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Role for p300 in Pax 8 Induction of Thyroperoxidase Gene Expression*

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The nuclear p300 protein functions as a co-activator of gene transcription. Here we show that p300 works as a co-activator of the transcription factor Pax 8 on the thyroperoxidase gene promoter. Consistent with its role as co-activator, p300 potentiates Pax 8-activated transcription. Furthermore, we provide evidence supporting the formation of a complex between both factors *in vivo* and *in vitro*. This interaction involves the amino-terminal and CH3 domains of p300 and the trans-activation domain of Pax 8 at its carboxyl-terminal end. We show that the CH3 domain is crucial for the co-activator role of p300 on the thyroperoxidase gene promoter. In agreement with our finding and with the ability of the adenoviral protein E1A to bind p300, we show that E1A down-regulates Pax 8 activity.

Thyroid follicular cells undergo a precise differentiation program to become the only cell type able to produce the thyroid hormones. Thyroid hormones are derived from the degradation of a very large precursor, thyroglobulin (Tg)[1], and to produce the final product Tg must first be iodinated by the thyroid-specific enzyme thyroperoxidase (TPO) (1). Hence Tg and TPO, as well as thyrotropin receptor and sodium/iodide symporter, are markers of thyroid differentiation (2, 3). Studies on the transcriptional regulation of these thyroid differentiation markers have resulted in the identification of a set of transcription factors crucial for the maintenance of the thyroid differentiation state. These factors include thyroid transcription factor 1 (TTF-1), thyroid transcription factor 2 (TTF-2), and Pax 8 (3). Each is expressed in a very restricted number of cell types, but their combined expression is limited to follicular cells of the thyroid gland. Their role in thyroid differentiation has been identified by targeted gene disruption in mice. Inactivation of any of the three transcription factors affects normal thyroid development. Null mice for the TTF-1 gene (4), as well as for the TTF-2 gene (5), die *in utero* and, in addition to having other developmental defects, do not develop a thyroid gland. Pax $8^{-/-}$ mice develop a reduced thyroid gland with normal calcitonin-producing parafollicular C cells but lacking differentiated follicular cells (6). Whereas TTF-1 is therefore required for the proper development of the thyroid gland and for the differentiation of both follicular and parafollicular cell types, Pax 8 is required only for the differentiation of the thyroxin-producing follicular cells. A direct correlation has been reported between the neoplastic transformation and dedifferentiation and the activity of the thyroid-specific transcription factors (7). Thyroid cells transformed with a variety of activated oncogenes lose hormonal control of the cell cycle, and the differentiation markers are suppressed. Such a role has been reported for v-*ras*, v-*src*, v-*raf*, and v-*mos* (8). Particularly interesting is the activity of the adenovirus E1A gene because it blocks the thyrocyte differentiation, but the neoplastic phenotype is achieved only after cooperation with other oncogenes (9). Consistent with its role in thyroid-specific gene expression and follicular differentiation, Pax 8 activity and expression have been correlated with thyrocyte transformation and oncogenic mediated dedifferentiation. In rat thyroid epithelial cells, the expression of RET/PTC1 oncogene as well as of polyoma middle T blocks tissue-specific gene expression, and in both cases Pax 8 gene expression is down-regulated (10, 11). Furthermore, it has been reported that exogenous over-expression of Pax 8 in thyroid cells transformed by polyoma middle T reactivates the entire differentiated program (11). Pax 8 belongs to the family of Pax genes constituting important developmental regulators characterized by a highly conserved paired domain at the amino terminus (12). Together with Pax 2 and Pax 5, Pax 8 forms a subfamily of the pax genes characterized by an activation domain and an inhibitory domain at the carboxyl-terminal end and by the presence of a partial homeodomain (12, 13). In thyrocytes, Pax 8 has been recognized as a positive trans-acting factor in Tg and TPO gene promoters (14, 15). Furthermore, it activates TPO and sodium/iodide symporter enhancers (16, 17). It is interesting to note that all the Pax 8 binding sites largely overlap the binding site of TTF-1 and that the binding of the two factors, *in vitro*, is mutually exclusive (15).

We have decided to focus on the role of Pax 8 in thyrocyte-specific transcription. Given the critical role played by Pax 8 in thyrocyte differentiation, an understanding of how this factor mediates transcription activation is essential if its role in establishing the thyroid follicular cell lineage and thyrocyte-specific transcription is to be understood. In this respect, nothing is known concerning the molecular requirements for Pax 8 transcriptional activation.

Increasing evidence of the trans-activation requirements of several transcription factors has established the crucial role of a class of proteins, termed co-activators or co-factors, that are not able to bind DNA directly but via multiple protein-protein interactions bridge the DNA-bound transcription factor to the RNA polymerase II complex, facilitating its recruitment on the

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[1] The abbreviations used are: Tg, thyroglobulin; TPO, thryoperoxidase; TTF, thyroid transcription factor; CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; TBP, TATA-binding protein; RI, Recruitment Index.

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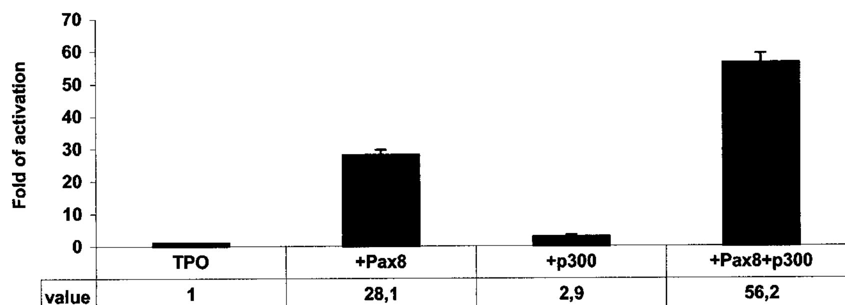

FIG. 1. **p300 cooperates with Pax 8 in TPO gene promoter activation.** HeLa cells were transiently transfected with TPO-pGL3 plasmid and were co-transfected with the vectors encoding the indicated factors as described under "Experimental Procedures." The relative luciferase activity of the cells transfected with TPO-pGL3 only was normalized to 1, and the other activities are expressed relative to this. Values of fold activation and standard deviations are reported.

promoter and thus transcription initiation (18, 19). p300 and CBP (CREB-binding protein) are the most widely characterized co-activator proteins (20, 21). They are ubiquitously expressed phosphoproteins and show an identity of 63% at the amino acid level. Although some differences in their activity have been reported (22), they are generally considered to be functionally homologous (23). p300 was cloned through its interaction with E1A of adenovirus, and CBP was first identified through its interaction with CREB (cAMP-response element-binding protein) (24, 25). These co-activators have been shown to potentiate the activity of several transcription factors, including the nuclear hormone receptors, AP-1, c-Myb, signal transducers and activators of transcription (STAT), NF-κB, and p53 and to interact also with basal transcription factors such as TF-IIB and the TATA-binding protein (TBP); see Refs. 20, 21, and 23 for additional examples of partners of these co-activators in protein-protein interactions. p300 and CBP are functionally conserved transcriptional co-activators that not only bind to histone acetyltransferase (P/CAF) but also show intrinsic acetyltransferase activity (26–28). Thus, they activate transcription by acting as multifunctional adaptor proteins and through acetylation of chromatin.

We searched for factors that might act as Pax 8 co-activators by testing candidate molecules for their ability to cooperate. We report here that p300 acts as a Pax 8 co-activator on the TPO gene promoter by a mechanism involving an interaction between the amino-terminal and CH3 domains of p300 and the carboxyl-terminal domain of Pax 8.

EXPERIMENTAL PROCEDURES

Plasmid Construction and Cell Transfection—TPO-pGL3 plasmid, termed also TPO-Luc, has been constructed by inserting the SacI-*Pvu*II fragment from p420TPOL (29) in pGL3 Basic (Promega) digested with *Sac*I and *Nco*I; the *Nco*I site was blunted with Klenow enzyme. TPO-Pm-pGL3 was constructed in the same way using the *Sac*I-*Pvu*II fragment from p420TPOL-Pm (29). To prepare pBlu-Pax8 plasmid, the Pax 8 gene was amplified from the plasmid C27B (32) by polymerase chain reaction using oligo(A) (5′-GCGGATCCCATGCCTCACAACTCGATC-3′) and oligo(B) (5′-GCGGATCCTGACAACTACAGATGGTC-3′). The amplified fragment was digested with *Bam*HI and cloned in pBluescript KS (Stratagene) digested with *Bam*HI. pGAL 4-Pax 8 was constructed inserting the *Eco*RI-*Xba*I fragment from pBlu-Pax 8 in pM vector (CLONTECH). pGAL 4-Pax8dl1–313, also termed pGAL 4-Pax 8dl1, was prepared by digesting pGAL 4-Pax 8 with *Pst*I and religating the deleted vector. pGAL 4-Pax 8dl331–457, also termed pGAL 4-Pax 8dl2, was constructed by excision of the *Sac*I-*Xba*I fragment from pGAL 4-Pax 8 plasmid followed by religation of the expression vector; the vector termini were blunted with T4 DNA polymerase. The new constructs were confirmed by restriction enzyme digestions. The ability to express the fusion proteins was tested by band-shift assays with the GAL 4 binding site and nuclear extracts of HeLa cells transfected with pGAL 4-Pax 8dl331–457 or pGAL 4-Pax8dl1–313 (data not shown).

HeLa cells were transfected by Effectrene reagent (Qiagen) following the protocol suggested by the manufacturer. The following amounts of the indicated plasmids were transfected: 0.4 μg, luciferase reporter plasmids; 10 ng, pCMV-Pax 8; 10 ng, E1A-expressing vector; 10 ng, the vector encoding for the E1A deletion mutant lacking the amino acids 2–36; 5 ng, pCMVβp300; 5 ng, p300dl33; and 10 ng, the plasmids encoding for the GAL 4 binding domain fusion proteins. The efficiency of transfection was assayed with 10 ng of pCMV-β-galactosidase plasmid. 48 h after transfections cell extracts were prepared. The luciferase activity was measured according to the Luciferase Assay System (Promega), and β-galactosidase assay was performed with the chlorophenol red-β-galactopyranoside substrate as previously reported (33). The values obtained for luciferase assays were corrected for transfection efficiency with the β-galactosidase assay. Transfection experiments were done in duplicate or in triplicate and repeated at least three times. For each experiment we report the mean of three independent experiments, and the standard deviations are shown.

Cell Extracts, Affinity Column, and Band-shift Assay—Quick cell extracts were prepared from transfected HeLa cells. Briefly, the transfected cells were washed in phosphate-buffered saline, scraped in TEN (50 mM Tris, 1 mM EDTA, 150 mM NaCl), and resuspended in 5 volumes of the packed cell volume in buffer A (30 mM Hepes (pH 7.9), 0.1 mM EGTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethanesulfonyl fluoride, 5% glycerol). Two cycles of freezing and thawing were performed, and NaCl was added to a final concentration of 450 mM. Extracts were incubated for 40 min on ice, centrifuged for 30 min in the cold room at 14,000 rpm in a Biofuge. Supernatant was collected, and protein concentration was determined with the Bio-Rad Assay using bovine serum albumin as standard.

The affinity column with the double strand oligonucleotide containing the GAL 4 binding site, 5′-GGATCGGAGGACAGTACTCCGCTAG-3′, was prepared as reported in Ref. 34 and equilibrated in buffer A plus 80 mM NaCl. The cell extracts were diluted with buffer A to decrease the NaCl concentration to 80 mM; 0.3 μg of poly(dI-dC) were added per μg of proteins and incubated for 15 min on ice. The affinity column (0.5-ml bed volume) was inoculated with the cell extract proteins and washed with 4 column volumes of buffer A plus 80 mM NaCl; the step elution was performed with 2 ml of buffer A plus 1 M NaCl. The flow-through, wash, and step elution fractions were concentrated on Centricon 30 and assayed in band-shift assays. The band-shift assays were performed as previously reported (16) using the double strand oligonucleotide with the Pax 8 binding site from the Tg promoter, oligo C (35). To obtain a protein fraction enriched for Pax 8 binding activity from the cell extract of HeLa cells over-expressing Pax 8, we loaded this cell extract on an affinity column with the double strand oligonucleotide C. This affinity column was processed as described above for the affinity column with the Gal 4 oligonucleotide. The fractions eluted from the oligo C affinity column were tested with band-shift assays with oligo C. Pax 8 binding activity was identified in the fraction of the elution step (data not shown).

RESULTS

Pax 8/p300 Synergism and Interaction—To study the Pax 8 requirements in transcriptional trans-activation, we used the strong activity shown by this factor on the TPO promoter in non-thyroid HeLa cells (15). As shown in Fig. 1, co-transfection of a plasmid containing the luciferase reporter gene under the control of the TPO gene promoter (TPO-Luc) with the Pax 8 encoding plasmid, results in strong activation of TPO promoter activity. Co-transfection of p300 and TPO-Luc results in very weak reporter gene expression, whereas the combined activity of p300 and Pax 8 results in cooperative activation of TPO gene promoter activity. We can demonstrate that this synergism requires Pax 8 to be bound on the promoter because the mutated TPO promoter, TPO-Pm (29), cannot be activated by Pax 8, and the co-expression of p300 and Pax 8 does not affect TPO-Pm promoter activity (data not shown).

To establish whether p300 and Pax 8 form a complex, we

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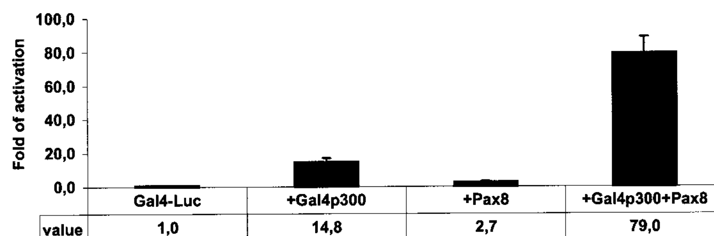
A



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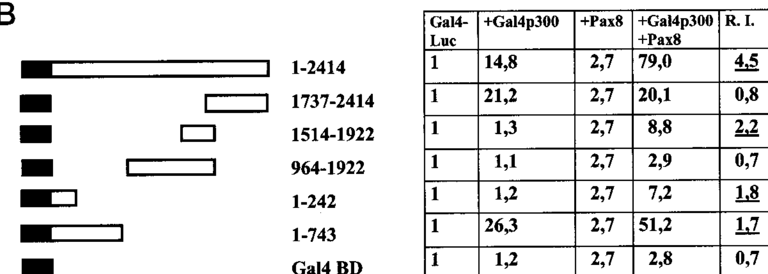

Gal4-Luc	+Gal4p300	+Pax8	+Gal4p300 +Pax8	R. I.
1	14,8	2,7	79,0	4,5
1	21,2	2,7	20,1	0,8
1	1,3	2,7	8,8	2,2
1	1,1	2,7	2,9	0,7
1	1,2	2,7	7,2	1,8
1	26,3	2,7	51,2	1,7
1	1,2	2,7	2,8	0,7

C

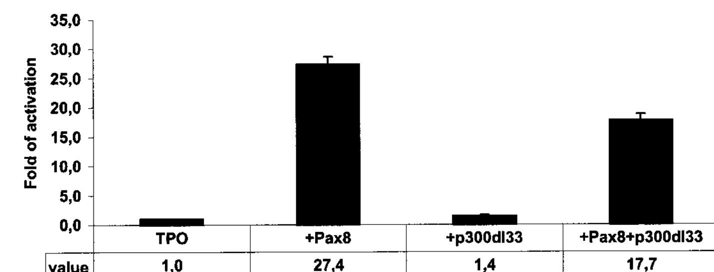

FIG. 2. **Pax 8/p300 interaction involves two domains of p300.** In *A*, the one-hybrid assay in HeLa cells is shown. In the GAL 4-Luc plasmid the promoter contains five GAL 4 binding sites in front of the E1B gene TATA box. The relative luciferase activity of the cells transfected with the GAL 4-Luc plasmid only was normalized to 1, and the other activities are expressed relative to this. In *B*, the GAL 4-p300 wild type and deletion mutants are schematically shown. They were used in the one-hybrid assay in HeLa cells to identify the p300 domain capable of interacting with Pax 8. The values of the one-hybrid experiments for each GAL 4-p300 deletion mutant are reported. Their standard deviations were negligible, and they have been omitted. The RI is reported, and values of RI > 1 are *underlined*. We have tested the ability of the plasmids encoding the GAL 4-p300 deletion mutants to express similar levels of the relative protein by performing bandshift assays with the protein cell extracts of transfected HeLa cells and with the oligonucleotide containing the Gal 4 binding site (data not shown). *C* shows that the p300 deletion mutant encoded by the p300dl33 plasmid and lacking the CH3 domain does not cooperate with Pax 8 to activate the TPO gene promoter in HeLa cells. This p300 mutant lacks the amino acids from 1737 to 1836 (24).

used the two-hybrid protocol in mammalian cells. Because Pax 8 is itself a transcription factor with its own trans-activation domain, our experiment is more similar to the one-hybrid experiment in HeLa cells (30). In Fig. 2*A* we show that the GAL 4-dependent promoter is activated by the GAL 4-p300 fusion protein, as has been reported by Yuan *et al.* (31). These authors have identified two trans-activation domains in the p300 molecule. We now show that although Pax 8 alone has no activity on the GAL 4-dependent promoter, GAL4-p300 is able to recruit Pax 8 on the promoter, resulting in a strong and synergic stimulation of the transcription of the reporter gene. This experiment therefore suggests that p300 and Pax 8 interact. To identify the domain of p300 involved in the interaction with Pax 8, we have used a panel of deletion mutants of GAL 4-p300 in a one-hybrid experiment in HeLa cells. To quantify the ability of the GAL 4-p300 deletion mutants to bind Pax 8, we introduce the Recruitment Index (RI) in Fig. 2*B*. RI is obtained by dividing the fold of activation obtained by the combined activity of Pax 8 and Gal4-p300 by the sum of the fold of activation obtained when Gal4-p300 and Pax 8 are over-expressed alone. An RI > 1 indicates that the Gal4-p300 wild type or deletion mutant is able to bind and recruit Pax 8 on the promoter. As shown in Fig. 2*B*, three GAL 4-p300 deletion mutants have an RI > 1. They identify two domains of p300 able to recruit Pax 8 on the promoter: a fragment of p300 including the amino acids 1514–1922, corresponding to the CH3 domain, and a fragment containing the amino acids 1–242 of p300. The ability of these two domains to recruit Pax 8 on the GAL 4-dependent promoter is much weaker than that of the full-length p300 protein, suggesting that the two domains in the intact protein could cooperate in Pax 8 binding or that a third domain of interaction, not identified with the p300 deletion mutants shown in Fig. 2*B*, exists. We show that the Gal4-p300 deletion mutant 964–1922, containing the CH3 domain, does not interact with Pax 8. We envisage the amino-terminal portion of this mutant as masking the CH3 domain or inducing a misfolding of it. However, the crucial role of the CH3 domain of p300 is also demonstrated by the experiment shown in Fig. 2*C*, in which the p300 deletion mutant, lacking the CH3 domain and encoded by the plasmid p300dl33, does not cooperate with Pax 8 in TPO promoter activation. Therefore, the CH3 domain of p300 is crucial for its cooperative activity with Pax 8.

To identify the Pax 8 domain involved in the interaction with p300, we performed the one-hybrid assay in HeLa cells using deletion mutants of Pax 8 fused in frame with the GAL 4 binding domain. Two Gal 4-Pax 8 deletion mutants, Gal 4-Pax 8 dl1 and Gal 4-Pax 8 dl2, were used as "bait" in the one-hybrid assay in HeLa cells. We show that GAL 4-Pax 8 dl2, containing amino acids 331–457 of Pax 8, is able to interact with p300 because it recruits the co-activator on the Gal 4-dependent promoter. Thus, the two trans-activation domains of p300 (31), recruited on the promoter by Gal 4-Pax 8 dl2, increase the promoter activity (Fig. 3*B*). Therefore, we demonstrate that the Pax 8/p300 interaction is mediated by the last 126 amino acids of the carboxyl-terminal end of Pax 8 and by two p300 domains, the first 242 amino acids at the amino terminus and the CH3 domain. To provide further evidence of Pax 8 and p300 interaction, we used a biochemical approach. Protein extracts pre-

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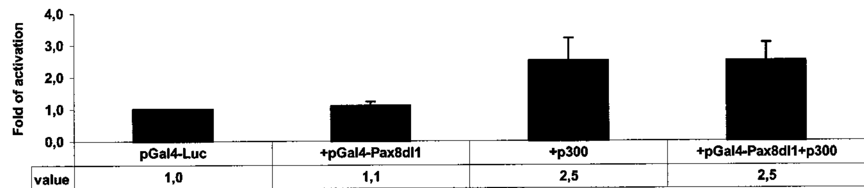

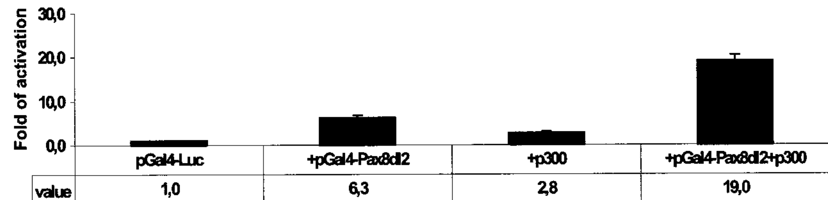

FIG. 3. **Pax 8/p300 interaction involves the carboxyl-terminal domain of Pax 8.** In the one-hybrid assay in HeLa cells, GAL 4-Pax 8 dl1 is not able to recruit p300 on the GAL4-dependent promoter (RI = 0.6) (*A*). *B* shows that, in the same assay, GAL 4-Pax 8 dl2 recruits p300 on the GAL 4-dependent promoter (RI = 2.0). The wild type and the deletion mutants of GAL 4-Pax 8 are schematically shown in *C*.

pared from HeLa cells transfected either with the plasmid encoding GAL 4-p300 or with the plasmid expressing Pax 8 were mixed with each other and used as the input of an affinity column with the double strand DNA oligonucleotide containing the GAL 4 binding site. We collected three fractions from the affinity column: the flow-through, the wash, and the elution step at 1 M NaCl. They were subsequently used in a band-shift assay to identify the fraction containing Pax 8. As shown in Fig. 4, *lane 6*, Pax 8 was identified in the elution step at high salt concentration. As a marker of the Pax 8/oligo C complex, we show in Fig. 4, *lane 7* the band-shift assay of Pax 8 purified from the cell extract of HeLa cells over-expressing Pax 8. The arrow shown in Fig. 4 indicates the Pax 8 complex with oligo C, and the same complex is present in the band-shift assay with the elution step fraction shown in *lane 6*. Therefore, in the presence of GAL 4-p300, Pax 8 binds the GAL 4 affinity column. In a control experiment where the same affinity column with the Gal 4 oligonucleotide had been loaded with the cell extract prepared from HeLa cells over-expressing Pax 8 (hence in the absence of GAL 4-p300), the binding activity of Pax 8 was identified in the flow-through fraction (data not shown). Thus, this experiment shows that, although Pax 8 does not bind the GAL 4 affinity column, in the presence of GAL 4-p300 it complexes on the column. This suggests, once more, the formation of a complex between Pax 8 and p300.

Pax 8 and E1A—Because it has been shown that E1A activity blocks the thyrocyte differentiation (9) and that E1A binds the p300 CH3 domain (24), we envisaged E1A and Pax 8 as

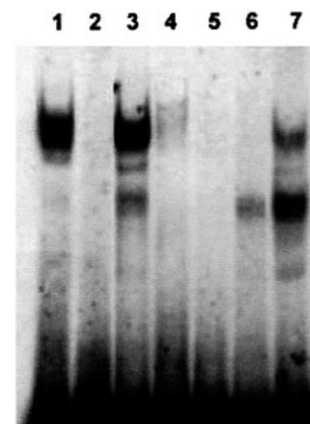

FIG. 4. **In vitro interaction of Pax 8 and p300.** Band-shift assay of the input protein cell extracts and fractions of the GAL 4 affinity column. The probe used in this assay was the double strand DNA oligonucleotide C containing the Pax 8 binding site (34). *Lane 1* is with proteins extracted from HeLa cells over-expressing GAL 4-p300. *Lane 2* is the control probe. *Lane 3* represents the band-shift assay in the presence of the proteins extracted from HeLa cells over-expressing Pax 8. We have found that the same strong band, shown in *lanes 1* and *3*, is also present in a band-shift assay performed with oligo C and a cell extract of HeLa cells (data not shown); therefore we consider this complex as unspecific and not related to Pax 8. *Lanes 4*, *5*, and *6* are the flow-through, the wash, and the elution step, respectively, of the GAL 4 affinity column. *Lane 7* shows the band-shift assay with the Pax 8 purified fraction (see "Experimental Procedures"). Thus, the enriched band of *lane 7*, indicated by the *arrow*, is the Pax 8/oligo C complex.

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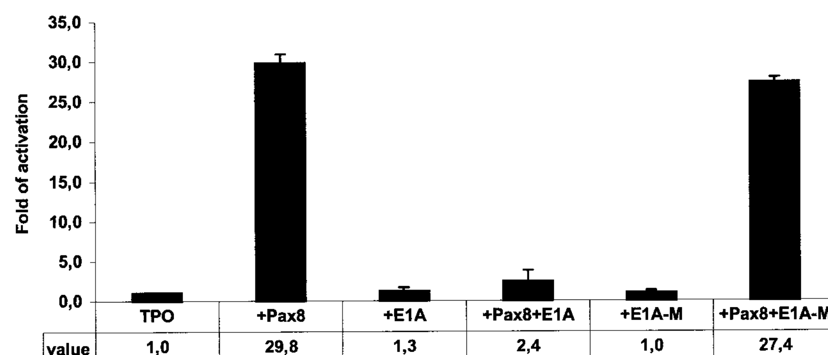

FIG. 5. **E1A inhibits Pax 8 activity.** The relative luciferase activity of the cells transfected with TPO-pGL3 only was normalized to 1, and the other activities are expressed relative to this. The reporter plasmid was co-transfected with the plasmid encoding the indicated proteins as described under "Experimental Procedures." E1A-M is the deletion mutant of E1A lacking the p300 binding domain, amino acids 2–36. Values of fold activation and standard deviations are reported.

competing for the binding of p300. To test this hypothesis we have over-expressed E1A and Pax 8 in the same assay. As shown in Fig. 5, E1A inhibits Pax 8 trans-activation activity, whereas the E1A deletion mutant (E1A-M), lacking the p300 interaction domain, does not affect Pax 8 activity. In this experiment we have used the plasmid encoding for E1A 12S; however, we have obtained a similar down-regulation of Pax 8 activity transfecting the E1A 13S-encoding plasmid (data not shown).

DISCUSSION

In this study we show that the paired domain-containing transcription factor Pax 8 can associate with the transcriptional co-activator p300 both *in vivo* and *in vitro*. This interaction involves the trans-activation domain of Pax 8 and both the amino-terminal domain and the CH3 domain of p300. We demonstrate that Pax 8/p300 interaction results in a synergic activation of TPO gene promoter activity. Therefore we suggest that p300 plays a crucial role in thyroid-specific gene expression and differentiation. In agreement with this hypothesis are results indicating that E1A is able to abrogate thyroid follicular cell differentiation with down-regulation of thyroid-specific gene expression. We now provide evidence of the mechanism of this repression. We show that E1A can repress the activation function of the most important transcription factor for TPO promoter activity. We suggest that repression by E1A results from the sequestration of a protein, p300, required for Pax 8-mediated activation. A similar mechanism has been proposed for the inhibition by E1A of the activity of several transcription factors, for example c-FOS (36) and STAT6 (37), in which p300/CBP is sequestered from the transcription factor by the adenoviral protein.

The results presented in this study indicate in the Pax8/p300 interaction a new critical event in TPO gene promoter activation. Although p300 is a pleiotropic protein, it shows two most relevant roles: the ability to acetylate, directly or indirectly, specific proteins and the ability to function as a bridging factor. More experiments are needed to clarify the molecular role of p300 on the TPO gene promoter; however, it is worth discussing an interesting correlation here. It has recently been reported that Pax 5, which belongs to the same subfamily of Pax genes as Pax 8, interacts directly with TBP (38). This interaction involves the rudiment of the homeodomain of Pax 5. As stated above, the same partial homeodomain is present also in the other elements of the Pax subfamily, Pax 2 and Pax 8 (12). Therefore, we could envisage Pax 8 interacting with TBP. Because p300 binds TBP (31), the formation of a complex with Pax 8/p300/TBP is likely. These multiple interactions would result in a stronger stabilization of the complex on the TPO gene promoter and therefore in its activation.

Although p300/CBP adaptor proteins are widely expressed, their concentration appears to be limiting (23), thus enabling them to integrate signals in the nucleus from different extracellular stimuli. It has been shown that TPO gene expression is regulated by Pax 8 (15) and by hormonal stimuli (39). TPO mRNA is very sensitive to thyrotropin regulation (2, 3). The induction of TPO gene transcription is rapid, cyclic AMP-dependent, and does not require protein synthesis (39). In contrast to the vast majority of cases, this induction is not mediated by CREB, because the TPO gene promoter lacks the cAMP-response element (3, 29). In this respect, our results suggest an interesting correlation. Because it has been shown that p300/CBP mediates cyclic AMP response (40) and because we now show that p300 cooperates with Pax 8 to activate the TPO gene promoter, we envisage thyrotropin regulation of TPO gene expression being mediated by p300. Consistent with this hypothesis, it has been shown that Pax 8 gene expression is increased by cyclic AMP (41); the higher concentration would favor Pax 8 in p300 recruitment and result in TPO gene expression activation. Experiments are in progress to address this question.

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