A zinc-dependent DNA-binding activity co-operates with cAMP-responsive-element-binding protein to activate the human thyroglobulin enhancer

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Footprinting experiments involving the human thyroglobulin gene enhancer and thyroid nuclear extracts revealed a protected region called X2, containing an incomplete cAMP-responsive element (CRE). Band-shift experiments identified two binding activities recognizing the X2 element: a CRE-binding protein (CREB)/activating transcription factor (ATF) relative that binds the half CRE motif and a second factor that interacts with a G-rich motif located just upstream from the CRE. The first factor appears to be CREB itself, as indicated by the supershifting when using an antibody directed against CREB, and the second DNA-binding activity involved was shown to be zinc-dependent and exhibited an apparent molecular mass of 42–44 kDa in South-Western blotting experiments. This factor may represent a novel

entity, which we named CAF, for ‘CREB Associated Factor’. Three copies of X2 sequence conferred a strong cAMP-dependent transcriptional activation to a heterologous promoter in transient transfection assay in cAMP-stimulated primary thyrocytes and HeLa cells. Transfection experiments of constructs containing the X2 element mutated in either the CRE or the G-rich site showed that both motifs were required for this transcription activating function. Moreover, the combination of several individual X2 elements mutated in either the CRE or the G-rich motif did not exhibit full transcriptional activity. This suggests that, in the context of the X2 element, CREB requires a close interaction with CAF to achieve both basal and cAMP-dependent transcriptional activation.

INTRODUCTION

Thyroglobulin gene transcription constitutes the first step in the biosynthesis of the thyroid hormones, which occurs in the terminally differentiated thyrocyte [1]. Like most of the other differentiated functions of the thyrocyte, thyroglobulin (Tg) gene transcription is controlled by the pituitary hormone thyrotropin (thyroid-stimulating hormone, TSH) via the intracellular cAMP pathway [2,3].

Transiently transfected Tg promoter constructs from various species exhibited proper cAMP-dependent regulation in primary cultured dog thyrocytes, which have proved to be a suitable experimental system for the study of thyroid gene expression *in vitro* [4]. Experiments using reporter constructs harbouring the bovine Tg promoter allowed the delimitation of the minimal promoter region involved in this control [5]. This region lacks a canonical cAMP-responsive element [6] and the molecular mechanisms underlying the cAMP-dependent activity of the Tg promoter are still uncharacterized.

The functional analysis of remote 5′-flanking sequences from the human Tg gene by transient transfection in the primary cultured thyrocytes led to the identification of a cAMP-responsive enhancer, located between −3.2 and −2.7 kb from the transcriptional start site (+1), and containing an atypical cAMP-responsive element (CRE) [7]. The 0.5 kb-long enhancer element could be divided into two functional subfragments of 0.2 and 0.3 kb respectively, the latter containing the CRE-like motif. Thyroid transcription factor 1 (TTF-1) binds to both of these subfragments at several positions. We report here that the CRE motif in the 0.3 kb subfragment constitutes a binding site for

CRE-binding protein (CREB) and an unknown factor, which we termed ‘CREB Associated Factor’ (CAF).

EXPERIMENTAL

Cell culture and transfection

Primary cultures of dog thyrocytes were established and maintained according to Roger et al. [8]. In brief, the thyroid tissue was sliced finely and incubated at 39 °C with 1.5 mg/ml collagenase and 0.4 mg/ml DNase I for 30 min with agitation. Intact and fragmented follicles in suspension were separated from isolated cells by low-rate centrifugations. The follicles were then seeded in dishes (diameter 6 cm) and kept in M medium [Dulbecco’s modified Eagle’s medium (DMEM)/F12/MCDB104 (2:1:1)/1 mM pyruvate/insulin (5 µg/ml)/ascorbic acid (40 µg/ml)/penicillin (100 i.u./ml)/streptomycin (100 µg/ml)/fungizone (2.5 µg/ml)] supplemented with 2% (v/v) fetal-calf serum (FCS) during 1 day. The cells were grown to confluence for 3–5 days in M medium containing 25 ng/ml epidermal growth factor and 10% FCS. A redifferentiating treatment with 10 mM forskolin was then performed during 16–24 h before the DEAE–dextran transfection step.

HeLa cells were cultured in DMEM supplemented with 10% FCS and antibiotics [penicillin (100 i.u./ml)/streptomycin (100 µg/ml)].

Transfected thyrocytes or HeLa cells were lysed after 3 days of culture in ‘control’ medium or in the presence of forskolin (10 µM). Chloramphenicol acetyltransferase (CAT) expression was measured as described previously [4].

Abbreviations used: ATF, activating transcription factor; bZip, basic leucine-zipper; CAF, CREB Associated Factor; CAT, chloramphenicol acetyltransferase; CRE, cAMP-responsive element; CREB, CRE-binding protein; DMEM, Dulbecco’s modified Eagle’s medium; DTT, dithiothreitol; EMSA, electrophoretic mobility shift assay; FCS, fetal-calf serum; HSV, Herpes Simplex Virus; mut, mutation of the CRE; Tg, thyroglobulin; TK, thymidine kinase; TSH, thyroid-stimulating hormone (thyrotropin); TTF-1, thyroid transcription factor 1; TTF-2, thyroid transcription factor 2.

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Nuclear extracts

Primary cultured thyrocytes and HeLa cells extracts

Cells grown to confluence in large dishes (400 cm²) were treated with 10 μ M forskolin for 3 days, and the nuclear extracts were prepared as described before [9]. The homogenization step was improved using a tissue homogenizer [conical 'double action' poly-grinder (glass), Delta-Bio].

Thyroid tissue extracts

Frozen pieces of thyroid tissue (10 g) were homogenized with a Teflon/glass Potter homogenizer [in 40 ml of buffer containing 6.14% (w/v) sucrose/10 mM Hepes, pH 7.9/10 mM KCl/0.1 mM EDTA/0.1 mM EGTA/0.75 mM spermidine/0.15 mM spermine/1 mM dithiothreitol (DTT)/0.5 mM PMSF/leupeptin (2 μ g/ml)/pepstatin (2 μ g/ml)], and the resulting cellular lysate was gauze-filtered. Crude nuclei were isolated by low-rate centrifugation (16000 g) and lysed, according to the method described in Shapiro et al. [10]. Nuclear proteins were resuspended in a final volume of 0.5 ml.

DNase I protection assay

A 0.22 kb fragment, overlapping the 0.2 and 0.3 kb enhancer subfragments (see Figure 2a), was used as probe. Footprinting experiments were performed as described by Civitareale et al. [11] in the presence of 50 mM KCl. In competition experiments, a 500-fold molar excess of a competing, unlabelled oligonucleotide was added to the binding mix, before the addition of nuclear proteins.

Mobility shift assay

Probes were prepared as described previously [12]. Nuclear extracts (1–3 μ l) or the CREB basic leucine-zipper (bZip) peptide (2 ng per lane, from Santa-Cruz) were preincubated for 15 min at 0 °C in 20 μ l (final volume) of binding buffer [for nuclear extracts: 20 mM Hepes, pH 7.9/50 mM KCl/0.1 mM EDTA/0.5 mM DTT/10% (v/v) glycerol/0.002% (v/v) Bromophenol Blue/0.5–1.5 μ g of poly(dI-dC); for CREB bZip peptide: 50 mM Tris, pH 8/12.5 mM MgCl₂/1 mM EDTA/1 mM DTT/10% glycerol/0.002% Bromophenol Blue/0.025 μ g of poly(dI-dC)]. The probe (0.2–0.4 ng, approx. 15000 c.p.m.) was then added to the mix, which was incubated for a further 15 min at 0 °C before loading on gel. In competition experiments, a 125–250-fold molar excess of unlabelled oligonucleotide was added to the preincubated mix, 1 min before the addition of the probe. Electrophoresis was performed on a 6% (19:1 or 39:1, w/v) polyacrylamide gel in 0.5×TBE at 200 V and 4 °C for 3 h (1×TBE is 89 mM Tris/89 mM boric acid/2 mM EDTA).

In supershifting experiments, 1 μ l of purified antibody or immune serum was added to the final incubated mix containing nuclear proteins, binding buffer, eventual competitor and probe. The incubation was prolonged for 1–2 h at 4 °C. The anti-CREB serum 244 was kindly provided by Dr. M. Montminy (The Salk Institute, La Jolla, CA, U.S.A.). The anti-AP2 antibody was purchased from Santa-Cruz.

Zinc depletion was performed by adding 2 μ l of 1,10-phenanthroline (Sigma) (final concentration 0.5 mM) to the preincubated mix containing nuclear proteins and binding buffer, and by further incubating for 10 min at 0 °C before the addition of the probe. To restore free Zn²⁺ concentrations, variable

amounts of zinc sulphate (final concentration 0.1–1 mM, added volume 1 μ l) were then added after the incubation following addition of phenanthroline, and the mix was incubated again for a further 10 min at 0 °C before the addition of the probe.

South-western blotting

Oligonucleotides were labelled using the method described for the preparation of band-shift probes [12]. Proteins from thyroid tissue nuclear extracts (30 μ l per lane, in Laemmli buffer, unboiled) were separated on a 10% gel by SDS/PAGE and electroblotted on nitrocellulose membrane (Schleicher & Schuell, PH 79, 0.1 μ m). The blot was incubated for 30 min at room temperature in a buffer containing 50 mM NaCl, 2 mM EDTA, 4 M urea, 0.1 mM DTT and 10 mM Tris/HCl, pH 7.5. After three successive 10 min incubations in a blocking/binding/washing buffer [Buffer 1, 10 mM Tris/HCl (pH 7.5)/1 mM EDTA/50 mM NaCl/0.02% (w/v) BSA/0.02% (w/v) Ficoll 400/0.02% (w/v) polyvinyl pyrrolidone], the membrane was incubated for 30 min in poly(dI-dC) solution (30 μ g/ml in Buffer 1) and washed again for 3×10 min. Cut membrane lanes were then immersed for 1 h in labelling solution [Buffer 1 with 1 μ g/ml poly(dI-dC), 10⁵ c.p.m./ml probe and a 200-fold molar excess of competing oligonucleotide], and washed for 5×5 min in Buffer 1. Membrane strips were dried and exposed to Hyperfilm (Amersham Hyperfilm-MP) for 3 days at –80 °C with an intensifying screen.

Sequence of the oligonucleotides used

X2 oligonucleotides

For X2L, X2 and mutated sequences see Figure 2(c). In each case (except for X2mG+mut oligonucleotide that was only designed for PCR mutagenesis), protruding TCGA bases in 5', and CATG in 3', were added to the non-coding strand to facilitate labelling and ligation.

Other oligonucleotides

CRE (from human gonadotropin α gene [13]): ATAAATTGA-CGTCATGGTAACATG; AP2 (from MT-IIA promoter [14]): ATGAAGTACCGCCGCGGCCGTGCATG; KE2 (E-box from immunoglobulin K enhancer [15]): GATCAAGGC-AGGTGGCCCACTAG; NF-KB (from immunoglobulin K enhancer [16]): ATCAGAGGGGACTTTCCGAGACATG.

DNA constructions

Constructs containing multimerized oligonucleotides

The 5' and 3' extremities of the double-stranded oligonucleotides presented *Sal*I and *Sph*I half restriction sites respectively (see Figure 2c). After 5' end phosphorylation of the single-stranded oligonucleotides using T4-polynucleotide kinase (BRL, 1 unit/ μ g oligo, in the presence of 0.7 mM ATP), and subsequent annealing, the oligonucleotides were partially ligated with T4 DNA ligase (BRL, 1 unit/ μ g oligo for 5–15 min at room temperature). The resulting trimers and pentamers were isolated after electrophoretic separation on a 5% polyacrylamide gel in 0.5×TBE, pH 8.3. These head-to-tail multimers were inserted in front of the Herpes Simplex Virus (HSV) thymidine kinase (TK) promoter/CAT fusion in the pBLCAT2 [17] vector.

Mutagenesis of the 0.3 kb enhancer containing construct using PCR

Each PCR reaction included the 0.3-TK-CAT → construct [7] as

template. [It contains the 0.3 kb subfragment inserted in front of the HSV TK promoter/CAT fusion in the pBLCAT2 vector in the natural (→) orientation relative to the CAT gene]. The two primers used were: a first primer of 20 bases encompassing the *SpeI* cloning site contained in an additional linker at the 5' border of the 0.3 kb enhancer fragment, and a second primer (60–80 bases), containing in each case both the desired X2 mutation and the internal *ScaI* restriction site of the 0.3 kb fragment. The resulting PCR products were subcloned in the parental 0.3-TK-CAT → construct cleaved by *SpeI* and *ScaI* (partial digestion). All mutagenized fragments were sequenced in order to exclude the presence of undesired base changes that could occur during the PCR step.

RESULTS

Identification of X2 footprint on the 0.3 kb human Tg enhancer element

The previous functional analysis of the 0.5 kb-long human Tg enhancer element by transient transfection in primary cultured dog thyrocytes had shown that a 0.3 kb-long subfragment exhibited full enhancer activity [7]. As shown on Figure 1, thyroid nuclear extracts generated a footprint which we called X2, located in this 0.3 kb enhancer element. Nuclear extracts from dog thyroid tissue, or forskolin-treated primary cultured dog

thyrocytes protected identical bases on the top strand (Figure 1a). The patterns appearing on the bottom strand contained additional hypersensitive sites: similar protections were obtained when using nuclear extracts from forskolin-treated thyrocytes (Figure 1b, lane 3), HeLa cells (lane 4) and human thyroid tissue (lane 6). Interestingly, the protected X2 sequence contained an atypical CRE-motif (TGACGT, see Figure 2), and a potential E-box-binding motif for basic helix–loop–helix transcription factors [15] (CAGGTG, see Figure 2).

The X2 element is recognized by a factor belonging to the CREB/ATF family and another-binding activity apparently related to factor AP2

In order to identify the factor(s) involved in the activity of the X2 sequence, electrophoretic-mobility-shift-assay (EMSA) experiments were conducted using nuclear extracts from dog thyroid. As appears on lane 2 of Figure 3, two major complexes (a and b) and a minor one (a') were detected when using extracts from forskolin-treated thyrocytes. (Identical results were obtained using extracts from dog thyroid tissue; not shown.) The competition experiments showed that the presence of the minor complex was clearly related to the presence of complex 'a'. Moreover, as this minor band exhibited variable intensities in different experiments, depending on the batch of nuclear extract used in each case, it appeared likely that it corresponded to a proteolysed form of complex 'a'. The addition of an excess of unlabelled X2 oligonucleotide (lane 3) resulted in the disappearance of all the complexes. Complex 'b' appeared significantly diminished when an oligonucleotide containing the palindromic CRE sequence (from the human gonadotropin α gene [13]) was used as competitor (lane 8), which suggested that this complex involved a member of the CREB/activating transcription factor (ATF) family of transcription factors. On the other hand, an oligonucleotide containing the binding site for the AP2 factor (from MT-IIA promoter [14]) was able to compete for the formation of complex 'a' and its related form (lane 9). The KE2 sequence (corresponding to the second E-box motif from the immunoglobulin K gene enhancer [15]) did not show any effect in the competition experiment (lane 10). When nuclear extracts from HeLa cells were used, similar competition results were obtained with the same competing oligonucleotides (not shown).

To delineate the DNA motifs of the X2 sequence that were critical for the formation of these complexes, we mutated the potentially relevant motifs present in the X2 element (see Figure 2c). The E-box motif was destroyed (X2mE), the half CRE was either destroyed (X2mut) or converted into a fully palindromic CRE (X2pal), and the string of four G residues located between these two motifs was also mutated (X2mG). The E-box mutation (X2mE; Figure 3, lane 7) lowered the affinity of the X2 sequence for both factors, as indicated by its reduced ability to compete for the formation of all complexes as compared with the wild-type sequence (lane 3). As expected, the X2mut sequence (no CRE) did not compete any more for formation of the CRE-based complex 'b' (lane 4). Conversely, the palindromic CRE present in X2pal mediated an efficient competition (lane 5). The X2mG sequence (disrupted G-tract) nicely competed out the formation of the CRE-based complex 'b' (lane 6), leaving intact complex 'a' and its associated form. The factor(s) involved in complex 'a' thus recognized the G-rich motif in the X2 sequence, which agrees with the fact that formation of this complex could be impaired in the presence of an excess of the similarly G-rich AP2-binding site (core sequence: CCGCC-CGCG).

Figure 1 Identification of the X2 footprint on the human Tg enhancer sequence

Footprinting results involving nuclear extracts from primary cultured dog thyrocytes, thyroid tissue, or HeLa cells. The two strands of a 0.22 kb DNA fragment were labelled at the 3' extremities. The half-boxes show the X2 protected regions, and the horizontal thick bars point to hypersensitive positions. (a) The coding strand was labelled (0.22c probe) and used with extracts from dog thyroid tissue ('Thyroid', lane 3) or from cultured thyrocytes under forskolin-stimulated conditions ('Fo Cell', lane 5). Other lanes: 'naked', naked DNA; 'G+A', Maxam–Gilbert G+A sequence ladder. (b) The non-coding strand was labelled (0.22nc probe), and used with extracts from cultured thyrocytes under forskolin-stimulated conditions (lane 3), from HeLa cells ('HeLa', lane 4), or from human thyroid tissue ('h.Thyroid', lane 6). Other lanes: 'naked', naked DNA; 'G+A', Maxam–Gilbert G+A sequence ladder.

Figure 3 Study of the two X2 element-binding activities

Mobility shift assay performed with labelled X2 oligonucleotide and forskolin-treated dog thyrocytes extracts. The horizontal bars point to the band shifts named 'a', 'a'' and 'b', and to free probe (fp). Lane 1, free probe; lane 2, no competitor added; lanes 3–10, the nature of the competing oligonucleotide used is indicated at the top.

Figure 4(b) (lanes 7–13), when increasing amounts of the CRE and X2 sequences were used to compete for binding of the bZip peptide on the CRE probe, approx. ten times more X2 oligonucleotide than CRE sequence was required to achieve a comparable extent of competition.

To verify that CREB contained in the thyroid nuclear extracts was also able to bind the X2 element, supershifting experiments were conducted using an antibody directed against CREB. The addition of the anti-CREB serum in a band-shift assay with X2 as probe resulted in the specific supershifting of complex 'b', which was supposed to involve a member of the CREB/ATF family (Figure 4c, lane 3). The addition of a control serum containing antibodies directed against Pax 8 protein [18] did not interfere with the formation of the complexes in the assay (lane 4), and the addition of an excess of unlabelled CRE oligonucleotide resulted, as expected, in the disappearance of the supershifted complex in the presence of anti-CREB serum (results not shown).

CAF, a zinc-dependent-binding activity that recognizes the G-rich motif of X2

As the factor involved in complex 'a' could bind the AP2

recognition sequence, we investigated the possibility that this factor was the AP2 protein itself. A purified antibody directed against AP2 factor was used in EMSA experiments involving thyroid nuclear extracts and the X2 sequence as probe. No supershifting of any of the complexes was observed (result not shown; the same antibody proved to be able to supershift one of the complexes obtained when using the same nuclear extract and AP2 oligonucleotide as probe). This indicated that the factor recognizing the G-rich motif in the X2 sequence was not immunologically related to AP2 factor. We called it CAF, for 'CREB Associated Factor'.

As an initial attempt to gather some information on the nature of this binding activity, EMSA experiments were undertaken in which the effect of zinc depletion on DNA binding was investigated. In the presence of 0.5 mM phenanthroline, complex 'a' and its related form were barely detectable, whereas complex 'b' was still observed (Figure 5a, lane 2). Restoring free Zn^{2+} availability by the addition of increasing amounts of zinc sulphate led to the re-appearance of complex 'a' (lanes 3–5). When the Zn^{2+} concentration reached values close to 1 mM, the formation of both complexes was dramatically reduced (lane 6), revealing a non-specific inhibition of DNA binding, a non-specific inhibition that has also been observed in other studies [19]. These results clearly showed that the DNA-binding activity of CAF required the presence of free Zn^{2+} ions and suggested that CAF could be a zinc-finger protein.

To determine the size of CAF, south-western blotting experiments were conducted using the X2 sequence as probe and blotted thyroid nuclear proteins. As appears on Figure 5(b), two main bands were detected ('1' and '2' on the Figure). The upper signal '1' was barely detectable when binding was conducted in the presence of an excess of unlabelled X2 (lane 5), but was not affected by the addition of an excess of the X2mG oligonucleotide (disrupted G-tract, lane 3). This indicated that this protein required the presence of an intact G-tract in X2 for binding, which corresponded exactly to what was expected for CAF. The size of this protein, as determined in individual experiments, ranged from 42 to 44 kDa. A band of similar molecular mass could also be detected when using the X2 sequence mutated in the CRE motif (X2mut) as probe (result not shown). The lower signal corresponding to a protein of 34 kDa did not appear to be highly specific, as it was not significantly reduced in the presence of an excess of unlabelled probe sequence (lane 5). No signal corresponding to the CREB protein, which we had shown is able to bind the CRE-like motif in X2, was observed in these experiments (no band disappeared in the presence of an excess of unlabelled CRE oligonucleotide; see lane 4). It could suggest that the experimental conditions used here did not allow the detection of DNA-binding activities requiring protein dimerization, as is the case for CREB.

The X2 sequence constitutes a functional activator element

The multimerization of enhancer fragments, called enhansons [20], often generates functional enhancer elements. To verify that the X2 element constituted a bona fide enhanson, three copies of this sequence (X2I, see Figure 2c) were inserted in front of the HSV TK promoter in plasmid pBLCAT2 [17], and assayed by transient transfection in primary cultured dog thyrocytes. The resulting construct provided a dramatic increase in the expression of the reporter gene in forskolin-treated cells compared with the pBLCAT2 vector (compare 3.X2I and TK constructs in Table 1, Expt. 1 and 2). A shorter X2 sequence performed equally well in the assay (3.X2 construct). These constructs were also largely more active than the parental plasmid (TK) when transfected in

Figure 4 Binding of the CREB bZip peptide and identification of CREB by supershifting

(a) Mobility shift assay performed with the CREB bZip peptide, using labelled X2 oligonucleotide. The horizontal bars point to the CREB bZip complex (bZ) and to free probe (fp). Lane 1, free probe; lane 2, no competitor added; other lanes, the nature of the competing oligonucleotide used is indicated at the top. (b) Mobility shift assay performed with the CREB bZip peptide, and the labelled CRE oligonucleotide. Lane 1, free probe; lanes 2 and 7, no competitor added; lanes 3–6, the nature of the competing oligonucleotide used is indicated at the top; other lanes, competition with increasing excess of competing CRE (lanes 8–10) or X2 (lanes 11–13) oligonucleotides, the competitor molar excess used being indicated at the top. (c) Supershift results obtained with X2 probe, forskolin-stimulated thyrocytes extract and CREB immune serum. An unrelated immune serum (Pax 8 [18]) was used as a control of antibody specificity. The horizontal bars point to the band-shifts named 'a' and 'b', to supershift (ss) and to free probe (fp). Lane 1, free probe; lane 2, thyroid extract alone; lanes 3 and 4, the nature of the antibody (Ab) used is indicated at the top.

HeLa cells (Exp. 3 and 4), where the maximal activation was also observed under forskolin conditions. These data indicated that the X2 element was likely to participate in the activity of the human Tg enhancer.

Both CRE and G-rich motifs are required for the functional activity of the X2 sequence

The existence of two different DNA–protein interactions involving two sequence motifs located very close to each other in the X2 element raised the questions of whether both factors could bind the X2 sequence at the same time and whether the binding of only one or both factors was required for the functional activity of the DNA element. To investigate these points we made use of the X2 mutated sequences, which could only bind the zinc-dependent CAF protein (X2mut) or CREB (X2mG). The trimerized oligonucleotides were inserted upstream from TK promoter in pBLCAT2, as was done before for the wild-type X2 sequence. The resulting constructs were assayed by transient transfection in thyrocytes and HeLa cells (Table 1). In both cellular systems, the independent mutations of the CRE-like

element (X2mut) or the G-rich motif (X2mG) completely abolished the enhancing activity of the X2 element. This demonstrated that neither of the two factors could provide activation independently of the other. When the other mutated sequences were assayed in the same experimental system, we observed that the transformation of the CRE-like site in a palindromic CRE (X2pal) had no deleterious effect on the activity of X2, as could be expected. We noticed that 3.X2pal construct was significantly more active than the 3.X2 construct in HeLa cells, whereas both constructs behaved almost identically in the thyrocytes. Considering the difference observed between the relative activities of 3.X2 and 2.CRE constructs in thyrocytes and HeLa cells respectively, this observation suggested the existence of a difference in CREB-related activity between these two cell types. On the other hand, the X2mE sequence (no E-box motif), which, for a still unclear reason, exhibited a largely reduced affinity for both factors in the EMSA experiments, consistently did not show any activating ability in the assay. To investigate whether the CRE motif of X2 would be able to function independently when separated from the other DNA motifs (G-tract and E-box), we designated the X2d oligonucleotide (see Figure 2c), which

Figure 5 Characterization of CAF

(a) Effect of zinc depletion on the band-shifts obtained with thyroid tissue nuclear extracts and X2 as probe. Restoration of the binding activity was performed with increasing concentrations of zinc sulphate (0.1–1.0 mM). The horizontal bars point to the band-shifts named 'a' and 'b' and to free probe (fp). Lane 1, thyroid extract alone; lane 2, + 0.5 mM phenanthroline; lanes 3–6, + 0.5 mM phenanthroline and ZnSO_4 in the concentrations indicated at the top. (b) South-western blotting results obtained with proteins from thyroid tissue nuclear extract and X2 as probe. In lane 2, the non-specific competition oligonucleotide NF-KB (ns) was added to obtain signals of comparable intensities with the other competition lanes. The horizontal bars point to protein bands named 1 and 2. Lane 1, Rainbow molecular-mass marker; lanes 2–5, the nature of the competing oligonucleotide used is indicated at the top.

contains the CRE motif and DNA sequences located downstream of it, up to a length identical with the other nucleotides. When assayed for function, it failed to reproduce a transcriptional activation of the TK promoter comparable with that of the X2 sequence, even in the presence of cAMP stimulation, in both cell types. Our results thus clearly indicated that both factors were required for the function of the X2 element.

However, because in the functional assay each construct harboured three identical copies of the individual oligonucleotides, we were left with the question of whether the two factors had to bind together on each copy of the sequence to provide activation, or if it only required the presence of both factors anchored to separated copies of the sequence on the construct. According to this second hypothesis, a construct harbouring copies of the X2 sequence mutated in either the CRE (X2mut) or the G-tract (X2mG) could eventually perform as well as the construct containing the wild-type X2 trimer in front of the TK promoter. Constructs containing mixed X2mut/X2mG trimers were thus generated. As six different mixed structures

could be expected, we first compared the CAT activities obtained, after transfection of thyrocytes, with four different pools of ten randomly selected clones, with the CAT activity obtained with a mixture composed of one part of 3.X2 construct and nine parts of 3.X2mut construct. None of the pools reached the level of activity obtained with the control mixture (results not shown). To exclude the possibility that we could have a biased representation of the different structures in the pools, the ten individual clones of one of these pools were sequenced and assayed individually. As shown in Table 2, most of the different structures (five out of six different possibilities) were obtained, and none of them displayed an activity similar to the wild-type 3.X2 construct. This result indicated that maximal activation required the binding of both factors on the same X2 unit, even if a moderate activation could be provided by the binding of the two factors as single entities on individual copies of the X2 sequence.

Accordingly, we expected that both X2mut and X2mG mutations would largely reduce the activity of a single X2 motif. We thus introduced the same mutations that were used in the

Table 1 Mutational analysis of trimerized X2 binding sites

Transient expression, in forskolin-treated or control primary cultured dog thyrocytes or HeLa cells, of pBLCAT2 [17] constructs containing, upstream from the HSV TK promoter, three copies of wild-type (3.X21 and 3.X2) or mutated X2 sequence (mutations described in Figure 2c). These mutants were transfected parallel to the parental pBLCAT2 plasmid (TK). A pBLCAT2 construct harbouring two copies of the 18 bp CRE-containing repeat from human gonadotropin α gene [13] was included as a control of the cAMP effect (2.CRE). The results of four representative experiments are shown, two in thyrocytes (Expt. 1 and 2) and two in HeLa cells (Expt. 3 and 4). CAT activities are expressed as percentage of 3.X2 CAT activity in forskolin conditions (mean of duplicates with half range error; background value of the assay deducted). The background and the 100% activity values of the assays were respectively, in c.p.m.: 763 and 125114 for Expt. 1, 363 and 78810 for Expt. 2, 601 and 61352 for Expt. 3, 385 and 58118 for Expt. 4. Parentheses indicate that the value is not significantly different from the background (less than twice the background value) or unreliable (large difference between the results of duplicates).

Transfected plasmid	3.X2 activity (%) in forskolin conditions			
	Thyrocytes, Expt. 1		Thyrocytes, Expt. 2	
	Control	Forskolin	Control	Forskolin
3.X21	(0.76 $\pm$ 0.28)	62.37 $\pm$ 10.85	(0.00 $\pm$ 0.05)	98.22 $\pm$ 12.10
3.X2	3.08 $\pm$ 0.53	100.00 $\pm$ 0.08	(0.38 $\pm$ 0.22)	100.00 $\pm$ 2.28
3.X2mE	(0.29 $\pm$ 0.04)	(0.53 $\pm$ 0.32)	(0.10 $\pm$ 0.03)	(0.53 $\pm$ 0.18)
3.X2mG	(0.12 $\pm$ 0.12)	(0.34 $\pm$ 0.08)	(0.51 $\pm$ 0.05)	(0.40 $\pm$ 0.03)
3.X2mut	(0.09 $\pm$ 0.09)	(0.43 $\pm$ 0.10)	(0.19 $\pm$ 0.02)	(0.82 $\pm$ 0.23)
3.X2pal	4.99 $\pm$ 0.65	91.07 $\pm$ 4.89	(0.58 $\pm$ 0.24)	115.62 $\pm$ 3.70
3.X2d	(0.19 $\pm$ 0.01)	(1.04 $\pm$ 0.25)	(0.42 $\pm$ 0.24)	1.67 $\pm$ 0.18
TK	(0.10 $\pm$ 0.03)	1.38 $\pm$ 0.59	(0.18 $\pm$ 0.03)	(0.24 $\pm$ 0.04)
2.CRE	(0.00 $\pm$ 0.00)	2.28 $\pm$ 0.40	(0.13 $\pm$ 0.34)	(4.31 $\pm$ 3.32)
HeLa cells, Expt. 3				
	Control	Forskolin	Control	Forskolin
3.X21	34.81 $\pm$ 4.08	113.94 $\pm$ 6.21	26.07 $\pm$ 4.14	65.48 $\pm$ 1.20
3.X2	32.82 $\pm$ 2.77	100.00 $\pm$ 12.35	51.35 $\pm$ 4.60	100.00 $\pm$ 3.54
3.X2mE	2.85 $\pm$ 0.19	4.55 $\pm$ 0.51	6.44 $\pm$ 1.83	8.72 $\pm$ 0.19
3.X2mG	(1.82 $\pm$ 0.36)	4.75 $\pm$ 1.56	4.03 $\pm$ 0.93	8.77 $\pm$ 0.85
3.X2mut	(0.39 $\pm$ 0.02)	(1.21 $\pm$ 0.08)	(0.69 $\pm$ 0.12)	(1.05 $\pm$ 0.21)
3.X2pal	125.02 $\pm$ 8.49	250.45 $\pm$ 5.13	117.80 $\pm$ 3.40	235.36 $\pm$ 4.54
3.X2d	5.92 $\pm$ 0.12	7.83 $\pm$ 0.58	7.81 $\pm$ 0.70	10.68 $\pm$ 1.18
TK	2.00 $\pm$ 0.52	3.65 $\pm$ 0.24	2.83 $\pm$ 0.23	3.24 $\pm$ 0.08
2.CRE	32.63 $\pm$ 2.19	67.02 $\pm$ 1.17	37.05 $\pm$ 1.76	71.91 $\pm$ 2.24

trimer constructs in the single X2 element present in the 0.3 kb enhancer sequence. As shown in Table 3, the absence of the G-rich motif (0.3/X2mG), or the CRE (0.3/X2mut) in the X2 sequence contained in the 0.3 kb enhancer region reduced significantly the activity of this element in transiently transfected thyrocytes. The remaining activity of the mutated constructs (25–50 % of that of the 0.3-TK wild-type construct) was likely to result from the presence in the 0.3 kb sequence of other activating DNA elements (e.g. the TTF-1-binding site) that contribute, with the X2 element, to the total activity of the Tg enhancer. If both G-rich and CRE motifs were suppressed (0.3/X2mG + mut), no further decrease in activity was observed, indicating that the weak activation resulting from the binding of one of the two factors on the X2 motif (CREB on X2mG or CAF on X2mut) could not be detected significantly in the context of the 0.3 kb element. As expected, a construct containing the X2pal mutation (0.3/X2pal) exhibited an activity similar to that of the wild-type sequence. By contrast, the introduction of the E-box mutation (0.3/X2mE) had an effect similar to that observed for the X2mut and X2mG mutations, as noted previously. Altogether, these results showed that the requirement for the-binding of both CREB and CAF, which appeared to be crucial for the activity of

Table 2 'Double-mutant' trimerized X2 oligonucleotides

Transient expression, in primary cultured dog thyrocytes under forskolin conditions, of ten individual trimer constructs resulting from ligation of two different mutated X2 sequences (X2mG and X2mut) in variable position and orientation (see the text), parallel to constructs containing the wild-type X2 and X2mut sequences. The trimers structure is indicated by the mutation present in each X2 monomer (mut or mG), and by the orientation of these monomers (arrows) relative to the 5' and 3' trimer extremities. The results of two representative experiments are shown. CAT activities are expressed as the percentage of 3.X2 CAT activity under forskolin conditions (mean of duplicates with half range error; background value of the assay deducted). The background and the 100% activity values of the assays were respectively, in c.p.m.: 395 and 26938 for Expt. 1, 649 and 91982 for Expt. 2. Parentheses indicate that the value is not significantly different from the background (less than twice the background value) or unreliable (large difference between the results of duplicates).

Structure of the trimer 5' $\rightarrow$ $\leftarrow$ $\rightarrow$ 3'		3.X2 activity (%) in forskolin conditions	
		Thyrocytes, Expt. 1	Thyrocytes, Expt. 2
mut-mut-mG	1	(1.96 $\pm$ 0.02)	2.70 $\pm$ 0.00
	2	(2.73 $\pm$ 0.44)	3.16 $\pm$ 0.05
	3	(3.44 $\pm$ 1.65)	3.76 $\pm$ 0.04
mG-mut-mG	4	3.52 $\pm$ 0.40	8.33 $\pm$ 2.04
	5	(4.51 $\pm$ 2.28)	6.04 $\pm$ 0.68
mut-mG-mG	6	(0.71 $\pm$ 0.08)	2.27 $\pm$ 0.18
mut-mG-mut	7	(1.53 $\pm$ 0.35)	1.90 $\pm$ 0.40
mG-mG-mut	8	(1.37 $\pm$ 0.22)	(0.94 $\pm$ 0.06)
	9	(1.14 $\pm$ 0.66)	(1.36 $\pm$ 0.00)
mG-mG-mG	10	(0.84 $\pm$ 0.37)	(0.56 $\pm$ 0.08)
mut-mut-mut	3.X2mut	(0.15 $\pm$ 0.06)	(0.71 $\pm$ 0.03)
X2-X2-X2	3.X2	100.00 $\pm$ 1.99	100.00 $\pm$ 2.90

Table 3 X2 mutagenesis in the 0.3 kb enhancer

Constructs containing different mutated motifs in the X2 element, within the 0.3 kb enhancer sequence, were assayed by transient expression in forskolin-stimulated or control primary cultured dog thyrocytes, parallel to the 0.3-TK-CAT $\rightarrow$ wild-type construct (0.3-TK). The mentioned X2 mutations in the mutated constructs are described in Figure 2(c). The results of two representative experiments are shown. CAT activities are expressed as the percentage of 0.3-TK activity under forskolin conditions (mean of duplicates with half range error; background value of the assay deducted). The background and the 100% activity values of the assays were respectively, in c.p.m.: 412 and 87088 for Expt. 1, 390 and 7642 for Expt. 2. Parentheses indicate that the value is not significantly different from the background (less than twice the background value) or unreliable (large difference between the results of duplicates).

Transfected plasmid	0.3-TK activity (%) in forskolin conditions			
	Thyrocytes, Expt. 1		Thyrocytes, Expt. 2	
	Control	Forskolin	Control	Forskolin
0.3-TK	(10.59 $\pm$ 4.51)	100.00 $\pm$ 0.57	19.11 $\pm$ 4.90	100.00 $\pm$ 3.68
0.3/X2mE	6.67 $\pm$ 0.00	23.09 $\pm$ 3.35	(7.36 $\pm$ 2.09)	19.76 $\pm$ 0.96
0.3/X2mG	7.24 $\pm$ 0.57	46.60 $\pm$ 6.89	(7.44 $\pm$ 0.53)	27.91 $\pm$ 6.06
0.3/X2mut	12.62 $\pm$ 2.15	77.03 $\pm$ 8.61	20.88 $\pm$ 2.30	51.22 $\pm$ 9.39
0.3/X2mG + mut	4.84 $\pm$ 0.15	21.78 $\pm$ 1.12	16.13 $\pm$ 1.48	40.68 $\pm$ 6.46
0.3/X2pal	12.02 $\pm$ 2.22	112.63 $\pm$ 12.06	20.30 $\pm$ 5.23	134.13 $\pm$ 7.72

the trimerized X2 DNA element, also applied when this X2 motif is present as a single copy in the context of the wild-type 0.3 kb Tg enhancer.

DISCUSSION

Our study has identified an atypical cAMP-responsive regulatory element contained in the human Tg gene enhancer. This X2 element appeared to be composed of two contiguous binding

sites: an incomplete CRE recognized by CREB and a G-rich motif bound by a still unknown factor, which we called CAF, for 'CREB Associated Factor'. CAF exhibited a molecular mass of 42–44 kDa in South-Western blotting experiments, and required the presence of Zn^{2+} ions for DNA binding. The functional analysis of the X2 element clearly showed that the presence of both factors was required for transcriptional activity *in vitro*, despite the fact that no ternary complex was observed in band-shift experiments. It is possible that the experimental conditions used did not allow us to detect the corresponding complex. Alternatively, stable binding of the X2 motif by CREB and CAF could require the presence of a co-activator, which could have been lacking in our nuclear extracts, or even could require a specific chromatin structure [21] that would not be reconstituted in the assay *in vitro*. The inability of the CRE-like motif contained in the X2 sequence to mediate transcriptional activation on its own and its reduced affinity for the CREB bZip peptide, as compared with the palindromic CRE, observed *in vitro*, suggest the existence of a close co-operation between CREB and CAF in the context of the X2 element. Other examples of co-operative interaction between CREB and unrelated factors have been described previously, e.g. in the retinoblastoma gene promoter [22], or in the porcine urokinase-type plasminogen activator (uPa) gene enhancer [23].

Both Tg and thyroperoxidase gene promoters are controlled by cAMP in the thyrocyte [24]. However, no classical CRE motif has been identified in these promoter regions. Rather, a thyroid-specific and cAMP-modulated-binding activity, called CMB [8] or thyroid transcription factor 2 (TTF-2) [11], is likely to be involved. Interestingly, the purification of TTF-2-binding activity by sequence-specific DNA affinity chromatography [25] led to the isolation of a zinc-finger protein of 60 kDa. The difference in size and the different natures of the respective binding sites make it very unlikely that CAF could correspond to this protein. In band-shift assay, no competition for complex 'a' formation was obtained with sequences corresponding to the binding site for TTF-2 [11] (results not shown). Furthermore, the recent cloning of TTF-2 cDNA revealed that it is a forkhead-domain-containing protein of 42 kDa [26,27]. The control of Tg gene activity by cAMP, at least in man, would then involve different classes of transcription factors acting through the regulatory region: the CREB-CAF complex located in the enhancer, and the unrelated TTF-2 factor within the proximal promoter. Very similar mechanisms of control by cAMP have been described for the enhancer and proximal promoter from the human adrenal CYP21A2 gene [28], where an adrenal-specific factor (ASP) mediates cAMP-dependent promoter activity, whereas a CREB-related protein binds to an imperfect CRE located in the upstream enhancer, conferring cAMP-dependent activity to this element also. In the thyroid also, the cAMP-dependent control on TSH receptor gene transcription has been shown to be mediated by CREB, acting in synergy with the factor TTF-1 [29] to activate the promoter [30].

A search for known transcription factors that could correspond to CAF failed to identify potential candidates. This opens the way to the purification and ultimate cloning of this apparently novel entity.

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