Negative cooperativity between juxtaposed E-box and cAMP/TPA responsive elements in the cholecystokinin gene promoter

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Abstract The promoter of the cholecystokinin (CCK) gene possesses evolutionary conserved juxtaposed E-box and cAMP/TPA responsive elements (CRE/TRE). We have examined the functional interaction of these two sites. As previously noted, c-Jun/c-Fos heterodimers greatly increase promoter activity through association with the CRE/TRE. Mutation of the E-box enhanced the activation by c-Jun/c-Fos, as well as stimulation by forskolin and bFGF, that acts through the CRE/TRE site. Moreover, c-Jun/c-Fos stimulation was inhibited by co-expression of c-Myc and Max. The results indicate that factors associating with the E-box exhibit a negative cooperative effect on the activation via the CRE/TRE element. We propose that this mechanism plays a significant role in CCK gene transcription and other genes with juxtaposed E-box and CRE/TRE.

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Key words: Cholecystokinin (CCK) gene promoter; Juxtaposed E-box and cAMP/TPA responsive elements (CRE/TRE); Negative cooperativity

1. Introduction

Cholecystokinin (CCK) is a peptide hormone and neurotransmitter primarily expressed in endocrine gut cells and neurons in the brain and gut (for review see [1]). Previous studies on the proximal promoter region of the CCK gene have identified a number of known transcriptional regulatory motifs including a TATA box, an Sp1 binding site, a basic region/helix-loop-helix/leucine zipper (bHLH-ZIP) E-box, and a combined cAMP-response and TPA-response element (CRE/TRE) [2,3]. The spacing and positioning of these elements are highly conserved in evolution from elasmobranch to man (Fig. 1). Only the shark promoter lack an E-box [4,5]. The conservation suggests that the motifs play a crucial role in the spatial and temporal control of CCK gene expression.

The E-box 5′-CACGTG-3′ motif is recognized by members of the bHLH-ZIP group of transcription factors. This family includes among other factors the Myc oncogenes (for review, see [6]), Max [7,8], as well as the upstream stimulatory factors (USF) 1 and 2 [9,10]. Protein-protein interaction within the bHLH-ZIP region results in the formation of homo- and heterodimers that bind the E-box motif, which can lead to either the activation or repression of gene transcription [6]. The CRE/TRE motif consists of a core consensus sequence of 5′-GCGTCA-3′ that is identical to the proenkephalin CRE-2 element [11] and the −296 element of the human c-fos gene [12]. Like the E-box, CRE/TRE elements are recognized by numerous transcription factors including CREB [13], ATF [14], c-Jun [15] and c-Fos [16]. Co-transfection experiments in SK-N-MC cells with a CCK promoter reporter construct have shown that CREB, c-Jun and c-Fos were all able to stimulate promoter activity through the CRE/TRE motif, with maximal stimulation when c-Jun and c-Fos were expressed concurrently [3].

Numerous genes posses both an E-box and an CRE/TRE in their proximal promoter region, including the murine AH receptor gene [17], the rat preprotachykinin-A gene [18], the rat tyrosine hydroxylase gene [19], the human ATF3 gene [20], the promoter region of the human preproenkephalin gene [11] and the human Alzheimer amyloid β-protein precursor gene [21]. Previously we raised the possibility that cross-talk between transcription factors binding the juxtaposed E-box and CRE/TRE motifs of the CCK gene promoter may play a crucial role in CCK gene transcription. Here we describe co-transfection studies involving c-Myc, Max, c-Jun, and c-Fos and provide evidence for negative cooperativity between the two sites upon binding of transcription factors.

2. Materials and methods

2.1. Expression and reporter plasmids

pCMV-Myc, pCMV-USF1 and pCMV-Max, encoding human c-Myc, human USF1 and murine Max (also known as Myn) respectively, in the pcDNA I vector (Invitrogen) have previously been described by Li et al. [22]. The pSV-c-Fos [23] and pRSV-c-Jun [24] expression constructs were obtained from Morten Johnsen (University of Copenhagen, Denmark).

Three previously described reporter constructs encompassing the proximal 100 bp of the human CCK promoter linked to the coding region of the bacterial chloramphenicol acetyl transferase (CAT) gene were used to monitor transcriptional activation/repression. pCCK-100 contains the wild-type CCK promoter in the pCAT-basic (Promega), whereas pCCK-100-ΔE-box and pCCK-100-ΔCRE/TRE possess mutated E-box (5′-CACGTG-3′ to 5′-TACATA-3′) and CRE/TRE motifs respectively (5′-CTGCA-3′ to 5′-CTGAA-3′).

2.2. Cell culture and transfections

All experiments were performed using the human neuroblastoma cell line SK-N-MC (obtained from the Department of Pathology, University of Uppsala, Sweden) which expresses CCK at a high level [25]. Cells were cultured in 40.5% Dulbecco's minimal Eagle's medium, 40.5% Ham's F-12 medium (GIBCO BRL) supplemented with 15% fetal calf serum (GIBCO BRL), 1% penicillin, 1% streptomycin, 1% glutamine and 1% non-essential amino acids. A total of 25 μg of DNA, including 5 μg of one of the three reporter constructs and 2 μg of pCMVβ-gal, were mixed with 2.5×10^6 exponentially growing cells using a calcium phosphate precipitation procedure [26]. When required, pBluescript (Stratagene) was to standardize the quantity of DNA used in the transfections. Briefly, the DNA was suspended in 1 ml 0.125 M $CaCl_2$, 25 mM *N*,*N*-bis(2-hydroxyethyl)-

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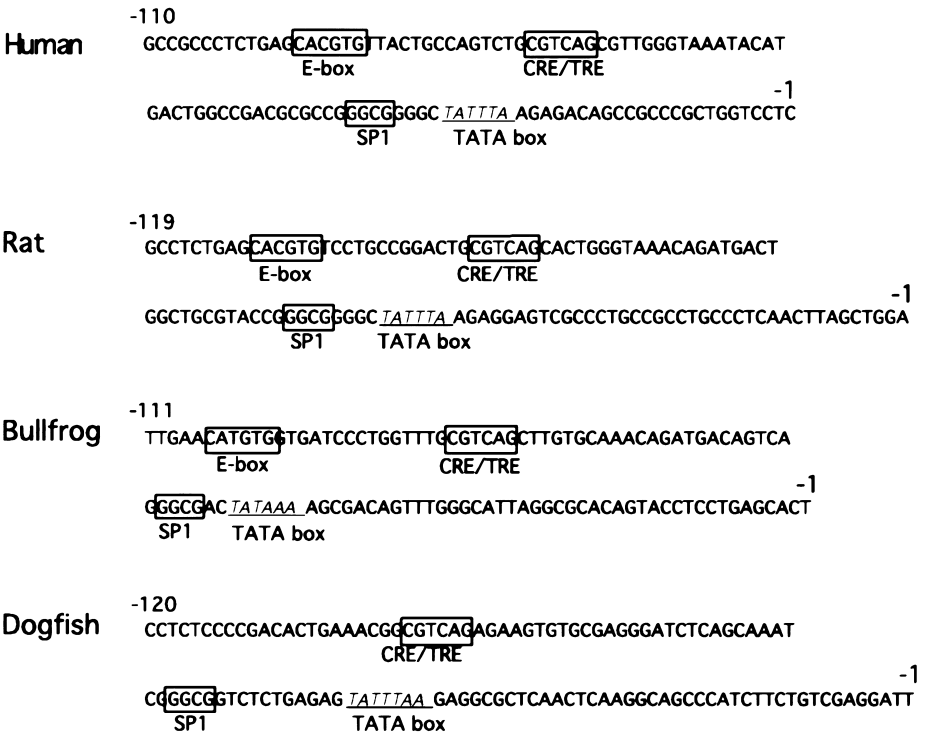

Fig. 1. Comparison of the proximal promoter regions of the human [36], rat [2], bullfrog [4] and dogfish [5] CCK genes. The consensus sequences for E-box, CRE/TRE, Sp1 and TATA box elements are underlined and labelled appropriately.

2-aminoethane-sulfonic acid (pH 6.95), 140 mM NaCl and 0.75 mM Na_2HPO_4 and left at room temperature for 20 min. This solution was then added to the cells and incubated overnight at 35°C in 3% CO_2 atmosphere. The following morning, fresh media were added and the cells incubated for 24 h at 37°C in 10% CO_2 atmosphere.

Stimulations were performed in fully complemented media and included 25 ng/ml basic fibroblast growth factor (bFGF) (Promega) and/or 10 μM forskolin (Sigma) added 6 h before harvesting of the cells.

2.3. CAT and β-galactosidase assays

Cell extracts were prepared from transfected SK-N-MC cells as outlined by Gorman et al. [27]. CAT activity was determined utilizing the CAT Enzyme Assay System with Reporter Lysis Buffer (Promega, USA) by following the procedure outlined by the supplier. β-galactosidase production was measured to normalize for transfection efficiency as previously described [28].

3. Results

3.1. Effect of bHLH-ZIP factors and CREB on CCK transcription

Independent expression plasmids encoding three members of the bHLH-Zip transcription factor family, c-jun and c-fos were transfected into SK-N-MC cells in conjunction with pCCK-100 to analyse their influence on reporter gene expression (Table 1). At the highest transfection rates USF1 (5 μg) resulted in a 3.6-fold stimulation of CAT activity above basal. In comparison c-Myc produced a 1.8-fold increase in promoter activity, whereas Max had no effect on promoter activity (data not shown). Co-expression of c-Myc and Max enhanced promoter activity 2.5-fold above basal. In accordance with the known binding site of bHLH-Zip transcription factors, mutation of the E-box within the proximal promoter region resulted in a reduction of USF1 mediated activation, as well as co-expressed c-Myc and Max, induced reporter gene expression (Table 1). Finally c-Jun and c-Fos increased transcription more than 40-fold via the CRE/TRE.

3.2. Mutation of E-box element stimulates activation via the CRE/TRE element

The influence of factors binding the E-box and CRE/TRE elements on activation via the juxtaposed element was examined firstly by mutation of the two elements in the CCK-100 construct (Table 1). Inactivation of the E-box resulted in a 2–3-fold enhancement of activation by c-Jun/c-Fos, whereas activation by USF1 was increased 2-fold by mutation of the CRE/TRE element. Promoter activity in the presence of c-Myc and Max was unchanged after mutation of the CRE/

Table 1
Effect of mutating the E-box and CRE/TRE binding sites on CCK gene promoter activity

Transcription factor	CCK-100 wild-type (fold induction)	CCK-100-ΔE-box (fold induction)	CCK-100-ΔCRE/TRE (fold induction)
c-Fos/c-Jun (2.5/2.5 μg)	42.7 (4.6)	90.0 (8.6)	5.2 (2.0)
USF1 (5 μg)	3.6 (0.4)	2.1 (0.1)	6.8 (0.4)
Myc/Max (3.75/1.25 μg)	2.5 (0.2)	1.7 (0.2)	2.1 (0.7)

The reporter constructs, pCCK-100 (wild-type), pCCK-100-ΔE-box and pCCK-100-ΔCRE/TRE were each co-transfected with either pRSV-c-Jun/pSV-c-Fos, pCMV-USF1 or pCMV-Myc/pCMV-Max. All data have been normalized to the level of β-galactosidase, and are presented as fold stimulation above CAT activity when reporter construct was transfected alone. Results are expressed as the mean of four experiments ± S.E.M.

TRE site and slightly reduced by mutation of the E-box (Table 1).

To examine the effect of the E-box motif on the CRE/TRE element under physiological conditions, SK-N-MC cells were transfected with wild-type pCCK-100, pCCK-100-ΔCRE/TRE, pCCK-100-ΔE-box reporter constructs and exposed to basic fibroblast growth factor (bFGF) or forskolin, that stimulates promoter activity via the CRE/TRE [29] (Fig. 2). CAT activity was stimulated 2-fold by bFGF, 10-fold by forskolin and 20-fold by bFGF and forskolin together. Following mutation of the CRE/TRE element, activation was almost completely inhibited, demonstrating that activation is mediated via the CRE/TRE element. However, as observed above, mutation of the E-box motif enhanced the stimulatory effect of each of the stimulants and their synergistic activation 2–3-fold.

3.3. *Co-expression of c-Myc and Max inhibits activation by c-Jun/c-Fos*

Since mutation of the E-box motif in the proximal promoter reporter construct resulted in enhanced promoter activity, we also examined the effect of expressing c-Myc and Max with c-Jun and c-Fos. The presence of c-Myc and Max greatly reduced the ability of c-Jun and c-Fos to activate reporter gene expression (Fig. 3). The repression was dependent on DNA binding, since c-Myc and Max expression no longer affected the ability of co-expressed c-Jun and c-Fos to stimulate CCK gene promoter activity when the experiment was repeated utilizing the pCCK-100-ΔE-box plasmid.

4. Discussion

The present study has examined the function of transcription factors binding to the evolutionary conserved and juxtaposed E-box and CRE/TRE elements in the human CCK gene promoter.

The mammalian CCK gene and the related gastrin gene are

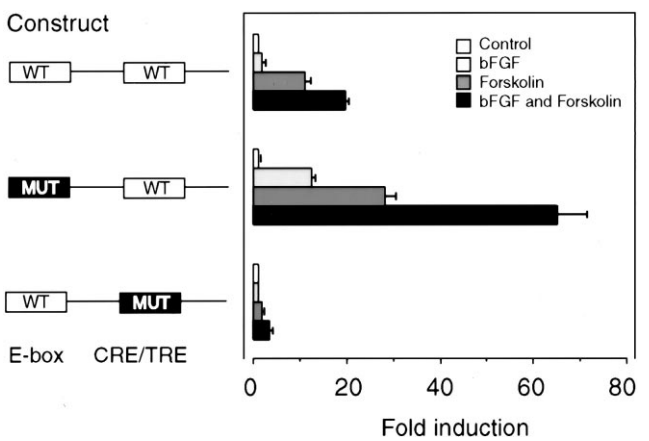

Fig. 2. The influence of mutating the E-box and CRE/TRE binding sites of the CCK promoter on bFGF and forskolin stimulated CAT activity. SK-N-MC cells transfected with either the pCCK-100, pCCK-100-ΔE-box or pCCK-100-ΔCRE/TRE reporter constructs were treated with bFGF (25 ng/ml), forskolin (10 μM) or both agents 6 h before harvesting of the cells. All data have been normalized to the expression level of β-galactosidase, and are presented as fold stimulation above CAT activity when reporter construct was transfected alone. Results are expressed as the mean of four experiments ± S.E.M.

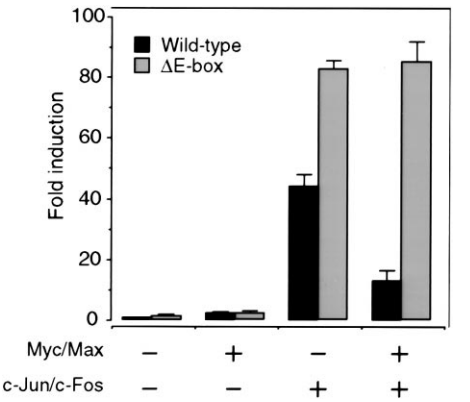

Fig. 3. Mutation of the E-box motif within the human CCK gene promoter abrogates the ability of c-Myc/Max to reduce c-Jun/c-Fos activation of CCK-CAT reporter gene expression. pCMV-Myc (3.75 μg) and pCMV-Max (1.25 μg) where transfected without and with 2.5 μg of both the pRSV-c-Jun and pSV-c-Fos expression constructs in the presence of the pCCK-100 or pCCK-100-ΔE-box reporter constructs. All data have been normalized to the expression level of β-galactosidase, and are presented as fold stimulation above CAT activity when reporter construct was transfected alone. Results are expressed as the mean of four experiments ± S.E.M.

believed to have originated via gene duplication events which occurred before, or perhaps during, the appearance of cartilaginous fish [5]. Both the gastrin and CCK genes in dogfish possess putative CRE/TRE binding sites whereas the gastrin genes from bullfrogs, mice and humans lack this motif [4,5]. Therefore it could be argued that the appearance of an E-box element in CCK genes and the loss of a CRE/TRE site in gastrin genes from higher vertebrates have played a role in the evolution of cell specific expression of these genes.

We examined the influence of factors binding to the E-box and CRE/TRE elements on the activation of the juxtaposed element in three situations. First, the activity of c-Jun/c-Fos and bHLH-ZIP factors was shown to be increased when the juxtaposed elements were mutated. Second, the effect of bFGF and forskolin, that activate the promoter via the CRE/TRE in a CREB dependent manner [29] was demonstrated to be enhanced following mutation of the E-box and finally co-expression of Myc and Max was shown to completely block activation by c-Jun/c-Fos. Taken together the results show that simultaneous binding of bHLH-ZIP and members of the CREB and AP-1 family of transcription factors exhibit a negative cooperative effect on the activation of the juxtaposed element. Consequently the amount of transcription factors such as c-Myc/Max and c-Jun/c-Fos may dictate in vivo CCK gene transcription.

How the negative cooperative effect is accomplished is unclear. The spacing between the E-box and CRE/TRE elements is conserved between the bullfrog and mammalian CCK promoters, with the core of the elements being separated by 12 bp or ~3.4 nm in ordinary B-DNA. Crystal data have shown that the bHLH-DNA binding domain binds to the major groove of the symmetrical CACGTG sequence in B-DNA [30,31]. The structure of the c-Jun/c-Fos binding complex is similar in the sense that the N-terminal region of the heterodimer also makes base specific contacts with the DNA in the major groove [32]. Although the effects of Myc and Max were dependent on binding of the heterodimer to DNA, suggesting that the negative cooperativity is the result of steric hindrance,

this is not directly supported by the crystallographic data or previous footprinting experiments [3]. Another possibility is therefore that the factors compete for putative co-factors or the general transcription complex e.g. the multiprotein complex TFII-D that associate with both USF [9], Myc [33] and members of the leucine zipper family [34,35].

The proximal promoter regions of many other genes possess both E-box and CRE/TRE binding sites. However we have not found studies similar to the present where the interplay of factors binding these motifs has been analyzed. Consequently our observation that proteins binding the E-box have a negative influence on promoter activation through the CRE/TRE element of the human CCK gene may have implications for many other genes which possess these regulatory elements in close proximity, such as the human amyloid β-protein precursor gene promoter [21], the murine AH receptor gene promoter [17], the rat tyrosine hydroxylase gene promoter [19] and the human activating transcription factor 3 gene promoter [20].

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