Pax8 has a key role in thyroid cell differentiation

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Transformation of rat thyroid cells with polyoma virus middle T antigen results in loss of the thyroid-differentiated phenotype, measured as the expression of the thyroglobulin (*Tg*), thyroperoxidase (*TPO*), and sodium/iodide symporter (*NIS*) genes. Among the transcription factors involved in the regulation of these genes, TTF-1 and TTF-2 were still detected at nearly wild-type levels, while a specific loss of the paired domain transcription factor Pax8 was observed. In this study, we used the PCPy cell line as a model system to study the role of Pax8 in thyroid differentiation. We demonstrate that the reintroduction of Pax8 in PCPy cells is sufficient to activate expression of the endogenous genes encoding thyroglobulin, thyroperoxidase, and sodium/iodide symporter. Thus, this cell system provides direct evidence for the ability of Pax8 to activate transcription of thyroid-specific genes at their chromosomal locus and strongly suggests a fundamental role of this transcription factor in the maintenance of functional differentiation in thyroid cells. Moreover, we show that Pax8 and TTF-1 cooperate in the activation of the thyroglobulin promoter and that additional thyroid-specific mechanism(s) are involved in such a cooperation. To identify the Pax8 domain able to mediate the specific activation of the thyroglobulin promoter, we transfected in PCPy cells three different Pax8 isoforms. The results of such experiments indicate that for the transcriptional activation of thyroid-specific genes, Pax8 uses an as yet unidentified functional domain.

Pax genes | gene expression

Terminally differentiated cells express genes whose products are undetectable in other cell types. The isolation of regulatory DNA sequences responsible for such cell-type-specific expression and the cloning of the cDNAs encoding cognate transcription factors revealed, in the majority of cases, that a combination of transcription factors is unique to a cell type. Thus, transcription of cell-type-specific genes appears to depend on synergy between transcription factors (1, 2). Frequently, transcription factors controlling the expression of genes specific to the terminal differentiation state of a given cell type play an additional role earlier in the development of the same cell type. As a consequence, the identification of factors controlling cell-type-specific gene expression also provides relevant information on earlier developmental stages in many organs and cell lineages. However, a direct assessment of the role of many transcription factors in cell-type-specific gene expression has proven difficult, given that mice lacking the relevant transcription factors often show complete absence of the implicated cell type.

To elucidate the mechanism(s) operating in the establishment and maintenance of cell-type-specific expression, we have been studying a model system represented by the thyroid follicular cells, the most abundant cell population of the thyroid gland. These cells are responsible for thyroid hormone synthesis and are characterized by the expression of thyroid-specific genes such as thyroglobulin (*Tg*) and thyroperoxidase (*TPO*), which are exclusively expressed in this organ, and by the expression of genes that have been detected only in a few tissues other than the thyroid, such as the TSH receptor (*TSHr*) and the sodium/iodide symporter (*NIS*) (2, 3). Tg and TPO promoters have been extensively studied, and three transcription factors have been cloned that specifically bind and activate these two promoters (3). 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 diencepahlon, thyroid, and lung (4); TTF-2 is a forkhead domain-containing protein expressed in pituitary and thyroid glands (5), and Pax8 is a member of the murine Pax family of paired domain-containing genes, expressed in the developing kidney, the neural tube, and the developing and adult thyroid (6). Thus, none of these transcription factors is expressed only in the thyroid, but their combination is unique to this organ and is likely to be responsible for early commitment and differentiation of thyrocytes.

In this study, we focused on the transcription factor Pax8. Pax8 binds to a single site on the promoters of the Tg and TPO genes and, in both promoters, the Pax8-binding site overlaps with that of TTF-1 (7). In the case of the Tg promoter, all mutations that abolish Pax8 binding also greatly reduce that of TTF-1, making it difficult to conclude which of the two factors is playing an important role for promoter expression at this site. Conversely, in the TPO promoter it has been possible to find a mutation that interferes only with Pax8 and that greatly reduces promoter activity, thus providing strong evidence for an important role of Pax8 in thyroid-specific gene expression (7). In addition, Pax8 was shown, in transient transfection assays, to activate transcription from the TPO and, albeit at a much lower level, the Tg promoters in nonthyroid cells. These data prompted us to propose these two thyroid-specific genes as putative targets for Pax8 action (7). Very recently, Pax8 has also been involved in thyroid-specific expression of the rat gene encoding for the sodium/iodide symporter (8). However, given both the exceedingly elevated concentration of transcription factors and of the cognate target promoters that are achieved in transient transfection assays, and given that Pax8-dependent transcriptional activation of the endogenous *Tg*, *TPO*, and *NIS* genes had not yet been reported, there was a need for direct evidence for Pax8-dependent transcription from the chromosomal Tg, TPO, and NIS genes. The finding that Pax8 knockout mice exhibit a total absence of the thyroid follicular cells (9), while indicating an essential role for this gene in thyroid organogenesis, could not contribute to a definitive assessment of the role of Pax8 in controlling the expression of the thyroid-differentiated phenotype, because the thyroid cells precursors disappear before the onset of the Tg, TPO, and NIS gene expression. Also, the association between mutations in PAX8 with human thyroid dysgenesis (10) underlines an important role for this protein in organogenesis but could not contribute to a further understanding of its involvement on the expression of thyroid-specific genes.

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Several thyroid-derived cell lines have been established, which maintain *in vitro* the expression of the differentiation markers. Among these cell lines, we have used the PC Cl3 cells (PC cells) (11) that express the *Tg*, *TPO*, *NIS*, and *TSHr* genes and depend on TSH for proliferation and for the expression of the differentiated phenotype (12). These cells also express the transcription factors TTF-1, TTF-2, and Pax8.

Transformed cell lines have been obtained by the expression of different oncogenes in PC cells (11, 13). In most cases, transformation causes loss of the differentiated phenotype, TSH-independent proliferation, and tumor formation on injection in nude mice. When the thyroid-specific transcription factors have been analyzed in these cell lines, often an impairment of their expression or activity has been found. However, more than one factor is usually affected, thus suggesting that complex changes in the transcriptional program have occurred. As a consequence of the complexity of the phenotype, a detailed study of the individual role of each factor in differentiation is often difficult.

In contrast, we report in this paper that transformation of PC cells with the polyoma middle T antigen (PCPy), resulting in loss of the differentiated down-regulation of Tg, TPO, and NIS expression and TSH-independent proliferation, is associated with specific loss of Pax8 expression. In contrast, TTF-1 is detected at wild-type levels, and TTF-2 is moderately reduced. We demonstrate in this paper that the introduction of Pax8 expression vectors in PCPy cells, followed by the synthesis of a functional Pax8 protein, is sufficient to activate expression of the endogenous Tg, TPO, and NIS, thus providing direct evidence that this transcription factor plays a key role in thyroid-specific expression of many genes. However, PCPy cells expressing Pax8 still show an altered growth potential, indicating that transformation affects the gene expression program and the proliferation of these cells by using two distinct mechanisms, only one of which involves Pax8.

We have investigated the mechanisms through which Pax8 reactivates Tg transcription. We obtained strong evidence that Pax8 acts directly on the Tg promoter and that the interaction with another thyroid-specific protein is required. Experiments aiming at the localization of the functional domains in Pax8 have been performed.

Materials and Methods

Plasmids. To generate ΔMT-Pax8, specific primers were used to amplify the entire coding region of mouse Pax8 by PCR. *Xho*I sites were added at 5′ and 3′ sites to facilitate cloning. The amplified product was cloned in the 32 ΔMT plasmid (14). The pCMV5-Pax8 plasmid has been previously described (7). Expression vectors encoding Pax8 splicing variants are described in ref. 15.

The plasmids used in transient transfection experiments have been previously described and are as follows: Tg-CAT (16) and Tg-CAT Acore (17).

Cell Culture and Transfection. PC and PCPy cell lines have been previously described (13).

Both cell lines were grown in Coon's modified F12 medium (Seromed, Milan) supplemented with 5% calf serum (GIBCO) and six hormones and growth factors as described by Ambesi-Impiombato and Coon (12).

Transfections of PCPy cells were carried out as previously described (18). To obtain stable clones, calcium phosphate–DNA precipitates were prepared with 10 μg of plasmid DNA containing either pCMV-Pax8 or ΔMT-Pax8 and 1 μg of a plasmid containing the neomycin resistance gene under the RSV promoter and 40 μg of calf thymus genomic DNA as a carrier (Boehringer Mannheim). Cells were selected with 300 μg/ml of G418 (Sigma). After 3 weeks, G418-resistant clones were isolated and expanded.

RNA Extraction and Northern Blot Analysis. Total RNA was prepared by the acid guanidium thiocyanate/phenol procedure (19) polyA$^+$ RNA was isolated with magnetic oligo-dT beads (Dynal, Oslo).

Either 30 μg of total RNA or 1 μg of polyA$^+$ RNA was 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) with 20× SSC, and hybridization and washing were carried out according to Church and Gilbert (20).

The blot was probed with rat cDNA fragments derived by restriction digestion of Tg, TPO, and NIS coding sequences, and labeled by random priming with [α-^{32}P] dATP and [α-^{32}P]dCTP (Amersham Pharmacia). A 300-bp fragment derived from the 3′ region of the rat GAPDH was used as control.

RNase Protection Assay. Total RNA from cultured cells was prepared by the acid guanidium thiocyanate/phenol procedure. The probe for RNase protection was synthesized by T3 polymerase transcription of *Eco*RI linearized TPO5′C plasmid, containing the TPO cDNA subcloned in Bluescript KS (Stratagene). A GADPH probe was used as control. Hybridization and RNase treatment were as described (21).

Reverse Transcription–PCR. Five micrograms of total RNA prepared from the different cell lines was used to synthesize cDNA by using the Superscript II Reverse Transcriptase kit (Life Technologies, Grand Island, NY) with random hexamers as primers. The cDNA was subsequently used as template for the PCR reaction with gene-specific primers. One-fifth of the total volume of the PCR reaction was analyzed on a 1% agarose gel.

Cell Proliferation and Soft Agar Assay. To evaluate cell growth, PC, PCPy, and PCPy-Pax8 cells were plated at 10^4 cells per 60-mm plate. The cells were grown in F12 medium with 0.2% calf serum either containing or not the six hormones and growth factors. The medium was changed every 24 h, and every 24 h cells were collected and counted.

Growth in soft agar was based on MacPherson and Montagnier methods (22). Briefly, each transfected cell line was seeded at 5×10^4 cells per 60-mm dish in growth medium containing 0.25% agar over a base layer of 0.6% and then incubated for 4 weeks. Cells were fed weekly with 1 ml of medium. The number of colonies per plate was divided by the number of cells plated to calculate the efficiency of colony formation. Colonies larger than 50 cells were scored as positive.

Results

Characterization of the PCPy Cell Line. The PCPy cell line is a transformed derivative of the differentiated thyroid cell line PC obtained by infection with a retrovirus carrying the polyoma middle T antigen (13). Polyoma middle T-mediated transformation results in loss of the differentiated phenotype, measured by the expression of thyroid-specific genes. Moreover, PCPy cells proliferate in a hormone-independent manner, show anchorage-independent growth, and are tumorigenic when injected in nude mice (23).

We analyzed RNA levels for the thyroid differentiation markers Tg, TPO, and NIS by Northern blot in the PC and PCPy cells. Tg, TPO, and NIS mRNA levels are severely reduced in PCPy cells in comparison to PC cells (Fig. 1). The reduction of Tg and TPO was previously reported (18).

We next checked for the presence of mRNAs encoding for TTF-1, TTF-2, and Pax8, which have been proposed to be crucial for Tg and TPO transcription.

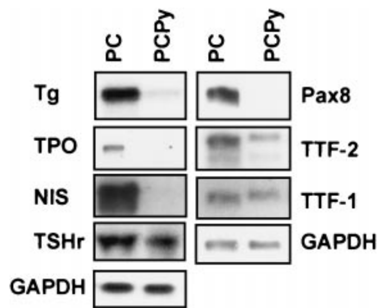

Fig. 1. Expression of differentiation markers in PCPy cells. Total RNA (30 μg) from PC cells and PCPy transformed cells was blotted onto nylon membranes and sequentially hybridized to the cDNA probes indicated on the side of the blots. The GAPDH probe was used for normalization.

The results obtained by Northern blot indicate that TTF-1 mRNA levels are similar in the two cell lines, TTF-2 mRNA is moderately reduced in PCPy cells, whereas Pax8 expression is severely affected by transformation (Fig. 1). In addition, TTF-1- and TTF-2-binding activities are comparable in PC and PCPy cells (data not shown).

It is worth noting that the TSH receptor is expressed in PCPy cells at levels comparable to those of PC cells (Fig. 1), even though PCPy have acquired the ability to grow in the absence of TSH.

Generation and Characterization of Stable Transfectants of PCPy Cells Expressing Pax8. To investigate whether the absence of Pax8 was responsible for the loss of differentiation of PCPy cells, we generated stable transfectants of PCPy cells with two different expression vectors encoding for Pax8: CMV5-Pax8 and ΔMT-Pax8 in which Pax8 is expressed under the control of a constitutive promoter or of an inducible metallothionein promoter, respectively.

Several independent clones were isolated, and Pax8 expression was determined by Northern blot analysis. As shown in Fig. 2*A*, three clones, named 4B1, 4B2, and 5B2, were able to express exogenous Pax8 at high levels, comparable to those of wild-type PC. The neomycin-resistant clone 4B3 did not express Pax8 and was used as a negative control.

In the three Pax8 positives clones (4B1, 4B2, 5B2), the endogenous Tg gene was reactivated, and the mRNA levels were comparable to wild-type PC cells, whereas Tg mRNA was undetectable both in PCPy cells and in the neomycin resistant clone (4B3) (Fig. 2*A*).

To confirm the importance of Pax8 for Tg transcription, we evaluated the effect of its expression also in the clones stably transfected with ΔMT-Pax8, which express Pax8 only on induction with $CdCl_2$. Fig. 2*B* shows the analysis by Northern blot of two of these clones (4A2, 4A3): Tg mRNA was detected only in Pax8 positive cells. Thus, these data obtained from PCPy cells carrying either a constitutive or an inducible Pax8 expression vector show that it is possible to reactivate transcription of the Tg gene in PCPy cells by the expression of Pax8, strongly supporting that this transcription factor is essential for Tg expression in thyroid cells.

Subsequently, we investigated the role of Pax8 in the expression of the other thyroid-specific differentiation markers. We evaluated TPO expression in PCPy-Pax8 clones by RNase mapping. TPO mRNA was undetectable in the control clone 4B3 (neomycin resistant but Pax8 negative) as well as in PCPy cells but was present at wild-type levels in Pax8-expressing clones (4B1 and 4B2) (Fig. 2*C*).

As shown in Fig. 1, NIS mRNA is undetectable in PCPy cells. We then analyzed the expression of NIS in PCPy-Pax8 clones,

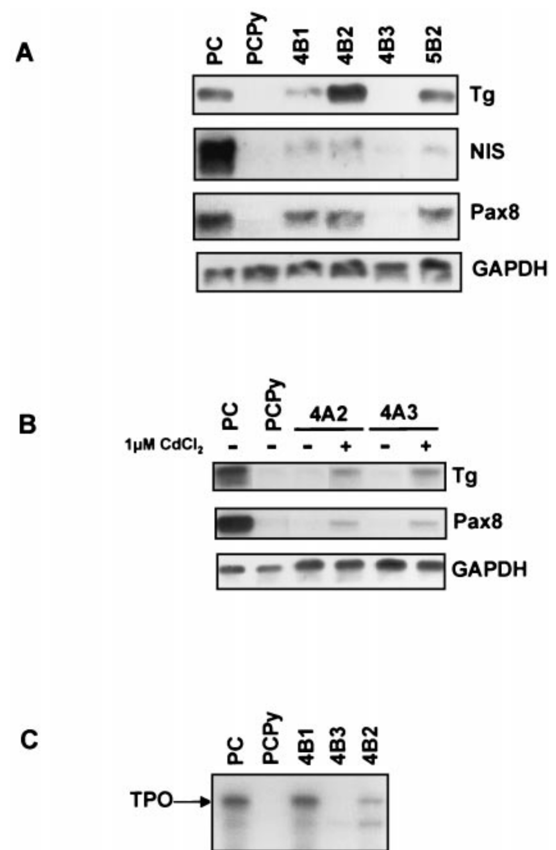

Fig. 2. Analysis of PCPy-Pax8 clones. (*A*) Total RNA (30 μg) from PC, PCPy cells, from three Pax8 positive clones (4B1, 4B2, 5B2), and from a negative clone (4B3) was blotted onto nylon membranes and sequentially hybridized with the cDNA probes indicated (*Right*). The GAPDH probe was used for normalization. (*B*) Total RNA (30 μg) from PC, PCPy cells, and two clones generated with inducible Pax8 (4A2, 4A3) was analyzed by Northern blot for the expression of endogenous thyroglobulin and Pax8, in the presence or absence of the induction with $CdCl_2$. The GAPDH probe was used for normalization. (*C*) Analysis of TPO expression by RNase protection on total RNA from PC, PCPy cells, two Pax8 positive clones (4B1, 4B2), and one negative clone (4B3).

and we found that, whereas its RNA is absent in the neomycin resistant clone 4B3, its transcription is reactivated in clones 4B1, 4B2, and 5B2, although only partially (Fig. 2*A*). These data support the evidence, recently published by our laboratory (8), of a role for Pax8 in the activation of the NIS gene transcription.

We then asked whether Pax8 was acting directly on the Tg promoter or indirectly promoting the synthesis of another protein that could activate transcription of the Tg gene. To distinguish between these two possibilities, we studied the time course of both Pax8 and Tg induction in one of the inducible PCPy-Pax8 clones (clone 4A2). Pax8 mRNA is induced already 2 h on $CdCl_2$ treatment, and Tg mRNA appears exactly at the same time (Fig. 3). Given the concomitant induction of the two genes and the short time required to see the Tg transcript, we conclude that the action of Pax8 on the Tg promoter is direct.

Pax8 Cooperates with TTF-1 in the Transcriptional Activation of the Thyroglobulin Promoter in Thyroid Cells. In transient transfection assays in HeLa cells, TTF-1 alone is able to activate transcription from a reporter construct in which the Tg minimal promoter is subcloned upstream of a CAT gene (Tg-CAT) (24). In contrast, in the same system, Pax8 is a poor activator of Tg promoter (7). Nevertheless, in PCPy cells, where TTF-1 is present at wild-type

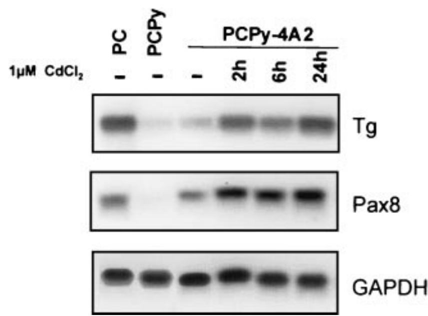

Fig. 3. Time course of Pax8 and thyroglobulin induction. One clone generated with inducible Pax8 (4A2) was stimulated for different times with $CdCl_2$, and total RNA was prepared. The expression of Pax8 and thyroglobulin was measured by Northern blot analysis. As shown, at 2 h of $CdCl_2$ induction, expression of both Pax8 and thyroglobulin is detected.

levels, Tg gene transcription is undetectable, and the addition of Pax8 causes a major activation.

To investigate whether Pax8 was acting on the same promoter region that was studied in transient transfection assays in HeLa cells, we transfected the reporter construct Tg-CAT in PC, PCPy, and PCPy-Pax8 cells. In Fig. 4, the activity of Tg-CAT in the different cell lines is reported as fold induction compared with the transcriptional activity of a basal promoter, a reporter construct containing only the TATA box of the E1b gene in front of the CAT gene. The activity of Tg-CAT is close to the control in PCPy cells, whereas in PCPy-Pax8 cells, the promoter is as active as in PC cells, indicating that in thyroid cells, Pax8 is required to activate Tg promoter, whereas TTF-1 alone is not sufficient.

To verify whether TTF-1 was indeed necessary for the activation of the minimal Tg promoter in thyroid cells, we also transfected the reporter construct Tg-CAT Acore, in which one of the binding sites for TTF-1 (site A in ref. 16), previously shown to be important for Tg promoter full activation, is abolished. As expected on the basis of TTF-1 role in Tg transcription, Tg-CAT Acore is not active in PC cells. When tested in PCPy-Pax8 cells, the Tg-CAT Acore promoter was shown to be inactive also in this cell environment, indicating that Pax8 is unable to activate Tg

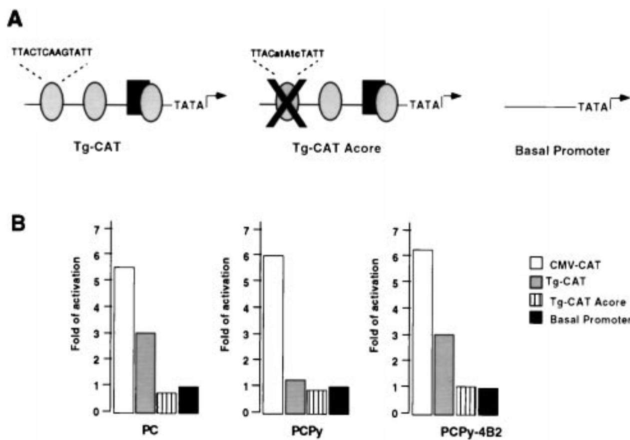

Fig. 4. Thyroglobulin promoter activity in PC, PCPy, and PCPy-Pax8 cells. (*A*) Schematic representation of the Tg reporter constructs, wild-type (Tg-CAT) and mutated in one of the TTF-1-binding site (Tg-CAT Acore). Gray ovals represent TTF-1, and the black rectangle represents Pax8. (*B*) PC, PCPy, and one PCPy-Pax8 clone (PCPy-4B2) were transiently transfected with 10 μg of wild-type Tg reporter construct (Tg-CAT) or of a mutated Tg reporter (Tg-CAT Acore), along with 2 μg of CMV-LUC. After normalization for LUC activity, the activity of the promoters was measured relative to that of a basal promoter.

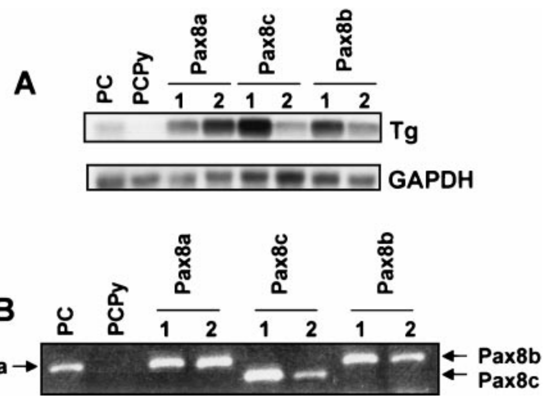

Fig. 5. Analysis of the transcriptional properties of Pax8 splicing isoforms. (*A*) Northern blot analysis of total RNA prepared from PCPy cells stably transfected with Pax8 splicing isoforms. Tg expression was analyzed in two different pools of clones for each transfected DNA. The GAPDH hybridization was used to normalize the amount of RNA present in each lane. (*B*) Reverse transcription–PCR analysis of cDNA derived from total RNA from the same cells as in *A*. Pax8 expression was analyzed in two different pools of clones for each transfected DNA.

promoter in the absence of TTF-1 (Fig. 4). These results clearly indicate that TTF-1 and Pax8 cooperate in the transcriptional activation of the thyroglobulin promoter in thyroid cells, providing evidence for such a mechanism in the regulation of thyroid-specific transcription.

Pax8 Splicing Isoforms Are Able to Reactivate Endogenous Tg Expression. Four Pax8 spliced transcripts have been described: Pax8a, Pax8b, Pax8c, and Pax8d (15, 25). Pax8a, the most relevant isoform, contains all of the 10 exons and encodes for the most abundant Pax8 species. Pax8b does not contain exon 8, whereas Pax8c lacks exons 7 and 8. In this latter isoform, as a consequence of the splicing of two exons, the shift in the reading frame generates a proline-rich C terminus and a premature termination of translation.

Studies on the Pax8 transcriptional properties showed that the C terminus of Pax8a is required for transactivation (26). In particular, the data obtained studying various Pax genes showed that the regulatory module of these proteins consisting of a potent transactivation region has been conserved among the members of the family and lies in the C-terminal region downstream amino acid 300 (26). Thus the C-terminal region of Pax8, as well as that of other Pax genes, represents the only domain of the protein involved in transcriptional activation that has been identified so far.

The four Pax8 variants encode for proteins that differ in the C terminus and thus could differ, as suggested in previous studies (15, 25), in transactivation properties. In all our experiments, we have used the full-length Pax8 cDNA, namely the Pax8a isoform. To evaluate the possibility that the C terminus of the protein could be the portion involved in Pax8 activation of the Tg promoter in thyroid cells, we tested whether Pax8a, Pax8b, and Pax8c behave differently with respect to Tg activation in PCPy cells (Pax8d was not included in this study).

We first demonstrated by bandshift assay that the isoforms Pax8a, -b, and -c were equally able to bind the same sequence derived from the Tg promoter (data not shown). Subsequently, we stably transfected PCPy cells with the expression vectors encoding for Pax8a, Pax8b, and Pax8c, and we isolated pools of clones. The expression of the different isoforms in the pools was checked by reverse transcription–PCR (Fig. 5*B*), and the pools were analyzed by Northern blot for the expression of the endogenous thyroglobulin gene. The results shown in Fig.5*A*

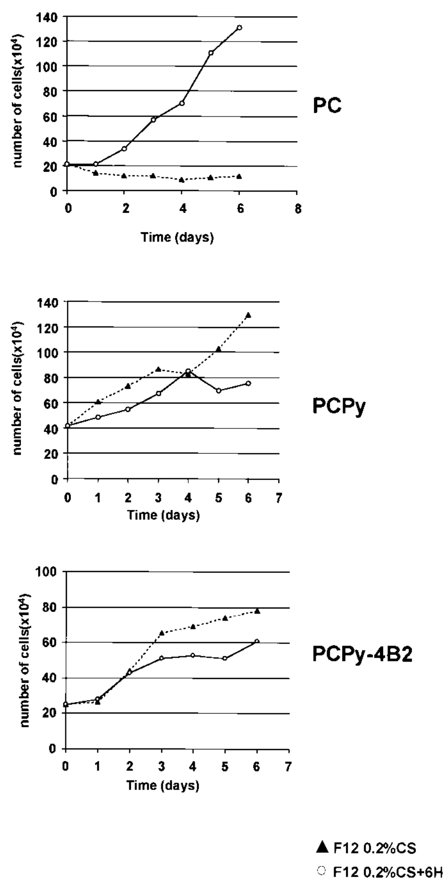

Fig. 6. Growth curves of PC, PCPy, and PCPy-Pax8 cells. PC, PCPy, and PCPy-Pax8 cells (clone 4B2) were grown in basal medium (0.2% calf serum) with or without the addition of the six-hormone mixture (6H), and the proliferation rate was measured as number of cells at different time points.

demonstrate that the three isoforms are equally capable of stimulating the expression of the endogenous Tg gene, suggesting that the C terminus of the protein is not involved in the thyroid-specific activation of the Tg promoter by Pax8.

Reexpression of Pax8 Does Not Influence Hormone- and Anchorage-Independent Growth of PCPy Cells. Thyroid cells in culture can proliferate only in the presence of TSH and insulin. After transformation by polyoma middle T antigen, PC cells become TSH and insulin independent. Because Pax8 is able to revert the transformed phenotype of PCPy cells for what concerns the expression of the thyroid-specific markers, we investigated whether Pax8 could revert the effects of transformation also for the growth parameters.

PC, PCPy, and PCPy-Pax8 cells were cultured in basal medium (F12 0.2% calf serum) with or without the addition of the six hormones (Fig. 6). Wild-type PC cells efficiently grew in medium containing the hormones, whereas no proliferation was obtained in basal medium. On the contrary, PCPy cells as well as PCPy-Pax8 clones could proliferate even in the absence of hormones.

PC cells are unable to form colonies in soft agar, whereas the colony-forming efficiency of PCPy cells is very high. Thus, we evaluated the anchorage-independent growth of PCPy-Pax8 cells, and we showed that PCPy-Pax8 cells are able to form colonies in soft agar with an efficiency comparable to that of PCPy cells (data not shown).

We conclude that the expression of the differentiated phenotype on one side and growth features and anchorage independence on the other side are under different genetic controls.

Discussion

The thyroid system is a very useful model to study tissue-specific transcription for several reasons. First, there are different thyroid follicular cell lines that retain in culture the differentiation features of thyroid follicular cells *in vivo*. In addition, the promoters of thyroid-specific genes, such as thyroglobulin and thyroperoxidase, have been characterized, and the promoter regions responsible for the thyroid-specific transcription have been identified. Three thyroid-specific transcription factors have been cloned: TTF-1, TTF-2, and Pax8.

In this manuscript, we show that in thyroid cells, Pax8 is a master gene for the regulation of the thyroid differentiated phenotype, important for the transcriptional activation of all of the differentiation markers analyzed such as thyroglobulin, thyroperoxidase, and sodium/iodide symporter.

Pax genes are a family of developmental control genes that encode transcription factors (27). Nine *Pax* genes have been identified so far in mouse and human, and in five of them mutations have been found associated with congenital human diseases or spontaneous mouse mutants. From the analysis of transgenic or knockout mice, it has become clear that *Pax* genes are key regulators during crucial steps of the organogenesis of kidney, eye, ear, nose, limb muscle, spinal cord, brain, and thyroid (27–30). In fact, mutant mice have revealed a crucial role of Pax genes in the development of various tissues. In particular, Pax8 knockout mice have a very small thyroid gland, which is deprived of the follicular cells (9), and patients suffering from congenital hypothyroidism have been shown to carry mutations in the *Pax8* gene (10). However, until now very little has been known about *in vivo* targets of the *Pax* genes. Functional targets have been suggested for Pax2, Pax5, and Pax8. Interestingly, an experimental system represented by the FRTL5-TA cell line has recently been described, in which the absence of Pax8 correlates with loss of the differentiated phenotype of these cells (31).

In addition, Pax8 has been demonstrated to be important during renal vesicle formation, and in particular it has been shown to regulate the expression of the *wt1* gene (32).

In the present work, we show that, in agreement with our earlier proposal that was solely based on transient transfection experiments, the *Tg* and *TPO* genes are indeed functional targets of Pax8. We have used the transformed thyroid cell line PCPy, which is a polyoma virus middle T transformed derivative of PC cells. This cell line has completely lost the differentiated phenotype, both as tissue-specific gene expression and as TSH dependence for proliferation. However, in PCPy cells, only the expression of Pax8, among the thyroid-specific transcription factors, is severely reduced.

We have transfected a Pax8 expression vector in the PCPy cells, and we have obtained several independent stable clones in which Pax8 was expressed. Importantly, the levels of Pax8 mRNA and protein (data not shown) are very similar to those observed in wild-type PC cells. The phenotype of these clones was analyzed for the expression of the thyroid-specific differentiation markers. We showed that in the PCPy cells reexpressing Pax8, the expression of *Tg* and *TPO* endogenous genes was restored at wild-type levels. The *NIS* gene is also activated, but its expression level remains clearly below of that observed in PC cells. These data are, to our knowledge, the first available evidence on Pax8 ability to activate transcription of genes in a physiological chromosomal context and hence validate the *Tg*, *TPO*, and *NIS* genes as bona fide targets of this transcription factor. However, it is important to note that important differences exist among the two sets of data. In transient assays, the TPO promoter was very efficiently activated, up to 80-fold, whereas the Tg promoter responded rather poorly to Pax8,

showing an activation of not more than 3-fold. On the contrary, in the PCPy cells, expression of Tg shows a very high inducibility by Pax8, certainly similar to that of TPO. Thus, given that in the PCPy system the entire Tg locus is available in the normal chromatic conformation and considering that in this cell system, transcriptional activation is observed at normal concentration of the transcription factor and target genes, we conclude that Pax8 must be considered essential also for Tg gene expression. The discrepancy between the transient expression data and those reported here could be most easily explained with the absence, in the Tg promoter used in the transient experiments, of a regulatory sequence that is important for Pax8 inducibility. However, we cannot exclude at present other explanations, such as an important role of chromatin conformation or the presence in PCPy cells of specific coactivators. The partial reactivation of the *NIS* gene observed in this study provides strong support to the role of Pax8 in NIS gene activation recently proposed. In fact, an upstream enhancer of the NIS promoter was identified, and Pax8 was shown to bind to this enhancer and to be involved in a functional protein–protein interaction with a still unknown factor that binds to a CRE-like sequence (8). However, our data also indicate that other factors not present in the PCPy cells are necessary for full expression of the *NIS* gene.

In addition, our results clearly indicate that TTF-1 and Pax8 cooperate in the transcriptional activation of the thyroglobulin gene promoter in thyroid cells. This observation is of interest considering our previous data showing that the binding site of Pax8 on the Tg promoter overlaps one of the binding sites of TTF-1 and that, at least *in vitro*, the two proteins cannot bind together to the same DNA region (7).

To localize the portion of the Pax8 protein involved in thyroid-specific activation of the Tg gene, we took advantage of the existence of different Pax8 isoforms. Interestingly, the results obtained with the splicing isoforms strongly indicate that the C-terminal portion of the protein containing the already known transactivation domain is dispensable for Pax8-dependent activation of the Tg promoter. Our current hypothesis is that a yet unidentified portion of the protein is able to confer the specificity of the transcriptional activation potential. The Pax8 role could be to recruit to the promoter another factor, like TTF-1 or a coactivator, as it has already been observed for other members of the Pax gene family (33, 34).

One of the features of differentiated rat thyroid cells is the requirement of TSH for proliferation (12). This requirement is lost in the transformed cell line PCPy, although the TSH receptor is present at wild-type levels and appears to be functional (data not shown). Interestingly, the expression of Pax8, although very effective in reestablishing a differentiated phenotype, is unable to revert the altered growth. Thus, PCPy-Pax8 clones remain TSH independent for growth and anchorage independent. We must conclude that polyoma middle T is able to bypass the TSH receptor and to induce cell growth and, independently, to interfere with the expression of Pax8. Polyoma middle T antigen is known to behave as an activated membrane receptor, which binds and activates different cellular tyrosine kinases. The tyrosine kinases in turn activate different pathways that lead to cellular proliferation in the absence of external stimuli. Interestingly, Pax8 has been proposed to have a role also in thyroid cell proliferation, as suggested by the observation that antisense treatment of thyroid cells inhibits Pax8 expression and reduces the ability to proliferate (35). However, our data indicate that the polyoma middle T antigen in thyroid cells alters differentiation via interference with Pax8 expression, whereas it induces growth by a Pax8-independent mechanism.

It is not yet clear whether *Pax* genes are involved in the maintenance of a differentiation decision or in early differentiation pathways, or both. In this manuscript, we show that Pax8 has a key role in cellular differentiation, being a direct mediator of the thyroid-specific expression of the genes exclusively expressed in the thyroid cell type. It is worth mentioning that the data presented in this manuscript are in agreement with the general opinion that *Pax* genes, thus Pax8 also, appear to play an important role in determining the differentiation state of the specific cell type in which they are expressed (28).

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