Synergistic transcriptional activation of the thyrotropin receptor promoter by cyclic AMP-responsive-element-binding protein and thyroid transcription factor 1

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In this study we have investigated the molecular mechanisms involved in hormonal induction of thyroid-specific transcription of the thyrotropin receptor (TSHr). A cyclic AMP-responsive element (CRE) has been characterized in the minimal TSHr promoter, and promoter activity shown to be also induced by thyroid transcription factor 1 (TTF-1). We here describe a co-operative effect between TTF-1 and CRE-binding protein on the

TSHr promoter. Moreover we have identified a second TTF-1-binding site in the minimal promoter, which does not activate TSHr promoter activity but is required for the co-operative activation of the promoter. This report describes a new aspect of thyroid-specific gene expression, namely, how a generic extra-cellular signal can be interpreted in a thyroid-specific way.

INTRODUCTION

Extracellular stimuli influence cell growth and differentiation. In differentiated organisms each cell type can respond with a specific and unrelated programme to an identical signal. Therefore the developmental history or identity of the target cell determines its specific response to a generic signal. Hence a central goal is to understand the molecular mechanisms involved in signal-transduction patterns integrated into the regulation of cell-specific gene expression. Signal transduction and differentiation are often both controlled at the transcriptional level by the induction and/or regulation of the activity of transcription factors [1–3]. Hence these proteins represent common ground for integration of the two pathways. Interesting in this respect are the reports on the co-operative activity between tissue-specific and ubiquitous factors. The co-operation of homeodomain (HD) proteins, which in many cases define cell identity, with proteins of the serum response factor family provides an example of how cell identity can be co-ordinated with response to external signals [4]. Thus synergism in transcription, i.e. the combined response of two transcription factors is greater than the sum of the responses from each of the elements, is a molecular mechanism that can integrate different pathways.

One of the best-studied pathways of signal transduction is the activation of protein kinase A (PKA) by the second messenger cyclic AMP (cAMP) [5,6]. Activated PKA modulates the activity of transcription factors that bind the cAMP-responsive element (CRE) found in the regulatory regions of the target genes. The best-known target of this enzyme is the transcription factor termed CREB (CRE-binding protein) [1,3]. CREB actually belongs to a large multigene family of transcription factors, including the ATF family and AP1 [6]. Although it is a ubiquitous factor it is also involved in tissue-specific gene expression [7]. It induces the expression of GHF-1/Pit-1, a pituitary-specific transcription factor [8], which activates growth hormone gene transcription. In other cases, it enhances the activity of tissue-

specific transcription factors. Somatostatin gene expression is specific to pancreatic islet cells and its transcription is mediated by the co-operative activity of CREB and the pancreatic-specific factor Isl-1 [9]. Similarly in liver, activation of the tyrosine aminotransferase gene is dependent on synergy between the liver-specific factors, HNF3 and HNF4, and CREB [7,10].

The thyroid cell provides a good experimental system for studying both the cAMP signal-transduction pathway and differentiation. The growth and differentiation of the thyroid gland depends on the cAMP cascade activated by thyrotropin (TSH) bound to its specific receptor (TSHr) on the plasma membrane of the thyrocyte [11].

The gene expression of the thyroid-differentiation markers, thyroglobulin (Tg) and thyroperoxidase (TPO), has been studied in FRTL5 cells [12]. They are regulated at the transcriptional level and both gene promoters have been studied [13,14]. Analysis of the *trans*-acting factors involved in thyroid-specific gene expression has revealed the role of a set of transcription factors, including thyroid transcription factor 1 (TTF-1) [15], thyroid transcription factor 2 (TTF-2) [16] and Pax 8 [17]. All three have a very restricted tissue distribution but their simultaneous presence is a specific feature of the thyroid cell [12].

Recently the TSHr promoter, a third thyroid-specific promoter, has been identified in the region between –199 and –39 bp upstream of the translation initiation site [18]. The minimal promoter lacks a TATA box and, like other TATA-less housekeeping gene promoters, has a high G+C content. However, in transfection assays, it is preferentially expressed in thyroid cells [18]. A *cis* DNA element similar to CRE has been identified in the minimal promoter, between –139 and –131 bp, and it has been shown that this element works as a constitutive enhancer of TSHr promoter activity [19]. We have shown previously that TTF-1 binds to the minimal TSHr promoter, from –187 to –177 bp, and is able to transactivate it in a co-transfection assay in non-thyroid cells, COS-7 [20].

As stated above, the binding of TSH to its specific receptor is

Abbreviations used: TSH, thyrotropin; TSHr, thyrotropin receptor; cAMP, cyclic AMP; CRE, cAMP-responsive element; CREB, CRE-binding protein; TTF-1, thyroid transcription factor 1; TTF-2, thyroid transcription factor 2; Tg, thyroglobulin; TPO, thyroperoxidase; HD, homeodomain; Mut-1, TSHr promoter mutated in the A region; Mut-2, TSHr promoter mutated in the B region; KCREB, a dominant negative CREB mutant.

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Figure 1 Schematic description of the TSHr promoter in DNase 1-protection experiments

The TSHr minimal promoter has been drawn as a continuous line. The dashed line represents the DNA sequence outside of the minimal promoter. The arrow indicates the start of translation. The open boxes show the protected sequences by the indicated proteins. The black boxes indicate the mutated regions. wt, Wild-type; TR/RXR, thyroid hormone receptor α 1/retinoic-X-receptor complex. The references are reported on the left; * indicates the present paper.

a critical event in the physiology of the thyrocyte. In FRTL5 cells, the induction of TSH-dependent cAMP levels stimulates expression of the Tg and TPO genes, whereas the transcription of the TSHr gene is down-regulated by this event [21]. We have previously shown that this inhibition is mediated by thyroid hormone and that the thyroid hormone receptor α 1 binds to the TSHr promoter in the region between -146 and -118 bp [22].

In the present paper we show that, in addition to the previously described TTF-1 binding site (site A) in the TSHr promoter, there is a second TTF-1-binding site (site B) lying between -134 and -116 bp. Both the TSHr promoter mutated at the B site and the wild-type promoter are activated by exogenous TTF-1 expression in COS-7 cells in a similar way. This result indicates that TTF-1 does not activate the TSHr promoter through the B site. Moreover we have found that there is a synergy between TTF-1 and CREB in the activation of the TSHr promoter. However, in order to co-operate functionally with CREB, both TTF-1-binding sites are required.

EXPERIMENTAL

Promoter mutagenesis

The mutated TSHr promoters, Mut-1 and Mut-2 have been described previously [20,22]. Briefly the wild-type sequence 5'-TCAAGT-3' (-184 to -179) was changed to 5'-GACCTG-3' in the Mut-1 promoter, and the wild-type sequence 5'-CTTG-3' (-125 to -122) was changed to 5'-AGGT-3' in the Mut-2 promoter. The plasmids containing the mutated TSHr promoters have been termed pGE-T200M1 and pGE-T200M2 respectively. They were constructed by the method of Ho et al. [23] using the wild-type TSHr minimal promoter plasmid, pGE-T200 [20], as template.

DNase 1-protection assay

Nuclear extracts from FRTL5 cells were prepared as described by Shapiro et al. [24]. Protein concentration was determined using the Bio-Rad protein assay using BSA as standard. DNase 1 footprinting assays were performed in buffer D (20 mM Hepes, pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 mM PMSF, 10% glycerol) with 100 mM KCl by the method of Lichtsteiner et al. [25]. The *SacI*-*HindIII* fragments of the plasmids pGE-T200, pGE-T200M1 and pGE-T200M2 were cloned in the plasmid pGEM3Zf(+) to obtain pG3-200, pG3-M1 and pG3-M2 respectively. TTF-1 HD was kindly provided by R. Di Lauro (Stazione Zoologica 'A. Dohrn', Naples, Italy).

Transfection assay

Transfection and co-transfection experiments in COS-7 cells were performed by the calcium phosphate-mediated protocol using 5 μ g of the indicated TSHr promoter plasmid. For the co-transfection assays, dose-response curves were produced to determine the optimal amounts of pCMV-TTF-1 [20] and pSV-CREB (kindly provided by Dr. P. Sassone-Corsi, Strasbourg, France [26]) to activate the TSHr promoter (results not shown); these were 0.5 and 1 μ g respectively for the two expression plasmids. The efficiency of transfection was assayed with 2 μ g of pCMV- β GAL [20]. Transfection, luciferase and galactosidase assays were performed as reported previously [20].

Materials

Materials used in the tissue culture media were obtained as reported by Civitareale et al. [20]. [α - 32 P]ATP and [γ - 32 P]ATP were purchased from Amersham Italia SRL. Enzymes were

purchased from Promega (Madison, WI, U.S.A.). Autoradiography was performed using Kodak X-R5 films.

RESULTS

Figure 1 shows schematically some of the *in vitro* binding studies of the TSHr promoter presented in this paper and in the literature.

Figure 2 DNase 1-protection analysis of the wild-type TSHr promoter

A pG3-200 *EcoRI*–*HindIII* fragment labelled at *HindIII* was used as substrate. DNA was labelled with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and T4 polynucleotide kinase in lanes 1, 2 and 3 and with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and Klenow enzyme in lanes 4, 5 and 6. Lanes 1 and 4 are G + A sequence reactions. Lanes 2 and 5 are DNase 1 digestions in the absence of proteins. The DNase 1-protections in the presence of FRTL5 nuclear extract are shown in lane 3. In lane 6 are shown the DNase 1 protections in the presence of TTF-1 HD. The regions of the DNase 1 protections are boxed and the numbers refer to their boundaries. As described in the text, the TTF-1 HD footprints on the TSHr promoter have been termed A, B and C as indicated.

Figure 3 TTF-1 HD titration on the TSHr promoter

The same DNA as in Figure 2 was used as substrate. This fragment was labelled at the *EcoRI* site with $[\alpha\text{-}^{32}\text{P}]\text{ATP}$ and Klenow enzyme and then digested with DNase 1 in the absence of proteins (lanes 2 and 3) or in the presence of increasing amounts of TTF-1 HD (lanes 4–8). A G + A sequencing reaction of the same fragment was used as size markers (lane 1). The boxes indicate the protected regions.

TTF-1 binds twice on the TSHr minimal promoter

In Figure 2 footprinting experiments are shown on the TSHr promoter using both crude FRTL5 nuclear extracts and purified TTF-1 HD overexpressed in *Escherichia coli*. This peptide reproduces the DNA-binding characteristics of the entire protein [15]. TTF-1 HD binds at three TSHr promoter regions which we have termed A, –188/–175 bp, B, –134/–116 bp and C, –33/–11 bp (Figure 2, lanes 5 and 6). As the C binding site is located outside the minimal promoter sequence [18], we did not consider it further in the present study. The A and C protections

Figure 4 Footprint analysis of the wild-type and Mut-1 TSHr promoter with TTF-1 HD

Lanes 1–3, wild-type TSHr promoter; lanes 4–6, Mut-1 promoter. The two *EcoRI*–*HindIII* fragments, wild-type and Mut-1, are derived from the plasmids pG3-200 and pG3-M1 respectively and both have been labelled with [α - 32 P]ATP and Klenow enzyme at the *EcoRI* site. Lanes 1 and 4, G + A reactions; lanes 2 and 5, DNase 1 control digestions; lanes 3 and 6, DNase 1 digestions in the presence of TTF-1 HD. The boxes indicate the protected regions.

have been identified also in the experiment with the FRTL5 nuclear extract (Figure 2, lanes 2 and 3) whereas the B protection is included within a larger protected area. Ikuyama et al. [19] have reported the same large footprint, –148/–117 bp, with FRTL5 nuclear extract (Figure 1, experiment f). This DNase 1 protection is centred on a CRE, –146/–131 bp, and purified proteins of the CREB family (CREB, ATF-2 and API) could only partially reproduce this protection over –148 to –124 bp (Figure 1, experiment e) [19]. Ikuyama et al. have also shown that the same promoter region, –148/–124 bp, was protected with a BRL nuclear extract (Figure 1, experiment g) [19], concluding

Figure 5 Footprint analysis of the Mut-2 TSHr promoter with TTF-1 HD

An *EcoRI*–*HindIII* fragment from pG3-M2 labelled at the *HindIII* site with [α - 32 P]ATP and the Klenow enzyme was used as substrate. Lane 1 is the G + A sequence reaction. The DNase 1 digestions in the absence and presence of TTF-1 HD are shown in lanes 2 and 3 respectively. The box indicates the protected region. The dashed line indicates the mutated B region of the TSHr promoter.

that the wider protection in the TSHr promoter with the FRTL5 nuclear extract could be due to a ubiquitous CREB-like factor plus a thyroid-specific protein. In agreement with this conclusion we now demonstrate that TTF-1 does interact with the promoter region from –134 to –116 bp. The B protection is shown in Figure 2 (lanes 5 and 6). Hence TTF-1 binds twice in the TSHr minimal promoter: the A site is distal and the B site proximal to the start codon. In a titration curve the two DNase 1 protections on the TSHr promoter appear at about the same concentration of TTF-1 HD, suggesting that the binding affinities of the A and B sites are similar (Figure 3).

We have mutated the TSHr promoter at either the A region (Mut-1) [17] or the B region (Mut-2) [19] and have performed DNase 1-protection experiments with TTF-1 HD (Figures 4 and 5 respectively). In Figure 4 the TTF-1 protections on the wild-type promoter are shown (lanes 2 and 3) and with the Mut-1 promoter (lanes 5 and 6). Figure 5 shows the TTF-1 HD protection on Mut-2. From these experiments we conclude that the A and B binding sites are independent of each other. TTF-1 binds to the A site in the absence of binding in the B region and vice versa (see Figure 1, experiments c and d). It is worth pointing out that the experiments shown in Figures 4 and 5 have been performed with the minimum amount of TTF-1 HD required to obtain complete protection in the wild-type TSHr promoter (see Figure 3, lane 7).

The TTF-1 proximal site is redundant

In a previous study we have shown that TTF-1 activates TSHr promoter activity [20]. All the transactivated expression was exerted through the A site. The TSHr promoter mutated in this region (Mut-1) is not activated by TTF-1 in co-transfection

Figure 6 Transfection experiments in COS-7 cells

The luciferase activity present in extracts of COS-7 cells transfected only with pGE-T200 was set at unity. The mutant promoters show in these cells an activity similar to wild-type. Dose-response curves for TTF1 and CREB vectors were produced to establish their optimal concentrations in the transactivation experiments (results not shown). The co-transfections of the TTF-1 and/or CREB expression plasmids are indicated. Activities are presented as means $\pm$ S.E.M. for at least three separate experiments, each experiment being performed in triplicate. □, Wild-type; ▨, Mut-1; ■, Mut-2.

experiments in non-thyroid cells, COS-7, [20] (Figure 6). We now consistently demonstrate that TTF-1 binding to the B site does not induce TSHr promoter activity. As shown in Figure 6, in transfection experiments in COS-7 cells, the Mut-2 promoter is transactivated by exogenous TTF-1 expression as efficiently as the wild-type promoter. This result indicates that, although TTF-1 binds to the B region, this binding *per se* does not produce TSHr promoter activation.

TTF-1 and CREB co-operative activation of the TSHr promoter

In Figure 6 we show that the transactivation of the wild-type TSHr promoter, transfected into COS-7 cells, achieved by the exogenous expression of TTF-1 together with that of CREB is greater than the sum of the activation triggered by either TTF-1 or CREB alone. Therefore there is a functional synergism in the activation of the TSHr promoter between TTF-1 and CREB. We also show that this co-operative activity relies on both TTF-1 binding sites. With Mut-1 or Mut-2, the synergy between the two factors is no longer detected (Figure 6); in both cases the transactivation obtained by simultaneous transfection of the plasmids encoding TTF-1 and CREB is similar to the sum of the activities produced by each transcription factor alone. The TSHr promoter Mut-1 is not activated by TTF-1 as previously reported [20], hence the binding of this factor on the B site must be

transcriptionally silent. In agreement with this conclusion the TSHr promoter Mut-2 is activated by TTF-1 as efficiently as the wild-type (Figure 6).

In order to look for a putative protein-protein interaction between TTF-1 and CREB, we immunoprecipitated FRTL5 nuclear proteins using anti-TTF-1 antibodies, and the precipitate was tested by immunoblot with anti-CREB antibodies (results not shown, the anti-TTF-1 antibodies and anti-CREB antibodies were kindly provided by R. Di Lauro and P. Sassone-Corsi respectively). No CREB was found in the precipitate, therefore we can exclude direct interaction between CREB and TTF-1.

DISCUSSION

In this paper we report that the thyroid-specific factor TTF-1 is able to co-operate with CREB to activate TSHr promoter activity. As CREB and TTF-1 in isolation showed only a modest activity on the promoter, it was not clear how it could be activated. We show that the functional co-operation between the two factors enhances TSHr promoter activity about 20-fold over the basal level, thus explaining the TSHr gene expression from a quantitative point of view also. Moreover our finding describes for the first time how a general signal mediated by the CRE can activate expression of a thyroid-specific gene.

In FRTL5 cells an increase in cAMP level modulates gene expression of all three thyroid-differentiation markers, Tg, TPO and TSHr [21]. Although studies on the Tg and TPO promoters have not revealed any CRE in their sequences [13,14], transcription of the two genes is nevertheless stimulated by cAMP [11,21]. In contrast, in FRTL5 cells, TSHr promoter activity is down-regulated by this second messenger [21], and this inhibition is mediated by the thyroid hormone receptor $\alpha 1$ [22].

Transcriptional co-operativity has been ascribed in some cases to specific protein-protein interaction of transcription factors resulting in an increased DNA-binding affinity [27,28]. A second possible mechanism assumes the simultaneous interaction of transcription factors with a common target involved in the general transcription machinery. This interaction would stabilize the preinitiation complex [29]. As demonstrated, we can exclude, in our system, a specific protein-protein interaction between CREB and TTF-1. Therefore, we envisage that the two factors interact with a third protein. This protein could bridge the activation domains of CREB and TTF-1 with the transcription machinery and thus recruit the RNA polymerase II complex to the TSHr promoter. With this hypothesis, CREB has distinct activation domains and each domain can interact with multiple target components of the general transcription machinery [30]. In particular, the constitutive activation domain of CREB shows three distinct regions required for transcription activation [30].

An important question that arises from our findings is how general in the thyroid cell is the functional co-operation between TTF-1 and CREB. FRTL5 cells have been stably transformed with a plasmid expressing a dominant negative CREB mutant, KCREB [31]. These transformed FRTL5 cells show a reduction in thyroid-specific iodine uptake, an increase in cell cycling and a reduction in TSH-stimulated thymidine incorporation. These effects are thyroid-specific because a non-thyroid cell line transformed with the same plasmid does not show these modifications [31]. From these results it is possible to conclude that the CREB thyroid-specific activity is not limited to the TSHr promoter. It would be interesting to know whether TTF-1 also mediates all or some of those events. Moreover, since TTF-1 is the most important transcription factor involved in Tg promoter activity [13], and since TSH-stimulated Tg induction was not modified in KCREB-transformed FRTL5 cells [31], it is possible

to conclude that CREB does not induce TTF-1 expression. Therefore CREB can interfere with TTF-1 only in modulating its activity, as reported in this study.

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