PAX 8 activates the enhancer of the human thyroperoxidase gene

Claudio ESPOSITO*, Stefania MICCADEI†, Adolfo SAIARDI‡¹ and Donato CIVITAREALE*§²

*Istituto di Medicina Sperimentale, CNR, Viale C. Marx 15, 00137 Roma, Italy, †Istituto Regina Elena, Laboratory of Cell Metabolism and Pharmacokinetics, Via della Messi d'Oro 156, 00158 Rome, Italy, ‡Istituto di Ricerche Farmacologiche Mario Negri, Consorzio Mario Negri Sud, Laboratory of Cellular and Molecular Endocrinology, Group of Gene Expression, Via Nazionale, 66030 Santa Maria Imbaro, Italy, and §Istituto Regina Elena, Laboratory of Molecular Pathology and Ultrastructure, Via delle Messi d'Oro 156, 00158 Rome, Italy

In this study we report on a novel natural target of the paired domain transcription factor PAX 8 in the enhancer element of the human thyroperoxidase gene, one of the most important thyroid differentiation markers. It is the primary enzyme involved in thyroid hormone synthesis and PAX 8 has been previously identified as an activating factor of the rat thyroperoxidase gene promoter. *In vitro*, PAX 8 binds a *cis* element of the human

enhancer and its exogenous expression induces the enhancer activity in co-transfection experiments in Cos-7 cells. When mutated at this binding site, the enhancer is no longer activated by PAX 8. Our finding strengthens the PAX 8 role in the maintenance of thyroid differentiation and in particular in the tissue-specific thyroperoxidase gene expression.

INTRODUCTION

Thyroperoxidase (TPO) (EC 1.11.1.7) is the primary enzyme involved in thyroid hormone synthesis. It catalyses iodide oxidation, iodination of tyrosine residues, and coupling of iodo-tyrosines to generate the iodothyronines T3 and T4 [1]. It is a thyroid-specific 100 kDa glycoprotein bound to the thyrocyte membrane [1]. The tissue-specific expression of TPO gene is regulated mainly at the transcriptional level [2,3] and its promoter has been extensively studied in both rat and human gene [2–5]. Tissue-specific expression of TPO gene promoter is induced by binding to *cis*-acting DNA elements of at least three transcription factors: Thyroid Transcription Factor 1 (TTF-1), Thyroid Transcription Factor 2 (TTF-2) and PAX 8 [2,6]. The same set of factors control the thyroid-specific expression of the thyroglobulin gene promoter [7]. In both promoters the PAX 8 binding site overlaps with the binding site of TTF-1 and their binding is mutually exclusive [6]. In co-transfection experiments in non-thyroid cells PAX 8 strongly activates the TPO promoter, while on this promoter TTF-1 shows a weaker activation [2,6].

Furthermore, human TPO (hTPO) gene transcription is also regulated by an enhancer element located approx. 5.5 kilobases upstream of the gene. This element is contained within a 230 base pair fragment and has three regions protected, in DNase 1 footprinting experiments, by thyroid-specific factor/s [8]. Mizuno et al. [9] have shown that TTF-1 binds the E2 sequence and activates the hTPO enhancer element.

In this paper we report on the role of PAX 8 in the activation of the human thyroid-specific enhancer of the TPO gene. We show that PAX 8 binds an enhancer *cis* element *in vitro* and through this site stimulates the enhancer activity in co-transfection experiments in COS-7 cells.

EXPERIMENTAL

Isolation of genomic sequences and plasmid constructions

The human TPO enhancer was amplified from HeLa cells genomic DNA by polymerase chain reaction (PCR) using the

oligos: A 5'-GGGATTCCATCTTTTATTCTAATGC-3' and B 5'-CTCGAGTGGCCATCCATGATTGGC-3'. The 230 base pair amplified DNA fragment was cloned by blunt-end ligation in pBluescript KS cut with *EcoRV* and checked by sequencing. From this new construct, pBLU-EN1, an *EcoRI*–*HindIII* fragment was purified and cloned again in pBLU-EN1 digested with *HindIII*. After the ligation reaction the *EcoRI* sites were blunted with Klenow enzyme and the plasmid blunt ends were ligated. We obtained two enhancer elements with the same orientation cloned in the pBluescript KS polylinker, pBLU-EN2. The *SalI*–*BamHI* restriction fragment from pBLU-EN2 was cloned in pGL3 Promoter (Promega, Madison, WI, U.S.A.), digested with the same enzymes, obtaining the plasmid p2XEn-WT. Thus, the luciferase gene transcription in p2XEn-WT is controlled by the SV40 promoter and by the two copies of the human TPO enhancer. The mutated enhancer element was obtained following the procedure described by Ho et al. [10]. The PCR steps were performed using pBLU-EN1 as template and oligo A with oligo AM 5'-CAGCGTATATAGGCCTTGGGA-AGAATGGGTCCTTCC-3' and oligo B with oligo BM 5'-CCCAAGGCCTATATACGCTGAAAGGGACTTGAGCAC-3', respectively. The mutated enhancer element was cloned in the *EcoRV* site of pBluescript KS. We obtained the plasmid p2XEn-Mut, with two copies of the mutated enhancer in the *SalI* / *BamHI* sites of pGL3 Promoter, following the same procedure described for p2XEn-WT. Therefore we have mutated the wild type sequence 5'-AGCTGCTCTATGAAGTGTGA-3' in 5'-AGC-GTATATAGGCCTTGGGA-3'.

PAX 8 expression and DNA-binding assays

The Pax 8 gene, plasmid C27B [11], was kindly provided by Prof. P. Gruss, Max Planck Institute, Gottingen, Germany. The Pax 8 gene was cloned in the prokaryotic expression plasmid Quex-9 (Qiagen, Chatsworth, CA, U.S.A.) and the PAX 8 protein was purified through a Ni²⁺-NTA-Agarose column following the Qiaexpressionist protocol as suggested by the manufacturer

Abbreviations used: TPO, thyroperoxidase; TSH, thyroid-stimulating hormone; TTF-1, thyroid transcription factor 1; TTF-2, thyroid transcription factor 2.

¹ Present address: Institut de Génétique et de Biologie Moléculaire et Cellulaire (I.G.B.M.C.), BP 163, 1 rue Fries, 67404 ILLKIRCH Cedex, C.U. de Strasbourg, France.

² To whom correspondence should be addressed at § (e-mail dciv@biocell.irmkant.rm.cnr.it).

(Qiagen, Chatsworth, CA, U.S.A.). The protein was further purified on a sequence-specific affinity column, prepared with the oligonucleotide C. Oligonucleotide C is a double stranded oligonucleotide with the PAX 8 binding site sequence found in thyroglobulin promoter [7]. In bind-shift experiments, purified PAX 8 was able to bind the C oligonucleotide and did not bind a second oligonucleotide with an unrelated sequence (data not shown).

Binding reaction and DNase 1 footprint were performed as previously reported [12]. The digested products were phenol extracted, ethanol precipitated and separated on a 6% sequencing gel. Maxam and Gilbert A + G sequencing reaction of the DNA probe were used to locate the footprinted region.

FRT nuclear extract was prepared according to Shapiro et al. [13]. Protein concentration was determined using the Bio-Rad (Richmond, CA, U.S.A.) Protein Assay using BSA as standard. The binding reaction for the band-shift assay with FRT nuclear extract was assembled on ice in buffer D (20 mM Tris/HCl, pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 phenylmethylsulphonyl fluoride, 10% glycerol) with 80 mM KCl, 5% Ficoll, 2 μ g poly (dI.dC) and 5 μ g nuclear protein. The reaction was started by adding 100 pg of labelled oligonucleotide. The binding reaction for the band-shift assay with the recombinant PAX 8 was performed in the same buffer but with 0.1 μ g of poly (dI.dC) and 2 μ g BSA. In competition experiments 50 ng of DNA competitor were added to the binding reaction prior to the addition of the labelled substrate. After 20 min at room temperature, samples were loaded onto a 6% acrylamide gel in 0.5 $\times$ Tris/Borate/EDTA buffer and electrophoresed at 200 V for 2.5 h at 4 $^{\circ}$ C. The double stranded oligonucleotides used were: E2-WT 5'-CAGCTGCTCTATGAAGTGTGAAGAA-3' derived from the hTPO enhancer sequence and containing the E2 site, E2-Mut 5'-CAGCGTATATAGGCCTTGGGAAGAA-3' the mutated sequence of the E2-WT as above described, C 5'-CACTGCCAGTCAAGTGGTTCTTGA-3' the TTF-1 and PAX 8 binding sequence derived from the rat thyroglobulin promoter and the double stranded oligonucleotide K 5'-TGAC-TAGCAGAGAAAACAAAGTGA + 3' [7] was used as control in competition experiments.

Transfection experiments

The COS-7 cells were transfected by the calcium phosphate-mediated protocol. The transfection experiments were performed in the six wells microtitre using 2 μ g of p2XEn-WT or p2XEn-Mut and 1 μ g of pCMV-Pax8 [6]. Dose-response curves for PAX 8 vector were performed to establish the optimal concentration: 0.02, 0.5, 1 and 1.5 μ g of this vector increased the luciferase activity, in the COS-7 extract co-transfected with p2XEn-WT, of about 1.8, 2.3, 3 and 2.8-fold, respectively. The efficiency of transfection was assayed with 1 μ g of pCMV- β Galactosidase. The luciferase and β Galactosidase activities were determined as previously described [14].

Materials

The materials used in tissue culture media were obtained from Gibco-BRL, chemicals were purchased from Sigma (St. Louis, MO, U.S.A.), [γ - 32 P]ATP and [α - 32 P]ATP was purchased from Amersham Italia SRL. Enzymes were purchased from BioLabs (Boston, MA, U.S.A.) and from Boehringer Mannheim (Germany). Autoradiography was performed using KODAK X-R5 films.

Figure 1 Footprint analysis of human TPO enhancer with PAX 8

The *Eco*RI/*Hind*III fragment from pBLU-EN1, labelled in *Eco*RI with Klenow enzyme and [α - 32 P]ATP, was used as substrate. Lane 1 is G + A sequence reaction. Lane 2 is DNase 1 digestion in absence of proteins. The DNase 1 protection in presence of PAX 8 is shown in lane 3. The box schematically indicates the protected region and its boundaries.

Table 1 Effect of PAX 8 on the hTPO enhancer

Assay of luciferase activity in COS-7 cells transfected with either the wild type hTPO enhancer or the mutated hTPO enhancer vector as indicated. The co-transfection of the PAX 8 expression vector is indicated as well. The luciferase values are reported as % of pGL3 Promoter vector activity. The results are presented as means $\pm$ S.E.M. for three separate experiments, each experiment was performed in duplicate.

Vector	— PAX 8	+ PAX 8
pGL3 Promoter	100	100 $\pm$ 6
p2XEn-WT	106 $\pm$ 5	315 $\pm$ 10
p2X En-Mut	120 $\pm$ 10	110 $\pm$ 6

RESULTS

A DNase 1 protection experiment with PAX 8, overexpressed in and purified to homogeneity from *E. coli*, and the 230 bp DNA fragment containing the human TPO enhancer is shown in Figure 1. A PAX 8 footprint is clearly detected between -121 and -152 base pairs. In order to demonstrate that PAX 8 binding plays a role in the human TPO enhancer activity, we have performed an *in vivo* study by means of transfection experiments in COS-7 cells. The results are shown in Table 1. The luciferase activity expressed by the SV40 promoter in the pGL3 promoter vector, transfected in Cos-7 cells, has been considered

Competitor	-	-	C	E2 WT	E2 Mut	K
FRT Extract	-	+	+	+	+	+
Probe	C	C	C	C	C	C

Figure 2 Band-shift competition experiment with FRT nuclear extract

The oligonucleotide C, labelled with [γ - 32 P]ATP and polynucleotide kinase, was incubated with FRT nuclear extract in presence or in absence of different competitors as indicated.

Figure 3 Band-shift competition experiment with recombinant PAX 8

The oligonucleotide E2-WT, labelled with [γ - 32 P]ATP and polynucleotide kinase, was incubated with PAX 8 expressed and purified from *E. coli* in presence or in absence of different competitors as indicated. The different complexes seen in this experiment correspond to PAX 8 degradation form or to premature termination during PAX 8 translation; we obtained the same pattern, in band-shift experiments, with recombinant PAX 8 and the C oligonucleotide, data not shown.

as 100% and PAX 8 does not affect its activity. On the contrary, PAX 8 activates the reporter gene expression in presence of the hTPO enhancer in the p2XEn-WT vector. Hence our results

A

5'-CACTGCCCACTCAAGTGTCTTGAA-3' rat Tg promoter
 5'-AGCTGCTCTATGAAGTGTGAAGAAT-3' hTPO enhancer
 5'-TGATGCCCACTCAAGCTTAGACAGG-3' rat TPO promoter

B

5'-AGCTGCTCTATGAAGTGTGAAGAAT3' hTPO enhancer
 5'-G-GCA-TGAAGCGTGAC-3' PAX 2/5/8

Figure 4 DNA sequences recognized by PAX 8

(A) Alignment of PAX 8 binding sites on rat Thyroglobulin and TPO promoters with the human TPO enhancer binding site presented in this study. The consensus sequence is underlined. (B) Comparison of the consensus recognition sequence for the paired domain of the PAX 2/5/8 shown by Czerny et al. [15,16] with the PAX 8 binding site in the hTPO enhancer.

indicate that PAX 8 can bind to human TPO enhancer and stimulates its activity.

In order to show that the binding of PAX 8 to the E2 *cis* element of the human TPO enhancer is important for enhancer activity we have mutated this sequence. The mutated enhancer is not able to bind PAX 8 *in vitro* (Figure 2 and Figure 3). We took advantage of the fact that the FRT cell line, derived from rat thyroid, does not maintain, in culture, the expression of the thyroid differentiation markers with the exception of PAX 8. Hence the FRT cell nuclear extract is a valuable source of PAX 8 to study its DNA binding features without the interference of TTF-1. Thus, the band-shift reported in Figure 2 (lane 2) shows the binding of PAX 8 on the oligonucleotide C. This complex is completed by the addition of unlabelled oligonucleotide C, Figure 2 lane 3, and by the addition of unlabelled oligonucleotide E2-WT, Figure 2 lane 4, but not by the unrelated oligonucleotide K, Figure 2 lane 6. These experiments indicate that PAX 8 binds specifically the sequences of both oligonucleotides C and E2-WT. Oligonucleotide E2-Mut fails to compete for the PAX 8 complex, Figure 2 lane 5. Hence, this experiment strongly indicates that the introduced mutations abolish PAX 8 binding. We have obtained the same result shown in Figure 3 using the recombinant PAX 8 expressed and purified from bacteria (Figure 4). PAX 8 binds the labelled E2-WT oligonucleotide, lane 2, and this complex is completed by the same unlabelled oligonucleotide, lane 3, but not by the unlabelled E2-Mut oligonucleotide. Taken together the DNA binding assays, footprint and gel retardation, demonstrate that PAX 8 binds *in vitro* the hTPO enhancer in the E2 regions. We show that PAX 8 binding on this *cis* element is crucial *in vivo*, the TPO enhancer containing the mutated sequence is not activated by the exogenous expression of PAX 8 in co-transfection experiments in COS-7 cells (Table 1).

To further support our results we show sequence homology amongst PAX 8 binding sites in rat thyroglobulin and TPO promoters [6] and the E2 site in the hTPO enhancer as shown by the sequence underlined in Figure 4A. The consensus recognition sequence of PAX 2 and PAX 5 (also termed BSAP) are reported as well [15,16] (Figure 4B). It is worth pointing out that PAX 2, 5 and 8 form a subfamily of the PAX genes sharing much conserved structure and function [17,18].

DISCUSSION

In the present study we provide evidence that PAX 8 binds the E2 site of the human TPO enhancer element by both footprinting

and band-shift assays *in vitro*. This factor is able to activate transcription from the co-transfected wild type TPO enhancer in COS-7. Furthermore, the mutated E2 site does not bind PAX 8 and the mutated enhancer is not activated by this transcription factor. Hence we show a correlation between binding and enhancer activity of PAX 8 on the hTPO enhancer. We conclude, therefore, that the E2 site is a new specific binding site for PAX 8 and that all its activity on the hTPO enhancer is mediated by the E2 *cis* element. This is the first report describing PAX 8 as an enhancer binding protein and strengthens the role of this factor in the maintenance of thyroid differentiation.

Mizuno et al. [9] have reported that TTF-1 binds the E2 site in the human TPO enhancer from -123 to -141 base pairs. Therefore, the PAX 8 and TTF-1 binding sites are largely overlapping. On the rat TPO and rat thyroglobulin promoters, the binding sites of TTF-1 and PAX 8 also overlap. By analogy it is very likely that the binding to the human TPO enhancer is also mutually exclusive. Although the interplay between these two factors is not clear, it is interesting to speculate that the regulation of the ratio and/or the activities of TTF-1 and PAX 8 could be crucial in the fine tuning of thyroid-specific gene expression. Thyroid-stimulating hormone (TSH) increases both TPO and thyroglobulin gene expression, mainly via cAMP [19]. In contrast with the regulation of the thyroglobulin gene, TSH activation of TPO gene transcription is rapid and does not require on-going protein synthesis [20]. It has been shown that TTF-2 plays an important role in this hormonal control [21] but it is also shown a TSH-dependent modulation of TTF-1 and PAX 8 activities. Recently it has been shown that, in FRTL5 cells, TTF-1 is down-regulated by TSH [22]. Conversely, PAX 8 expression is increased by cAMP in primary cultured dog thyrocyte [23]. Thus, although no typical cAMP Responsive Element (CRE) has been found in the regulatory sequences of TPO gene, its expression can be stimulated by TSH through the modulation of the most important transcription factor involved in both the TPO promoter [6] and, as reported in this study, TPO enhancer activities.

Hence, although the human TPO enhancer has not been extensively characterized yet, it is interesting to note that its thyroid-specific activity relies on the same factors identified and required for the activity of the thyroid-specific promoters.

We thank M. Price and H. Francis-Lang for critical reading of the manuscript. This study was supported in part by Telethon, grant No E.254 to D.C., and by Italian Association for Cancer Research (AIRC, Milan, Italy) and the Italian National Research Council (Progetto Finalizzati ACRO, contract No 93.02354.39) and Convenzione CNR-Consortio Mario Negri Sud and by Italian Ministry of Health (Progetto finalizzato 'Basi genetiche delle malattie tiroidee').

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