An upstream stimulatory factor (USF) binding motif is critical for rat preprotachykinin-A promoter activity in PC12 cells

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We demonstrate the presence of a functional E box motif in the proximal rat preprotachykinin-A (rPPT) promoter. This element (spanning nucleotides –67 to –47) exhibits the sequence 5'-CACGTG-3' which is recognized and bound by the basic helix-loop-helix family of regulatory proteins. We also show that at least one of the factors bound to this rPPT promoter element in both HeLa and PC12 nuclear extract is the ubiquitously expressed transcription factor, the upstream stimulatory factor (USF).

Mutation of this element by insertion of a 10 bp linker into the E box motif, in an rPPT promoter fragment spanning –865 to +92, destroys the ability of this promoter fragment to support reporter gene expression in a PC12 cell model of rPPT promoter activity. The data indicate that this rPPT E box element is likely to function as an important *cis*-regulatory domain in the rPPT promoter.

INTRODUCTION

Tachykinins are a family of biologically active neuropeptides which are widely but selectively distributed throughout the nervous system, where they may function as neurotransmitters [1]. The rat preprotachykinin-A (rPPT) gene encodes the neuropeptides substance P, neurokinin A, neuropeptide K and neuropeptide γ , which are derived by alternative splicing of primary RNA transcripts and post-translational processing of the peptide precursors [2]. The rPPT gene is also expressed in a variety of non-neuronal cells, including pituitary [3], adrenal gland [4], thymus [5], Leydig cells [6] and eosinophils [7]. Expression of this gene is regulated by a variety of stimuli including nerve growth factor [8,9], conditioned media [10,11], inflammation [12] and cocaine [13]. It is likely that many of these stimuli act at the level of transcription. Therefore, this gene offers an excellent opportunity to study both tissue-specific and stimulus-inducible gene expression. The major problem for analysis of rPPT promoter activity has been the lack of a clonal cell line which either expressed the endogenous gene (to significant levels) or allowed expression of a reporter gene supported by fragments of the rPPT promoter ([14] and C. F. Morrison and J. P. Quinn, unpublished work). However, we have recently generated PC12 cell line models for analysis of the latter, which are detailed below.

The promoter region spanning nucleotides –865 to +92 is sufficient for expression of a reporter gene when microinjected into adult rat dorsal root ganglia (DRG) neurons grown in culture or electroporated into PC12 cells [14–16]. Candidate DNA sequences which might act as transcriptional regulators of the rPPT promoter have been previously suggested from DNA sequence analysis [17,18]. However, not all candidate DNA regulatory sequences suggested from consensus sequences have been demonstrated to be actually bound specifically by tran-

scription factors and to modulate transcription levels. We have set out to determine transcription factor interactions within the promoter and to determine their role in rPPT gene expression, both tissue-specific and stimulus-inducible. Exonuclease analysis of a 3.8 kb fragment of the rPPT promoter has revealed the existence of multiple sequence-specific protein–DNA interactions [19]. All but one of these sites occurred in similar locations within the rPPT promoter, independent of the source of cell extract used, suggesting that the use of material from clonal cell lines should allow us to determine several of the *cis*-acting elements within the rPPT promoter. DNAase I footprint analysis has further delineated some of these sites [20,21] and has allowed us to further characterize potential *cis*-acting regulatory elements likely to be involved in the control of rPPT promoter activity.

This analysis has determined multiple elements which might act as activator or enhancer elements. These elements, where tested, have functioned to support reporter gene expression in both clonal cell lines and primary cultures of DRG neurons [22,23]. We have also by a similar analysis demonstrated that repression of the rPPT promoter in clonal cell lines (as assayed by reporter gene expression) is associated with multiple silencer regions [14,23]. Definition of these repressor elements was determined from DNAase I footprint analysis followed by subsequent functional analysis. Silencer or repressor domains have been demonstrated in a number of other neuropeptide genes studied [24–26]. One of the rPPT silencer elements which appears to act as a dominant repressor of rPPT promoter activity in PC12 cells is located between the TATA box and the major transcriptional start site [14]. For this reason, we have used an alternative approach, based on our characterization *in vitro* of protein–DNA interactions within the rPPT promoter, to determine putative functional elements. We have excised domains from the rPPT promoter identified as sites for sequence-specific DNA binding by transcription factors and inserted them into a

Abbreviations used: rPPT, rat preprotachykinin-A; USF, upstream stimulatory factor; bHLH, basic helix-loop-helix; DRG, dorsal root ganglia; IPTG, isopropyl β -D-thiogalactoside; EMSA, electrophoretic mobility-shift assay; CRE, cyclic AMP response element; AP1, activator protein 1; CAT, chloramphenicol acetyltransferase; DMEM, Dulbecco's modified Eagle's medium; CGRP, calcitonin gene-related peptide.

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heterologous promoter construct linked to a downstream reporter gene. This allows us to determine both the ability of these domains to stimulate transcription and the signal transduction pathways which may regulate that activity. This approach has been used successfully in the identification of multiple cyclic AMP response element/activator protein 1 (CRE/AP1)-like enhancer elements present within the rPPT promoter [22,23].

A strong candidate transcriptional regulator appears in the rPPT gene spanning -67 to -47 relative to the major transcriptional start site of the rPPT gene. This element is similar to the E box consensus sequence CANNTG, originally defined as a *cis*-regulatory element of immunoglobulin gene expression [27]. An oligonucleotide corresponding to this region has previously been demonstrated to support reporter gene expression in HeLa and PC12 cells when linked to a heterologous minimum promoter [14]. E box elements are recognized and bound by a family of proteins which have in common a basic helix-loop-helix (bHLH) domain [28,29]. These proteins have frequently been shown to be involved in the control of tissue-specific gene expression and the determination of cell lineage. They include c-Myc and related proteins, ubiquitously expressed and related regulatory proteins E12/E47 and the upstream stimulatory factor (USF), the muscle-specific factor MyoD, the *Drosophila* neurogenic achaete-scute complex and the murine neurogenic proteins MASH and NSCL [29-36]. Heterodimerization between ubiquitous and cell-lineage-specific family members has been proposed as a mechanism for the regulation of tissue-specific gene expression.

This paper sets out to determine the transcription factors which might bind the proximal E box motif spanning nucleotides -67 to -47, and whether this element plays a role in our PC12 model of rPPT promoter activity.

MATERIALS AND METHODS

Electrophoretic mobility-shift assays (EMSA)

Nuclear extract was prepared [37] and EMSA was performed as described previously [38,39]. Briefly, each reaction contained 0.5 ng of ³²P-3'-end-labelled double-stranded oligonucleotide probe. This end-labelled probe was mixed with 20 µg of protein extract which had been pre-incubated on ice with 1 µg of poly(dI-dC)·poly(dI-dC). Where indicated, antibodies were added at this point. Antibodies to the bHLH proteins c-Myc and USF were purchased from Santa Cruz Biotechnology, Inc. The anti-USF antibody will recognize both forms of the protein (43 and 44 kDa) which have been characterized. Reactions were incubated for 15 min at room temperature and separated on a 6% non-denaturing polyacrylamide (29:1) gel.

Preparation of bacterial cell extract

A bacterial vector expressing the bHLH protein Max was the gift of Drs. F. Fisher and C. R. Goding (Marie Curie Institute, Oxted, Surrey, U.K.). Expression of Max directed by the T7 promoter was induced in the lambda lysogen BL21(DE3) (40 ml of culture) at $A_{600} = 0.3$ by addition of isopropyl β-D-thiogalactoside (IPTG) to a final concentration of 0.4 mM. Incubation at 37 °C was continued for 2 h after which the bacterial cells were pelleted and extracts prepared. As a control, BL21(DE3) lacking the Max expression construct were treated in the same way. Extracts were prepared by resuspending the bacterial pellets in 2 ml of lysis buffer (25 mM Hepes, pH 7.9/5 mM 2-mercaptoethanol/10% glycerol/0.1 mM EDTA/1 mM PMSF) and sonicated on ice for three 15 s periods. Supernatants were recovered following centrifugation at 40000 rev./min (TLA 100.2 rotor in TL100 ultracentrifuge) for

10 min at 4 °C. Extracts were stored at -70 °C and 20 µg of protein was used in EMSA.

Oligonucleotide probes and competitors

The oligonucleotides used in this study correspond to the following double-stranded DNA sequences. The E box spanning -67 to -47 within the rPPT promoter; oligonucleotides 472/3:

5'TCGAGGAGAGTGTACGTGGCTCTCC3'

3'CCTCTCACAGTGCACCGAGAGGAGCT5'

The A-T rich sequence from the rPPT promoter spanning -672 to -651; oligonucleotides 782/3:

5'TCGATTTCCCCTAAATGTAAACAAA3'

3'AAAGGGGATTACATTTTGTGTTAGCT5'

The somatostatin CRE [40]:

5'GATCGTGACGTCAGG 3'

3'CACTGCAGTCCCTAG5'

The oligonucleotide pairs representing the rPPT E box and the somatostatin CRE were radiolabelled by extension to the 3'-ends and used as probes in EMSA.

Construction of reporter gene constructs

Chloramphenicol acetyltransferase (CAT) reporter gene constructs PPT22 and MINPPT have been described previously [16]. Briefly, these constructs contained rPPT promoter fragments spanning -865 to +92 (PPT22) and -47 to +92 (MINPPT). The construct PPT22 was linearized at the E box sequence CACGTG at -60 in the rPPT promoter by the enzyme *Eco*72I which cleaved the sequence CACGTG between the central C and G residues. This linear PPT22 molecule was religated in the presence of a 10 bp *Bam*H1 linker oligonucleotide (CCGG-ATCCGG). The insertion of a single copy of this linker into this proximal E box motif was confirmed by DNA sequence analysis. This mutated rPPT promoter construct was termed p22mut.

Cell growth and electroporation

PC12 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 15% heat-inactivated horse serum and 5% heat-inactivated calf serum. HeLa cells (for preparation of nuclear extract) were grown in DMEM supplemented with 10% heat-inactivated calf serum. PC12 cells were transfected by electroporation in their normal growth medium using a Bio-Rad gene pulser apparatus with a capacitance extender. Each electroporation contained 20 µg of reporter construct and 20 µg of pCH110. The reporter gene construct contained the CAT gene and pCH110 (Pharmacia) supported expression of the β-galactosidase gene by the SV40 promoter. Electroporation parameters were determined empirically for PC12 cells; 10×10^6 cells were electroporated at 200 V/960 µF. Each electroporation was then divided into four populations seeding 2.5×10^6 cells/collagen-coated 35 mm tissue-culture dish, two of which were appropriate were exposed to a combination of forskolin and potassium-evoked depolarization whereas the other two served as controls for the basal level of transcriptional activity before exposure to stimuli. Forskolin was used at 10 µM final concentration. Depolarization of PC12 cells was achieved using final concentrations of 10 mM CaCl₂ and 40 mM KCl. Transfection efficiencies were normalized by assay for β-galactosidase activity.

Experiments were performed three times in duplicate using cells of the same or close passage number. Levels of CAT expression were analysed using a CAT (Kit, Boehringer Mannheim), according to the manufacturer's instructions.

RESULTS

An E box element exists within the rPPT proximal promoter

DNAase 1 footprinting analysis of the rPPT promoter had previously indicated a putative E box element spanning -67 to -47 relative to the major transcriptional start site [14,20]. An oligonucleotide corresponding to this element had previously been demonstrated to support reporter gene expression when linked to a heterologous minimum promoter [14]. This element exhibits the consensus sequence 5' CACGTG3' required for strong DNA binding by characterized E box-binding proteins belonging to either the ubiquitous USF factors or the c-Myc sub-family of bHLH proteins [41–43]. Synthetic oligonucleotides (472/3), representing this protected rPPT promoter sequence, were used to further characterize this region *in vitro*.

To confirm that this E box element can be recognized and bound by E box-binding proteins, oligonucleotides representing the rPPT E box were used as a probe in EMSA using the well-characterized bHLH protein Max, expressed as a fusion protein in a bacterial cell extract. Max is a member of the c-Myc sub-family of bHLH proteins which recognizes and binds to E box elements, with preference for the canonical sequence 5' CACGTG3' [44,45]. EMSA using bacterial cell extracts (Figure 1) shows that a complex is formed with the rPPT E box probe only when Max is present in the extract (compare lanes 1 and 2). The sequence specificity of this interaction is confirmed by the fact that complex formation is only competed by homologous

Figure 1 The rPPT E box is recognized and bound by the bHLH protein, Max

The rPPT E box (oligonucleotides 472/3) was used as a probe in EMSA. Lanes 2–8 contain BL21 bacterial cell extract where Max is expressed as a fusion protein. Lane 1 contains BL21 extract from IPTG-induced cells only. Homologous competitor (oligonucleotides 472/3) was included at a molar excess of 10, 50 and 100 in lanes 3, 4 and 5 respectively. Heterologous competitor (oligonucleotides 782/3) was included at a molar excess of 10, 50 and 100 in lanes 6, 7 and 8 respectively.

Figure 2 The rPPT E box is recognized and bound by specific proteins in both HeLa and PC12 cells

EMSA using HeLa (a) or PC12 (b) cell extract and the rPPT E box probe are shown. Homologous competitor (oligonucleotides 472/3) was included at a molar excess of 10, 50 and 100 in both (a) and (b), lanes 2, 3 and 4 respectively. Heterologous competitor (oligonucleotides 782/3) was included at a molar excess of 50, 100 and 200 in both (a) and (b), lanes 5, 6 and 7 respectively.

(lanes 3–5) and not heterologous (lanes 6–8) oligonucleotide sequences. These data confirm that the rPPT proximal promoter contains an E box element spanning -67 to -47 relative to the transcriptional start site which is recognized and bound by a bHLH protein in a sequence-specific manner.

This motif can support reporter gene expression in both HeLa and PC12 cells when linked to a heterologous minimum promoter [14]. The interaction of this motif with transcription factors was therefore analysed in both HeLa and PC12 cells. The rPPT E box is bound specifically by multiple protein complexes present in both HeLa and PC12 cells. The formation of at least two complexes between HeLa cell proteins and the rPPT E box probe was observed (indicated by the arrow in Figure 2a). These complexes were displaced only by homologous oligonucleotide competitor (lanes 2–4), indicating their sequence specificity. These results indicate that the rPPT E box is recognized and bound with high affinity by sequence-specific DNA-binding proteins present in HeLa cells. A similar pattern was observed in PC12 cells (Figure 2b) where complex formation was competed by unlabelled homologous (lanes 2–4), but not heterologous (lanes 5–7), oligonucleotide competitor. The mobility of complexes binding the rPPT E box appears similar in both cell types, although it is more diffuse in PC12 cell extract.

The rPPT E box is recognized and bound by the bHLH transcription factor USF which is present in both HeLa and PC12 cells

Sequence-specific DNA-binding to a *cis*-regulatory element may be dictated by a combination of: (1) the precise sequence of the core DNA motif; (2) DNA sequences flanking the core; and (3) the repertoire of transcription factors present. The core sequence of the rPPT E box element suggests that it is likely to be recognized by one or more members of the c-Myc sub-family of bHLH proteins or the USF factor. The potential involvement of

Figure 3 USF binds the rPPT E box

(a) EMSA using HeLa and PC12 cell extracts and the rPPT E box as a probe. Reactions were pre-incubated with antibodies to c-Myc (lanes 2 and 5) or USF (lanes 3 and 6) as indicated. (b) EMSA using HeLa cell extract and a probe representing the somatostatin CRE is shown. Reactions were pre-incubated with antibodies to c-Myc (lane 2) and USF (lane 3) as indicated.

two proteins from this group, c-Myc and USF, was tested in EMSA by the use of antibodies. Figure 3(a) shows EMSA using the rPPT E box as a probe with extracts from HeLa cells (lanes 1–3) and PC12 cells (lanes 4–6). Recognition of the rPPT E box probe (lanes 1 and 4) was specifically competed by addition of anti-USF antibody (lanes 3 and 6) but remained unaffected by the addition of antibody specific for c-Myc (lanes 2 and 5). Competition, rather than super-shift, of the protein–DNA complex by anti-USF antibody has also been observed previously [46]. The specificity of competition by the anti-USF antibody is confirmed in Figure 3(b) which shows EMSA using HeLa cell extract and a probe representing the somatostatin CRE. Recognition of this probe by HeLa cell proteins (lane 1) remains unaffected by the addition of antibody specific for either c-Myc (lane 2) or USF (lane 3). These results indicate that the rPPT E box is recognized and bound by the bHLH transcription factor USF which is present in both HeLa and PC12 cells.

The proximal rPPT E box element functions as a major determinant of rPPT promoter activity in PC12 cells

We had previously demonstrated that an rPPT promoter fragment spanning –865 to +92 could support reporter gene expression under certain conditions in PC12 cells. These involved either removal of a dominant repressor from the region spanning the TATA box to the transcriptional start site [14], or the ability of the rPPT promoter to respond to the synergistic action of multiple stimuli, including that of forskolin and potassium-evoked depolarization [16]. We used the latter of these two models to determine whether the E box motif spanning –67 to –47 played a role in the response of the promoter to forskolin and depolarization. To do this we made a mutation of this E box element in the context of the reporter construct pPPT22 which

Table 1 Mutation of the proximal E box motif in the rPPT promoter eliminates promoter activity in PC12 cells

The constructs PPT22, p22mut and MINPPT were electroporated into PC12 cells which were then exposed to the synergistic action of forskolin and potassium-evoked depolarization (F/D). The amount of CAT produced is given in fold increase over basal growth conditions. Experiments were done three times in duplicate. The error indicates the standard deviation.

Construct	Fold increase in response to F/D
PPT22	60 ± 10
p22mut	5 ± 2
MINPPT	1

contains the rPPT promoter fragment from –865 to +92. This mutation was made by insertion of a 10 bp linker into the E box motif as described in the Materials and methods section; this construct was termed p22mut. As can be seen in Table 1, the pPPT 22 construct as before was able to support high expression of the reporter gene, whereas little or no reporter gene activity was directed by the construct, p22mut, containing the mutation.

DISCUSSION

An E box motif, present within a region spanning nucleotides –67 to –47 in the rPPT proximal promoter, has been shown to be recognized by the well-characterized bHLH protein Max *in vitro*. We have also shown that this element is bound in a sequence-specific manner by the bHLH transcription factor USF which is present in both HeLa and PC12 cells. This is consistent with our previous functional analysis which showed that this E box motif functions as a constitutive activator of transcription in HeLa cells and in the neuronal-derived cell line PC12 [14]. This motif plays a role in rPPT promoter activity, as witnessed by mutation of this element within a promoter fragment spanning –865 to +92 in a reporter gene construct which supported little or no expression in a PC12 model of rPPT promoter activity.

USF, originally purified as a complex from HeLa cells [41,47,48], comprises two polypeptides of apparent molecular mass 43 and 44 kDa which both belong to the bHLH protein family, and the cDNAs encoding these have subsequently been cloned [49,50]. Both USF polypeptides are expressed ubiquitously although the relative ratios of homo- and hetero-dimers formed between the two polypeptides has been shown to vary between different cell lines and a putative tissue-specific variant has been observed in muscle [49]. Although USF is a ubiquitously expressed transcription factor it has been implicated in playing a role in tissue-specific gene expression. In particular a recent study has suggested a role for this molecule in the cardiac muscle gene expression programme [51]. There is also evidence that bHLH molecules are important for regulation of calcitonin gene-related peptide (CGRP) [52–54]. CGRP, like PPT, is expressed in a sub-population of neurons in rat DRG and can be regulated by nerve growth factor *in vivo* and in primary neuronal cultures.

Specificity of binding to the rPPT E box may be due to the precise sequence of the E box element itself, or may involve recognition of flanking sequences in addition to the core motif. The strong preference exhibited for the rPPT E box sequence 5' CACGTG 3' is typical of that displayed by members of the c-Myc family of transcription factors, many of which are present in HeLa cells, and includes Max and its heterodimeric partners c-Myc, Mad and Mxi 1 as well as USF. The presence of a T residue immediately 5' or an A residue immediately 3' of this core sequence has been shown to be highly unfavourable for binding

by c-Myc/Max heterodimers *in vitro* but does not affect binding by Max homodimers [42,43]. It is interesting to note that the rPPT E box is 5'-flanked by a T residue, suggesting that it will be recognized well by Max homodimers but poorly by heterodimers of Max and c-Myc. Our EMSA data are consistent with this hypothesis. Clearly, sequences flanking the core E box motif are important for selective transcription-factor binding. Inverted repeated sequences flank the rPPT E box and are protected from DNAase I digestion as well as the core motif [20]. It is possible that these sequences may contribute to the specificity and/or the affinity of factors binding to the E box, which may regulate the functional activity of this region of the rPPT promoter *in vivo*. This would be consistent with previous data which demonstrated that the two other E box motifs which are present 5' of the transcriptional start site do not support reporter gene expression when assayed as individual elements although they do bind bHLH proteins in EMSA ([23] and J. M. Paterson and J. P. Quinn, unpublished work).

The precise repertoire of transcription factors present in the cell is likely to modulate the function of the rPPT E box. An interesting feature of the bHLH E box-binding family of regulatory proteins is their capacity to form either homodimeric or heterodimeric complexes with certain other members of the family. These properties are also held by other families of regulatory proteins including the AP1/CREB/ATF (ATF, activator transcription factor), C/EBP and octamer-binding families of transcription factors [55,56]. Interaction between transcription factors from different families has been documented [57–59] and been shown to synergistically regulate transcription [52]. Synergy between overlapping AP1 and E box motifs has been shown to be required for tissue-specific expression of the rat tyrosine hydroxylase gene [60]. This flexibility of interaction between transcription factors may facilitate tissue-specific regulation of gene expression. Using such a mechanism, it is conceivable that the E box element at position –60 may have a role in the tissue- and/or stimulus-specific regulation of rPPT gene expression operating at the level of transcription. HLH proteins, in synergy with octamer-binding transcription factors, have been shown to regulate cell-specific expression of the calcitonin/CGRP gene in thyroid C cells [52]. The observation of multiple protein complexes observed in EMSA might reflect binding of the rPPT E box motif by different homo- or hetero-dimeric combinations of factors or, alternatively, post-translational modification of a specific factor or heterodimer. It is also possible that the relative ratios of USF proteins in different cell types contributes to this. Alternatively, the ubiquitous bHLH protein USF may interact with a more discretely distributed factor.

Mutation of the proximal E box motif in the rPPT promoter fragment spanning –865 to +92 completely eliminated the ability of this fragment to support reporter gene expression in PC12 cells in response to the synergistic action of forskolin and potassium-evoked depolarization. This was surprising to us given that we had previously demonstrated other activator domains in this region of the promoter [22,23]. Indeed one of these elements, spanning –200 to –180, has been demonstrated to support further increased levels of gene expression in a heterologous reporter gene construct in PC12 cells exposed to forskolin and depolarization [22]. Therefore, the function of the E box-binding complex at this site in the promoter in response to such stimuli might be either to synergize with other promoter elements or to counteract the action of the repressor elements described above within the rPPT promoter. Further characterization of the rPPT promoter will allow us to understand the role of the bHLH group of proteins in both tissue-specific and stimulus-induced expression of this gene.

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