Regulation of human prohormone convertase 2 promoter activity by the transcription factor EGR-1

Erik JANSEN, Torik A. Y. AYOUBI, Sandra M. P. MEULEMANS and Wim J. M. VAN DE VEN[1]

Laboratory for Molecular Oncology, Center for Human Genetics, University of Leuven and Flanders Interuniversity Institute for Biotechnology, Herestraat 49, B-3000 Leuven, Belgium

Prohormone convertases are involved in the tissue-specific endoproteolytic processing of prohormones and neuropeptide precursors within the secretory pathway. In the present study, we have isolated genomic clones comprising the 5′-terminal region of the human prohormone convertase 2 (PC2) gene and established characteristics of the *PC2* promoter region. The proximal promoter region is very G + C-rich and does not contain a canonical TATA box or a CAAT box. Transient expression assays with a set of human *PC2* gene fragments containing progressive 5′ deletions demonstrate that the proximal promoter region is capable of directing high levels of neuroendocrine-specific expression of reporter gene constructs. In addition, we show that the transcription factor EGR-1 interacts with two distinct elements within the proximal human *PC2* promoter region. Transfection experiments also demonstrate that EGR-1 is able to enhance *PC2* promoter activity.

INTRODUCTION

A variety of regulatory peptides and proteins are generated from inactive precursors by endoproteolytic processing. This endoproteolytic cleavage, generally at sites consisting of paired basic amino acid residues, is a common post-translational modification of membrane and secretory proteins on the exocytotic transport route. Such proteins include precursors of peptide hormones, neuropeptides, growth factors, coagulation factors, serum albumin, cell surface receptors, adhesion molecules and viral glycoproteins. All these proteins play important roles in a large variety of different biological processes, and their function depends on proteolytic cleavage of their respective precursor molecules (for reviews, see [1,2]). In mammals, seven prohormone- and proprotein-processing enzymes responsible for this cleavage have been molecularly characterized.

The mammalian prototype of this enzyme family is furin [3,4], which is involved in the cleavage of precursor molecules within the constitutive secretory pathway. Structure and expression of the *FUR* gene, encoding furin, have been analysed in detail ([5] and references therein). It has been shown that the *FUR* gene is expressed in a wide variety of tissues and cell lines. Recently, we have shown that *FUR* gene transcription is regulated by multiple promoter regions [6]. In addition to furin, the very recently cloned member of the prohormone convertase (PC) enzyme family, LPC [7], described as PC7 by Seidah et al. [8], also exhibits a ubiquitous expression pattern and is capable of processing substrates within the constitutive secretory pathway.

In contrast with this, PC1 [9,10], also described as PC3 [11], and PC2 [9,12] are neuroendocrine-specific [13]. Recently, we have shown that neuroendocrine-specific human *PC1* gene expression and its hormonal regulation are directed by the proximal promoter region [14,15]. PC1 and PC2 have been shown to be involved in the tissue-specific processing of prohormones and neuropeptide precursors, e.g. proglucagon, proinsulin, prosomatostatin, proenkephalin and pro-opiomelanocortin (POMC) in the regulated secretory pathway [16–22]. It has been demonstrated that the cell-type-specific processing of the multifunctional precursor protein POMC by PC1 and PC2 is downregulated in anti-sense transfection experiments in pituitary corticotroph-derived AtT-20 cells [23]. It has very recently been shown that PC1 and PC2 are involved in the differential processing of the proneurotensin/proneuromedin precursor protein [24].

Understanding the mechanisms governing neuroendocrine-specific expression of the human *PC2* gene requires knowledge of the promoter functions of the gene. In this paper, we report the cloning and sequencing of the promoter region of the human *PC2* gene. Upon transfection analysis the *PC2* promoter has been shown to direct neuroendocrine-specific expression of the luciferase reporter gene. In addition, evidence is provided that *PC2* promoter activity is regulated by the zinc finger transcription factor EGR-1 [25]. We report the identification of two distinct binding sites for EGR-1 in the proximal region of the *PC2* promoter and demonstrate that EGR-1 functions as a transcriptional activator after binding to these sites.

MATERIALS AND METHODS

Library screening and sequence analysis

A human genomic library in Lambda FIXII (Stratagene) was screened with a ^{32}P-labelled probe spanning 266 bp from the 5′ end of the published human *PC2* cDNA sequence [9,12]. Inserts were subcloned into pGEM vectors (Promega) and characterized by Southern-blot analysis by standard procedures [26]. The 5 kb nucleotide sequence of the human *PC2* gene containing 4.5 kb of 5′-flanking region, the complete first exon and part of the first intron was determined in both senses of the DNA using the AutoRead Sequencing and Automatic Laser Fluorescence protocols (Pharmacia). Evaluation of the sequence data was performed with computer programs in the Lasergene package (DNASTAR). Scanning of the promoter region for consensus binding sites of transcription factors was performed using Signal Scan 3.0 [27] and Transcription Factor Data Base release 7.3 [28].

Abbreviations used: EMSA, electrophoretic mobility-shift assay; PC, prohormone convertase; POMC, pro-opiomelanocortin; RT-PCR, reverse transcriptase PCR.

[1] To whom correspondence should be addressed.

The 4.5 kb nucleotide sequence reported in this paper has been submitted to the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under the accession number U73595.

Northern-blot analysis and reverse transcriptase (RT)-PCR

Total RNA was isolated from AtT-20, β-TC3 and COS-1 cells using the guanidinium thiocyanate procedures [29]. RNA (20 μg) was size-fractionated through a 1% agarose gel, blotted on to Hybond-N membranes (Amersham), and hybridized to a human *PC2* cDNA probe by standard procedures [26]. Polyadenylated RNA was selected using the poly-ATtract mRNA isolation system (Promega). RT-PCR, allowing amplification of the complete EGR-1-coding region of 1.6 kb, was performed with the first-strand cDNA synthesis primer 5′-TTAGCAAATTTCAATTGTCCTGGGAGAAAAGG-3′ and PCR primers 5′-ATGGCAGCGGCCAAGGCCGAG-3′ and 5′-GATGTCTCCGCTGCAGATCTCTGACCC-3′. The products were analysed on a 1% agarose gel. The integrity of the PCR products was verified by nucleotide sequence analysis using standard procedures [26].

Reporter plasmid construction

Human *PC2*-luciferase fusion gene expression plasmids were constructed by subcloning into the polylinker of the promoterless luciferase-encoding plasmid pGL2-Basic (Promega). The 3′ end of all promoter constructs is at position +137 bp relative to the position of the major transcription-start site [30].

Conditions for cell culture and transfection

AtT-20 pituitary corticotroph cells (ATCC; CRL 1795), β-TC3 insulinoma cells [31] and COS-1 kidney fibroblasts (ATCC; CRL 1650) were cultured according to the suppliers' protocols. DNAs were purified using anion-exchange chromatography (Nucleobond AX; Machery-Nagel). Unless otherwise indicated, cells were propagated in the prescribed medium supplemented with 10% fetal calf serum. Cells were transfected using cationic liposomes (Lipofectamine; Life Technologies) according to the manufacturer's protocol. Cells were harvested 24 h after the start of the transfection, and luciferase reporter enzyme activity driven by the various human *PC2* promoter fragments was determined with the Luciferase Assay System (Promega) using a Monolight 2010 luminometer (Analytical Luminescence Laboratory). For each experiment, luciferase activity was determined in triplicate wells and normalized for transfection efficiency in the different cell lines. The results are expressed as the mean of four individual transfection experiments. In transfection experiments in which the effect of EGR-1 on *PC2* promoter activity was analysed, β-TC3 cells were shifted to serum-free medium 48 h before the start of the transfection. The effect of the EGR-1 transcription factor on *PC2* promoter activity was analysed by co-transfecting plasmids containing the *PC2* promoter (1 μg/well) with 100 ng of the plasmid pCMVzif [32], which directs EGR-1 expression. In control experiments pCMVzif was replaced by 100 ng of the corresponding empty cloning vector pCMV-5 [32].

In vitro transcription/translation and EMSA analysis

The EGR-1-encoding DNA fragment as present in pCMVzif was subcloned into pcDNA3 (In Vitrogen), and full-length EGR-1 was synthesized using rabbit reticulolysate in the coupled *in vitro* transcription/translation system T7-TNT (Promega) according to the manufacturer's protocol.

Binding assays were carried out for 10 min at room temperature in a 20 μl reaction mixture containing 25 mM Hepes, pH 7.9, 60 mM KCl, 2 mM $MgCl_2$, 0.1 mM EDTA, 0.5 mM dithiothreitol, 4 mM spermidine, 0.1 mM $ZnCl_2$, 50 μg/ml double-stranded poly(dI-dC), 10% glycerol and 100 μg/ml BSA. Labelled double-stranded probe (0.5 ng) was added, and the incubation was continued for 20 min. For antibody-supershift experiments, binding mixtures were incubated with monospecific anti-EGR-1 antibody (Santa Cruz; Santa Cruz, CA, U.S.A.). Protein–DNA complexes were analysed on 4% non-denaturing polyacrylamide gels at 4 °C in 0.5 × Tris/borate/EDTA, and visualized by autoradiography.

Complementary oligonucleotides corresponding to the human *PC2* gene 5′-flanking DNA sequences −216 to −195 bp, relative to the major transcription start site (BS-1, 5′-AAAGGGGCGGGGGGCGCGGGCTC-3′) and sequences −129 to −108 bp (BS-2, 5′-GGGTGGGCGGGGGGCGGGCCCGG-3′) were obtained from Pharmacia. Double-stranded oligonucleotides were radiolabelled using [γ-^{32}P]ATP (6000 Ci/mmol; Dupont–NEN) and T4 polynucleotide kinase (Boehringer-Mannheim) and used as probes in the above binding assays.

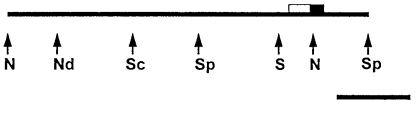
RESULTS

Cloning and nucleotide sequence analysis of the 5′-flanking region of the human *PC2* gene

On screening of a human genomic library with a human *PC2* 5′ cDNA fragment, four independent clones were isolated and shown to be overlapping. These clones cover 12 kb of the 5′-flanking region, the complete first exon and the 5′ part of intron 1. Fragments comprising 4.5 kb of the upstream region, the complete first exon and part of the first intron were subcloned for sequencing purposes. Figure 1 (top) shows a schematic representation of the 5′ part of the human *PC2* gene and flanking region.

Sequence analysis of the total 4.5 kb nucleotide sequence revealed the presence of two segments within the distal promoter region showing significant homology (> 95%) with human repetitive elements. In more detail, a repeat homologous to the THE1B family of repeats is identified at −3.6 kb, relative to the translation-initiation codon, and an Alu repeat is present at −1.9 kb.

The major transcription-start site of the human *PC2* gene is located 312 bp upstream of the translation-initiation codon with a minor transcription-start site located 28 bp more downstream




```
-232 GAGCTCTCCGGTGCTGAAAGGGGCGGGGGGCGCGGGCTCGCTGCGCTGCGCTGCGCTGCCG
-172 TGCGCCCCCAGAAGGCGGAGGCGGCCGGGCCGGGCCGGGCCGGGGGTGGGCGGGGGCGGG
-112 CCCGGGGCGGCCCAGGGCGGGGCCCGCCGCTTCCCCCGCCCTCATTGGCCGCTGGCGGAC
 -52 TCACGTCACCCGAGCTGGTGCTCTCCCCCAGCCCCTAGGGAATTGGAGCTGAGGAGGAGC
  +9 TGAAAATGCAGATTTAGCATCAAGCACAGACCTACACTCGCTCTTTCTCTCCGGTACACA
 +69 CAGCTCCCCACATTCGCACCCCTGCCCGCGCGCCGGGCCGCCTGACTGCACGGCTTCCCC
+129 TCCAGCCAGATGCTGGAGAACACACACTGATTCGCTGCTTTCCAAGACCCTGTTCAGTCT
+189 CTTTCTCTATACAAAGATTTTTTTAAAAACTATATATAAGAATTCTTTATTTGCACCCTC
+249 CCTCCGAGTCCCCTGCTCCGCCAGCCTGCGCGCCTCCTAGCACCACTTTTCACTCCCAAA
+309 GAAGGATGAAGGGTGGTTGTGTCTCCCAGTGGAAGGCGGCCGC
```

Figure 1 Structure of the 5′-flanking region of the human *PC2* gene

Top, restriction map of a DNA fragment containing the first exon and adjacent sequences. Exon 1 is represented as a box with the shaded area corresponding to coding sequences. Relevant restriction endonuclease sites are indicated: N, *Not*I; Nd, *Nde*I; S, *Sac*I; Sc, *Sca*I; Sp, *Sph*I. Bottom, DNA sequence of the proximal promoter of the human *PC2* gene. The major transcription-start site is underlined. The translation-initiation codon is indicated in bold. Potential EGR-1-binding sites are double underlined.

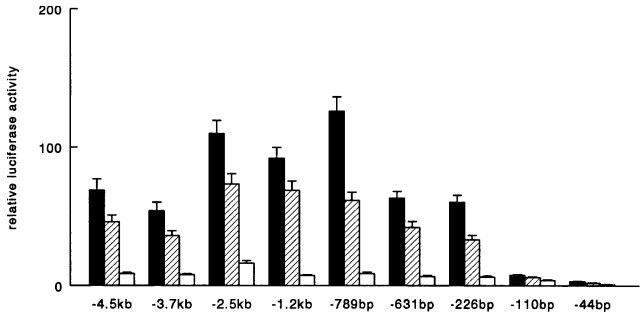


Figure 2 Deletion analysis of the human *PC2* gene promoter

PC2 gene fragments containing progressive 5′ deletions and a common 3′ end at nucleotide +137 relative to the transcription-start site were inserted upstream of the promoterless luciferase gene in the pGL2-Basic vector. Promoter activity of each *PC2*–luciferase fusion gene in β-TC3 (solid bars), AtT-20 (hatched bars) and COS-1 cells (open bars) was determined. Values are normalized for transfection efficiency and represent the mean ± S.E.M. for four independent transfection experiments, each of which was performed in triplicate.

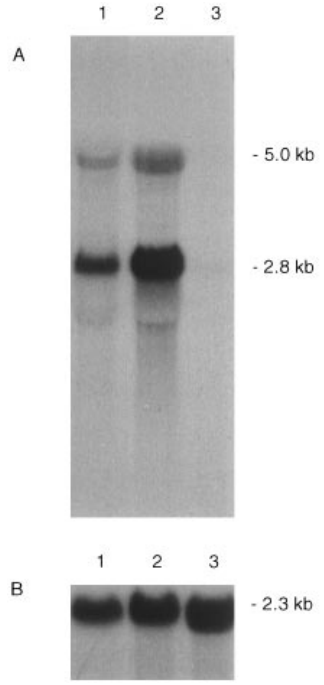


Figure 3 Northern-blot analysis of *PC2* gene expression

(**A**) Northern blot of 20 μg of total RNA isolated from AtT-20 cells (lane 1), β-TC3 cells (lane 2) and COS-1 cells (lane 3). Hybridization with a ^{32}P-labelled human *PC2* cDNA probe. (**B**) Same Northern blot, subsequently hybridized with a mouse actin cDNA probe.

[30]. Examination of the nucleotide sequence of the proximal 5′-flanking DNA revealed that the most prominent feature is the high G+C content ($\geqslant 80\,\%$) of the proximal 200 bp, directly upstream of the major transcription-start site and the absence of canonical TATA or CAAT boxes within the proximal 5′-flanking region. Scanning of the proximal region for potential transcription-factor-binding sites revealed the presence of two potential binding sites for the zinc finger transcription factor EGR-1. These sites were identified at −210 bp and −123 bp (5′-GCGGGGGCG-3′), relative to the position of the major transcription-start site (Figure 1, bottom). Although the expression of this transcription factor, which is also known as Zif268 [33], NGFI-A [34], Krox-24 [35] and TIS 8 [36], has been extensively studied, only a few genes have been identified as specific target genes [32,37–39]. A detailed functional analysis of the human *PC2* promoter is presented in the next sections of this report.

Neuroendocrine-specific expression directed by the human *PC2* gene promoter

To test whether the 5′-flanking region of the human *PC2* gene is capable of directing neuroendocrine-specific gene expression, a fusion gene construct was made containing 4.5 kb of 5′-flanking sequence of the human *PC2* gene fused to the promoterless luciferase gene as present in pGL2-Basic. The resulting construct was assayed for promoter activity into several cell lines of neuroendocrine as well as non-neuroendocrine origin. As a negative control, the promoterless luciferase-encoding plasmid pGL2-Basic was used in parallel transfections of each cell line. In all cell lines tested, transfection of the pGL2-Basic DNA resulted in very low basal activity. As shown in Figure 2, the highest levels of *PC2* promoter-driven luciferase activity were observed in β-TC3 insulinoma cells and slightly lower levels were observed in AtT-20 cells. The relative expression of the transfected reporter genes coincides with the observed relative expression levels of the endogenous *PC2* gene, which is the highest in β-TC3 cells, as determined by Northern-blot analysis (Figure 3). In addition, luciferase activity was 8–10-fold lower in the non-neuroendocrine COS-1 cells that do not express the endogenous *PC2* gene (Figure 3). With two other non-neuroendocrine cell lines, 3T3-L1 and HeLa, which also do not express the endogenous *PC2* gene, similar results were obtained as illustrated by a comparable 9–11-fold lower promoter activity (results not shown). This neuroendocrine-specific expression of the *PC2* promoter construct suggests that the 4.5 kb of 5′-flanking sequence contains signals for directing cell-type-specific expression of the human *PC2* gene. As shown in Figure 2, sequential deletion of the promoter region between position −4.5 and −789 bp resulted in a slight increase in promoter activity. However, additional deletion of the *PC2* promoter region up to −631 bp resulted in a 2-fold fall in luciferase activity in β-TC3 cells, whereas this effect was less pronounced in AtT-20 cells. Further reduction of the upstream sequence to −226 bp did not clearly affect promoter activity, but additional 5′-deletion up to −110 bp decreased promoter activity by 6-fold in β-TC3 cells and 3-fold in AtT-20 cells, indicating that the DNA sequence between −226 and −110 bp contains positive regulatory elements, particularly active in β-TC3 cells and enhancing *PC2* expression. No significant difference was observed in COS-1 cells. Further reduction of the *PC2* promoter region up to −44 bp resulted only in a 1.5-fold fall in promoter activity. The activity of this minimized promoter construct is almost identical with the activity of the promoterless pGL2-Basic plasmid.

In summary, the region between −226 and −1 represents a minimal core promoter that is capable of directing neuroendocrine-specific expression of the human *PC2* gene.

Regulation of *PC2* promoter activity

Close inspection of the nucleotide sequence of the proximal *PC2* promoter region revealed the presence of two potential EGR-1 transcription-factor-binding sites. The EGR-1 transcription factor binds with high affinity to the sequence 5′-GCGGGGGCG-3′. This sequence is located within the proximal *PC2* promoter region at nucleotide positions −210 and −123 bp (Figure 1B).

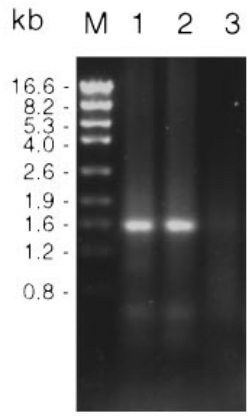


Figure 4 RT-PCR analysis of endogenous EGR-1 expression

RT-PCR with EGR-1-specific primers was performed on RNA isolated from β-TC3 cells (lane 1), AtT-20 cells (lane 2) and COS-1 cells (lane 3). Lane M, molecular-mass markers.

Table 1 Regulation of human *PC2* promoter activity by EGR-1

Human *PC2*-promoter–luciferase reporter constructs containing sequences up to −226 or −110 bp and a common 3′ end at nucleotide +137 were tested in transient transfection experiments of β-TC3 cells. The effect of the EGR-1 transcription factor on *PC2* promoter activity was analysed by co-transfecting with the plasmid pCMVzif, which directs EGR-1 expression. In control experiments, pCMVzif was replaced by the corresponding empty cloning vector pCMV-5. Results are means ± S.E.M.

	Relative luciferase activity	
Construct	−EGR-1	+EGR-1
−226 bp	35 ± 2.9	215 ± 15
−110 bp	14 ± 1.5	13 ± 1.2

This is interesting, since deletion of the proximal *PC2* promoter region from −226 bp up to −110 bp resulted in a 6-fold fall in promoter activity in β-TC3 cells, when assayed in transient transfection experiments (Figure 2). This suggests a potential role for the zinc finger transcription factor EGR-1 in the regulation of *PC2* promoter activity. By means of RT-PCR, expression of the endogenous EGR-1 gene was detected in β-TC3 cells and AtT-20 cells (Figure 4).

To investigate the potential role of EGR-1 in *PC2* promoter regulation, we performed transient transfection experiments with β-TC3 cells in which the −226 and −110 bp *PC2* promoter–luciferase reporter constructs were co-transfected with a plasmid directing EGR-1 expression. As shown in Table 1, co-transfection with the expression plasmid encoding EGR-1 resulted in a 6–8-fold activation of the −226 bp *PC2* promoter construct, as compared with the control transfections in which the empty cloning vector was co-transfected. However, this transcriptional activation by EGR-1 is completely absent when the proximal promoter region is deleted up to −110 bp. This lack of transcriptional activation by EGR-1 coincides with the deletion of the potential binding sites for EGR-1 located within this region at positions −210 and −123 bp. To investigate the observed EGR-1 activation in more detail, we tried to specifically mutate EGR-1-binding sites 1 and 2 by site-directed mutagenesis. However, we were not successful, probably because of the GC-rich nature of both sites and flanking sequences (Figure 1B). Altogether, the above data suggest the EGR-1 is involved in the

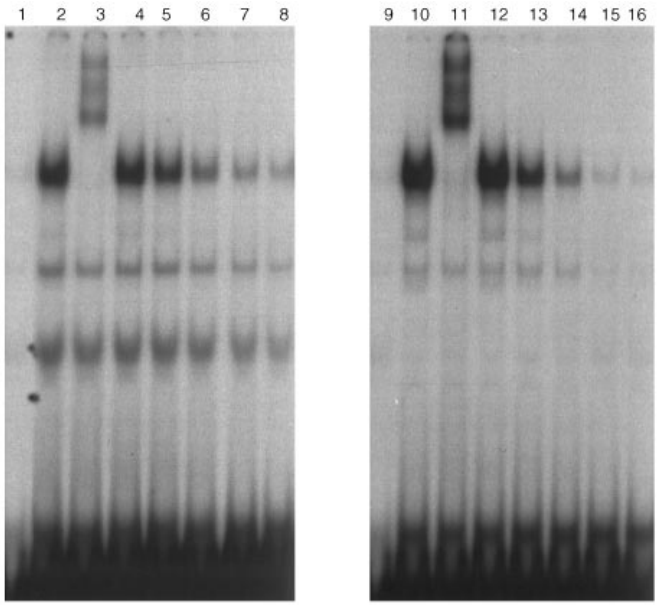


Figure 5 EMSA analysis of the binding of EGR-1 to sequence elements within the proximal region of the human *PC2* promoter

Radioactive labelled double-stranded oligonucleotides BS-1 (lanes 1–8) and BS-2 (lanes 9–16) comprising the putative EGR-1-binding sites 1 and 2 respectively served as probes in the binding experiments. Lanes 1 and 9 represent negative control incubations in which unprogrammed reticulolysate was added to the probe. Lanes 2–8 and lanes 10–16, lysate containing EGR-1 was added to the probe. Lanes 3 and 11, immunoshifts in which EGR-1-specific antibody was added to the incubation mixtures. In the competition experiments (lanes 5–8 and lanes 13–16) unlabelled BS-1 and BS-2 were added in a 10-, 50-, 500- and 1000-fold molar excess respectively.

regulation of *PC2* promoter activity. To investigate whether the observed transcriptional activation of the *PC2* gene is an effect of the interaction of EGR-1 with the proximal promoter elements, we analysed the protein–DNA interactions at both potential EGR-1-binding sites 1 and 2.

Binding of EGR-1 to the proximal promoter region of the *PC2* promoter

To test whether EGR-1 binds to the sequence motifs (Figure 1B) at −210 to −202 bp (site 1) and −123 to −115 bp (site 2) of the proximal promoter region of the human *PC2* gene, we performed electrophoretic mobility-shift assays (EMSAs). In initial experiments we tested nuclear extracts prepared from β-TC3, AtT-20 and COS-1 cells for EGR-1-binding activity using probes comprising sites 1 and 2. However, in these experiments it was found that EGR-1-binding activity was below detection levels (results not shown). The same observations have been made by others [32,38]. Therefore we synthesized the EGR-1 protein by *in vitro* transcription/translation as outlined in the Materials and methods section. Reticulocyte lysate containing the newly synthesized EGR-1 protein was used to assay the binding of the EGR-1 protein to DNA. As an internal control we used reticulocyte lysate without the EGR-1 protein. Radioactive labelled double-stranded oligonucleotides comprising site 1 and site 2 (BS-1 and BS-2 respectively) served as probes in the EMSAs. The results of the binding experiments with probes BS-1 and BS-2 are shown in Figure 5. In the control experiments in which lysate without EGR-1 was added to the probe (lanes 1 and 9), no shift was observed. However, when we used the pro-

grammed reticulocyte lysate containing EGR-1 protein synthesized *in vitro*, a specific protein–DNA complex was observed with both probe BS-1 and probe BS-2 (lanes 2 and 10 respectively). As can be seen in lanes 4–8 and lanes 12–16, complex-formation was efficiently competed for by the addition of an excess of unlabelled BS-1 and BS-2 respectively as specific competitor. To identify the binding protein more specifically, we performed an immunoshift experiment. As can be seen in lanes 3 and 11, the addition of EGR-1-specific antibody resulted in a supershift of the protein–DNA complexes with BS-1 and BS-2 respectively. This provided additional evidence for the binding of EGR-1 to both site 1 and site 2 within the proximal region of the human *PC2* promoter.

DISCUSSION

In an effort to define DNA-regulatory elements controlling neuroendocrine-specific expression of the human *PC2* gene, we cloned and sequenced the 5′-terminal region of the human *PC2* gene, including 4.5 kb of 5′-flanking sequences, the complete first exon and the 5′-part of intron 1. It has been shown that most of the human *PC2* transcripts originate from a single major transcription-start site which is located 312 bp upstream of the translation-start codon [30]. Analysis of the upstream sequence reveals the absence of canonical TATA or CAAT boxes. Moreover, the immediate 5′-flanking region is extremely GC-rich. Apart from housekeeping genes, the above promoter characteristics are also features of an increasing number of cell-type-specific genes, including neuroendocrine-specific genes encoding, e.g., m4 cholinergic muscarinic receptor [40], synapsin I [41] and aldolase C [42].

In addition, it is interesting to note that the human *PC2* promoter is clearly distinct from the human *PC1* promoter, which was analysed in detail in our laboratory [14,15]. This clear lack of similarity may be responsible for the observed differences in developmental onset of expression and differential expression patterns of both genes as observed in tissues and cell lines, resulting in the tissue-specific processing of the respective substrates [13,43–45].

In the present study, we have analysed the 5′-flanking region of the human *PC2* gene for promoter activity. It was demonstrated that this promoter region is highly active in neuroendocrine cells and displays only low activity in non-neuroendocrine cells. Deletion analysis clearly indicated that the core promoter region between −226 and −1 bp, relative to the translation-initiation codon, exhibits substantial neuroendocrine specificity. In addition, in a previous report we demonstrated that neuroendocrine-specific human *PC1* expression is also directed by its proximal promoter region [14]. This aspect of core promoter selectivity has also been observed in several other neuroendocrine-specific genes, like the genes encoding synapsin I [41], synapsin II [38], insulin [46] and POMC [47]. Our data from the transient transfection experiments with the human *PC2* promoter constructs are somewhat different from the results reported in an initial study by Ohagi et al. [30]. In their experiments, they analysed several human *PC2* promoter constructs up to −789 bp for transcriptional activity in β-TC3 cells. It was shown that 5′-deletion of the promoter from −789 to −631 bp resulted in a 2.6-fold fall in promoter activity which is comparable with our data (Figure 2). However, on additional 5′-deletion to −226 bp, promoter activity decreased 2.4-fold. This latter effect was not observed in our experiments. In addition, in contrast with our data, Ohagi et al. [30] did not detect a substantial decrease in promoter activity when the proximal region was further reduced up to −110 bp. This discrepancy may be due to differences in promoter constructs and methods used for measuring reporter gene activity. In more detail, the promoter constructs used by Ohagi et al. [30] comprise a more downstream region up to and including 10 codons of the human PC2-coding region. This may interfere with reporter gene expression. In addition, these investigators used the *CAT* reporter gene to detect promoter activity in transient transfection analysis. The −226 bp construct already displayed low activity in their experiments. This could have interfered with the detection of reduced *PC2* promoter activity resulting from the −226 to −110 bp deletion.

The results of our promoter-deletion analysis pointed towards the presence of important regulatory elements within the *PC2* proximal promoter between −226 and −110 bp, since deletion of this region resulted in a drastic fall in promoter activity, which was specifically observed in the neuroendocrine cell lines. Subsequent inspection of the nucleotide sequence revealed the presence of two potential EGR-1-binding sites: site 1 at position −210 bp and site 2 at position −123 bp. This zinc finger transcription factor, EGR-1, has been shown to interact with a GCGGGGGCG sequence motif. On DNA binding, both activity and repression of transcription have been observed, depending on cell type and promoter context [48]. The results of our deletion analysis of the *PC2* promoter suggest that EGR-1 may be involved in the up-regulation of *PC2* promoter activity. This is interesting, since this protein is known to be induced by various extracellular signals and has been shown to be involved in the transcriptional regulation of several neuroendocrine-specific genes. Moreover, the activities of the proximal promoters of genes encoding acetylcholinesterase [37], synapsin I and -II [32,38] and phenylethanolamine *N*-methyltransferase [39] are regulated by EGR-1. Recently, it was shown that the pituitary-specific promoter of the luteinizing hormone β-subunit gene was activated by EGR-1 [49].

To assess the functional significance of the potential EGR-1-binding sites within the proximal human *PC2* promoter, we performed transfection experiments to investigate the effect of EGR-1 on *PC2* promoter activity. We demonstrated that EGR-1 transactivates the human *PC2* promoter. Deletion of the EGR-1 sites completely abrogated promoter activity. Further evidence for the involvement of EGR-1 in *PC2* promoter regulation was provided by the results of binding experiments. In these EMSAs, we demonstrated the binding of EGR-1 protein to both sites 1 and 2 within the proximal human *PC2* promoter. Therefore the binding and transactivation data reported in this study suggest that EGR-1 plays a role in the regulation of the *PC2* gene. Apart from the reported EGR-1 sites, potential binding sites for Sp1, CTF and ATF/CREB are also found within the human *PC2* promoter region. However, thus far we have no indications for the involvement of these factors in the regulation of *PC2* promoter activity. Most probably, neuroendocrine-specific expression of the *PC2* gene requires a concerted action of multiple *trans*-acting factors. From the data presented in this paper, it is clear that the minimal *PC2* promoter confers neuroendocrine specificity, which might be the effect of multiple DNA-binding proteins interacting with the *PC2* promoter and also with each other and most likely with the basic transcription apparatus.

In summary, this paper describes the neuroendocrine-specific expression of the human *PC2* promoter and its transcriptional activation by transcription factor EGR-1. Further analysis is required to gain a better understanding of the precise role and function of EGR-1 in *PC2* promoter regulation.

We thank Dr. Douglas Hanahan for kindly providing the β-TC3 cell line and Dr. Gerald Thiel for providing the EGR-1 expression plasmids. We are grateful to Marleen

Willems for cell culture. This work was supported by the Geconcentreerde Onderzoekacties 1992–1996, EC-contract ERBCHBGCT 920091, the Vlaams instituut voor de bevordering van het wetenschappelijk-technologisch onderzoek in de industrie (IWT) and the Stichting Technische Wetenschappen (STW-22.2726).

REFERENCES

1 Barr, P. J. (1991) Cell **66**, 1–3
2 Steiner, D. F., Smeekens, S. P., Ohagi, S. and Chan, S. J. (1992) J. Biol. Chem. **267**, 23435–23438
3 Van de Ven, W. J. M., Voorberg, J. T. L., Fontijn, R., Pannekoek, H., Van den Ouweland, A. M. W., Van Duijnhoven, J. L. P., Roebroek, A. J. M. and Siezen, R. J. (1990) Mol. Biol. Rep. **14**, 265–275
4 Van de Ven, W. J. M., Creemers, J. W. M. and Roebroek, A. J. M. (1991) Enzyme **45**, 257–270
5 Van de Ven, W. J. M., Van Duijnhoven, J. L. P. and Roebroek, A. J. M. (1993) Crit. Rev. Oncogen. **4**, 115–136
6 Ayoubi, T. A. Y., Creemers, J. W. M., Roebroek, A. J. M. and Van de Ven, W. J. M. (1994) J. Biol. Chem. **269**, 9298–9303
7 Meerabux, J., Yaspo, M.-L., Roebroek, A. J. M., Van de Ven, W. J. M., Lister, T. A. and Young, B. D. (1996) Cancer Res. **56**, 448–451
8 Seidah, N. G., Hamelin, J., Mamarbachi, M., Dong, W., Tadros, H., Mbikay, M., Chrétien, M. and Day, R. (1996) Proc. Natl. Acad. Sci. U.S.A. **93**, 3388–3393
9 Seidah, N. G., Gaspar, L., Mion, P., Marcinkiewicz, M., Mibikay, M. and Chrétien, M. (1990) DNA **9**, 415–424
10 Seidah, N. G., Marcinkiewicz, M., Benjannet, S., Gaspar, L., Beaubien, G., Mattei, M. G., Lazure, C., Mbikay, M. and Chrétien, M. (1991) Mol. Endocrinol. **5**, 111–122
11 Smeekens, S. P., Avruch, A. S., Lamendola, J., Chan, S. J. and Steiner, D. F. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 340–344
12 Smeekens, S. P. and Steiner, D. F. (1990) J. Biol. Chem. **265**, 2997–3000
13 Seidah, N. G., Chrétien, M. and Day, R. (1994) Biochimie **76**, 197–209
14 Jansen, E., Ayoubi, T. A. Y, Meulemans, S. M. P. and Van de Ven, W. J. M. (1995) J. Biol. Chem. **270**, 15391–15397
15 Jansen, E., Ayoubi, T. A. Y., Meulemans, S. M. P. and Van de Ven, W. J. M. (1997) J. Biol. Chem. **272**, 2500–2508
16 Benjannet, S., Rondeau, N., Day, R., Chrétien, M. and Seidah, N. G. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 3564–3568
17 Thomas, L., Leduc, R., Thorne, B. A., Smeekens, S. P., Steiner, D. F. and Thomas, G. (1991) Proc. Natl. Acad. Sci. U.S.A. **88**, 5297–5301
18 Bennett, D. L., Bailyes, E. M., Nielsen, E., Guest, P. C., Rutherford, N. G., Arden, S. D. and Hutton, J. C. (1992) J. Biol. Chem. **267**, 15229–15236
19 Smeekens, S. P., Montag, A. G., Thomas, G., Albiges-Rizo, C., Carroll, R., Benig, M., Phillips, L. A., Martin, S., Ohagi, S., Gardner, P., Swift, H. H. and Steiner, D. F. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 8822–8826
20 Rouillé, Y., Westermark, G., Martin, S. K. and Steiner, D. F. (1994) Proc. Natl. Acad. Sci. U.S.A. **91**, 3242–3246
21 Brakch, N., Galanopoulou, A. S., Patel, Y. C. and Seidah, N. G. (1995) FEBS Lett. **362**, 143–146
22 Johanning, K., Mathis, J. P. and Lindberg, I. (1996) J. Neurochem. **66**, 898–907
23 Bloomquist, B. T., Eiper, B. A. and Mains, R. E. (1991) Mol. Endocrinol. **5**, 2014–2024
24 Rovère, C., Barbero, P. and Kitabgi, P. (1996) J. Biol. Chem. **271**, 11368–11375
25 Sukhatme, V. P., Cao, X., Chang, L. C., Tsai-Morris, C.-H., Stamenkovich, D., Ferreira, P. C. P., Cohen, D. R., Edwards, S. A., Shows, T. B., Curran, T., Le Beau, M. M. and Adamson, E. D. (1988) Cell **53**, 37–43
26 Sambrook, J., Fritsch, E. F. and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd edn., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
27 Prestridge, D. S. (1993) Comput. Appl. Biosci. **9**, 113–115
28 Ghosh, D. (1993) Nucleic Acids Res. **21**, 3117–3118
29 Chomczynski, P. and Sacchi, N. (1987) Anal. Biochem. **162**, 156–159
30 Ohagi, S., LaMendola, J., LeBeau, M. M., Espinosa, III, R., Takeda, J., Smeekens, S. P., Chan, S. J. and Steiner, D. F. (1992) Proc. Natl. Acad. Sci. U.S.A. **89**, 4977–4981
31 Efrat, S., Linde, S., Kofod, H., Spector, D., Delannoy, M., Grant, S., Hanahan, D. and Baekkeskov, S. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 9037–9041
32 Thiel, G., Schoch, S. and Petersohn, D. (1994) J. Biol. Chem. **269**, 15294–15301
33 Christy, B. A., Lau, L. F. and Nathans, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 7857–7861
34 Milbrandt, J. (1987) Science **238**, 797–799
35 LeMaire, P., Revelant, O., Bravo, R. and Charnay, P. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 4691–4695
36 Lim, R. W., Varnum, B. C. and Herschman, H. R. (1987) Oncogene **1**, 263–270
37 Li, Y., Camp, S., Rachinsky, T. L., Bongiorno, C. and Taylor, P. (1993) J. Biol. Chem. **268**, 3563–3572
38 Petersohn, D., Schoch, S., Brinkmann, D. R. and Thiel, G. (1995) J. Biol. Chem **270**, 24361–24369
39 Ebert, S. N., Balt, S. L., Hunter, J. P. B., Gashler, A., Sukhatme, V. and Wong, D. L. (1994) J. Biol. Chem. **269**, 20885–20898
40 Wood, I. C., Roopra, A., Harrington, C. and Buckley, N. J. (1995) J. Biol. Chem. **270**, 30933–30940
41 Sauerwald, A., Hoesche, C., Oschwald, R. and Killimann, M. W. (1990) J. Biol. Chem. **265**, 14932–14937
42 Thomas, M., Makeh, I., Briand, P., Kahn, A. and Skala, H. (1993) Eur. J. Biochem. **218**, 143–151
43 Marcinkiewicz, M., Ramla, D., Seidah, N. G. and Chrétien, M. (1994) Endocrinology **135**, 1651–1660
44 Scopsi, L., Gullo, M., Rilke, F., Martin, S. and Steiner, D. F. (1995) J. Clin. Endocinol. Metab. **80**, 294–301
45 Neerman-Arbez, M., Cirulli, V. and Halban, P. A. (1994) Biochem. J. **300**, 57–61
46 Walker, M. D., Edlund, T., Boulet, A. M. and Rutter, W. J. (1983) Nature (London) **306**, 557–561
47 Jeannotte, L., Trifiro, M. A., Plante, R. K., Chamberland, M. and Drouin, J. (1987) Mol. Cell. Biol. **7**, 4058–4064
48 Gashler, A. L., Swaminathan, S. and Sukhatme, V. P. (1993) Mol. Cell. Biol. **13**, 4556–4571
49 Lee, S. L., Sadovsky, Y., Swirnoff, A. H., Polish, J. A., Goda, P., Gavrilina, G. and Milbrandt, J. (1996) Science **273**, 1219–1221

Received 28 January 1997/10 June 1997; accepted 4 July 1997