Hormonal control of the transcription factor Pax8 and its role in the regulation of thyroglobulin gene expression in thyroid cells

A Mascia, L Nitsch, R Di Lauro¹ and M Zannini

CEOS, CNR and Dipartimento Biologia e Patologia Cellulare e Molecolare, Via Pansini 5, 80131 Naples, Italy

¹Stazione Zoologica A Dohrn, Villa Comunale, 80121 Naples, Italy

(Requests for offprints should be addressed to M Zannini, Dipartimento di Biologia e Patologia Cellulare e Molecolare, Università di Napoli Federico II, via Pansini, 5–80131 Naples, Italy; Email: stella@alpha.szn.it)

Abstract

The transcription factor Pax8 plays an important role in the expression of the differentiated phenotype of thyroid follicular cells. It has recently been shown that Pax8 is necessary for thyroglobulin (Tg) gene expression in the fully differentiated rat thyroid cell line PC. We have used the PC model system to investigate the role of Pax8 as a mediator of TSH regulation of Tg gene expression. We have demonstrated that Pax8 expression, as well as Tg expression, is severely reduced in cells grown in the absence of hormones and serum. The re-addition of TSH or forskolin to the culture medium is able to restore to wild-type levels the expression of both Pax8 and Tg. We have determined that the action of TSH/forskolin on Pax8 is at the transcriptional level. However, the re-expression of Pax8 can be observed several hours before that of Tg,

suggesting that either another factor is needed or that Pax8 itself must be post-translationally modified by a newly synthesized protein to become active. To distinguish between these two possibilities we have stably transfected into PC cells an exogenous Pax8 that is expressed independently of TSH. Our results indicate that in these cells the Tg promoter is still dependent on TSH despite the constitutive presence of Pax8. Furthermore, we also show that in this condition Tg gene transcription requires *de novo* protein synthesis. In conclusion, TSH regulates the expression of Pax8 at a transcriptional level and also regulates the activity of Pax8 by controlling the expression of one or more as yet unknown factors.

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Introduction

Thyrocytes, the most abundant cell population in the thyroid gland, represent a useful model system to study the mechanisms for establishment and maintenance of cell-type-specific patterns of gene expression. Thyrocytes are mainly involved in the synthesis of thyroid hormones and are characterized by the expression of specific genes such as thyroglobulin (Tg) and thyroperoxidase (TPO), which are exclusively expressed in this cell type (Damante & Di Lauro 1994, Damante *et al.* 2000). Tg is a huge protein of 330 kDa that is secreted by thyrocytes and is accumulated in the follicular lumen where it is iodinated and is the precursor to thyroid hormones. The promoter of the Tg gene has been extensively studied and three transcription factors have been cloned that specifically bind and activate this promoter (Damante & Di Lauro 1994, Damante *et al.* 2000). The three factors are TTF-1 (thyroid transcription factor-1), TTF-2 (thyroid transcription factor-2) and Pax8. TTF-1 is a homeodomain-containing protein expressed in embryonic diencephalon, thyroid and lung (Lazzaro *et al.* 1991); TTF-2 is a forkhead domain-containing protein expressed in pituitary and thyroid (Zannini *et al.*

1997), and Pax8 is a member of the murine Pax family of paired domain-containing genes, and it is expressed in kidney, in the developing excretory system and in the thyroid (Plachov *et al.* 1990).

The main physiological stimulus regulating thyroid function is given by thyrotropin (TSH) produced by the pituitary gland. Like many other hormones, TSH exerts its function through a G protein-coupled receptor, the TSH receptor (TSHR), which relies on the associated G protein to transmit and amplify the signal inside the cell. In rat thyroid cells, TSH-mediated activation results primarily in cAMP production that is likely to play a crucial role in the onset of differentiation both *in vitro* and *in vivo*. Indeed cAMP agonist, for instance forskolin (FSK), can mimic TSH-induced differentiation. The presence of TSH is also required for the maintenance of the differentiation status, since withdrawal of TSH for a prolonged time causes loss of thyroid-specific gene expression in cultured cells. Several studies have led to the conclusion that TSH regulates the expression of the Tg gene by determining its rate of transcription (Avvedimento *et al.* 1984, Van Heuverswyn *et al.* 1984, 1985, Santisteban *et al.* 1987). A number of *in vitro* studies, in which cultured thyrocytes

were used, have shown that lack of TSH leads to a drastic reduction in Tg mRNA and that re-addition of TSH is able to reactivate Tg transcription (Chebath *et al.* 1979, Tosta *et al.* 1983, Nagayama *et al.* 1989, Roger *et al.* 1997). *In vivo* studies in the rat also suggested that TSH controls Tg gene transcription via the interaction with its receptor on thyrocytes, and that cAMP is a physiological mediator of this effect (Van Heuverswyn *et al.* 1984, 1985). However, no cAMP-responsive elements have been found in the rat Tg promoter region (Gerard *et al.* 1989, Lee *et al.* 1991). The molecular mechanism by which TSH controls Tg gene expression, and the potential involvement of the transcription factors which are known to bind to its promoter, have not been elucidated yet (Santesteban *et al.* 1992, Van Renterghem *et al.* 1995, 1996, Kambe *et al.* 1996, Ortiz *et al.* 1997). In addition, it must be said that there is a significant variability among the experiments performed in the different model systems in the extent of Tg regulation by TSH. This variability is possibly related to the different hormonal backgrounds, in particular to the presence or absence of serum or insulin in the culture conditions, and to the length of time of TSH deprivation.

We have focused our studies on the role played by the transcription factor Pax8 that is expressed in the thyrocytes of either embryonic or adult thyroid gland, as well as in differentiated thyroid cells in culture. Pax8 was initially shown to bind to and to activate transcription from the TPO promoter in non-thyroid cells, thus identifying for TPO the first target for Pax8 action (Zannini *et al.* 1992). Pax8 also binds to a single site on the Tg promoter, and was recently shown to activate Tg gene expression through a thyroid-specific mechanism (Pasca Di Magliano *et al.* 2000). In addition, Pax8 appears to be also involved in the thyroid-specific expression of the rat sodium/iodide symporter (NIS) gene (Ohno *et al.* 1999). Interestingly, in Pax8 knockout mice the thyroid gland is much smaller than normal and lacks follicles (Mansouri *et al.* 1998). Thyroid follicular cells are absent in these mice and the expression of thyroid-specific markers, such as Tg and TPO, cannot be detected (Mansouri *et al.* 1998). These *in vivo* studies led to the conclusion that Pax8 is needed for proper differentiation of thyrocytes. Mutations in the Pax8 gene have also been associated with congenital hypothyroidism in humans (Macchia *et al.* 1998). Patients carrying the mutations are affected by thyroid dysgenesis, indicating an important role for this gene in thyroid organogenesis.

A correlation between low levels of expression of Pax8 and lack of thyroid differentiation has been proposed by the study of the transformed FRTL5/TA thyroid cells (Van der Kallen *et al.* 1996). Moreover, a direct demonstration that Pax8 plays a key role in the control of thyroid-specific gene expression has been recently obtained by studying the transformed cell line PcPy (Pasca Di Magliano *et al.* 2000). Very little is known about Pax8 transcriptional and post-transcriptional regulation.

Recently, it has been published that in FRTL5 rat thyroid cells (Fabbro *et al.* 1998, Medina *et al.* 2000) as well as in canine thyroid cells in culture (Van Renterghem *et al.* 1996) TSH regulates Pax8 synthesis.

We have investigated the role of Pax8 as a mediator of TSH action in thyrocytes. To this purpose we used as a model system rat thyrocytes in culture, the PC cells that express thyroid-specific genes such as Tg, TPO, NIS and TSH receptor (Fusco *et al.* 1987), and the thyroid-specific transcription factors TTF-1, TTF-2 and Pax8. We show here that in PC cells TSH regulates the expression of Tg and Pax8 genes at a transcriptional level by a cAMP-mediated mechanism. The delayed reactivation of Tg with respect to Pax8 gene suggested that another factor is required or that Pax8 protein needs to be modified to become active. By studying stable clones of PC cells expressing constitutively an exogenous Pax8, we determined that in the absence of TSH the Tg promoter is transcriptionally inactive despite the presence of Pax8. We also demonstrated that under these conditions Pax8 is inactive and that TSH-dependent *de novo* protein synthesis is required for its activation, thus indicating the requirement for one or more additional factor(s).

Materials and Methods

Plasmids

The pEF-Pax8-myc tag construct was generated by PCR amplification of the mouse Pax8-coding region with a 5' primer and a 3' primer containing at their ends the BamHI and EcoRI sites respectively. The fragment was then subcloned into the corresponding sites of the expression vector EFplink that was previously modified with the addition of a myc tag by R. Treisman (ICRF, London, UK). The construct was sequenced to control the fidelity of the frame between the myc tag and the inserted fragment.

The plasmids used in transient transfection experiments have been previously described and were as follows: pTACAT3 (Sinclair *et al.* 1990), and CP5-CAT (Missero *et al.* 1998).

CMV-CAT and RSV-LUC plasmids were used for control activities and for normalization in the transient transfection respectively.

The DNA of all the plasmids was prepared by Qiagen cartridges (Qiagen, Milan, Italy) and used for cell transfection.

Cell culture and transfection

The PC cell line has been previously described (Fusco *et al.* 1987). Both PC cells and PC-Pax8-myc tag stable clones were cultured in Falcon plastic dishes (Becton Dickinson Labware, Lincoln Park, NJ, USA) as described

by Ambesi-Impiombato & Coon (1979). The complete medium consisted of Coon's modified Ham's F12 medium (Sigma Chemical Co., Milan, Italy) supplemented with 5% calf serum (Life Technologies, Karlsruhe, Germany) and six hormones (6H) and growth factors as described by Ambesi-Impiombato & Coon (1979). The starvation medium consisted of Coon's modified Ham's F12 medium supplemented only with 0.2% BSA.

Transfections were carried out with the Lipofectin reagent (Life Technologies) as suggested by the manufacturer with minor modifications. Briefly, 3 h prior to transfection the medium of subconfluent cells, cultured either in complete medium or in starvation medium, was replaced with OPTIMEM 1 (Life Technologies). The DNA/liposome ratio was 1:5 in all the experiments.

For transient transfection, cells were collected after 48 h to determine either the levels of CAT protein with a CAT enzyme-linked immunosorbent assay kit (Boehringer, Monza, Italy) or LUC activities as described previously (Zannini *et al.* 1997).

To obtain stable clones, PC cells were transfected with plasmid pEF-Pax8-myc tag and a plasmid containing the neomycin-resistance gene under the RSV promoter (ratio 20:1). Cells were selected with 300 µg/ml G418 (Sigma). After 2 weeks, G418-resistant clones were isolated, expanded and examined by immunofluorescence.

RNA extraction and Northern blot analysis

Total RNA was prepared by the acid guanidinium thiocyanate/phenol procedure (Chomczynski & Sacchi 1987).

Thirty micrograms of total RNA were electrophoresed on a 1% agarose gel containing 3.7% formaldehyde and 20 mM morpholinepropane sulfonic acid buffer. RNA was then blotted onto nylon membranes (Hybond-N; Amersham-Pharmacia, Milan, Italy) with 20 × SSC, and hybridization and washing were carried out according to Church & Gilbert (1984).

The blot was probed with rat cDNA fragments derived by restriction digestion of Tg- and Pax8-coding sequences, and labeled by random priming with [α -³²P]dATP and [α -³²P]dCTP (Amersham Life Science, Amersham, Bucks, UK). A 300 bp fragment derived from the 3' region of rat glyceraldehyde-3-phosphate dehydrogenase was used as control.

RT-PCR

One nanogram of total RNA prepared from the different cell lines was used to synthesize cDNA using the Superscript One-Step RT-PCR system (Life Technologies) with gene-specific primers. The primers used to amplify the Tg transcript were TG-2 and TG-4 (Mascia *et al.* 1997), which map in the first and second exon of the Tg gene respectively. The reactions were set up according

to the protocol of the kit, and the conditions used were as previously described (Mascia *et al.* 1997). An aliquot of the total volume of the PCR reactions was analyzed on a 1% agarose gel.

Cell extracts and Western blot

PC and PC-Pax8-myc tag cells were cultured in 100 mm plastic dishes in complete medium or for 7 days in starvation medium. Cells were washed twice with ice-cold PBS and scraped in 1 ml of 40 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl. Cell extracts were prepared as previously described (Zannini *et al.* 1992), and protein concentration was determined using the Bio-Rad protein assay (Bio-Rad). Forty to eighty micrograms of total extract for Pax8, or 10 µg for Tg, were solubilized in reducing Laemmli buffer, boiled for 5 min and analyzed by SDS-10% PAGE or SDS-7% PAGE. Gels were blotted onto Immobilon P (Millipore, Bedford, MA, USA) in a Bio-Rad apparatus (Bio-Rad, Milan, Italy) for 16–24 h.

Immunodetection was performed using an anti-Pax8 antibody, an anti-myc antibody and an anti-Tg antibody diluted in 150 mM NaCl, 20 mM Tris-HCl, pH 7.5 containing 0.5% non-fat dry milk (Bio-Rad) at 1:1000, 1:500 and 1:2500 respectively. Subsequently, the filters were developed using an enhanced chemiluminescence detection method (Amersham) according to the manufacturer's directions.

Immunofluorescence

Cells were cultured directly on 12 mm diameter glass coverslips in complete medium or in starvation medium. Where indicated, TSH (1 mM) or FSK/3-butyl-1-methylxanthine (IBMX) (10 µM/1 mM) was added at different times. To visualize Tg, Pax8-myc tag or Pax8, the cells were fixed for 20 min with 4% paraformaldehyde (Sigma) in PBS containing 0.9 mM calcium and 0.5 mM magnesium (PBS CM) at room temperature, washed twice in 50 mM NH₄Cl in PBS CM and twice in PBS CM. Cells were permeabilized for 5 min in 0.5% Triton-X 100 (Bio-Rad) in PBS CM and washed twice, for 10 min, in 0.2% gelatin (Sigma) in PBS CM.

Cells were then incubated for 1 h with the primary antibodies diluted in 0.5% BSA (Sigma) in PBS. After three washes with 0.2% gelatin in PBS CM, cells were incubated for 20 min with the appropriate rhodamine- or fluorescein-tagged goat anti-mouse or anti-rabbit secondary antibody (Jackson ImmunoResearch, West Grove, PA, USA), diluted 1:50 in 0.5% BSA in PBS. To visualize actin filaments, permeabilized cells were incubated with a 1:70 dilution of rhodamine-conjugated phalloidin (Sigma) for 20 min. After final washes with PBS, the coverslips were mounted on a microscope slide using a 50% solution

of glycerol in PBS and examined with a Zeiss Axiophot microscope (Zeiss, Milan, Italy). NIS was visualized as described (Paire *et al.* 1997).

Antibodies and reagents

The rabbit polyclonal antibody to rat Tg was described in Mascia *et al.* (1997); the polyclonal anti-Pax8 antibody was raised against a peptide derived from Pax8-coding region and was described in De Vita *et al.* (1998); rabbit anti-serum to rat NIS (pAb 716) was a kind gift of B Rousset (Faculté de Médecine Lyon, RTH Laennec, Lyon Cédex, France); anti-c-myc (9E10) monoclonal antibody was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA); horseradish peroxidase-linked anti-rabbit or anti-mouse antibodies were from Amersham. BSA, TSH, FSK and IBMX were from Sigma.

Results

TSH deprivation causes loss of expression of Pax8, Tg and NIS genes in cultured rat thyroid cells

To investigate the role of TSH in the expression of thyroid-specific genes, differentiated rat thyroid PC cells were cultured for 7 days in serum-free medium containing either 0.2% BSA alone, or 0.2% BSA plus 6H or TSH. The expression of the thyroid-specific transcription factors Pax8 and TTF-1, and of the thyroid-specific genes Tg and NIS was analyzed by indirect immunofluorescence (Fig. 1). A strong reduction in fluorescence staining of Pax8 (Fig. 1b), Tg (Fig. 1e) and NIS (Fig. 1h) was observed in cells cultured in the absence of hormones with respect to cells cultured in the presence of 6H (Fig. 1a, d and g). Staining of the three proteins in the presence of 0.2% BSA plus TSH alone was comparable with that in the presence of 6H (Fig. 1c, f and i). No significant change in TTF-1 staining was detected under any of the culture conditions tested (Fig. 1j–l). TSH deprivation caused, as expected (Tramontano *et al.* 1982, Westermark & Portor 1982, Kurihara *et al.* 1990), a dramatic change in cell shape and microfilament organization (Fig. 1m–o). The same results were also obtained in the differentiated rat thyroid cell line FRTL5 and when serum was present in the culture medium (data not shown). These data indicate that TSH regulates the expression of Pax8, Tg and NIS genes in rat thyroid cells in culture.

Pax8 and Tg gene expression is coordinately induced by TSH stimulation

To determine if Pax8 and Tg gene expression was induced with similar kinetics by TSH stimulation, PC cells were cultured for 7 days in medium lacking serum and hormones until both Pax8 and Tg proteins had disappeared.

The cells were then treated with TSH for different lengths of time (6, 24 or 48 h) and Pax8 and Tg proteins were detected by indirect immunofluorescence (Fig. 2). Pax8 nuclear staining was strongly reduced after serum and hormone starvation (Fig. 2a), could be observed already at 6 h of TSH treatment (Fig. 2b) and did not significantly increase thereafter (Fig. 2c and d). At variance, Tg staining could be detected only after 24–48 h of TSH treatment (Fig. 2g and h). We then asked if the TSH effect was mediated by cAMP. To address this question the same experiment was performed with cells treated with FSK/IBMX instead of TSH. Also in this condition, Pax8 was detected after 6 h of FSK/IBMX treatment (Fig. 2j), while 24 h of FSK/IBMX treatment were needed before Tg became detectable (Fig. 2o). Similar results were obtained by treating cells with cAMP derivatives, such as 8-Br-cAMP (data not shown). The induction of Pax8 and Tg expression was also analyzed by Western blot (Fig. 3A). In good agreement with the immunofluorescence data, in PC cells, after 7 days of starvation, a 6 h treatment with TSH was sufficient to induce the synthesis of Pax8 protein. At least 24 h were indeed needed to detect the presence of Tg within the cells. The expression of TTF-1 did not appear to significantly change during starvation or upon TSH treatment (Fig. 3A).

To determine whether TSH regulates the expression of Pax8 at the mRNA level, PC cells were starved of serum and hormones for 7 days and were then treated with TSH for different times. Total RNA was extracted and the appearance of Pax8 and Tg mRNAs was analyzed by Northern blot (Fig. 3B). Pax8 and Tg mRNAs were not detected following the 7 days of starvation while, after 6 h of TSH stimulation, Pax8 mRNA could already be seen within the cells. Only after 24 h of TSH treatment was a transcript corresponding to Tg mRNA detected (Fig. 3B). The kinetics of appearance of Pax8 and Tg mRNAs are in good agreement with those of the corresponding proteins, indicating that the reactivation of the two genes is not concomitant and that Pax8 gene is already transcribed and translated several hours before the Tg gene. These results suggest, on the one hand, that Pax8 protein might be needed before Tg gene expression occurs; on the other hand, the long lag between the expression of the two genes might indicate that additional events are required, other than Pax8 protein synthesis, to fully activate the molecular machinery that promotes Tg gene transcription.

Pax8 does not restore Tg gene expression in TSH-starved PC cells

Several lines of evidence indicate that Pax8 is a transacting factor strictly required for Tg gene transcription (Fabbro *et al.* 1998, Pasca Di Magliano *et al.* 2000). To investigate whether Pax8 presence was indeed sufficient to reactivate Tg gene expression in the absence of TSH stimulation, the expression vector pEF-Pax8-mycTag, which codes for a

Figure 1 Detection of Pax8, Tg, NIS and TTF-1 by immunofluorescence in PC cells. PC cells were plated on glass coverslips in complete culture medium. The day after, medium was changed and fresh medium containing 0.2% BSA plus 6H (a, d, g, j, m), 0.2% BSA alone (b, e, h, k, n) or 0.2% BSA plus 1 mU/ml TSH (c, f, i, l, o) was added. After 7 days of further culture, cells were fixed with formaldehyde, permeabilized with Triton X-100 and stained by immunofluorescence using the anti-Pax8 (a–c), anti-Tg (d–f), anti-NIS (g–i) or anti-TTF-1 (j–l) antibody. The nuclear staining of Pax8 (a), the cytoplasmic staining of Tg (d) and the membrane staining of NIS (g) are lost in the absence of 6H (b, e, h) but retained in the presence of TSH alone (c, f, i). No significant change in TTF-1 staining was observed in the different culture conditions (j, k, l). Actin microfilaments were prominent in the absence (n) and disappeared in the presence (m, o) of TSH. Bar=50 μ m.

Figure 2 Time course of Pax8 and Tg re-expression in TSH or FSK/IBMX-treated PC cells. Cells were cultured for 7 days in starvation medium (a, e, i, m) and then treated with 1 mU/ml TSH (b–d, f–h) or 10 μ M FSK plus 1 mM IBMX (j–l, n–p) for 6 h (b, f, j, n), 24 h (c, g, k, o) or 48 h (d, h, l, p). Cells were fixed, permeabilized with 0.1% Triton X-100 and stained by indirect immunofluorescence using a polyclonal anti-Pax8 (a–d, i–l) antibody (1:300) or a polyclonal anti-Tg (e–h, m–p) antibody (1:5000). Pax8 nuclear staining was seen by 6 h of TSH (b) or FSK/IBMX (j) treatment. The intensity of staining was not much increased after 24 h (c, k) or 48 h (d, l). Tg staining could only be detected after 24 and 48 h of TSH (g, h) or FSK/IBMX (o, p) treatment. Bar=50 μ m.

Figure 3 Detection of Pax8 and Tg proteins and mRNAs in PC cells after starvation followed by TSH stimulation. (A) PC cells were cultured in regular medium (lane C) or maintained in starvation medium for 7 days (lane 0) and then treated with 1 mU/ml TSH for different times (1, 2, 6, 12, 24 and 48 h), and analyzed by Western blot to detect Pax8 and Tg proteins. The same amount of total extract was loaded in each lane. Proteins were separated by SDS-10% PAGE (Pax8 and TTF-1) or SDS-7% PAGE (Tg). Pax8 protein was detected in PC control cells cultured in the presence of 6H (lane 0) and in PC cells treated with TSH for 6, 12, 24 and 48 h following 7 days of starvation. Instead, Pax8 was not detected 1 or 2 h after starvation. Tg was detected at levels comparable with those of control cells (lane C) only after 24 and 48 h of TSH treatment. Tg was not detected immediately after starvation (lane 0) or after 1, 2, 6 and 12 h of TSH stimulation. TTF-1 was expressed at similar levels in all culture conditions. (B) PC cells were cultured in starvation medium for 7 days and then treated with 1 mU/ml TSH for 6, 12, 24 and 48 h. RNA was extracted and analyzed by Northern blot using the Pax8 and Tg probes indicated in the Material and Methods section. Following the 7 days of starvation (time 0) both Pax8 and Tg mRNAs were almost undetectable compared with control (C). Pax8 mRNA was detected after 6 h of TSH stimulation while Tg mRNA became detectable at 24 h.

Pax8 protein with a myc tag at the N-terminal under the control of a viral promoter, was generated. The presence of the tag allowed us to distinguish between exogenous and endogenous Pax8. When transiently transfected into HeLa cells, pEF-Pax8-myc tag was able to induce the expression of a CAT gene under the control of an artificial promoter consisting of five Pax8-binding sites (CP5-CAT) thus indicating that the protein was transcriptionally active (data not shown). pEF-Pax8-myc tag was then transiently transfected into PC cells cultured for 7 days in starvation medium. Forty-eight hours after transfection, cells

were examined for the expression of Pax8 and Tg by indirect immunofluorescence. Cells that expressed Pax8 (myc tag-positive after transfection) were still negative for Tg when probed with the anti-Tg antibody (Fig. 4), suggesting that Pax8 was not sufficient to promote Tg gene expression. To confirm this observation and to further analyze the role of Pax8, stable clones of PC cells, expressing pEF-Pax8-myc tag, were obtained by co-transfecting the pRSV-NEO plasmid and by indirect immunofluorescence screening with an anti-myc antibody. The expression of Pax8 and Tg, in two representative clones cultured in complete medium or in starvation medium for 7 days, was analyzed by Western blot (Fig. 5). Pax8-myc tag, revealed by the anti-myc antibody, was not detected in wild-type PC cells (lanes 1 and 2) while it was detected either in the presence or in the absence of serum and hormones in the two PC-Pax8-myc tag clones (lanes 3–6). When analyzed with the anti-Pax8 antibody, PC cells expressed Pax8 only in the presence of hormones (lanes 1 and 2), while a Pax8 band was always detected in the PC-Pax8-myc tag clones independently of culture conditions (lanes 3–6). Tg was expressed only in cells cultured in complete medium (lanes 1, 3, 5) and was not detected in cells cultured in starvation medium even if Pax8-myc tag was present (lanes 4, 6). These results indicate that Pax8 is not sufficient to activate Tg gene expression in PC cells cultured in the absence of TSH. Thus either Pax8 is inactive and needs to be activated by a TSH/cAMP-dependent pathway or some factors are needed, together with Pax8, to promote Tg gene expression.

Activation of the cAMP pathway is not sufficient to promote Pax8-dependent transcription in starved PC-Pax8 cells

We then tested the hypothesis that a modification induced by the TSH/cAMP signaling cascade was needed to activate Pax8. To this purpose PC-Pax8 cells were cultured in the absence of serum and hormones and then stimulated with FSK/IBMX for different times (Fig. 6). Although the Pax8 protein was always present, still the Tg protein could not be detected at any time of FSK/IBMX stimulation (Fig. 6). These results indicate that Pax8 is unable to promote the expression of the Tg gene when cells are maintained in the absence of TSH, even if the cAMP signaling pathway has been activated, and suggest that, under these experimental conditions, Pax8 might be transcriptionally inactive. We therefore tested whether the transfected Pax8 was able to activate transcription from a reporter gene under the control of the Tg promoter (pTA-CAT3) or from the CP5-CAT reporter construct. Both pTA-CAT3 and CP5-CAT were active in PC cells and in PC-Pax8 cells cultured in complete medium while they were inactive when cells were cultured in starvation medium (Fig. 7A and B). To evaluate if, under the same experimental conditions, signaling through the TSH/

Figure 4 Transfection of Pax8 in PC cells cultured for 7 days in starvation medium does not reactivate Tg gene expression. PC cells were plated on glass coverslips, cultured for 7 days in starvation medium, and transfected with pEF-Pax8-myc-tag. Forty-eight hours later cells were fixed with paraformaldehyde, permeabilized with Triton X-100 and stained with a polyclonal anti-Tg (1:5000) and a monoclonal anti-myc (1:50) antibody. No Tg-positive staining (b) was detected in cells that were positive for myc staining (a). Occasional staining of Tg was observed either in transfected or in non-transfected cells. Bar = 30 μ m.

cAMP pathway could activate the Pax8 protein, PC-Pax8 cells were cultured in starvation medium for 7 days, transiently transfected with pTA-CAT3 or CP5-CAT and stimulated with FSK/IBMX for different times (Fig. 7C). No increase in CAT activity was observed over the basal level up to 6 h of FSK/IBMX stimulation. These data indicate that, in cells starved of serum and hormones, Pax8 is transcriptionally inactive and that the cAMP-signaling cascade cannot activate it.

Protein synthesis is required to activate Pax8-dependent Tg gene expression in TSH-starved PC cells

To determine if additional factors, together with Pax8, were needed to activate TSH-dependent Tg gene expression, PC-Pax8 cells were cultured in starvation medium

and stimulated with FSK/IBMX for different lengths of time in the presence or in the absence of cycloheximide. The expression of Tg in the various conditions was determined by RT-PCR. When cycloheximide was added to the medium, Tg mRNA could not be detected at any time, up to 24 h, regardless of the presence or absence of FSK/IBMX (Fig. 8, lanes 7–12). On the contrary, Tg mRNA was clearly detected already at 12 h stimulation with FSK/IBMX in PC-Pax8 cells grown in the absence of cycloheximide (Fig. 8, lane 5). These data strongly indicate that the synthesis of protein(s), other than Pax8, is therefore required to activate the TSH-dependent Tg gene expression.

Discussion

The role of TSH in the regulation of the thyroid differentiated phenotype has been studied in several different model systems (Dumont *et al.* 1992). It has been demonstrated that the effects of TSH are largely mediated by the cAMP-dependent pathway and the relevance of the cAMP cascade in thyroid follicular cells has been well established (Dumont *et al.* 1992, Roger *et al.* 1995, Dremier *et al.* 1997). The expression of several thyroid-specific genes such as Tg (Avvedimento *et al.* 1984, Santisteban *et al.* 1987), TPO (Magnusson & Rapoport 1985, Nagayama *et al.* 1989), TSHR (Kohn *et al.* 1995) and NIS (Kogai *et al.* 1997, Ohmori *et al.* 1998, Ohno *et al.* 1999, Spitzweg *et al.* 1999) has been shown to be modulated by TSH/cAMP. While in the case of the TSHR a CRE site has been shown to play an important role (Ikuyama *et al.* 1992, Shimura *et al.* 1993), and in the NIS upstream enhancer element a CRE-like site has been recently identified (Ohno *et al.* 1999), the situation is more complicated for the Tg promoter. In fact, a CRE-like

Figure 5 Western blot analysis of Pax8 and Tg proteins in PC cells and PC-Pax8 clones stably expressing Pax8-myc-tag. PC cells (lanes 1, 2) and two clones of PC-Pax8 cells expressing Pax8-myc-tag (lanes 3–6) were cultured in regular medium (lanes 1, 3, 5) or in starvation medium for 7 days (lanes 2, 4, 6). Proteins in the cell homogenates were analyzed by Western blot as in Fig. 3 using an anti-Pax8 antibody (Pax8), an anti-myc antibody (myc) or an anti-Tg antibody (Tg).

Figure 6 Pax8 and Tg expression in PC-Pax8 cells stimulated with FSK/IBMX. PC-Pax8 cells were cultured in regular medium (a, e) or in starvation medium for 7 days (b, f) and then stimulated for 3 h (c, g) or 5 h (d, h) with FSK/IBMX. Pax8 protein was detected by indirect immunofluorescence in PC-Pax8 cells by using an anti-myc tag antibody (a–d). The expression of Tg was analyzed by using a polyclonal anti-Tg antibody (e–h). Bar=40 μ m.

consensus sequence is present in the dog and human Tg promoter (Christophe *et al.* 1989, Berg *et al.* 1996), but no typical cAMP-responsive element has been identified in the rat Tg promoter regions. Recently, it has been suggested that the thyroid-specific transcription factors TTF-1, TTF-2 and Pax8 could be the mediators of the TSH/cAMP action (Kambe & Seo 1996). Whether the promoters of any of the thyroid-specific transcription factors contain a CRE-like sequence remains to be established. Interestingly, in FRTL5 cells Pax8 expression has been demonstrated to be increased by TSH (Fabbro *et al.* 1998, Medina *et al.* 2000) and in dog thyrocytes cAMP was shown to moderately increase Pax8 protein and mRNA levels (Van Renterghem *et al.* 1996).

Pax8 has been shown to be critically involved in determination and maintenance of the differentiation state of thyrocytes (Mansouri *et al.* 1998, Pasca Di Magliano *et al.* 2000). In this paper we show that also in PC rat

thyroid cells in culture the expression of Pax8 is under the control of TSH/cAMP and that this control operates at a transcriptional level. We have observed that in PC cells cultured in the absence of TSH, Pax8 mRNA and protein rapidly disappear, whereas upon TSH stimulation both mRNA and protein are synthesized within 6 h. Since Tg synthesis is under TSH control and Pax8 plays a key role in the regulation of thyroid-specific gene expression, we have tested the hypothesis whether Pax8 could be the mediator of the TSH action on Tg expression. There are several lines of evidence from the literature and from our own work supporting this hypothesis: (i) Pax8 binds to the Tg promoter and activates transcription from Tg promoter reporter constructs in both thyroid and non-thyroid cells (Zannini *et al.* 1992, Fabbro *et al.* 1998); (ii) Pax8 is essential for Tg gene expression both in PC cells (Pasca Di Magliano *et al.* 2000) and in the mouse (Mansouri *et al.* 1998); (iii) Pax8 gene expression, as well as Tg gene

Figure 7 FSK/IBMX stimulation does not induce pTA-CAT3 and CP5-CAT promoter activity. One microgram of CMV-CAT, 10 μ g of pTA-CAT3 or 10 μ g of CP5-CAT were transfected, together with 2 μ g of RSV-LUC, into PC-Pax8 cells (A) and into PC wild-type cells (B) cultured either in complete medium (hatched bars) or in starvation medium (open bars). The relative activity of the pTA-CAT3 and CP5-CAT promoters is with reference to that of the CMV-CAT promoter (grey bars) which was used as control and arbitrarily set at 100. Data were normalized for transfection efficiency by determining the LUC activity and represent the average of three independent experiments. (C) PC-Pax8 cells were cultured in starvation medium for 7 days and then transfected with the same reporter constructs as in (A) and (B). Cells were stimulated with FSK/IBMX for 0.5, 1, 2, 4 and 6 h. pTA-CAT3 (hatched bars) and CP5-CAT (open bars) relative activity is with reference to that of the CMV-CAT promoter (grey bar). The data represent the average of two independent experiments.

expression, is controlled by TSH; and (iv) the TSH-dependent induction of Pax8 takes place several hours before that of Tg.

We then expressed exogenous Pax8, driven by a viral promoter, in PC cells grown in the absence of TSH and we measured Tg mRNA levels. Our results clearly indicate that Pax8 is not the only factor that mediates TSH action on Tg gene expression. In TSH-starved PC thyroid

cells in culture, in fact, the expression of Pax8, either in transient or in stable transfectants, is not sufficient to promote Tg gene expression. Since the reactivation of the cAMP pathway, in TSH-starved cells, fully restores Tg synthesis, we tested whether a cAMP-dependent modification was needed to activate Pax8 protein. Our experiments demonstrate that this is not the case; in thyroid cells on TSH starvation a cAMP-dependent

Figure 8 Requirement of protein synthesis in Tg gene activation. PC-Pax8 cells were cultured in complete medium (lane 2) or maintained for 7 days in starvation medium (lane 3) and treated for 6, 12 or 24 h with FSK/IBMX in the absence (lanes 4–6) or in the presence (lanes 7–9) of cycloheximide. Tg mRNA was detected by RT-PCR using two oligonucleotides located respectively in the first and second exon of the Tg gene. RT-PCR products were separated on a 1% agarose gel. Tg mRNA can be detected in control cells cultured in complete medium (lane 2) or after 12 and 24 h of FSK/IBMX treatment in the absence of cycloheximide (lanes 5, 6). Tg mRNA was not detected in HeLa cells (lane 1), in PC-Pax8 cells cultured in starvation medium for 7 days (lane 3) and in cells maintained in the presence of cycloheximide either treated (lanes 7–9) or not treated (lanes 10–12) with FSK/IBMX.

modification of Pax8, or of any other factor involved in Tg gene expression, is not sufficient to activate the Tg gene promoter. We suggest that an additional factor yet unknown, whose synthesis is induced by cAMP, is necessary, together with Pax8, to promote Tg gene transcription. Furthermore, we demonstrated, by using a Pax8-dependent artificial promoter, that Pax8 protein is transcriptionally inactive in the absence of cAMP stimulation.

How can we explain Pax8 inability to activate transcription in TSH-deprived PC cells? Several hypotheses can be made. On the one hand, it can be hypothesized that one or more factors required to activate Pax8 are missing in TSH-starved thyroid cells. We demonstrated that to restore Pax8 transcriptional activity, the TSH/cAMP-stimulated synthesis of additional protein(s) is required. This putative factor(s) could act directly on Pax8, since in TSH-starved thyroid cells, Pax8, as well as being unable to activate a Tg gene promoter, is also unable to activate an artificial promoter consisting of five Pax8-binding sites, thus suggesting that Pax8 itself is inactive. In addition, it also possible that a yet unidentified factor, whose expression is controlled by the TSH/cAMP pathway, is necessary together with Pax8 to activate the Tg promoter. On the other hand, we can speculate that other factors are inactivating Pax8 either by directly binding or by reversibly modifying it. Some of the presumptive inhibitors and/or activators are tissue-specific proteins and some of them are likely to directly interact with Pax8. The identification of Pax8 interacting proteins will clarify this issue.

The Pax8-binding site on the Tg promoter overlaps one of the TTF-1-binding sites, thus it could be argued that

TTF-1 is a candidate for acting as a Pax8 modulator. It has been suggested that a specific ratio of TTF-1/Pax8 is needed to activate Tg gene promoter (Fabbro *et al.* 1998). It has also been indicated that TTF-1 expression is under hormonal regulation; TSH, possibly in the presence of insulin or serum, was shown to decrease TTF-1 levels (Saito *et al.* 1997). In our experiments, however, no change in TTF-1 levels are observed in the different culture conditions. Another potential candidate could be the transcription factor TTF-2, since it is a thyroid-specific transcription factor and its expression also depends upon TSH (Ortiz *et al.* 1997, Zannini *et al.* 1997). However, the data on TTF-2 present in the literature are quite controversial. Initially TTF-2 was suggested to be important for Tg transcription (Santisteban *et al.* 1992) but subsequently it was demonstrated that in both thyroid and non-thyroid cells TTF-2 acts as a promoter-specific repressor (Perrone *et al.* 2000). In addition, we have to consider that in our experiments Pax8 is not able to activate transcription from the CP5 promoter, whose activation depends only upon Pax8.

When is the TSH/cAMP control of Pax8 established? Pax8 is a factor that plays a double role; in the mouse, during embryonic development, it is required for correct thyroid organogenesis, as indicated by the finding that Pax8 knockout mice do not have thyroid follicular cells (Mansouri *et al.* 1998), and it is responsible for the maintenance of differentiated functions in thyroid cells in culture (Pasca Di Magliano *et al.* 2000). Although not proven, these two functions are possibly independent as suggested by the fact that the expression of a functional Pax8 at E10.5 does not induce Tg gene expression while already playing a role in thyrocytes survival and proliferation. The role of Pax8 in follicular cell formation during organogenesis might be TSH-independent; mutations in the TSH receptor (*hyt/hyt* mouse) (Green *et al.* 1988, Anthony *et al.* 1993) that impair the cell response to TSH and cause hypothyroidism do not impair thyroid follicle formation or Tg and Pax8 expression (M De Felice, unpublished observations). Therefore, surprisingly, Pax8 appears to be functionally active during thyroid development despite the lack of thyrocyte response to TSH. Interestingly, it has recently been reported that the thyroid-specific expression of a dominant-negative CREB isoform in transgenic mice causes impairment of thyroid function, failure of follicular cells differentiation, and alteration of the expression of thyroid-specific genes and TTFs (Nguyen *et al.* 2000). Presumably the effect of the dominant-negative CREB is an inhibition of the TSH signaling pathway downstream of the receptor itself. Because CREB can be activated by various other signaling cascades (Andrisani 1999), the apparent discrepancy between the data obtained in the *hyt/hyt* mouse model and in the CREB dominant-negative transgenics suggested that in the former the selective inhibition of the TSH pathway at the level of the receptor is not

sufficient to impair CREB, while instead it occurs in the latter.

Loss-of-function mutations of the TSH receptor have been implicated in the pathogenesis of thyroid diseases. In some patients carrying an inactivating mutation of the TSH receptor, plasma Tg levels were in the normal range and were in fact disproportionately high considering the small size of the patients' thyroids (Gagne *et al.* 1998). It is known that TSH is not required for the development and migration of the thyroid anlage, but it is essential for thyroid growth and function. The possible mechanisms for Tg secretion in the absence of TSH action have been discussed in the literature (Abramowicz *et al.* 1997). The presence of circulating Tg in these patients may indicate that some synthesis of Tg is still occurring. Alternatively, it could result from incomplete polarization of thyrocytes, from abnormal routing of Tg, or from intercellular leakage from dysplastic follicles. Of note, recently it has been reported the case of a patient carrying a new loss-of-function mutation of the TSH receptor, with no detected levels of circulating Tg (Tonacchera *et al.* 2000).

Thus, the mechanisms by which TSH and cAMP regulate thyroid differentiation are at present still not completely understood, and we should like to suggest that TSH-mediated regulation of transcription is likely to be a complex phenomenon involving several different mechanisms.

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