

Mutant TSH receptor constructs devoid of the ectodomain displayed a phenotype compatible with partial activation, thus confirming the inhibitory effect of the ectodomain on the serpentine portion already suggested by the partial activation of the receptor by a trypsin treatment. However, activation of cAMP production in cells transfected with truncated constructs was much lower than after full stimulation of the wild type receptor by TSH or by mutation of Ser281 (307). These observations led to the following model for activation of the TSHr (Fig. 1-14) (307).

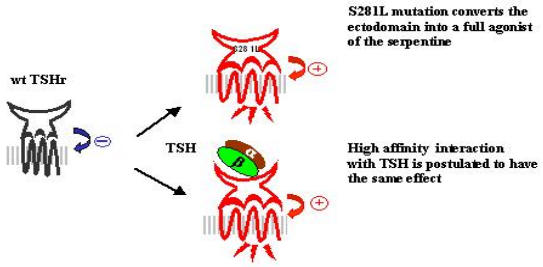



***Fig. 1-14*** *: Model for activation of the thyrotropin receptor. Interactions between the ectodomain and the serpentine domains are implicated in the activation mechanism and functional specificity. The TSH receptor is represented with its ectodomain containing a concave, hormone binding structure facing upwards, and a transmembrane serpentine portion. The basal state of the receptor is characterized by an inhibitory interaction between the ectodomain and the serpentine domain (indicated by the (-) blue sign). The ectodomain would function as a tethered inverse agonist of the serpentine portion. Mutation of Ser281 in the ectodomain into leucine switches the ectodomain from an inverse agonist into a full agonist of the serpentine domain (indicated by the (+) red sign). Binding of TSH (indicated by dimeric structure) to the ectodomain is proposed to have a similar effect, converting it into a full agonist of the serpentine portion. The serpentine portion in the basal state is represented as a compact, black structure. The fully activated serpentine portion is depicted as a relaxed red structures with arrows indicating activation of Gs.*

In the resting state, the ectodomain would exert an inhibitory effect on the activity of an inherently noisy rhodopsin-like serpentine, qualifying pharmacologically as an inverse agonist of the serpentine. Upon activation, by binding of the hormone, or secondary to mutation of S281 in the hinge region, the ectodomain would switch from inverse agonist to full agonist of the serpentine portion. The ability of the strongest S281 mutants to fully activate the receptor in the absence of hormone, suggests that the ultimate agonist of the serpentine domain would be the “activated” ectodomain, with no need for an interaction between the hormone and the serpentine domain.

This model in which the “real” agonist of the serpentine domain would be the activated ectodomain is conforted by the identification of a monoclonal antibody recognizing the ectodomain and endowed with inverse agonistic activity (308).

Activation by Chorionic Gonadotropin

As indicated above, the sequence similarity between TSH and hCG, and between their receptors, allows for some degree of promiscuous stimulation of the TSH receptor by hCG during the first trimester of pregnancy, when hCG levels reach peak values. The inverse relation between TSH and hCG observed in most pregnant women is clear indication that their thyroid is subjected to the thyrotropic activity of hCG (163). Whereas this situation is usually associated with euthyroidism, thyrotoxicosis may develop in cases of excessive hCG production (as it occurs in twin pregnancies or hydatiform moles), or in rare patient harboring a mutant TSH receptor with increased sensitivity to hCG (309).

Activation by Autoantibodies

Autoantibodies found in Graves' disease and some types of idiopathic myxedema can stimulate (TSAb) or block (TSBAb) TSH receptor, respectively. Epitopes recognized by TSAbs are being identified from precise mapping of binding site of murine or human monoclonal antibodies endowed with TSAb activity (288;310). However, the mechanism implicated in activation of he receptor by TSAbs (and by TSH) are still unknown. Although most TSAbs do compete with TSH for binding to the receptor (263), and despite similarity in interaction surfaces (288), the precise targets of the hormone and autoantibodies are likely to be different, at least in part. It has indeed been shown that sulfated tyrosine residues, which are important for TSH binding, are not implicated in recognition of TSH receptor by TSAbs (294). Also, contrary to TSH, most TSAbs from Graves' patients display a delay in their ability to stimulate cAMP accumulation in transfected cells (200). The recent availability of TSAb preparations purified from individual patients will hopefully allow to explore these issues in a direct fashion (311).

Activation by Thyrostimulin

Thyrostimulin has been identified as a novel agonist of the TSH receptor. As glycoprotein hormones, it is composed of two subunits, coined a2 and b5 and activates the receptor with a lower EC50 than human TSH. It is produced by the pituitary in corticotrophs but its physiological significance remains mysterious (312;313).

Downregulation of the TSH receptor

Desensitization of some G protein–coupled receptors has been shown to involve phosphorylation of specific residues by G protein receptor kinases (homologous desensitization) or PKA (heterologous desensitization) enzymes (219). When compared with other G protein–coupled receptors, the TSH receptor contains few phosphorylatable serine or threonine residues in its intracellular loops and C-terminal tail, which probably accounts for

the limited desensitization observed after stimulation by TSH. Acute desensitization of the receptor in the presence of TSH, presumably by phosphorylation, is weak and delayed (314). Its internalization is rapidly followed by recycling to the cell surfaces while the hormone is degraded in lysosomes (315). Similarly a weak downregulation, confounded by the long life of both TSHR mRNA and protein, takes place but has little functional role (285). On the other hand in the presence of constant stimulation the cAMP accumulation is downregulated mostly as a consequence of phosphodiesterase induction (316). The persistence of hyperthyroidism in patients submitted to constant TSH stimulations in TSH secreting pituitary adenomas or TSAb stimulation in Graves disease testifies to the weakness of these negative regulations.

The TSHr contains six sites for N-glycosylation, of which four have been shown to be effectively glycosylated (271). The functional role of the individual carbohydrate chains is still debated. It is likely that they contribute to the routing and stabilization of the receptor through the membrane system of the cell.

Alone amongst the glycoprotein hormone receptors, the TSHr undergoes cleavage of its ectodomain, severing it from the serpentine domain (267). This phenomenon has been related to the presence in the TSHr of a 50 amino acid “insertion” for which there is no counterpart in the FSHr or LH/CGr. Current data indicate that the initial cleavage step, by a metalloprotease, takes place around position 314 (within the 50 aminoacid insertion), followed by nibbling of the aminoterminal extremity of the serpentine-containing portion (317;318). The aminoterminal and serpentine portions of the resulting dimer remain bound to each other by disulphide bounds. The functional meaning of this TSHr-specific postranslational modification remains unclear. Whereas all wild type TSHr at the surface of thyrocytes seem to be in cleaved form, it has been shown that non cleavable mutant constructs are functionally undistinguishable from cleaved receptors, when expressed in transfected cells (267). Noteworthy, when transiently or permanently transfected in non-thyroid cells, the wild type human TSHr is present at the cell surface as a mixture of monomer and cleaved dimer.

The TSH receptor is specifically inserted into the baso-lateral membrane of thyrocytes. This phenomenon involves signals encoded in the primary structure of the protein, as it is conserved when the receptor is expressed in the MDCK cell, a polarized cell of non-thyroid origin (319).

Dimerization.

AS most rhodopsin-like GPCRs, the glycoprotein hormone receptors have been demonstrated to dimerize/oligomerize by a variety of experimental approchaes including

FRET, BRET and functional complementation of mutants (320-324). The physiological significance of this phenomenon remains unknown, but it has been shown to be associated with allosteric properties of the dimers/oligomers (325). TSH binding to the TSHr displays strong negative cooperativity, which is classically considered to account for a shallow concentration action curve, extending over two orders of magnitude.
The possibility that TSHr are present at the cell surface as “dimers of cleaved dimers” has recently been raised following demonstration that most rhodopsin-like GPCRs do dimerize (326). Functional complementation of mutants in the ecto- and the serpentine domains has been observed after co-transfection of FSH receptor constructs in cells (327), demonstrating the possibility of dimerization for glycoprotein hormone receptors. Preliminary data have been provided for the TSHr (320), but definitive demonstration of the functional significance of its dimerization is still missing.

Desensitization of some G protein-coupled receptors has been shown to involve phosphorylation of specific residues by G protein receptor kinases (GRK) (homologous desensitization) or protein kinase A (heterologous desensitization) enzymes (267). As compared with other G protein-coupled receptors, the TSH receptor contains few phosphorylatable serine or threonine residues in its intracellular loops and carboxyl-terminal tail, which probably accounts for the limited desensitization observed after stimulation by TSH and for the continuous TSAb action which accounts for Graves disease.

## The TSH Receptor Gene.

The gene coding for the human TSH receptor has been localized on the long arm of chromosome 14 (14q31) (328;329). It spreads over more than 60 kb and is organized into 10 exons displaying an interesting correlation with the protein structure (330). The extracellular domain is encoded by a series of 9 exons, each of which corresponds to one or an integer number of leucine rich repeat motifs (see below). The C-terminal half of the receptor containing the C-terminal part of the ectodomain and the serpentine domain is encoded by a single large exon. This finding is reminiscent of the fact that many G protein–coupled receptor genes are intronless. A likely evolutionary scenario derives from this gene organization (330): the glycoprotein hormone receptor genes would have evolved from the condensation of an intronless classic G protein–coupled receptor with a mosaic gene encoding a protein with leucine rich repeats. Triplication of this ancestral gene and subsequent divergence led to the receptors for LH/CG, FSH, and TSH. The existence of 10 exons in both the TSH and FSH receptor genes (as opposed to the 11 exon LH/CG receptor), suggests the following phylogeny : one ancestral glycoprotein hormone receptor

duplicating in the LH receptor and in the ancestor of TSH and FSH receptor which lost one intron. The latter later duplicated in TSH and FSH receptors.

The gene promoter has characteristics of "housekeeping" genes in that it is GC-rich and devoid of TATA boxes; in the rat it was shown to drive transcription from multiple start sites (279). Recent association studies have provided convincing evidence that the TSHr gene is one amongst many genetic determinants implicated in the development of Graves'disease (331).

## CONTROL OF THYROID FUNCTION

### Thyroid Hormone Synthesis

Thyroid hormone synthesis requires the uptake of iodide by active transport, thyroglobulin biosynthesis, oxidation and binding of iodide to thyroglobulin, and within the matrix of this protein, oxidative coupling of two iodotyrosines into iodothyronines. All these steps are regulated by the cascades just described.

Iodide Transport

Iodide is actively transported by the iodide $Na^+/I^-$ symporter (NIS) against an electrical gradient at the basal membrane of the thyrocyte and diffuses, following the electrical gradient, by a specialized channel (pendrin or another channel) (332;333) from the cell to the lumen at the apical membrane. The opposite fluxes of iodide, from the lumen to the cell and from the cell to the outside, are generally considered to be passive and nonspecific. At least five types of control have been demonstrated (248;249;332).

1. Rapid and transient stimulation of iodide efflux by TSH in vivo, which might reflect a general increase in membrane permeability. The cascade involved is not known.

2. Rapid activation of iodide apical efflux from the cell to the lumen by TSH. This effect, which contributes to the concentration of iodide at the site of its oxidation, is mediated, depending on the species, by $Ca^{2+}$ and/or cAMP (228;332). In human cells it is mainly controlled by $Ca^{2+}$ and therefore by the TSH effect on phospholipase C.

3. Delayed increase in the capacity (Vmax) of the active iodide transport NIS in response to TSH. This effect is inhibited by inhibitors of RNA and protein synthesis and is due to activation of iodide transporter gene expression. This effect of TSH is reproduced by cAMP analogues in vitro and is therefore mediated by the cAMP cascade (124). mRNA expression is enhanced by TSH and cAMP and decreased by iodide (261;334). TSH enhancement of thyroid blood flow, more or less delayed depending on the species, also contributes to increase the uptake of iodide (124). Iodine levels in the thyroid are also inversely related to blood flow (335).

4. Rapid inhibition by iodide of its own transport in vivo and in vitro. This inhibitory effect requires an intact transport and oxidation function, that is, it fulfills the criteria of an XI effect. After several hours the capacity of the active transport mechanism is greatly impaired (adaptation to the Wolff-Chaikoff effect) (249). The mechanism of the first effect is unknown but probably initially involves direct inhibition of the transport system itself (akin to the desensitization of a receptor), followed later by inhibition of NIS gene expression and NIS synthesis (akin to the downregulation of a receptor) (261).

5. Inhibition by iodide of thyroid blood flow. This effect may be direct as it takes place in patients treated with thyroperoxidase inhibitors and therefore does not fit the XI paradigm. By decreasing the iodide input it decreases the uptake.

Iodide Binding to Protein and Iodotyrosine Coupling

Iodide oxidation and binding to thyroglobulin and iodotyrosine coupling in iodothyronines are catalyzed by the same enzyme, thyroperoxidase, with $H_2O_2$ used as a substrate (336). The same regulations apply to the two steps. $H_2O_2$ is generated by a NADPH oxidase system of which two proteins DUOX or THOX have been identified by cloning (337;338). The system is very efficient in the basal state inasmuch as little of the iodide trapped can be chased by perchlorate in vivo. Also, in vitro and in vivo the amount of iodine bound to proteins mainly depends on the iodide supply. Nevertheless, in human thyroid in vitro, stimulation of the iodination process takes place even at low concentrations of the anion, thus indicating that iodination is a secondary limiting step. Such stimulation is caused in all species by intracellular $Ca^{2+}$ and is therefore a consequence of activation of the $Ca^{2+}$-$PIP_2$ cascade. In many species, phorbol esters and diacylglycerol, presumably through protein kinase C, also enhance iodination (339). It is striking that in a species such as the human, in which TSH activates the $PIP_2$ cascade, cAMP inhibits iodination, whereas in a species (dog) in which TSH activates only the cAMP cascade, cAMP enhances iodination. Obviously in the latter species a supplementary cAMP control was necessary (339;340).

Thyroperoxidase does not contain any obvious phosphorylation site in its intracellular tail. On the other hand, all the agents that activate iodination also activate $H_2O_2$ generation, and inhibition of $H_2O_2$ generation decreases iodination, which therefore suggests that iodination is an $H_2O_2$ substrate–driven process and that it is mainly controlled by $H_2O_2$ generation and iodide supply (339;341). Congruent with the relatively high $K_m$ of thyroperoxidase for $H_2O_2$, $H_2O_2$ is generated in disproportionate amounts with regard to the quantity of iodide oxidized. Negative control of iodination by iodide (the Wolff-Chaikoff effect) is accompanied and mostly explained by the inhibition of $H_2O_2$ generation. This effect of $I^-$ is relieved by perchlorate and methimazole and thus pertains to the XI paradigm (254;339).

Iodotyrosine coupling to iodotyrosines is catalyzed by the same system and is therefore subject to the same regulations as iodination. However, coupling requires that suitable tyrosyl groups in thyroglobulin be iodinated, that is, that the level of iodination of the protein be sufficient. In the case of severe iodine deficiency or when thyroglobulin exceeds the iodine available, insufficient iodination of each thyroglobulin molecule will preclude iodothyronine formation whatever the activity of the $H_2O_2$ generating system and thyroperoxidase. On the other hand, when the iodotyrosines involved in the coupling are present, coupling is controlled by the $H_2O_2$ concentration but independent of iodide (336). In this case, $H_2O_2$ control has a significance even at very low iodide concentrations.

$H_2O_2$ generation requires the reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH) as a coenzyme and is thus accompanied by NADPH oxidation. Limitation of the activity of the pentose phosphate pathway by $NADP^+$ insures that NADPH oxidation for $H_2O_2$ generation causes stimulation of this pathway. Also, excess $H_2O_2$ leaking back into the thyrocyte is reduced by glutathione (GSH) peroxidase, and the oxidized GSH (GSSG) produced is reduced by NADPH-linked GSH reductase. Thus both the generation of $H_2O_2$ and the disposal of excess $H_2O_2$ by pulling NADP oxidation and the pentose pathway lead to activation of this pathway—historically one of the earliest and unexplained effects of TSH (124;339).

On the long-term, in vivo or in vitro, the activity of the whole iodination system obviously also depends on the level of its constitutive enzymes. In human thyrocytes H2O2 generation but not TPO is stimulated by direct activation of DUOX by the Gq-PLC-$Ca^{++}$ cascade which TPO expression, but not DUOX is upregulated by the TSH-cAMP cascade (342). It is therefore not surprising that activation of thyrocytes by the cAMP cascade increases the corresponding gene expression whereas dedifferentiating treatments with EGF and phorbol esters inhibit this expression and thus reduce the capacity and activity of the system. Apparent discrepancies in the literature about the effects of phorbol esters on iodination are mostly explained by the kinetics of these effects (acute stimulation of the system, delayed inhibition of expression of the involved genes).

## Thyroid Hormone Secretion

Secretion of thyroid hormone requires endocytosis of human thyroglobulin, its hydrolysis, and the release of thyroid hormones from the cell. Thyroglobulin can be ingested by the thyrocyte by three mechanisms (124;343-345).

In *macropinocytosis*, which is the first, pseudopods engulf clumps of thyroglobulin. In all species this process is triggered by acute activation of the cAMP/PKA cascade and therefore by TSH. Stimulation of macropinocytosis is preceded and accompanied by an enhancement of thyroglobulin exocytosis and thus of the membrane surface (341;346;347). In dog thyroid

slices (220) and even (254) primary cultures, TSH and PKA activation acutely induces phagocytosis (348), which appears as the *in vitro* manifestation of the macropinocytosis of thyroglobulin involved in stimulated thyroid hormone secretion. This process might be mediated by inactivation of the Rho family small G proteins (Fortemaison Endocrinology 2005), resulting in microfilament depolymerization and stress fiber disruption accompanied by dephosphorylation of cofilin (349) and myosin light chains (350).

By *micropinocytosis*, the second process, small amounts of colloid fluid are ingested. This process does not appear to be greatly influenced by acute modulation of the regulatory cascades. It is enhanced in chronically stimulated thyroids and thyroid cells early mobilization ro the membrane and later with induction of vesicle transport proteins Rab 5 and 7 (351) (352;353). It probably accounts for most of basal secretion.

A third (hypothesized) process is *receptor-mediated endocytosis*; it is enhanced in chronically stimulated thyroid cells (354-356). The protein involved could be megalin (357) or and asyaloglycoprotein. This process probably accounts for the transcytosis of low hormonehenic thyroglobulin (358).

Contrary to the last named, the first two processes are not specific for the protein. They can be distinguished by the fact that macropinocytosis is inhibited by microfilament and microtubule poisons and by lowering of the temperature (below 23°C) (124;314). Whatever its mechanism, endocytosis is followed by lysosomal digestion with complete hydrolysis of thyroglobulin. The main iodothyronine in thyroglobulin is thyroxine. However, during its secretion a small fraction is deiodinated by type I 5 and in man type II 5 -deiodinase to triiodothyronine ($T_3$), thus increasing relative $T_3$ (the active hormone) secretion (359).

The free thyroid hormones are released by an unknown mechanism, which may be diffusion or transport. The iodotyrosines are deiodinated by specific deiodinases and their iodide recirculated in the thyroid iodide compartments. Under acute stimulation, a release (spillover) of amino acids and iodide from the thyroid is observed. A mechanism for lysosome retention of poorly iodinated thyroglobulin on *N*-acetylglucosamine receptors and recirculation to the lumen has been proposed. Under normal physiologic conditions, endocytosis is the limiting step of secretion, but after acute stimulation, hydrolysis might become limiting with the accumulation of colloid droplets. Secretion by macropinocytosis is triggered by activation of the cAMP cascade and inhibited by $Ca^{2+}$ at two levels: cAMP accumulation and cAMP action. It is also inhibited in some thyroids by protein kinase C downstream from cAMP. Thus the $PIP_2$ cascade negatively controls macropinocytosis (239).

The thyroid also releases thyroglobulin. Inasmuch as this thyroglobulin was first demonstrated by its iodine, at least part of this thyroglobulin is iodinated; thus it must originate from the colloid lumen. Release is inhibited in vitro by various metabolic inhibitors and therefore corresponds to active secretion (346;360). The most plausible mechanism is

transcytosis from the lumen to the thyrocyte lateral membranes (347). As for thyroid hormone, this secretion is enhanced by activation of the cAMP cascade and TSH and inhibited by $Ca^{2+}$ and protein kinase C activation. Because thyroglobulin secretion does not require its iodination, it reflects the activation state of the gland regardless of the efficiency of thyroid hormone synthesis. Thyroglobulin serum levels and their increase after TSH stimulation constitute a very useful index of the functional state of the gland when this synthesis is impaired, as in iodine deficiency, congenital defects in iodine metabolism, treatment with antithyroid drugs, and the like (361). Regulated thyroglobulin secretion should not be confused with the release of this protein from thyroid tumors, which corresponds in large part to exocytosis of newly synthesized thyroglobulin in the extracellular space rather than in the nonexistent or disrupted follicular lumen. In inflammation or after even mild trauma, opening of the follicles can cause unregulated leakage of lumen thyroglobulin.
Transcytosis or leakage from the lumen yields iodinated thyroglobulin while exocytotic thyroglobulin is not iodinated.

### Functional Heterogeneity

It has long been known that at any given time the function of the thyroid follicles is not homogeneous. For instance, after injection of radioiodide, some follicles will incorporate important amounts of radioiodine while others will not incorporate at all. Similarly, after stimulation with TSH in in vivo thyroids or in in vitro incubated slices, some cells will develop pseudopods for macropinocytosis whithin 15 min while others submitted to the same stimulus will only respond after one to two hours (112;362).
In a beautiful study, Gérard et al (363) (364) showed in human thyroids that while some follicles exhibit marked expression of pendrin, TPO and THOX, others did not. The expressing follicles were those containing iodinated thyroglobulin. They correspond to larger capillary networks and to the expression in the follicular cells of vascular regulators nitric oxide synthase and endothelin. This shows the existence of active and inactive angiofollicular units. It suggests that over time angiofollicular units cycle from active to inactive states and that this is controlled by the follicular cells. It would be interesting to know if the inactive state corresponds to a lower sensitivity to TSH.

## CONTROL OF THYROID-SPECIFIC GENE EXPRESSION

The study of specific gene expression and proliferation at the biochemical and mechanistic level requires long term in vitro incubations, i.e. cell culture. It is therefore easy and tempting to rely on cell lines such as the rat thyroid FRTL-5, WRT and PCCl3 cells. However these cells, sometimes different from one lab to another, are different from each other and are very

different from the cells in vivo, especially the human cells. Primary cultures are closer to the in vivo situation but they are difficult to obtain and not as reproducible. However, in monolayers, but not in reorganized follicles, follicular structure is fully and cell polarity and structure are partially lost. As most authors generalize the results of work done on their pet systems to "The Thyroid" the literature is very confusing and full of contradictions (243). Among the various possibilities demonstrated in such systems only those validated in vivo in transgenic animals and in human cells interest us.

A positive in vivo effect of TSH on general protein synthesis has been well documented. This effect is mimicked by cAMP agonists and is part of the trophic effect of TSH on the thyrocyte. It involves stimulation of transcription and translation; however, the detailed mechanisms implicated are not known. In cells in culture there is no such effect and the TSH cyclic AMP cascade has rather an inhibitory effect. Stimulation of protein synthesis and hypertrophy of the cell in culture results from IGF1 action on PI3 kinase (365). The in vivo effect of TSH might be mediated by IGF-1.

The so-called thyroid-specific genes encode proteins that are either found in the thyroid exclusively, like thyroglobulin and thyroperoxidase, or that, although being also found in a few additional tissues, are primarily involved in thyroid function, like TSH receptor and sodium/iodide symporter. The transcription of these genes in the thyroid appears to rely on the coordinated action of a master set of transcription factors that includes at least the homeodomain protein TTF-1 (also known as Nkx 2.1), the paired-domain protein Pax 8, and perhaps also the forkhead-domain protein TTF-2 (also known as FoxE1) (366;367). Loss of function mutant mice for TTF-1, Pax 8 or TTF-2 have been generated and allowed to identify a crucial role for these transcription factors in the development of the thyroid also. However, as none of these animals develop a normal mature thyroid, they could not be used to investigate the exact role of these key factors in the control of gene expression in the mature thyroid. Most of the work concerning this last aspect has been conducted either in primary cultures of thyrocytes (368) or in immortalized thyroid cell lines like FRTL-5 and PCCl3 (369). Although the data gathered to date agree on most basic aspects, significant differences have sometimes been observed between primary versus immortalized cell models (370). Part of these discrepancies may result from the existence of occasional species-specific differences (371).

The main regulator of thyroid function, the TSH signal, which is predominantly conveyed inside the cell by cAMP and PKA, upregulates the expression of transcription factor Pax 8, both in primary cells (372) and established cell lines (373). However, mice genetically deprived of TSH or of functional TSH receptor do not show reduced amounts of Pax 8 in their thyroids as compared to wild type animals (156) suggesting that compensatory mechanisms may ensure an adequate production of this factor when thyroid development takes place in

the absence of the normal physiological stimulus. Besides this control on the amount of Pax 8 protein, there is no firm evidence that TSH, or cAMP, exerts any other control at the level of the master thyroid transcription factors identified presently (370;374;375). The expression of several other transcription factors was shown to be upregulated, often at least transiently, in response to TSH/cAMP in the thyroid, namely c-myc (376), c-fos (376), fos B, jun B, jun D (377), CREM (378), NGFI-B (379) and CHOP(380), for example. An hypothetical role in the control exerted by TSH/cAMP on the expression of the thyroid-specific genes has been proposed for some of these factors (378;380), but no final link has been established yet (381). It is noteworthy that in addition to its control on the transcription of the individual thyroid-specific genes, which is detailed below, TSH also regulates gene expression by acting at some post-transcriptional steps, as shown in the case of thyroglobulin (382). Finally, many effects of TSH and cAMP on gene expression (including on thyroid-specific genes such as thyroglobulin) might be rather indirect and depend in part on the profound modifications of cell morphology and cytoskeleton that result from PKA activation (182)(218).
TGF-β has been shown to downregulate the expression of thyroid-specific genes (383;384). It seems to involve a reduction in the level of Pax 8 activity that is mediated by Smad proteins (384;385). In human thyroid primary cultures, TGF-β inhibits most effects of cAMP on gene expression (189). As above, this might be related in part to an inhibition of morphological effects of TSH/cAMP. In all the species tested so far, EGF strongly represses thyroglobulin and thyroperoxidase gene expression as well as iodide transport (165;182;285;386;387). FGF has a similar action in some species including bovine (165). The mechanisms have not been explored. The apparent dedifferentiation induced by EGF in dog thyrocytes is associated with an enhanced vimentin expression and a progressive induction of a fusiform fibroblast-like morphology, which is suggestive of an epithelial-mesenchymal transition (179) (388). This process is reversible after elimination of EGF and re-addition of TSH.

## Thyroglobulin

The regulatory DNA elements of the thyroglobulin gene have been characterized in several species (366;371;389). The proximal promoter, as defined in transfection experiments, extends over 200 base-pairs and contains binding sites for transcription factors TTF-1, TTF-2 and Pax 8 (see Fig. 1-15). An upstream enhancer element containing binding sites for TTF-1 has been identified in beef and man (390). In the latter, the enhancer region is longer and harbors additional binding sites for TTF-1 and cAMP responsive element binding (CREB) protein (391). Both TTF-1 and Pax 8 proteins were individually shown to exert a major control on thyroglobulin gene transcription (392;393). By contrast, TTF-2 activity appears to be

dispensable as the thyroglobulin gene is expressed in cells devoid of TTF-2 protein (392). Synergism in the transcriptional activation of the gene by TTF-1 and Pax 8 appears to rely on a direct interaction between these two factors (394), and on their coordinated action involving both the enhancer and proximal promoter sequences (395). The known thyroglobulin gene regulatory elements were shown to be sufficient to drive the thyroid-restricted expression of a linked gene in living mice (396). This thyroid-restricted expression likely results from the requirement for the simultaneous presence of both TTF-1 and Pax 8, which occurs in thyroid only. It is associated with the tissue-specific demethylation of thyroglobulin gene sequences (397). Demethylation of the DNA is supposed to relieve the constitutive silencing of the gene (398).

Thyroglobulin gene transcription has been shown to require the presence of circulating TSH in the adult rat (399) and to be highly dependent on an elevated cAMP level in dog thyroid tissue slices, in primary cultured cells (400), and, to a much lower extent, in immortalized thyroid cell lines like FRTL-5 (401). Although they are devoid of classical cAMP-responsive element (CRE), the proximal promoter sequences are essentially involved in this control, as indicated by the observation of TSH/cAMP-induced changes in their chromatin structure (402) and their TSH/cAMP-dependent activity in transfection experiments (403). It has however been demonstrated recently that the onset of thyroglobulin gene expression during thyroid development takes place normally in mouse strains deprived of either circulating TSH or functional TSH receptors (155;156). This may be consistent with the observation that the thyroglobulin gene displays a low level of cAMP-independent transcription in primary cultured thyrocytes (400), which might depend on insulin, as observed in different culture models (404-406). In primary cultures of dog thyrocytes, the transcriptional activation of the thyroglobulin gene by cAMP after transient TSH withdrawal is also delayed as compared to that of the thyroperoxidase gene (400), and, unlike thyroperoxidase gene expression, it requires an active protein synthesis (400). The increase in Pax 8 concentration consecutive to TSH/cAMP stimulation of the thyrocyte is not sufficient to account for the observed control on thyroglobulin gene transcription, as TSH is still required for transcriptional activation even in cells expressing high levels of Pax 8 protein (373). Thus, besides TTF-1 and Pax 8, at least one additional, still unidentified, factor is likely to play a key role in the control of thyroglobulin gene expression, as also suggested by the observation that, in the course of thyroid development, both TTF-1 and Pax 8 are present well before thyroglobulin gene is expressed.

**Comment [U2]:** PKA induit la Tg (Dremier 2007)

In addition to the full length thyroglobulin mRNA, a shorter transcript accumulates in the rat thyroid in response to TSH stimulation (407). This transcript results from differential splicing and polyadenylation of the primary transcript, and encodes a protein limited to the very N-terminal part of thyroglobulin. As this truncated protein still contains a major hormonogenic

site(408), it could suggest that, in conditions in which the balance of thyroid metabolism would favor hormone synthesis over iodine storage (e.g., shortage of iodine), the rat thyrocyte would manufacture a shorter thyroglobulin with a preserved hormonogenic ability but lacking many of the nonhormonogenic tyrosines.

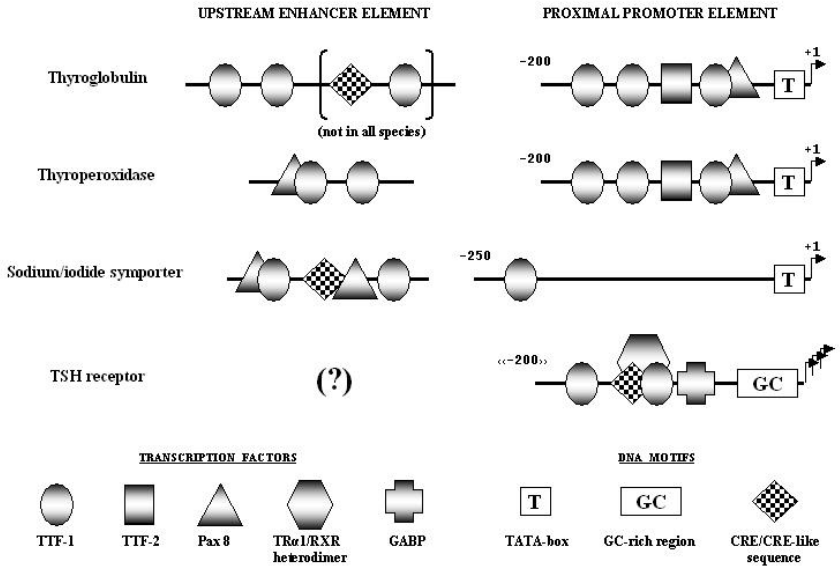



***Fig. 1-15.*** *Schematic of the known regulatory elements of thyroglobulin, thyroperoxidase, sodium/iodide symporter and TSH receptor genes. The organization of the proximal promoter and upstream enhancer elements of the different genes is depicted as determined in the studied so far. Coordinates of the proximal promoters are in base pairs and refer to the transcription start site as +1. The positions of the upstream enhancer elements relative to the transcription start site are not indicated as they vary extensively among the different species.*

## Thyroperoxidase

In the species studied so far, the architecture of the proximal promoter region of the thyroperoxidase gene strikingly resembles that of the corresponding region of the thyroglobulin gene (409;410) (see Fig. 1-15). The upstream enhancer element also encompasses a pair of TTF-1 binding sites and contains an additional binding site for Pax 8, as compared to its counterpart in the thyroglobulin gene (411;412). Here again, the combination of the upstream enhancer and proximal promoter supports the synergistic action of TTF-1 and Pax 8 on gene transcription (395).

Despite the existence of this high similarity, thyroperoxidase gene transcription is more tightly and more rapidly controled by TSH and cAMP than that of the thyroglobulin gene in primary cultured thyrocytes, and does not require a new protein synthesis (400;413). Contrary to the

thyroglobulin gene also, the thyroperoxidase gene is not expressed in the absence of circulating TSH or functional TSH receptors in intact animals (156). On the other hand, the constitutive hyperactivation of the cAMP cascade leads to an increased expression of the gene as compared to the normal situation (414). In spite of their lack of a classical CRE, the proximal promoter sequences have been shown to mediate this TSH/cAMP control on transcription in transfection experiments (409). Low doses of iodide also decrease thyroperoxidase gene expression in vivo, while the expression of thyroglobulin remains unaffected (261). Thus, apart from their basic dependence on the presence of the transcription factors TTF-1 and Pax 8, which insures their shared thyroid-restricted expression, the thyroperoxidase and thyroglobulin genes distinguish themselves significantly regarding the control of their transcription. It has been postulated recently that the hormone-induced developmental activation of the thyroperoxidase gene involves the concerted action of TTF-2 and NF-1, both of which bind neighbouring sequences in the gene promoter (see figure 1-15) resulting in the initial opening of the chromatin structure of the promoter (354).
The existence of a major thyroperoxidase mRNA isoform has been detected in man (415). It appears to encode a protein devoid of its normal enzymatic activity.
It has been postulated recently that the hormone induced developmental activation of the thyroperoxidase gene involves the concerted action of TTF2 and NF1, both of which bind neighbouring sequences in the gene promoter (see figure 1-15) resulting in the initial opening of the chromatin structure of the promoter (416). There are indications that, as in the case of the DUOXes, the expression of thyroperoxidase at the membrane of the thyrocyte is cell specific and may require specific factors (417).

## Sodium/iodide Symporter

Although the sodium/iodide symporter plays a key role in thyroid hormonogenesis, the expression of the corresponding gene is not restricted to the thyroid. Accordingly, the proximal promoter sequences identified so far do not exhibit a thyroid-specific activity in vitro (418;419), even if this activity may be marginally increased in the presence of TTF-1 (420). The robust and appropriately controlled expression of this gene in the thyroid seems to be mediated essentially by the upstream enhancer element which contains binding sites for both TTF-1 and Pax 8, and a cAMP responsive element (CRE)-like DNA motif which is involved in the control by TSH/cAMP (333;421) (see Fig. 1-15). As for the thyroperoxidase gene, TSH signaling is indispensable for sodium/iodide symporter gene transcriptional activation in vivo (155;156), and iodide downregulates the expression of the gene (261). A very similar control is thus exerted on the expression of thyroperoxidase and sodium/iodide symporter genes in the thyroid in spite of the fact that their known regulatory regions bear only limited

resemblances.

## Thyropin Receptor

Like the gene described above, the TSH receptor gene is also expressed in tissues other than the thyroid. Again, the promoter elements identified presently, which include binding sites for thyroid hormone receptor (TR)-α1/retinoid-X receptor (RXR) heterodimer (422), GA-binding protein (GABP) (423), cAMP responsive element binding (CREB) protein(422)and TTF-1 (424) (see Fig. 1-15), do not display a clear thyroid-specific activity in transfection experiments, as could be expected. Contrary to the promoters described so far, the promoter of the TSH receptor gene does not contain a TATA-box motif, but encompasses a GC-rich region preceding the multiple neighbouring transcription start sites. Consistent with the presence of TTF-1 binding sites in the promoter region, the TSH receptor genes exhibits a decreased activity in animals expressing reduced level of TTF-1 (425). No other regulatory DNA element specifically involved in the thyroid-specific expression of this gene has been identified as yet. On the other hand, DNA demethylation events in the promoter region have been observed in thyroid cells expressing the TSH receptor gene, as compared to non-expressing cells (423).

The control exerted on the expression of the TSH receptor gene in the thyroid seems to be more complex than the ones described previously. Discordant effect of TSH/cAMP on the expression of this gene have been reported depending on the nature of the experimental system used (426). The presence in the promoter region of a CRE-like DNA motif which appears to be able to bind the CREB protein(422), a transcriptional activator directly activated by cAMP, as well as the CREM isoform ICER(378), a transcriptional repressor induced by cAMP, could explain both reported increase and decrease in gene expression following TSH stimulation, depending on the relative amounts of these factors (and likely of other CRE-binding proteins also) preexisting in the studied cells and the kinetics of the individual observations. Moreover, the binding site of the TRα1/RXR heterodimer identified in this promoter encompasses the CRE-like motif (see figure 1-15), which may add a further level of complexity depending on the availability of thyroid hormone in the experimental system.

On the other hand, the possible existence of species-specific differences could also account for the occurrence of seemingly discrepant reports (285). The accumulation of TSH receptor mRNA requires an active protein synthesis in primary cultured dog thyrocytes (285), which is reminiscent of what was observed for the thyroglobulin gene (see above). Considering the

facts that, after the TSH receptor gene, the thyroglobulin gene is the most affected in its expression by a reduced TTF-1 availability as compared to the other known thyroid-specific genes(425), and that, alike the TSH receptor gene itself, the thyroglobulin gene is activated independently of the TSH/TSHR signaling during thyroid development(155;156), it suggests that these two genes may share at least partially similar control mechanisms.

Control of TSH receptor gene expression has been studied in the FRTL5 cell line (426-428), the canine thyrocyte in primary culture (285), cultured human thyrocytes (429;430), and human thyroid cancer (365;431). The general conclusion emerging from these studies is the extreme robustness of TSH receptor gene expression as compared with the other markers of thyroid cell differentiation (thyroglobulin and thyroperoxidase). In the dog, levels of TSH receptor mRNA remain virtually unchanged in animals subjected for 28 days to hyperstimulation by TSH secondary to treatment with methimazole or to TSH withdrawal achieved by administration of thyroxine (285). In the same study, the effect of TSH or forskolin has been investigated in dog thyrocytes in primary culture. This experimental system has the advantage that the differentiation state of the cells can be manipulated at will: cAMP agonists maintain expression of the differentiated phenotype, whereas agents such as EGF, tetradecanoyl phorbol acetate (TPA), and serum lead to "dedifferentiation" (386). The results demonstrate that the dedifferentiating agents reduce accumulation of the receptor mRNA. However, contrary to what is observed with thyroglobulin and thyroperoxidase mRNA, the inhibition is never complete. TSH or forskolin is capable of promoting reaccumulation of the receptor mRNA, a maximum being reached after 20 hours. As with thyroglobulin but at variance with the thyroperoxidase gene, this stimulation requires ongoing protein synthesis (285). Chronic stimulation of cultured dog thyrocytes by TSH for several days does not lead to any important downregulation in mRNA. Similar data have been obtained with human thyrocytes in primary culture (285;430). By contrast, negative regulation of receptor mRNA accumulation has been observed in immortal FRTL5 cells after treatment with TSH or TSAB (426;428). This difference versus human and canine cells must probably be interpreted in the general framework of the other known differences in phenotype and regulatory behavior of this cell line as compared with primary cultured thyrocytes (see below) (243). The presence in the promoter region of a CRE-like DNA motif which appears to be able to bind the CREB protein (422), a transcriptional activator directly activated by cAMP, as well as the CREM isoform ICER (378), a transcriptional repressor induced by cAMP, could explain both reported increase and decrease in gene expression following TSH stimulation, depending on the relative amounts of these factors (and likely of other CRE-binding proteins also) preexisting in the studied cells and the kinetics of the individual observations. Moreover, the binding site of the TRα1/RXR heterodimer identified in

this promoter encompasses the CRE-like motif (see Fig. 1-15), which may add a further level of complexity depending on the availability of thyroid hormone in the experimental system.

The effect of malignant transformation on the amounts of TSH receptor mRNA has been studied in spontaneous tumors in humans (365;431), in a murine transgenic model of thyroid tumor promoted by expression of the simian virus-40 large T oncogene(432), and in FRTL5 cells transformed with v-*ras* (427). In the two last models, expression of the TSH receptor gene was suppressed: the tumor or cell growth became TSH independent. In the transgenic animal model, loss of TSH receptor mRNA seemed to take place gradually, with early tumors still displaying some TSH dependence for growth. In the human tumors a spectrum of phenotypes was observed. As expected, anaplastic tumors had completely lost the receptor mRNA, as well as other markers of thyrocyte differentiation (thyroglobulin and thyroperoxidase). In papillary carcinoma, variable amounts of TSH receptor mRNA were invariably found (431), even in the tumors that had lost the capacity to express the thyroglobulin or thyroperoxidase genes (431). These data agree well with the observations of thyrocytes in primary culture: expression of the TSH receptor gene is robust and it persists in the presence of agents (or after several steps in tumor progression) that promote extinction of the other markers of thyroid cell differentiation. This evidence leads to the conclusion that the basic marker of the thyroid phenotype is probably the TSH receptor itself, which makes sense: the gene encoding the sensor of TSH—the major regulator of thyroid function, growth, and differentiated phenotype—is virtually constitutive in thyrocytes. From a pragmatic viewpoint, these data provide a rationale for the common therapeutic practice of suppressing TSH secretion in patients with a differentiated thyroid tumor (433).

## Thyroid oxidases

Two distinct genes, DUOX-1 and -2 (also known as ThOX1 and ThOX2), both significantly related to the gene encoding the phagocyte NADPH oxidase gp91$^{Phox}$, are expressed in the thyroid essentially, but not only, at least for ThOX2(434;435). In the dog, ThOX mRNAs accumulate in response to TSH/cAMP stimulation (434). This effect is much less apparent in man (434), and in the rat conflicting results were obtained in vivo and in the established FRTL-5 cell line respectively (435). The proximal promoter sequences of both human ThOX1 and ThOX2 genes have been delineated using a functional assay (436). These promoters do not exhibit a thyroid cell-restricted activity in vitro, and are not controled positively by TSH/cAMP, as could be expected. No known regulatory DNA motif could be identified obviously within these promoter sequences. Notably, like the promoter of the TSH receptor gene, both of these promoters are devoid of a TATA-box motif. Recently, genes encoding proteins required for the functional maturation of the thyroid oxidases were identified in the

close vicinity of both ThOX genes, and named DUOXA-1 and -2 respectively (416). Within each ThOX-DUOXA pair, the two genes are arranged in a head to head configuration, the distance separating the putative transcription starts being in the range of one hundred of base pairs only. Each DUOXA is necessary for expression of the corresponding DUOX (i.e. DUOXA1 for DUOX1, DUOXA2 for DUOX2) at the membrane (416).

## CONTROL OF GROWTH AND DIFFERENTIATION

### Thyroid Cell Turnover

The thyroid is composed of thyrocytes (70%), endothelial cells (20%), and fibroblasts (10%) (proportions measured in dog thyroid) (110). Human thyroids: 80% follicular cells for 20% stromal endothelial cells and fibroblasts 80% (437;437). In a normal adult the weight and composition of the tissue remain relatively constant. Because a low but significant proliferation is demonstrated in all types of cells, it must be assumed that the generation of new cells is balanced by a corresponding rate of cell death (152;437;438). The resulting turnover is on the order of one per 5 to 10 years for human thyrocytes, that is, six to eight renewals in adult life, as in other species. In one child the turnover was 2 per year (438). Normal cell population can therefore be modulated mainly at the level of proliferation but also secondarily of cell death. In growth situations, that is, either in normal development or after stimulation, the different cell types grow more or less in parallel, which implies coordination between them (164;185;439;440). Because TSH receptors and iodine metabolism and signaling coexist only in the thyrocyte in thyroid, this cell, sole receiver of the physiologic information, must presumably control the other types of cells by paracrine factors such as FGF, IGF-I, NO, and the like (164). The successful isolation of human thyroid endothelial cells will allow a more detailed study of these interactions (441). TSH has been demonstrated to upregulate the production of vascular endothelial cell growth factor (VEGF) by human thyrocytes (442). It is interesting in this regard that the vascular support of the follicles reflects their activity suggesting the concept of angiofollicular units (363;364).

### The Mitogenic Cascades

The study of the control of thyroid cell proliferation has been much confused by the unwarranted extrapolation of data obtained in different model systems, including different rat thyroid cell lines at different stages in their evolution, to the human thyroid. Sentences like

"Agent X stimulates pathway Y in PCCl3 cells, thus human thyroids could ne treated by agent Z which inhibits agent X action" are not acceptable even if only implied (243).

In the thyroid at least three families of distinct mitogenic pathways have been well defined (Fig. 1-16): (1) the hormone receptor–Gs-adenylyl cyclase–cAMP-dependent protein kinase system, (2) the hormone receptor–tyrosine protein kinase pathways, and (3) the hormone receptor–Gq-phospholipase C cascade (152;443). The thyroid also autoregulates its size by an unknown mechanism. Thyroxin treated dogs, and humans, compensate the loss of one thyroid lobe independently of TSH (261).

The receptor–tyrosine kinase pathway may be subdivided into two branches; some growth factors, such as EGF, induce proliferation and repress differentiation expression, whereas others, such as FGF in dog cells or IGF-I and insulin, are either mitogenic or are necessary for the proliferation effect of other factors without being mitogenic by themselves, but they do not inhibit differentiation expression (179;404). In human thyroid cells, IGF-I is required for the mitogenic action of TSH or EGF but by itself it only weakly stimulates proliferation (236). In dog and human thyrocytes in primary cultures, after induction of insulin receptors by TSH, physiological concentrations of insulin permit the proliferative action of TSH (170;171). In PCCl3 and rat cells, and in mouse thyroid in vivo (168), IGF-I is weakly mitogenic per se (444), whereas in pig thyroid cells it produces a strong effect (445).

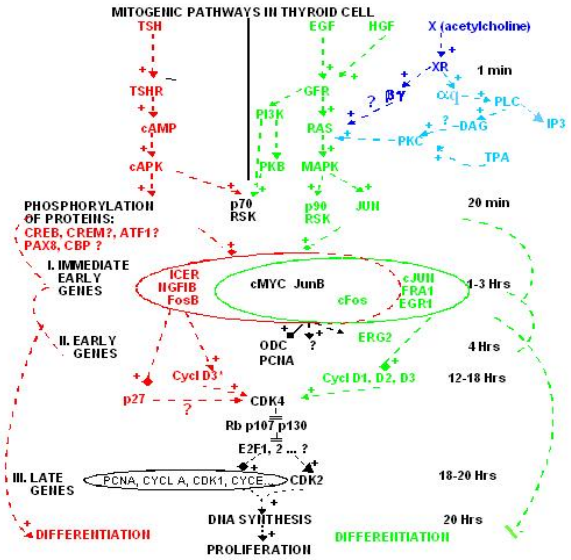



***Fig 1-16.*** *Mitogenic pathways in the thyroid. Data from the thyroid cell systems are integrated into the present general scheme of cell proliferation cascades. In the first line, known activator of various cascades in dog and human thyroid cells are shown. Various levels indicate a time sequence and postulated causal relationships from initial interaction of extracellular signal with its receptor to endpoints: proliferation and differentiation expression.*

*In dog but not in human thyroid cells, acetylcholine through muscarinic receptors activates the phospholipase C cascade. PCNA, proliferating cell nuclear antigen; cAPK, cyclic adenosine monophosphate-dependent kinase; CDK, cyclin-dependent kinase; DAG, diacylglycerol, ——→⁺, stimulation; ——/⁺, inhibition; ; ——♦⁺, induction; GFR, growth factor receptor; ODC, ornithine decarboxylase; PI3K, phosphatidylinositol 3-kinase; PKB, protein kinase B; PLC, phospholipase C; RSK, ribosomal S6 kinase.*

It should be noted that TSH directly stimulates proliferation while maintaining the expression of differentiation. Differentiation expression, as evaluated by NIS or by thyroperoxidase and thyroglobulin mRNA content or nuclear transcription, is induced by TSH, forskolin, cholera toxin, and cAMP analogues (152). These effects are obtained in all the cells of a culture, as shown by in situ hybridization experiments (404). They are reversible; they can be obtained either after the arrest of proliferation or during the cell division cycle (387;404). Moreover, the expression of differentiation, as measured by iodide transport, is stimulated by concentrations of TSH lower than those required for proliferation (236).

All the proliferation effects of TSH are mimicked by nonspecific modulators of the cAMP cascade, that is, cholera toxin and forskolin (which stimulate adenylate cyclase), cAMP analogues (which activate the cAMP-dependent protein kinases), and even synergistic pairs of cAMP analogues acting on the different sites of these two kinases (222;236;446). They are reproduced in vitro and in vivo by expression of the adenosine $A_2$ receptor, which is constitutively activated by endogenous adenosine (414), and by constitutively active Gsα (447) and cholera toxin (448). They are inhibited by antibodies blocking $G_s$ (449). Inhibition of cAMP-dependent protein kinases (PKA) inhibits the proliferation and differentiation effects of cAMP (450;451). Moreover, stimulation of PKA by selective cAMP analogs that do not activate EPAC proteins is sufficient to fully mimic mitogenic effects of TSH and forskolin in dog thyrocytes (450). There is, therefore, no doubt that the mitogenic and differentiating effects of TSH are mainly and probably entirely mediated by cAMP-dependent protein kinases. A complementary role of the Rap guanyl nucleotide exchange factor EPAC and of Rap has been proposed in rat thyroid cell lines (452;453) but not observed in canine thyroid primary cultures (450).

EGF also induces proliferation of thyroid cells from various species (152;164;236). However, the action of EGF is accompanied by a general and reversible loss of differentiation expression assessed as described above (386). The effects of EGF on differentiation can be dissociated from its proliferative action. Indeed, they are obtained in cells that do not proliferate in the absence of insulin and in human cells, in which the proliferative effects are weaker, or in pig cells at concentrations lower than the mitogenic concentrations (152).

Finally, the tumor-promoting phorbol esters, the pharmacologic probes of the protein kinase C system, and analogues of diacylglycerol also enhance the proliferation and inhibit the

differentiation of thyroid cells. These effects are transient because of desensitization of the system by protein kinase C inactivation.

Activation of the Gq/phospholipase C (PLC)/PKC cascade by a more physiological agent such as carbamylcholine in dog thyroid cells does not reproduce all the effects of phorbol esters. In particular, prolonged stimulation of this cascade by carbamyl choline permits the cAMP-dependent mitogenesis of dog thyrocytes (454), but unlike phorbol esters it does not induce proliferation in the presence of insulin (455). The Ras protooncogene is strongly activated by phorbol esters but more weakly by carbachol (246). Thus we cannot necessarily equate the effects of phorbol esters and prolonged stimulation of the PLC cascade. The dedifferentiating effects of phorbol esters do not require their mitogenic action either. Thus the effects of TSH, EGF, and phorbol esters on differentiation expression are largely independent of their mitogenic action (152).

In several thyroid cell models, very high insulin concentrations are necessary for growth even in the presence of EGF. We now know that this prerequisite mainly reflects a requirement for IGF-I receptor (152;234;444;456). It is interesting that in FRTL5 cells, as in cells from thyroid nodules, this requirement may disappear, probably because the cells secrete their own somatomedins and thus become autonomous with regard to these growth factors (234;457). By contrast, in primary cultures of normal dog and human thyrocytes, very low concentrations of insulin, acting on insulin receptors, are sufficient to support the mitogenic effects of TSH and cAMP when insulin receptors have been induced to high levels by TSH (170;171). This puzzling regulation, which is reminiscent of the induction of insulin receptors during the differentiation of adipocytes, suggests that thyroid might well be revealed as a more specific target of circulating insulin than hitherto recognized.

In the action of growth factors on receptor protein tyrosine kinase pathways, the effects on differentiation expression vary with the species and the factor involved: from stimulation (e.g., insulin, as well as IGF-II in dog and FRTL5 cells) (405) to an absence of effect (458), to transitory inhibition of differentiation during growth (FGF and HGF in dog cells (179;459) to full but reversible dedifferentiation effects (EGF in dog and human cells) (235;386). Ret/PTC rearrangements, activating mutations of Ras, as well as oncogenic mutation of B-Raf, which are responsible of most differentiated carcinoma, constitutively activate the signaling cascades of growth factors (460-462).

The kinetics of the induction of thymidine incorporation into nuclear DNA of dog thyroid cells is similar for TSH, forskolin, EGF, and TPA. Whatever the stimulant, a minimal delay of about 16 to 20 hours takes place before the beginning of labeling, that is, the beginning of DNA synthesis (463). This time is the minimal amount required to prepare the necessary machinery. For the cAMP and EGF pathways, the stimulatory agent has to be present during this whole prereplicative period; any interruption in activation (e.g., by washing out the

stimulatory forskolin) greatly delays the start of DNA synthesis (464). This limitation explains why norepinephrine and prostaglandin E, which also activate the cAMP cascade, do not induce growth and differentiation: the rapid desensitization of their receptors does not allow a sustained rise in cAMP levels.

The three main types of mitogenic cascade, specifically, the growth factor–protein tyrosine kinase, phorbol ester–protein kinase C, and TSH-cAMP cascades, are fully distinct at the level of their primary intracellular signal and/or the first signal-activated protein kinase (152).

Iodide actually inhibits the cAMP and the $Ca^{2+}$-phosphatidylinositol cascades and in a more delayed and chronic effect decreases the sensitivity of the thyroid to the TSH growth response. These effects are relieved, according to the general paradigm of Van Sande, by perchlorate and methimazole (254;465).

Steps in the Mitogenic Cascades (Fig. 1-17)

The phenomenology of EGF, TPA, and TSH proliferative action cells has been partially elucidated using dog thyroid primary cultures (152;243;368). The mechanisms of TSH/cAMP mitogenic effects have also been investigated using immortal rat thyroid cell lines (FRTL-5, WRT and PC Cl3 cells) (244). Whereas the signaling cascades involved in the action of growth factors and IGF-I are likely to be well conserved in the different thyroid systems, as generally observed in the other cell types, the mechanistic logics of cell cycle regulation by cAMP has disappointingly turned out to strongly diverge in the various thyroid in vitro models (243). These divergences do not only reflect species differences (152). Among the apparently similar rat thyroid cell lines, or even among different subclones of FRTL-5 cells, major differences have been observed (243). For instance, the PI3 kinase/PKB cascade is activated by cAMP in WRT cells (466), but inhibited by cAMP in PC Cl3 cells (467). The induction of c-jun by TSH/cAMP in FRTL-5 cells and its repression by cAMP in WRT cells (468) as in dog (469) and human thyrocytes likely reflect major differences in upstream signaling cascades, and should result in divergent expression of downstream target genes, such as cyclin D1. Cyclin D1 synthesis, an accepted endpoint of mitogenic cascades, is indeed induced by cAMP in FRTL-5 and PC Cl3 cells, but rather repressed by cAMP in dog and human thyroid primary cultures (243;470). The reasons for such discrepancies are unclear. Some signaling features, when they lead to selective proliferative advantages, might have been acquired during the establishment and continuous cultures of the cell lines and stabilized by subcloning. Many mechanisms demonstrated in the dog thyroid primary culture system so far apply to normal human thyrocytes (470), but much remains to be defined (243). In the following lines, we thus rely mostly on these systems.

Three biochemical aspects of the proliferative response occurring at different times of the prereplicative phase have been considered. The pattern of protein phosphorylation induced

within minutes by TSH is reproduced by forskolin and cAMP analogues. It totally diverges from the phosphorylations induced by EGF and phorbol esters (471). EGF, HGF and phorbol ester actions rapidly converge on the activation of Ras (246) and the resulting activation of p42/p44 MAP kinases and $p90^{RSK}$ (232;233;245). PI-3-kinase and its effector enzyme PKB are activated for several hours only by insulin and IGF-I, the effect of EGF being short lived (232). This activity is therefore the one specific feature of insulin action and presumably the mechanism of the facilitating effect on mitogenesis. In dog thyrocytes, only HGF can trigger cell proliferation in the absence of insulin/IGF-I; this is explained by the fact that only this factor strongly activates both PI3 kinase and MAP kinase cascades (232). Only insulin, IGF-I and HGF also markedly enhance general protein synthesis and induce cell hypertrophy (472). By contrast, TSH and cAMP are very unique as mitogens, as they do not activate Ras, the PI3kinase/PKB pathway, or any of different classes of MAP kinases in dog thyrocytes (232;233;245;246). TSH and cAMP also do not activate MAPkinases in human thyrocytes (233). The phosphorylation and activation of $p70^{S6K}$ and thus likely of mTOR cascade constitutes the only early convergence point of growth factor and cAMP-dependent mitogenic cascades (232;473). A recent study has demonstrated the crucial role of this cascade for TSH-elicited thyroid follicular hyperplasia *in vivo* in mice (474). Indeed, as found in dog thyroid primary cultures (232) and PCCl3 cells (Blancquaert and Roger, unpublished), TSH stimulates in mice the mTOR/ $p70^{S6K}$ axis without activating PKB, and a rapamycin derivative abrogates the hyperplastic (but, interestingly, not the hypertrophic) responses to TSH (474). The cAMP-dependent mitogenesis and gene expression also appears to require the phosphorylation by PKA and activity of CREB/CREM transcription factors (475;476).

As in other types of cells, EGF and TPA first enhance c-fos and c-myc mRNA and protein concentrations in dog thyrocytes. On the other hand, TSH and forskolin strongly, but for a short period, enhance the c-myc mRNA concentration and with the same kinetics as the enhancement of the c-fos mRNA concentration by EGF/TPA. In fact, cAMP first enhances and then decreases c-myc expression. This second phenomenon is akin to what has been observed in the fibroblast, in which cAMP negatively regulates growth. As in fibroblasts, EGF and TPA enhance c-jun, junB, junD, and egr1 expression. However, as in fibroblasts, activators of the cAMP cascade decrease c-jun and egr1 expression. c-Jun is therefore not, as has been claimed, a gene whose expression is universally necessary for growth (377;469).

The investigation of the pattern of proteins synthesized in response to the various proliferation stimuli has suggested very early that the proliferation of dog thyroid cells is controlled during G1 phase by at least two largely distinct, cAMP-dependent or cAMP-independent, pathways (232;477). Recent microarray analyses have confirmed and extended this concept in human thyrocytes (133;478). Nevertheless, the different mitogenic

cascades are expected to finally modulate the level and activity of proteins that are the primary regulators of the cell cycle machinery.

As generally considered, mitogenic signals regulate mammalian cell cycle by stimulating the accumulation of D-type cyclins and their assembly through a ill-defined mechanism with their partner the cyclin-dependent kinases (cdk) 4 and 6. These complexes operate in mid-to-late G1 phase to promote progression through the restriction point, and thus commit cells to replicate their genome (479). In the current model, this key decision depends on the initiation by cyclin D-cdk complexes of the phosphorylation of the growth/tumor suppressor protein pRb, which triggers the activation of transcription factors, including those of the E2F family, the synthesis of cyclin E and then cyclin A, and cdk2 activation by these cyclins. Activated cdk2 in turn further phosphorylates pRb and other substrates and initiates and organizes the progression through the DNA synthesis phase (479). The down regulation of cdk inhibitors of the CIP/KIP family, including $p27^{kip1}$, by mitogenic factors and/or their sequestration by cyclin D-cdk complexes participate to cdk2 activation, but their proposed role of adaptor and/or nuclear anchor for cyclin D-cdk complexes suggests positive influences on cell cycle progression as well (480). These mechanisms have been well studied in dog thyroid cells (Fig. 1-17). As expected, the different mitogenic stimulations (TSH, cAMP, growth factors) require the activity of cdk4 (481), and converge on the inactivating phosphorylation of pRb and related proteins p107 and p130 (482), on the phosphorylation and nuclear translocation of cdk2, and on the induction of cyclin A and cdc2 (483). These effects are dependent on insulin action (482;484). What is strikingly different between the cascades is the mechanism of D-type cyclin-cdk4 activation. TSH, unlike all the other known mitogenic factors, does not induce the accumulation of cyclins D (485), but it paradoxically stimulates the expression of the cdk "inhibitor" $p27^{kip1}$ (486). However the predominant cyclin D3 is required for the proliferation stimulated by TSH, but not in the proliferation of dog thyrocytes stimulated by EGF or HGF that induce cyclins D1 and D2 in addition to increasing cyclin D3 levels (485). The formation and the nuclear translocation of essential cyclin D3-cdk4 complexes depend on the synergistic interaction of TSH and insulin (484;485). These complexes are absent from cells stimulated by TSH or insulin alone. Paradoxically, in the absence of insulin TSH inhibits the basal accumulation of cyclin D3 (484). On the opposite insulin alone stimulates the required cyclin D3 accumulation and it overcomes in large part the inhibition by TSH (484), but it is unable to assemble cyclin D3-cdk4 complexes in the absence of TSH. In the presence of insulin, TSH (cAMP) unmasks some epitopes of cyclin D3 and induces the assembly of cyclin D3-cdk4 complexes and their import into nuclei (484;485) where these complexes are anchored by their association with $p27^{kip1}$ (487;488). This also sequesters p27 away of cdk2 complexes (487), thus contributing to cdk2 activation. Moreover, cAMP exerts an additional crucial function in very late G1

phase to stimulate the enzymatic activity of cyclin D3-cdk4-p27 complexes, which involves the stimulation of the activating Thr172-phosphorylation of cdk4 (489). TGFβ selectively inhibits the cAMP-dependent proliferation of dog thyrocytes by preventing the association of the cyclin D3-cdk4 complex with nuclear $p27^{kip1}$and the Thr172-phosphorylation of cdk4 (487;488) (490)

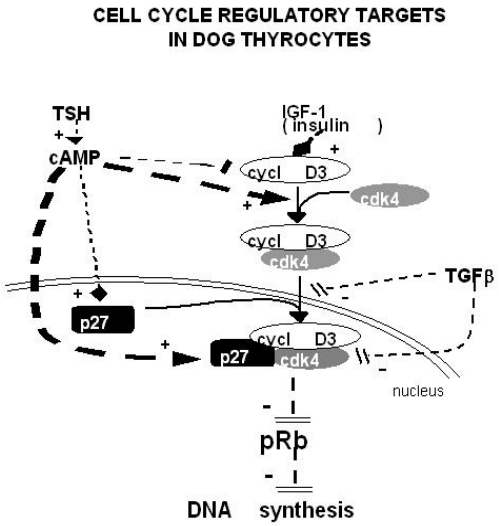



***Fig. 1-17*** *: Targets of cell cycle regulatory effects of TSH, insulin/IGF-1 and TGFβ, as demonstrated in the dog thyroid primary culture system. Diamond/rectangle arrowheads represent inductions/repressions: the other dashed arrows are activations (+) and inhibitions (-). TSH (cAMP) does not induce cyclins D but assembles and then activates the cyclin D3-cdk4-p27 holoenzyme. IGF-1 and insulin allow the accumulation of the required cyclin D3. TGFβ inhibits the nuclear translocation of the cyclin D3-cdk4 complex, its association to p27 and its activation by TSH(cAMP). See text for full explanation.*

The investigation of cell cycle regulatory proteins has thus clearly established that both cdk4 activation and pRb phosphorylation result from distinct but complementary actions of TSH and insulin, rather than from their interaction at an earlier step of the signaling cascades (454;484) (Fig. 1-17). Together with the fact that the necessary increase of cell mass before division depends on insulin/IGF-I but not TSH (472), these observations provide a molecular basis for the well established physiological concept that in the regulation of normal thyroid cell proliferation, TSH is the “decisional” mitotic trigger, while locally produced IGF-I and/or circulating insulin are supporting “permissive” factors (152). Of note, in all these experiments,

the facilitative action of insulin can be replaced by activation of the Gq/PLC cascade by carbamylcholine (454).

Studies of protein phosphorylation, proto-oncogene expression, and cell cycle regulatory proteins in dog thyrocytes allow discrimination between two models of cAMP action on proliferation in this system: a direct effect on the thyrocyte or an indirect effect through the secretion and autocrine action of another growth factor. If the effect of TSH through cAMP involved such an autocrine loop, one would expect to find faster kinetics of action of the growth factor and at least some common parts in the patterns of protein phosphorylation and protein synthesis induced by cAMP and the growth factor. The results do not support such an hypothesis, at least for the growth factors tested (152) (Fig. 1-16). Moreover, the data on cAMP action in the dog and human thyrocyte systems do not support a major role for various mechanisms involving cross-signaling of cAMP with growth factor pathways, as claimed in rat thyroid cell line studies (reviewed and discussed in (243)). Indeed, in primary cultures of normal human thyrocytes, EGF+serum increases cyclin D1 and p21 accumulation, and it stimulates the assembly and activity of cyclin D1-cdk4-p21. By contrast, TSH (cAMP) represses cyclin D1 and p21, but it stimulates the activating phosphorylation of cdk4 and the pRb-kinase activity of preexisting cyclin D3-cdk4 complexes (470). Cyclin D1 or cyclin D3 are thus differentially used in the distinct mitogenic stimulations by growth factors and TSH, and potentially in hyperproliferative diseases generated by the overactivation of their respective signaling pathways.

The validity of these concepts in vivo has been established by using transgenic mice models. The expression in thyroid of oncogene E7 of HPV-16, which sequestrates pRb protein, leads to thyroid growth and euthyroid goiter. Expression in the thyroid of the adenosine $A_2$ receptor, which behaves as a constitutive activator of adenylyl cyclase, induces thyroid growth, goitrogenesis, and hyperthyroidism (414). Similar, albeit weaker phenotypes are obtained in mice expressing constitutive Gs (the G protein activating adenylyl cyclase) (447) or cholera toxin (448). A contrario, the expression in thyroid of a dominant negative CREB provokes a marked thyroid hypotrophy, suggesting the crucial role of CREB and its activating phosphorylation by PKA (476). By contrast, transgenic mice overexpressing both human IGF-I and IGF-I receptor in their thyroid (TgIGF-I–TgIGF-IR) and the downregulation of PTEN the PIP3 3’ phosphatase develop only a mild thyroid hyperplasia and respond to some extent to a goitrogenic effect of antithyroid drugs while maintaining a comparatively low serum TSH level. This indicates some autonomy of these thyroids, as in acromegalic patients, and a much greater sensitivity to endogenous TSH (168). Very recently, thyrocyte-specific deficiency of $Gq/G_{11}$ (the G proteins activating PLC□) in mice was shown to impair not only the TSH-stimulated iodine-organification and thyroid hormone synthesis, but also TSH-dependent development of goiter (491). It remains to be defined whether this impaired

follicular cell hyperplasia could result in part from the lack of induction of VEGF and angiogenesis (491) which normally accompany goitrogenesis. Nevertheless, the phenotype of these mice underscores the role in TSH-dependent goitrogenesis of PLC, which is activated by TSH but even more strongly by neurotransmitters. Noteworthy, section of inferior laryngeal nerve in rats was similarly found to impair both thyroid function and growth stimulated by TSH (492). Moreover, activation of Gq /PLC by carbamycholine can facilitate cAMP-dependent mitogenesis in dog thyrocytes cultured without insulin or IGF-I (454). On the other hand, expression of Ret, which is a rearranged constitutive growth factor receptor, in papillary thyroid carcinoma (PTC), leads to growth, cancer, and hypothyroidism (493;494).

## Proliferation and Differentiation (Fig 1-18)

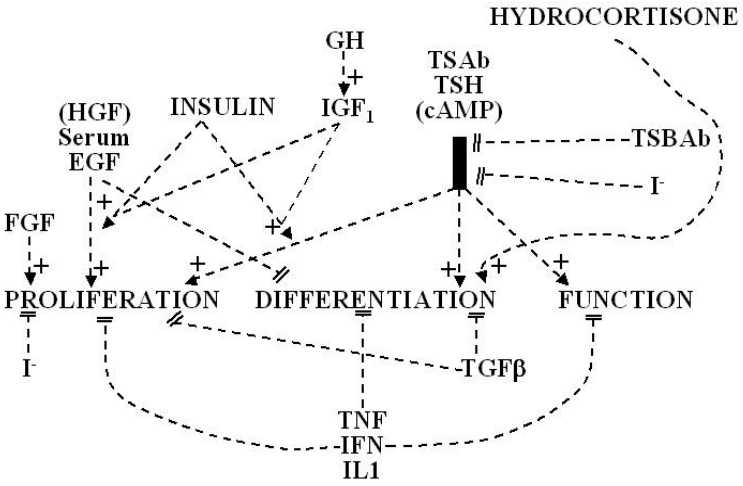



***Fig 1-18.*** *Main controls of the principal biologic variables of the human thyrocyte. EGF, epidermal growth factor; FGF, fibroblast growth factor; GH, growth hormone; HGF, hepatocyte growth factor; $I^-$, iodide; IGF-I, insulin-like growth factor; IFN, interferon; IL-1, interleukin-1; TGF$\beta$, tumor growth factor-$\beta$; TNF, tumor necrosis factor; TSAb, thyroid-stimulating immunoglobulins; positive control (stimulation); $\rightarrow^+$: negative control (inhibition) $---/$.*

The incompatibility at the cell level of a proliferation and differentiation program is commonly accepted in biology. In general, cells with a high proliferative capacity are poorly differentiated, and during development such cells lose this capacity as they progressively differentiate. Some cells even lose all potential to divide when reaching their full differentiation, a phenomenon called terminal differentiation. Conversely, in tumor cells, proliferation and differentiation expression are inversely related. Activation of Ras and p42/p44 MAPkinases, induction of c-jun, sustained expression of c-myc, induction of cyclin

D1 and down regulation of $p27^{kip1}$, all have been shown to be causatively associated not only with proliferation, but also with loss of differentiation in a large variety of systems, sometimes independently of proliferation effects. It is therefore not surprising that in thyroid cells the general mitogenic agents and pathways, phorbol esters and the protein kinase C pathway, EGF, and in calf and porcine cells, FGF and the protein tyrosine kinase pathway, induce both proliferation and the loss of differentiation expression. The effects of the cAMP cascade are in striking contrast with this general concept. Indeed, TSH and cAMP induce proliferation of dog thyrocytes while maintaining differentiation expression; both proliferation and differentiation programs can be triggered by TSH in the same cells at the same time (404). This situation is by no means unique because neuroblasts in the cell cycle may also simultaneously differentiate. It is tempting to relate this apparent paradox to the unique characteristics of the cAMP-dependent mitogenic pathway, such as the lack of activation (or even the inhibition) of the Ras/MAPkinase/c-jun/cyclin D1 cascade, as demonstrated in dog and human thyrocytes. For instance, if one generalization could be made about proto-oncogenes, it is the dedifferentiating role of c-myc. A rapid and dramatic decrease in c-myc mRNA by antisense myc sequences induces differentiation of a variety of cell types. It is therefore striking that in the case of the thyrocyte, in which activation of the cAMP cascade leads to both proliferation and differentiation, the kinetics of the c-myc gene expression appears to be tightly controlled. After a first phase of 1 hour of higher level of c-myc mRNA, c-myc expression is decreased below control levels. In this second phase, cAMP decreases c-myc mRNA levels, as it does in proliferation-inhibited fibroblasts. It even depresses EGF-induced expression. The first phase could be necessary for proliferation, whereas the second phase could reflect stimulation of differentiation by TSH (376;495). The specific involvement of cyclin D3 in the cAMP-dependent mitogenic stimulation of dog and human thyrocytes, but not for their response to growth factors, is also interesting in this context (470;485). Indeed, unlike cyclins D1 and D2, cyclin D3 is highly expressed in several quiescent tissues in vivo, and its expression is not only stimulated by mitogenic factors but also induced during several differentiation processes associated with a repression of cyclin D1 (496). We have recently shown that the differential utilization of cyclin D1 or cyclin D3 affects the site specificity of the pRb-kinase of cdk4, including in dog and human thyrocytes (470;470). In addition to inhibiting E2F-dependent gene transcription related to cell cycle progression, pRb plays positive roles in the induction of tissue-specific gene expression by directly interacting with a variety of transcription factors, including Pax8 in thyroid cells (497). Whether, the selective utilization of cyclin D3 in the TSH cascade, associated with a more restricted pRb-kinase activity, could allow the preservation of some differentiation-related functions of pRb thus remains to be examined.

We now consider the distinct cAMP-dependent mitogenic pathway, which appears to be adjuncted to the more general mechanisms used by growth factors, as pertaining to the specialized differentiation program of thyroid cells (240). In dog thyrocytes, the proliferation in response to serum or growth factors specifically extincts their capacity to respond to TSH/cAMP as a mitogenic stimulus (498). Similarly, in less differentiated thyroid cancers generated by the subversion of growth factor mechanisms, the TSH-dependence of growth is generally found to be lost.

Because the cell renewal rate is very low in the thyroid (once every 8 years in adults), the role of apoptosis is unimportant. However, under different circumstances the apoptotic role can greatly increase, such as after the arrest of an important stimulation in vitro (499) and in vivo (500) (501;502).

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