1 Introduction

Diseases of the thyroid, in general and of pathological growth in particular, are extremely common, and present one of the most frequent problems encountered in endocrinologic practice. An epidemiological survey carried out in Germany between 1993 and 1994 revealed as much as 50 % of the adult population to suffer from an enlargement of the thyroid gland, 20 to 30 % of these actually requiring some form of treatment (Pfannenstiel et al., 1997). A more recent ultrasonographic screening named “Papillon” was carried out among the German working population between 2001 and 2002, including 214 companies or institutions with 96,278 healthy volunteers. In this study abnormal thyroid findings were discovered in 33.1% of participants, with nodular disease in 9.1% and goitre prevalence in 9.7% of the participants (Reiners et al., 2004). In contrast, a similar survey started in 1997 and finished in 2001, examining 3,915 healthy individuals from Pomerania, a north-eastern region of Germany of (“Study of Health in Pomerania, SHIP”), a formerly iodine-deficient area, revealed even more dramatic results: in as much as 62.0% of the disease-free population, at least one yet unknown thyroid disorder was detected, 36.1% presented with goitre, and in 19.6% one or more nodules were found (Völzke et al., 2005).

Indeed, although our understanding of the mechanisms leading to thyroid pathology is still far from complete, iodine deficiency is most often blamed as cause of goitre development, a causal relationship postulated already as early as 3000 before Christ. Inadequate thyroid hormone synthesis due to lack of iodine results in chronically elevated release of thyroid-stimulating hormone (TSH). TSH release from the pituitary gland is itself controlled by the so-called TSH-releasing-hormone (TRH) produced by neurosecretory cells of the hypothalamus, and these in turn are regulated through a negative feed-back loop by 3,5,3`-triiodo-L-thyroxine (T_3). This results in a tight coupling between TSH-release and the level of circulating thyroid hormone.

At low concentrations, TSH enhances thyroid hormone output by supporting the function of preexisting thyrocytes: several genes, whose products are essential to thyroid hormone synthesis, including those for peroxidase, thyroglobulin, and the iodide symporter, are upregulated by TSH stimulation. Prolonged stimulation, however, results in thyroid cell proliferation, an increase in thyroid mass permitting a more efficient scavenging of serum iodide.

Experiments such as those of Wynford-Thomas *et al.* (Wynford- Thomas et al., 1982), who demonstrated in goitrogen-treated rats that thyroid proliferation follows the level and circadian rhythm of TSH, or those of Peter et al. (Peter et al., 1985), who showed TSH-increasing treatments to markedly enhance the growth of human thyroid tissue transplanted into nude mice, established TSH to be recognized as the major element controlling thyroid growth *in vivo*.

The TSH receptor, a 200-kDa integral membrane protein, belongs to the family of G protein-coupled receptors, and as such possesses the characteristic seven transmembrane helices located at its carboxyl terminus. In comparison to other members of this family it contains a rather large amino-terminal, extracellular hormone-binding domain, a typical feature of the glycoprotein receptors. The TSH- receptor has also been named a „noisy“ receptor, since it shows a relatively high intrinsic activity.

G protein-coupled receptors transmit their signal through a superfamily of small, heterotrimeric GTP-binding proteins. In the inactive state GDP is bound to the α-subunit of these proteins. Ligand binding to the respective receptor then leads to conformational changes that allow the exchange of GTP for the bound GDP. In this activated state the α-subunit will dissociate from the βγ-dimer, and both parts of the trimer set free in this way transmit the activation signal to different effector systems. Signal transduction by this system is terminated by hydrolysis of the bound GTP by the intrinsic GTPase activity of the α- subunit, which acts like an inbuilt timer. The consequent reassociation of the three subunits reestablishes the inactive state. To date, four families of G proteins, $G_{s\alpha}$, $G_{i\alpha}$, $G_{q/11}$, and G_{12}, have been described. Effector specificity of these proteins is transferred by the respective α-subunits, which correspondingly are called α_s, α_i, α_q, α_{11}, and α_{12}.

The TSH-receptor has been reported to interact with members of all four subfamilies (Laugwitz et al., 1995). However, so far only the down-stream transduction pathways of $G_{s\alpha}$ and $G_{q/11}$ are known in some detail for the thyroid. In many cell types, $G_{s\alpha}$ has been shown to elevate intracellular cAMP-levels by activation of adenylate cyclase, and in some cases also regulation of Na^+- and Ca^{2+}- channels by $G_{s\alpha}$ has been demonstrated. There is also clear *in vitro* evidence for the employment of the $G_{s\alpha}$-adenylate cyclase- cAMP dependent protein kinase (PKA) cascade in TSH-stimulated thyrocytes: (1) TSH-independent activation of $G_{s\alpha}$ by cholera toxin can mimic the effects of TSH, (2) TSH causes a significant rise in intracellular cAMP-levels in less than five minutes, and (3) direct activation of adenylate

cyclase by forskolin or of PKA by the stable cAMP-analogue dibutyryl cyclic AMP can reproduce most of the effects of TSH on cell growth, proliferation, morphology, and differentiation (Roger et al., 1982, Roger et al., 1987, Roger, Taton et al., 1988, Roger and Dumont, 1984, Wynford- Thomas et al., 1987).

In some species (including man) TSH also stimulates the phosphoinositol pathway by activating phospholipase C_β via $G_{q/11}$ proteins (Allgeier et al., 1994). Phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol (DAG) and inositol 1,4,5- triphosphate (IP_3). IP_3 induces influx of Ca^{2+} from endoplasmatic reticulum stores, and Ca^{2+} together with DAG promotes activation of conventional PKCs at the plasma membrane. This pathway is a stimulator of H_2O_2 generation, and hence an important element in the control of follicular cell function. However, while TSH stimulates the cAMP pathway at concentrations expected *in vivo*, the concentrations (at least *in vitro*) required for activation of phospholipase C are much higher (Van Sande et al., 1990).

By the mid-eighties the importance of the cAMP pathway as predominant mediator of TSH-effects had been well characterized and several cAMP-regulated genes identified. However, the link between PKA-activation and cAMP-induced gene expression was still missing.
Elucidation of this connection came from studies of the somatostatin (SST) promoter: SST is a 14-amino acid peptide synthesized by specialized cells in the pituitary, pancreatic islets, and gastrointestinal tract that modulates secretion of other regulatory peptides in a paracrine fashion. SST secretion is increased by cAMP and agents that stimulate adenylate cyclase. Deletion analysis of the SST promoter in PC12 rat pheochromocytoma cells led to the discovery of an 8-bp palindromic element, 5´- TGACGTCA- 3´, that is critical for cAMP-sensitivity of this promoter. Subsequently, employing affinity chromatography, a 43-kDa protein could be purified from PC12 nuclear extracts that bound this „cAMP response element“ (CRE) called consensus sequence with very high specificity, discriminating even against the strongly related TRE-site (Montminy et al., 1987). Soon after its purification, the sequence of the CRE-binding protein (CREB) was published independently by Hoeffler *et al*. (Hoeffler et al., 1988), who cloned the human CREB cDNA by screening a human cDNA expression library with a double-stranded ^{32}P-labelled CRE, and Gonzalez *et al*. (Gonzalez et al., 1989), who cloned the rat CREB cDNA after sequence analysis of the purified protein. The putative proteins of 327 and 341 amino acids encoded by the isolated human and rat

cDNA clones, respectively, were found to be highly conserved, differing mainly by the presence of a 14-amino acid insert, termed the α-peptide, in the longer form.

On the basis of functional aspects, the CREB protein may be considered a bipartite molecule: the carboxyl terminal part, comprising not more than a stretch of about 30 amino acids, suffices for specific DNA-binding, while the much larger amino terminal part contains all the motifs needed for transcriptional activation.

The CREB-DNA-binding domain puts it into the large class of transcription factors known as „leucine zipper“ proteins. The common feature of these proteins is an α-helix motif at their carboxyl terminus carrying a repeat of several leucines at regular intervals of seven residues. As an α-helix contains 3.6 residues per turn, leucine sidechains seven residues apart will repeat along one face of the helix in an almost straight row. When two such helices are aligned in parallel, packaging of hydrophobic leucine sidechains will force them into a coiled-coil structure resembling a twisted ladder with the peptide backbones making the sides, and the interdigitating leucines forming the rungs. In this way dimerization of leucine zipper proteins is brought about
(see Fig. 1 for illustration).
Adjacent to this dimerization domain lies a lysine- and arginine-rich stretch of amino acids, the so-called „basic domain“ (BD). This is the actual DNA-binding part, with the positive charges interacting directly with the acidic DNA; it confers consensus sequence specificity to the individual leucine zipper protein. As first proposed by Vinson *et al.* (Vinson et al., 1989), the whole structure is shaped like a T: the vertical stoke representing the coiled-coil zipper and the horizontal bars formed by the basic regions on either side. Dimerization through the leucine zipper is essential for optimal positioning of the DNA-binding domains, the whole construct entrapping the DNA like a clamp, the basic regions wrapping around the major grooves in opposite directions corresponding to the dyad symmetry of the CRE consensus site.

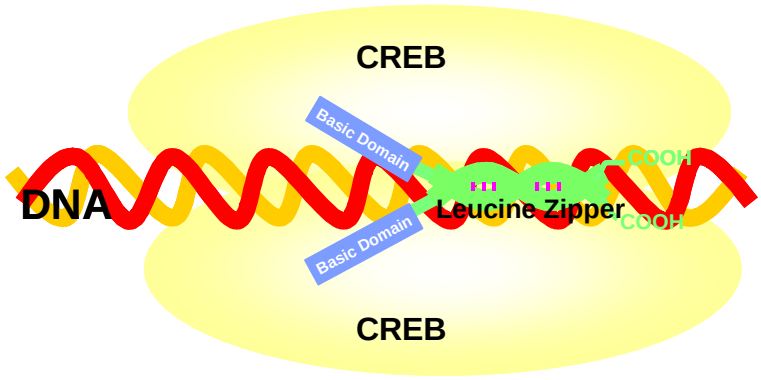


FIG.1 Dimerization and DNA- binding of CREB

Once bound to its respective consensus sequence within a promoter or enhancer region , in order for induction of gene expression to occur, a transcription factor still needs to assemble and fix the diverse parts of the complex eukaryotic transcription machinery around the transcription initiation site. This process is called transactivation. The CREB protein follows at least two different strategies, one intrinsic, the other inducible, to accomplish this goal.

Firstly, depending on the splicing variant (see below), CREB proteins may possess up to two so-called „Q-domains“. Q-domains derive their name from their high content of glutamines, they are constitutively active, and are found in many other transcription factors, for instance Sp 1 and the Oct-1 and Oct-2 protein. For the Q2-domain of CREB (residues 165-252), physical interaction with components of the general RNA polymerase II transcription machinery including TFIIB and the TAF_{II} 130 subunit of TFIID has been demonstrated. A second glutamine-rich region (Q1) within the amino terminus of CREB also contributes to basal transcription activation, although the magnitude of this contribution is much lower.

Secondly, and finally representing the actual target of the cAMP-pathway, the CREB protein contains a unique, inducible transactivation domain referred to as „KID“ (kinase inducible domain) or „P-Box“ (phosphorylation box). Transactivation through the KID is an indirect process requiring the recruitment of an additional protein, suitably called „CREB-binding protein“ (CBP). CBP is a large, nuclear protein of 265 kDa, and was originally isolated from a human thyroid cDNA expression library by screening with phosphoCREB, for phosphorylation of CREB at a particular serine residue (Ser 133) within the 60-residue KID region is an absolute prerequisite for its recognition by CBP. When phosphorylated at Ser133 the KID can assume a structure consisting of two α-helices that kink close to the

phosphorylation site. The helix carboxyl terminal to the kink may then bind tightly within a hydrophobic pocket created by a palisade of three α-helices within a corresponding region at the amino terminus of CBP known as the „KID interaction domain“ (KIX). Once bound, CBP serves as a molecular bridge that allows upstream transcription factors, such as CREB, to recruit and stabilize the RNA polymerase II (Pol II) transcription complex to the TATA box (see also Fig. 2). However, although CBP can associate with Pol II directly, *in vivo* interaction may be indirect. Thus, screening of a cDNA expression library with a specific carboxyl terminal region of CBP (the C/H3 domain) found important for interaction with Pol II, identified yet another factor, RNA helicase A (RHA) that binds to the C/H3 domain and appears to mediate its interaction with Pol II (Nakajima et al., 1997).

In addition to recruiting Pol II, CBP also contributes to CREB-mediated transcription by affecting the chromatin structure. CBP possesses an intrinsic histone acetyltransferase (HAT) activity and associates with another HAT-containing factor termed p/CAF. By catalyzing acetylation of lysine residues in the amino termini of histones, CBP and p/CAF alter the chromatin structure in a fashion believed to make the DNA template more accessible to the transcriptional machinery

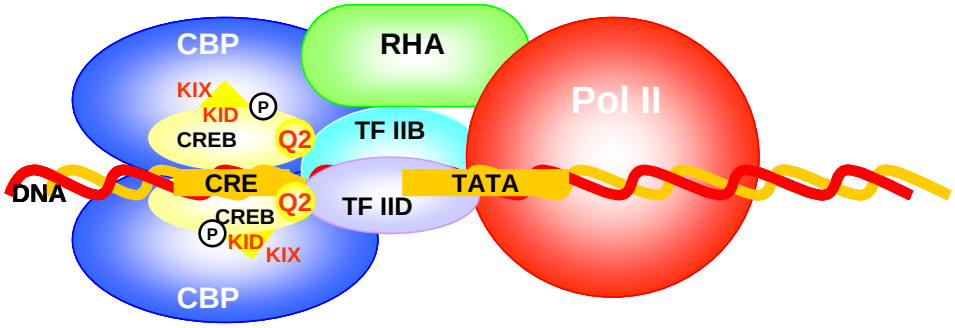


FIG.2 Transcriptional activation by CREB.

Taken together, it appears the CREB protein is composed of a series of more or less independently functioning domains or modules, each with its own specific properties. It can easily be envisaged how by alternative splicing and/or use of alternative promoter sites a whole set of CREB-proteins with variable degrees of transactivation potential may be constructed *in vivo*. A particularly good, and well-studied example of this modular shuffle is

the so-called „cAMP response element modulator“ (CREM). The CREM protein shows extensive sequence identity with CREB, particularly in exons encoding the transactivation and DNA-binding domains (i.e. 84 %, 83 %, 80 % and 96 % amino acid identity in exons E, F, H and I, respectively). However, in contrast to CREB, which shows only minor splicing variation, CREM is not expressed as a uniform protein across different tissues. As outlined in Fig. 3, at least a dozen CREM isoforms are derived from the single gene locus by transcriptional and posttranscriptional mechanisms.

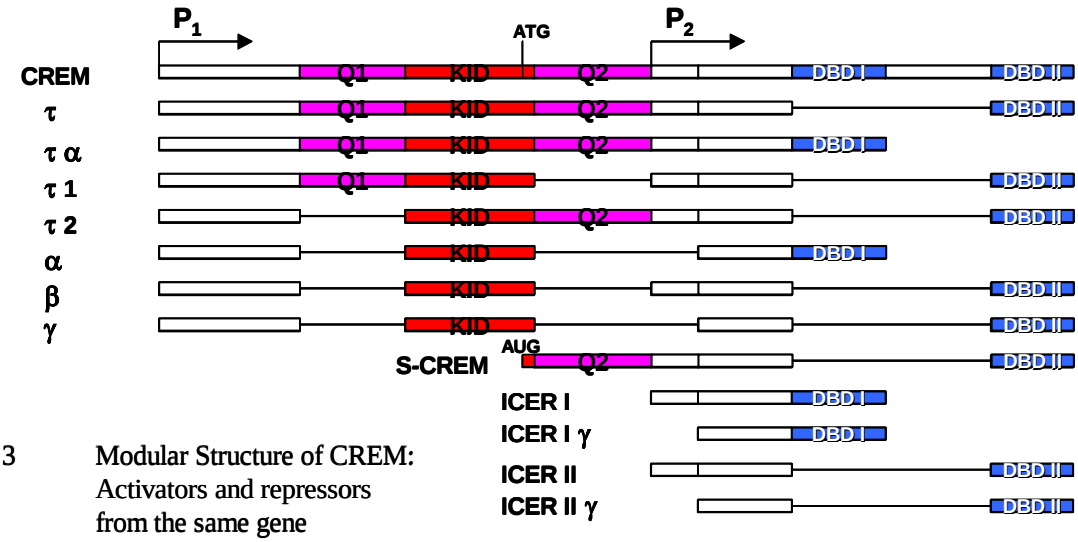


FIG.3 Modular Structure of CREM: Activators and repressors from the same gene

Firstly, alternative splicing of the two Q-domains gives rise to either CREM isoforms with strong transactivation activity (i.e. CREM τ, τα and τ2, comprising the Q2- domain) or weaker activity (i.e. CREM τ1, possessing only Q1) or even no intrinsic transactivation potential at all (i.e. CREM α, β and γ, missing both Q-domains).

In addition, the CREM gene encodes for two distinct DNA-binding domains (DBDs). Of these, DBD I shows 95 % identity with the CREB-DBD, while DBD II with 75 % identity is less similar. By gel retardation assays with the CRE-binding sites from 10 different genes, Laoide *et al.* were able to demonstrate distinct binding efficiencies of these two alternatively spliced DBDs, with DBD II binding more strongly than either DBD I or CREB to various CREs (Laoide et al., 1993).

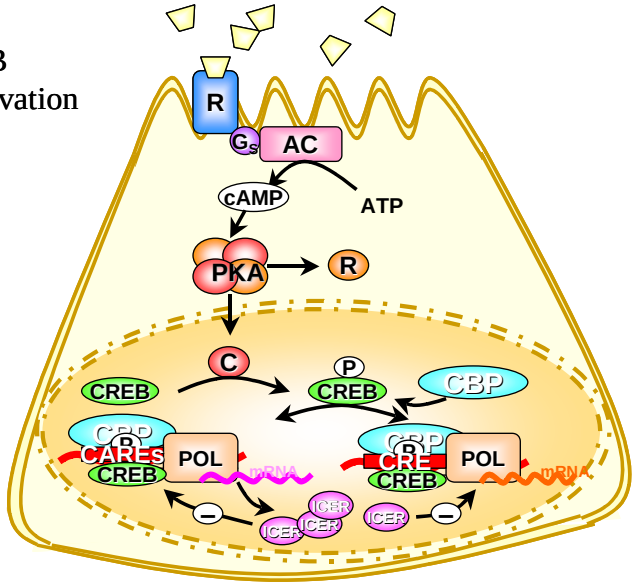
Secondly, the CREMτ mRNA transcript harbours two alternative translation start codons, both with good Kozak consensus sequences, the first at corresponding amino acid position +3, the second at position +152. Translation initiation from this down-stream AUG results in production of a truncated, 21- kDa protein termed S („short“)-CREM. S-CREM lacks most of the KID-region including the essential phosphoacceptor serine at position 117. It does possess basal transactivation activity, but represses PKA-stimulated gene induction from CRE.

And last, and most strikingly, the CREM-gene also contains an alternative promoter (P_2) located on an intron within its 3`-end. Transcription from P_2 generates the shortest CREM isoforms described so far of 12 and 13,5 kDA, including essentially only the DBD. These so-called „inducible cAMP early repressors“ (ICERs) function as pure, competitive inhibitors at the CRE-site. Interestingly, usage of P_2 is itself under control of two pairs of closely spaced CRE-like elements termed „cAMP autoregulatory elements“ (CAREs). ICER induction thereby constitutes an effective negative feedback mechanism.

As all isoforms produced from CREB, CREM, and the third closely related gene in this family, ATF-1 („activating transcription factor 1“), are able to homo- or heterodimerize with each other, a very fine, cell- and developmentally specific tuning of CRE-controlled gene expression is achieved. On the other hand, knock-out experiments also indicate a good degree of redundancy in this complex system (Blendy et al., 1996; Hummler et al., 1994). This „fail-but-proof“ principle further underlines the pivotal role CRE-binding factors may play *in vivo*.

In Fig. 4 the steps of the „classic“ pathway of CRE-binding protein-stimulated gene expression are summarized:

FIG.4 Induction of CREB upon G-protein activation



The cascade is initiated by ligand binding to a G protein-coupled receptor, thereby inducing activation of $G_{s\alpha}$, which in turn transduces the signal to membrane-bound adenylyl cyclase (AC). Formation of cAMP from ATP by activated AC results in a swift increase of intracellular cAMP levels. This permits binding of cAMP to the regulatory subunits of „cAMP-dependent-protein kinase“ (PKA), the conformational changes leading to disintegration of the tetramer.

Liberated catalytic subunits of PKA then translocate into the nucleus, and here catalyze phosphorylation of Ser133 in the KID of nuclear CREB. Phosphorylated CREB bound to the promoter of CRE-regulated genes subsequently fixes CBP to the complex. The combined transactivation potential of the two strongly facilitates assembly of the transcription initiation complex, thereby increasing the transcription rate of CRE-controlled genes. However, at the same time ICER transcription will be induced from the internal CREM-promoter. Accumulation of ICER copies in the nucleus will finally terminate the cascade by closing the negative feedback loop.

First evidence for the employment of this „classic“ CREB-induction pathway in TSH-stimulated thyrocytes came from studies on the homologous desensitization of the TSH-receptor. When thyroid cells are exposed to TSH for prolonged times, initially a fast and transient up-regulation of TSH-receptor expression occurs, however, this is followed by a subsequent dramatic decrease of TSH-receptor mRNA levels within a few hours (Saji et al., 1992). Indeed, within the TSH-receptor promoter a good CRE-consensus sequence was found

(TGAGGTCA, positions -139 to -132, Ikuyama et al., 1992). As shown by Lalli and Sassone-Corsi, TSH-receptor down-regulation is paralleled by a concomitant increase in ICER production and ICER-binding to the CRE-site in the TSH-receptor promoter (Lalli and Sassone-Corsi, 1995).

Further support for an important role of CRE-binding factors in the regulation of thyroid function came from the experiments of Woloshin *et al.* (Woloshin et al., 1992), who transfected FRTL-5 cells, a rat thyroid cell line that has retained many features of a differentiated phenotype (Ambesi-Impiombato et al., 1980), with a dominant negative CREB mutant. Although the maximal inhibition of CRE-directed transcription achieved in this system was only 60 %, a 4-fold reduction in TSH-stimulated iodide uptake was observed in cells expressing the mutant CREB-form. Interestingly, transfected cells in addition exhibited an increase in cell cycle length of 31 %, as well as an 18-40 % reduction in TSH-stimulated thymidine incorporation.

Other TSH-induced genes reported to contain CRE-sites within their promoter sequences include the 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (Bifulco et al., 1995) and the thyroglobulin gene, although the physiological significance of the latter remains questionable, as the thyroglobulin promoter comprises only „half“ of the CRE consensus sequence (TGACGT) and Berg *et al*. failed to show any effect of this site on gene transcription (Berg et al., 1996).

Moreover, many genes of generalized importance, like c-fos, the Na^+/K^+-ATPase, cyclin D1 or the cyclin A gene, have meanwhile been reported to be CRE-controlled.

Interestingly, the list of CRE-regulated genes includes factors expressed at opposite ends of the cell cycle. In multicellular organisms cell proliferation is necessarily a highly coordinated and tightly controlled process, with differentiated cells being mostly found in a non-dividing, „quiescent“ state (G_0). When eukaryotic cells are induced to progress towards cell division, they normally have to transverse a fixed sequence of events, during which they are strongly dependent on provision of specific environmental factors for progression to occur (G_1). For instance, transition from G_0 to G_1 requires the presence of a particular class of growth factors, known as „competence“ factors. Competence factors are cell type-specific, and, in addition to inducing the expression of the so-called „immediate early“ group of genes (including, for instance, the CRE-regulated c-*fos*), they also promote differentiated functions.

By the time cells reach the stage of „competence“, they have become susceptible to other growth factors, like the „epidermal growth factor“ (EGF) or the „insulin-like growth factor 1“ (IGF-1), which are non-cell-type-specific and characterized by a more general range of

activity. Members of this second class of growth factors typically activate tyrosine kinase receptors, which in turn employ such diverse factors as protein kinase C (PKC), MAP kinases or Ras to transduce the signal. External control of cell division ends in late G_1 at the so-called „restriction (R) point“. The (R) point marks the begin of assembly of the DNA replication machinery, and passage beyond this point commits the cell to commence through S and M phases.

Internal control of the cell division cycle is obtained by phase-specific expression and degradation of a group of proteins termed „cyclins“, which represent positive regulators of a certain family of protein kinases („cyclin-dependent protein kinases“, Cdks). Thus, in G_1 D cyclins predominate, until in late G_1 cyclin E appears and persists up to the G_1/S- boundary, from where on cyclin A will be formed, lasting throughout S, G_2 and M phase, but being accompanied by B cyclins in G_2 and M.

Consequently, CRE-binding proteins are involved in the regulation of cell cycle-controlling factors in early G_1 (e.g. c-*fos*), throughout G_1 (e.g. cyclin D1), as well as in early S phase (e.g. cyclin A). Interestingly, Desdouets *et al*. were able to demonstrate the cell cycle dependency of CREB phosphorylation and ICER II expression in synchronized fibroblasts, both occurring exclusively during G_1 (Desdouets et al., 1995). Moreover, different complexes of CRE-binding factors were identified on these various promoters by gel retardation assays. For instance, ATF-1, ATF-2 and CREB/M hetero- and homodimers were found to bind to the cyclin A promoter CRE in different cell systems (Yoshizumi et al., 1995; Desdouets et al., 1995). In contrast, the cyclin D1 promoter was shown to bind homodimers of ATF-2, heterodimers of ATF-2/CREB/M, and, interestingly, also heterodimers of ATF-2 and Jun. Cross-family dimerization between Fos and Jun with members of the CRE-binding protein family was previously shown *in vitro* to alter DNA-binding specificity, thus an ATF-2/Jun dimer was found to bind with higher affinity to an Enk-2 site (TGCGTCA) than either to a CRE or TRE site (Hai and Curran, 1991).

In this context, it is important to note that CREB-phosphorylation is by no means a prerequisite for PKA. Indeed, the KID region harbours a multitude of serine kinase recognition sites, and CREB and CREM phosphorylation at serines 133 and 117, respectively, has been demonstrated for such important regulators as PKC, CamK II and IV, cdc2, RSK 1-3, and G-kinase *in vitro* and *in vivo* (Yamamoto et al., 1988; De Groot et al., 1993; Gudi et al., 2000). Lately, there is also *in vivo* evidence for CREB phosphorylation by MAPKAP-K2 and MSK1, however, de Groot *et al*. had previously shown that MAP kinases fail to

phosphorylate CREM *in vitro* (Xing et al., 1998; Deak et al., 1998; De Groot et al., 1993). Correspondingly, CREB was found to be the target of many diverse signal transduction cascades. In addition, many other serine and threonine phosphorylation sites have meanwhile been described in CREB, CREM, ICER, and CBP, regulating protein-protein interactions, DNA affinity or protein stability. For instance, phosphorylation of CREM by casein kinase I or II at several serine and/or threonine residues within the KID was found to result in a 3-fold enhancement of CRE-binding (De Groot et al., 1993). On the other hand, casein kinase II phosphorylation of so-called PEST sequences may cause an increased rate of protein degradation by the ubiquitin-proteasome pathway, as for instance described for the transcription factor IκBα (Lin et al., 1996; Rechsteiner and Rogers, 1996). In addition, ICER protein phosphorylation at serine 41 by MAPK was shown to promote its degradation *via* the ubiquitin-proteasome system, shortening the half-life of ICER by 4-5 hours (Yehia et al., 2001).
Taken together, the CRE-binding proteins obviously represent the final target of a complex network of cellular signal transduction events, with cross-talk between differentiation and growth promoting pathways being possible at all steps.

In the thyroid, TSH fulfills the role of a typical competence factor, supporting thyroid differentiation and hypertrophy, while during prolonged stimulation also inducing the first steps towards cell division by activation of „immediate early“ genes like *c-fos* and *c-myc*. For a long time, however, it was highly controversial, whether or not TSH might be able to cause thyroid growth directly. Now there is plenty, and overwhelming, evidence, obtained *in vivo* and *in vitro*, in favour of an indirect mechanism, where thyroid cell division requires the TSH-induced synthesis of further autocrine and paracrine growth factors. Firstly, animals treated with inhibitors of thyroid hormone synthesis, show a lag phase of 3 days in rats and 6 days in mice before follicular cell growth begins (Wollman and Breitman, 1970). During this time, expression of fibroblast growth factor 2 (FGF-2), FGF receptor 1 and insulin-like growth factor I (IGF-I) was shown to be induced in goitrogen-treated rats (Logan et al., 1992; Patel et al., 1996; Becks et al., 1994). Also, in hypophysectomized rats, TSH by itself was found insufficient to stimulate thyroid growth, but required adequate levels of another factor, as for instance insulin, for a full thyroid growth response (Isler, 1974; Jolin et al., 1970; Jolin et al., 1974). Moreover, removal of submaxillary glands, a tissue particularly rich in epidermal growth factor (EGF), in mice, has been reported to lead to thyroid regression (Suarez Nunez, 1970), whereas perfusion of fetal sheep with EGF results in a considerable enlargement of the

thyroid gland (Thorburn et al., 1981). On the other hand, in patients with hypopituitarism, increases in serum IGF-I effected by growth hormone replacement are not associated with an increase in thyroid mass in the absence of TSH (Cheung et al., 1996). These *in vivo* observations are supported by corresponding findings made *in vitro*: thus, several groups, working with either primary monolayer cultures or follicles in suspension culture isolated from thyroids of various species, failed to show an acute mitogenic response to TSH alone (see, for example, Gärtner et al., 1985; Westermark et al., 1979; Heldin and Westermark, 1988; Eggo et al., 1984). Rather, it was demonstrated that TSH in serum-free medium stimulates thyrocyte growth only in the presence of either insulin in high concentrations or IGF-I (Dumont et al., 1991; Eggo et al., 1989, Eggo et al., 1990; Williams et al., 1987; Wynford-Thomas et al., 1987; Roger et al., 1983; Ollis et al., 1989), and that the growth response can be abolished by antibodies recognizing either IGF-I and IGF-II or blocking the type I IGF receptor (Maciel et al., 1988). Furthermore, the different growth factors act synergistically, for instance, the concomitant addition of EGF and IGF-I to the culture medium does elicit more than just an additive effect on growth promotion (Gärtner et al., 1990).

Taken together, a picture emerges, where progress through G_1 in the thyroid may be controlled by the consecutive, but overlapping action of several auto- and paracrine loops: (1) low levels of TSH-stimulation increase the sensitivity of thyroid cells to TSH, possibly this is the result of TSH-receptor upregulation through the CRE within its promoter (Takasu et al., 1977); (2) in dog thyroid and in FRTL-5 cells the insulin-receptor was shown to be upregulated by TSH (Burikhanov et al., 1996). Also, TSH and forskolin induce IGF-I mRNA expression (Tode et al., 1989; Hofbauer et al., 1995). Interestingly, in osteoblasts this upregulation of the IGF-I promoter was shown to be mediated via a CRE-site (Thomas et al., 1996). IGF-I in turn, is stimulatory to transcription of the TSH-receptor cDNA (Saji et al., 1992), and this is mediated by an IRE („insulin-responsive element“) within the TSH-receptor promoter region (Shimura et al., 1994); (3) acute TSH treatment causes a transient down-regulation of EGF receptor binding in human thyroid cells (Di Carlo et al., 1990), whereas chronic treatment results in an increase of EGF receptor number in porcine thyroid cells (Atkinson and Kendall-Taylor, 1987). EGF in turn, enhances cAMP generation during short stimulation periods (Kraiem et al., 1995), but decreases TSH-induced cAMP formation and causes downregulation of TSH-receptors during prolonged treatment (Waters et al., 1987).

In addition to promoting cell growth, tyrosine kinase receptor agonists also influence thyroid differentiated functions. Thus, along with TSH and IGF-I, and to a lesser extent also insulin and IGF-II, were shown to enhance thyroid peroxidase and thyroglobulin expression, iodine uptake and organification, and TSH-stimulated cAMP formation (Santisteban et al., 1987; Eggo et al., 1990; Brenner-Gati et al., 1989). IGF-I and insulin were also found to modulate the level of the thyroid-specific transcription factor „thyroid transcription factor 2“ (TTF-2), and this may be the mechanism underlying upregulation of thyroglobulin mRNA (Santisteban et al., 1992).
In contrast, EGF (and all other tyrosine kinase receptor ligands examined so far) has profound inhibitory effects on thyroid differentiation expression, opposing the effects of TSH-stimulation (Waters et al., 1987). Interestingly, the effects of EGF on cell proliferation and differentiation suppression can be mimicked by phorbol ester treatment (Roger et al., 1986). Phorbol esters like „12-O-tetradecanoyl-phorbol-12-acetate“ (TPA) are naturally found in croton oil, and are potent tumour promoters. They structurally resemble DAG, but, in contrast to the latter, are not readily degraded, thereby inducing persistent activation of classic and novel PKCs.

While TSH appears to be the driving force behind diffuse goitre formation, there is now compelling evidence indicating disturbances in tyrosine kinase and PKC functions as the cause of nodular and malignant transformation. Thus, increased enzymatic activity of PLC and abnormally high levels of particulate PKC were demonstrated in thyroid carcinoma (Kobayashi et al., 1993; Hatada et al., 1992). Seemingly in contrast to this, Shaver *et al.* reported failure of TSH to activate PKC in 50 % of high risk thyroid cancer tissues (Shaver et al., 1993). An interesting explanation for this paradox came from Broecker *et al*., who were able to show that in two thyroid carcinoma cell lines an initially inhibitory effect of TSH on inositol phosphate generation could be turned into a stimulatory one by pretreatment of cells with PKC inhibitors (Broecker et al., 1997). They concluded that an excessive secretion of autocrine growth factors might be responsible for the observed high basal PKC activities. In support of this hypothesis they were latter able to prove the existence of overstimulatory loops in these cell lines (Broecker et al., 1998).

Indeed, overexpression of legitimate (e.g. EGF- or HGF- („hepatocyte growth factor“-)) and illegitimate (e.g. NGF- („nerve growth factor“-)) tyrosine kinase receptors often accompanied by simultaneous secretion of the corresponding ligands is an extremely common phenomenon

in thyroid tumours, and appears to parallel malignancy (Aasland et al., 1990; Lemoine et al., 1991; Heldin et al., 1988). However, the genetic basis of these aberrations are with few exceptions (e.g. intrachromosomal rearrangements in the case of the NGF receptor (Radice et al., 1991) not known. Moreover, while the signal cascades induced by the TSH receptor have meanwhile been elucidated in some detail, the effector systems employed by these tyrosine kinase growth factor receptors are still hardly known for the thyroid.

Hence, the aim of this work was to clarify whether (1) CRE- binding proteins are end points of tyrosine kinase receptor cascades in the thyroid, (2) whether there are differences in CREB/M- phosphorylation patterns in thyroid carcinoma as compared to primary cells, (3) whether phosphorylation of CREB/M through these pathways also leads to gene induction, and, finally, (4) employing specific inhibitors to elucidate part of the protein kinase cascades involved.