The human vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor 1 (VPAC1) promoter: characterization and role in receptor expression during enterocytic differentiation of the colon cancer cell line Caco-2Cl.20

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The basic organization of the human vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor (VPAC) 1 promoter was investigated after cloning the 5'-flanking region (1.4 kb) of the VPAC1 gene from a human genomic library. Subsequent functional analysis of various deletions of the 5'-flanking sequence, subcloned upstream of a luciferase reporter gene, was carried out in HT-29 cells. The minimal promoter region identified encompasses the -205/+76 sequence and contains a crucial CCAAT box (-182/-178) and a GC-rich sequence. Moreover a region (-1348/-933) containing a silencer element was identified. We previously showed that the expression of the VPAC1 receptor binding site is strictly dependent upon the enterocytic differentiation of human colon cancer Caco-2 cells [Laburthe, Rousset, Rouyer-Fessard, Couvineau, Chantret, Chevalier and Zweibaum (1987) *J. Biol. Chem.* **262**, 10180–10184]. In the present study we show that VPAC1 mRNA increases dramatically when Caco-2Cl.20 cells differentiate, as measured by RNase protection assays and reverse

transcriptase-PCR. A single transcript species of 3 kb is detected in differentiated cells by Northern-blot analysis. Accumulation of VPAC1 receptor mRNA is due to a 5-fold increase of transcription rate (run-on assay) without a change in mRNA half-life (9 h). Stable transfections of various constructs in Caco-2Cl.20 cells and subsequent analysis of reporter gene expression, during the enterocytic differentiation process over 25 days of culture, further indicated that the -254/+76 5'-flanking sequence is endowed with the regulatory element(s) necessary for transcriptional regulation of VPAC1 during differentiation. Altogether, these observations provide the first characterization of the basic organization of the human VPAC1 gene promoter and unravel the crucial role of a short promoter sequence in the strict transcriptional control of VPAC1 expression during differentiation of human colon cancer Caco-2 cells.

Key words: epithelium, intestine, PACAP, VIP.

INTRODUCTION

The neuropeptide vasoactive intestinal peptide (VIP) is widely distributed in the central and peripheral nervous systems and has a large spectrum of biological actions [1]. VIP interacts with two different receptors now referred to as human vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor (VPAC) 1 and VPAC2 [2], because they display very similar affinity for VIP and another regulatory peptide, the pituitary adenylate cyclase-activating peptide (PACAP) [3]. VPAC receptors belong to the class II G-protein-coupled receptor (GPCR) family [4,5], which include receptors for a family of peptides structurally related to VIP, such as glucagon and secretin as well as peptides unrelated to VIP, such as parathyroid hormone (PTH) and calcitonin [4]. This subfamily has been recently extended to the putative epidermal growth factor module-containing mucin like-peptide hormone receptor ('EMR1') [6], the leucocyte activation antigen CD97 [7] and the α -latrotoxin receptor [8].

Structure–function relationship studies of human VPAC receptors have provided evidence that the N-terminal extra-

cellular domain is crucial for VIP binding and correct delivery of the receptor to the plasma membrane [9–11]. Other functional domains for VIP binding [12,13] and peptide selectivity [14] have also been identified in extracellular loops and the third transmembrane segment of the human VPAC1 receptor. Finally, constitutively active mutants of the human VPAC1 receptor have been generated by point mutations at junctions of the second [15] and fourth [16] transmembrane segments with intracellular loops. While the functional roles of several domains of the VPAC1 receptor protein are now documented, very little is known about the VPAC1 receptor gene and its regulation. It is known that the human VPAC1 receptor gene has a complex organization with many introns [17], like genes encoding all class II GPCRs [4]. The human VPAC1 receptor gene spans 22 kb and is composed of 13 exons [17]. Although the 5'-flanking region of the human VPAC1 receptor gene has been sequenced [17], no functional studies are currently available regarding the basic properties of the receptor promoter.

It has been known for many years that differentiation of intestinal epithelial cells is associated with significant changes in

Abbreviations used: AU, arbitrary units; CMV, cytomegalovirus; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; GPCR, G-protein-coupled receptor; GRE, glucocorticoid response element; RT-PCR, reverse transcriptase-PCR; PACAP, pituitary adenylate cyclase-activating peptide; PTH, parathyroid hormone; PTHrP, PTH-related peptide; VIP, vasoactive intestinal peptide; VPAC, vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide receptor; VIPR-RP, VPAC1 receptor repressor.

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The nucleotide sequence data reported here have been submitted to the GenBank®, EMBL, DDBJ and GSDB Nucleotide Sequence Databases under the accession number AJ012074.

expression of cell surface receptors [18–23], including VIP receptors [24,25]. Moreover, intestinal epithelial cells represent a physiologically relevant target of VIP since the neuropeptide promotes water and electrolyte secretion and mucus output, and also exerts control over metabolism and proliferation in intestinal epithelium [4,26]. We have shown previously that expression of VPAC1 receptors is strictly dependent upon enterocytic differentiation of human colon cancer Caco-2 cells [25]. Differentiated cells express a high amount of VPAC1 receptors whereas undifferentiated cells show a very low density of receptors [25]. The accumulation of VPAC1 receptors during Caco-2 cell differentiation represents the crucial event in the development of the adenylate cyclase response to VIP [25]. The Caco-2 cell model represents an invaluable cellular model [26] for the study of the regulation of human VPAC1 receptor expression inasmuch as it is reminiscent of the situation found in normal human small intestine where mature villus cells express more VPAC1 receptors [intestinal epithelial cells and colon cancer express the VPAC1 but not the VPAC2 receptor subtype (J.-J. Maoret, unpublished work)] than proliferative and undifferentiated crypt cells [24].

In this context, the present study has a 2-fold objective (1) to analyse the basic organization of the human VPAC1 receptor gene promoter and (2) to investigate the mechanism whereby VPAC1 receptors accumulate upon enterocytic differentiation of Caco-2 cells. We provide evidence for regulation of mRNA level by strict transcriptional control, and we have characterized the crucial role of the human VPAC1 promoter in this regulation.

MATERIALS AND METHODS

Materials

All chemicals and reagents were purchased from Sigma (St Louis, MO, U.S.A.), Bioprobe (Paris, France), Interchim (Asnières, France), Gibco-BRL (Cergy-Pontoise, France) or Promega (Madison, WI, U.S.A.) unless otherwise indicated. Radioactive isotopes (^{125}I , ^{32}P and ^{35}S) were purchased from Amersham (Les Ulis, France). Native VIP was purchased from Neosystem (Strasbourg, France). ^{125}I -VIP was labelled as previously described [11].

Cell culture

The Caco-2Cl.20 cell clone was obtained by cloning of parental Caco-2 cells (A.T.C.C. no. HTB-37; passage 20) using the limiting dilution method. Briefly, a monodispersed cell suspension was distributed to microtest plates at a mean ratio of 0.9 cells/well. Among several clones obtained clone 20, referred to as Caco-2Cl.20, was used in further studies. Cultured cell lines HT-29 (A.T.C.C. no. HTB-38; passages 165–172) and Caco-2Cl.20 (passages 6–15) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal calf serum (FCS) for HT-29 cells and 20% FCS/1% (v/v) non-essential amino acids for Caco-2Cl.20 cells.

Immunofluorescence studies, measurement of enzyme activities and cell layer resistivity

Indirect immunofluorescence using monoclonal anti-(dipeptidyl peptidase IV) antibody or polyclonal anti-(sucrase isomaltase) antibody [27] was performed on cells grown on glass coverslips according to a previously published technique [15]. Optical sections through Caco-2Cl.20 cells were performed in the plane perpendicular (xz plane) to the cell monolayer using a Leica TCS 4D confocal fluorescence microscope. After preparation of cell homogenates [25], sucrase isomaltase (EC 3.2.1.48) and dipeptidyl peptidase IV (EC 3.4.14.5) activities were determined

as described in [27]. Cell-layer resistivity was measured using a Millicell-ERS resistivimeter (Millipore, Bedford, MA, U.S.A.) on cells grown on Transwell clear membrane (Corning, Brumath, France).

Ligand binding assay

Cell homogenates and ligand binding assays were prepared as previously described [15]. Specific binding was calculated as the difference between the amount of ^{125}I -VIP bound in the absence and in the presence of 1 μM unlabelled VIP. Protein was determined using the Bio-Rad protein assay (Bio-Rad, Hercules, CA, U.S.A.).

Detection of mRNA: RNase protection assay, Northern-blot analysis and reverse transcriptase (RT)-PCR

For the RNase protection assay, cDNA encoding the human VPAC1 receptor, subcloned into a pcDNA I vector, was linearized by *NheI* before transcription. A human β -actin cDNA subcloned into a pcDNA I vector (Invitrogen, Leek, The Netherlands) served as a template for an antisense probe of human β -actin mRNA. Templates were transcribed with Sp6 RNA polymerase, yielding antisense probes of 414 bases for the human VPAC1 receptor and 156 bases for human β -actin. The protected fragments were 354 bases and 123 bases, respectively. The RNase protection assay was performed as described in [28].

RT-PCR experiments and Northern-blot analysis for the VPAC1 receptor were performed exactly as described in [29]. Amplification of mRNA encoding the G_{α} subunit was performed as previously described [29] using 5'-GCAATTATTACTGTTTCGGTTGGCT-3' as the sense oligonucleotide and 5'-GCGTTCTTTACGAACAGCCAAGCC-3' as the antisense oligonucleotide.

Measurement of RNA stability and run-on assays

RNA stability was determined by incubating Caco-2Cl.20 cells at 37 °C for various periods in the presence of actinomycin D (10 $\mu\text{g}/\text{ml}$). Total RNA was then extracted as described above and quantified using the RNase protection assay. Results were expressed as % of control (time 0 of incubation with actinomycin D) and the VPAC1 receptor half-life was determined when 50% of VPAC1 receptor mRNA was detectable.

Isolation of nuclei and run-on assays were performed exactly as described in [30]. Blots were autoradiographed and quantified using the NIH image 1.62 software. Results were expressed as follows:

$$\frac{\text{AU}_{\text{VPAC1 receptor}} - \text{AU}_{\text{pcDNA I}}}{\text{AU}_{\beta\text{-actin}} - \text{AU}_{\text{pcDNA I}}}$$

where $\text{AU}_{\text{VPAC1 receptor}}$, $\text{AU}_{\beta\text{-actin}}$ and $\text{AU}_{\text{pcDNA I}}$ represent the absorbance in arbitrary units (AU), corresponding to hybridization of the labelled RNA with the VPAC1 receptor probe, the β -actin probe and the pcDNA I vector, respectively.

Promoter cloning and plasmid constructions

A total of 4×10^5 clones of a human placental genomic library in a pWE15 cosmid vector (Stratagene, La Jolla, CA, U.S.A.) were screened, under standard conditions of plaque hybridization [29], with a ^{32}P -labelled cDNA probe encoding the human VPAC1 receptor N-terminal region (bases 1–440). One positive clone (≈ 10 kb size) was selected. The reporter gene eucaryotic expression vector pSNeoLuc was constructed from

the pcDNA3 (Invitrogen) vector by excision of the cytomegalovirus (CMV) promoter (pSNeo vector) and addition of the firefly luciferase reporter gene.

The *Bam*HI–*Sst*II fragment of the 5′-flanking region of the VPAC1 receptor was fused immediately 5′ to the firefly luciferase reporter gene into a pSNeoLuc vector. It was fully sequenced by the dideoxy-chain-termination method using an automatic sequencer (Genome Express, Grenoble, France). The resulting p1424Luc clone corresponding to the sequence –1348/+76 of the human VPAC1 receptor gene was used to perform 5′ deletion constructions. The resulting plasmids were named according to the size of their respective VPAC1 receptor promoter fragments as follows: p1008Luc was obtained by digestion of p1424Luc with *Bst*XI, blunt-ended and religation; p822Luc was obtained by digestion with exonuclease III using the Exo mung bean deletion kit (Stratagene); p330Luc was constructed by digestion of p1424Luc with *Bam*HI–*Xho*I, blunt-ended and religation; p224Δ330Luc was constructed by digestion of p1424Luc with *Xho*I and religation; p295Luc was constructed by digestion of p330Luc with *Stu*I, blunt-ended, digested with *Xba*I and inserted into a pSNeo vector; p330Δ130Luc was constructed by digestion of p330Luc with *Sma*I–*Kpn*I and inserted into a pSNeoLuc vector; p281Luc, p249Luc, pX1T, pX1C1 and pX1C2 were obtained by site-directed mutagenesis as described in [9]; p130Luc was obtained by digestion of p1424Luc with *Sma*I–*Xba*I and inserted into the pSNeo vector. Construction of p4100Luc was carried out by selecting another positive clone with a larger size (10 kb). All constructions were verified by direct sequencing.

Cell transfection and luciferase activity measurement

Cells (HT-29 or Caco-2Cl.20) were transfected with 15 µg of pSNeoLuc or 15 µg of promoter-luciferase gene construct and 1 µg of pRL, containing cDNA coding *Renilla* luciferase, using the calcium phosphate/DNA co-precipitation method following PROFECTION kit instructions (Promega). For Caco-2Cl.20 cells which had to be followed over 25 days during the enterocytic differentiation process, stable transfections were performed by addition of 1 g/l G418 (Gibco-BRL) to the culture medium. After selection, resistant cells were grown in DMEM supplemented with 20 % FCS and 1 % non-essential amino acids for up to 5 days (undifferentiated cells) or 20–25 days (differentiated cells). Luciferase activities (firefly luciferase and *Renilla* luciferase) were measured in cell extracts using a dual-luciferase reporter assay system kit (Promega). *Renilla* luciferase activity in these same extracts was measured to correct for variations in transfection efficiency. Relative luciferase activity of various promoter constructions was calculated as the ratio between firefly luciferase and *Renilla* luciferase activities for each transfection and then reported as % of the ratio determined for the p1424Luc construction. Background luciferase activity was determined with the promoterless vector, pSNeoLuc.

Electrophoretic mobility-shift assays

Cell nuclear extracts were prepared using the method described by Dignam et al. [31]. Synthetic oligonucleotides used for electrophoretic mobility-shift assays corresponded to CCAAT box 1 and 2 sequences. These sequences and those of competitor oligonucleotides used are shown in Table 1. Double-stranded oligonucleotides were ³²P-labelled using T4 kinase in the presence of [γ-³²P]ATP and purified from unincorporated radioactivity by passage through spin-columns (Pharmacia Biotech, Orsay, France). Samples containing 30000 c.p.m. of 5′-end labelled double-stranded oligonucleotides were in-

Table 1 Oligonucleotides used in electrophoretic mobility-shift assays

Name	Sequence
wtC1	5′-AAGGTGTGGGATTGGCAAGAGAGA-3′
wtC2	5′-CCACCCATCCATTGGCTAGATCCGC-3′
muC1	5′-AAGGTGTGGGATCCCAAGAGAGA-3′

cubated for 30 min on ice with 5 µg of various nuclear extracts and 2 µg of poly(dI-dC) in binding buffer [20 mM Hepes buffer/50 mM NaCl/5 mM MgCl₂/1 mM dithiothreitol/20 % (w/v) glycerol/0.1 mM PMSF/2 mM levamisole/0.1 µg/ml aprotinin/0.1 µg/ml bestatin/5 mM benzamidine, pH 7.9]. For competition experiments, a 100-fold molar excess of unlabelled double-stranded oligonucleotides was preincubated with nuclear extracts for 15 min on ice and then ³²P-labelled probe was added for an additional 30 min incubation. All samples were electrophoresed on 6 % non-denaturing TBE (45 mM Tris/borate/1 mM EDTA) acrylamide gels for 2 h at 150 V. Gels were dried and autoradiographed overnight at –80 °C with an intensifying screen.

RESULTS

Cloning of the VPAC1 receptor promoter

In order to determine the basic properties of the VPAC1 receptor promoter, the 5′-flanking region of the VPAC1 receptor gene was cloned. A total of 4 × 10⁵ clones of a human placental genomic library were screened with a ³²P-labelled cDNA probe, coding for the N-terminal region (bases 1–440) of the receptor. Six positive clones were further characterized by restriction mapping and Southern-blot analysis using a ³²P-oligonucleotide probe corresponding to bases 1–63 of the VPAC1 receptor. A fragment of a clone referred to as L1 and representing 1.5 kb upstream of the initiation codon was characterized and fully sequenced (Figure 1). Consensus TATA box, CCAAT boxes and Sp1 sites, as well as other putative regulatory sequences, were identified and are indicated together with the ATG codon in Figure 1.

Functional characterization of the minimal VPAC1 receptor promoter

To determine whether this cloned sequence had promoter activity, a 1424 bp fragment from nucleotide –1348 to +76 (Figure 1) was subcloned upstream of a luciferase reporter gene (p1424Luc). The purified DNA was transfected into human colon cancer HT-29 cells, which express endogenous VPAC1 receptor independently of the time of cell culture and allow reporter gene studies to be undertaken in transiently transfected cells [32]. As shown in Figure 2, the 5′-flanking region of the VPAC1 receptor gene directed a high level of luciferase expression. This activity was about 33-fold higher than the background level determined by transfection of the pSNeoLuc promoterless plasmid (Figure 2). It should be noted that a luciferase construct containing the first 4.1 kb of the VPAC1 sequence upstream of the initiation codon (p4100Luc) exhibited the same level of promoter activity as the p1424Luc construct (Figure 2), supporting the idea that upstream sequences did not contain further functional regulatory element(s), at least when transfected into HT-29 cells.

The sequence borders of VPAC1 promoter activity were determined by using a set of progressively shorter 5′ deletions of

GCCGTTACTA GTGGATCCCC GGGTACCGAG CTCCTGCGCG CCTGCTGTTC -1298
 AGCCAAAGCC TAGCGCGGGA CCTCTTTCAG GTAGCCGCAC ATTCTGGTCA -1248
 GGGCTCTGGG ACCCGCTGCC CTCTCCGCTC CTCACCTGAA CTCGCTCTCT -1198
 CCTTCTGTCA CACGGCTCCG CCAGCCACTC TATCTTGCCG CCGGACCCAC -1148
 ATCTCCAGCC TCGATTGTGC AGGCTCGAGC CCGTAGAAGG AGGGCTAGCA -1098
 GCCACCTGCG CTTGGTCCAC AAGAAGACGC GCTCCAGGGG CGCAGAGCTG -1048
 GTGACCCCGC TCCCATGTC AGGGACGAAT AAACGAGAAA AGGGGCTGAC -998
 CCGGTGTAC CCAGCGGCG AGCAGCGGCT CTTGGAAGC TCAGCACCAA -948
 AGGCAGGCG CCAGGGTCTT GGCCCTCTCT TGGACCCAC TATCCCCAGC -898
 TGTAATAATAG GGAATGACAG AGGAGGACGA CGTCTGTGGT TCGTTTATAG -848
 TCAGTGTGCG AGTCGGCCTG CGGGCTGGGA GCAGACCTCG CTCGCCACGC -798
 GAGGGCGCTG GCACCTTACG CCAGCCTCCC TGGGGAGGTG CCCTGGACGA -748
 GACCCGCGAG GGTGCGGACA CCTCATCTCT CCTGGAGCAG GTCTCCCGAG -698
 GCTTGAAGGG CCAGGGCACC CAACAGGTGC TGAGCGCGCC CCACCAAGTC -648
 ACCCGGGAGC AGGGAACACC AAGGAACCCA GAGTCGGGCG TCTTTCGAGC -598
 CCTCGCTTCC TCTGCGCTGG CTTCTTCGCG GCGAAGGATG AATTGGGGTT -548
 GTTGAAATAT GATCAACAA CCCGATCTCT GTCAGCGCCT GGACACTTGC -498
 TGGGCCCCAG CTCGACGCTT GGGAGATGAA GTGGCACTTC CTGTCTCGTA -448
 ATCGGATTTC CGCGCACGAA CCTCGGAGAC GCACAGGTGG GAAATGGGGA -398
 GGCTGGCGGG GAGGCTCCTT GGAGGAGGTG GCTCCGAGGG TAGACATGAG -348
 GAGCGCCCTG GGATGGGGCT GAGGGTGGAT ACAGAGGTGG GAGCTTGCGA -298
 AGGTCGTGAG CTAAGCATGG AAGGATGGAA TGTGTTCCGG TCTCGAGATT -248
 CCCGGGTGAT GAGCCCGGAG GGGCGAAGGC CTATAGATGT CTCACGACGG -198
 AAGACCAAGG TGTGGGATTC GCAAGAGAG AGCAGAGCTT CCTCACTGCG -148
 NFkB AP-2 Sp1 CCAAT Sp1
 AGGGGCTCCC CAGCCCCGCC CCATACCTGC GCGCGCGGCC CGCTCCACCC -98
 CCAAT Sp1 CREB AP-2 Sp1
 CATCCATTGG CTAGATCCCG CGCCGCTCAG ACGAGCCCCC GGGGCCCGCC -48
 AP-2 ATF
 CCCAGCCCTG ACGTGCCTCT CTTAGCTCTG GGGCCACAGC GCCAGCGCCA 2
 CTCTGCCAGG CTCGCCGCA TCGCCCGCCT GGTGCGCCGC CCGCCAGCTC 52
 TTTCGCCGCG CGGGGCCGCC CGCCGCGGGCTCAGGGCAGACATGCGCCGCCCA ...

Figure 1 Nucleotide sequence of the 5'-flanking region of the VPAC1 receptor gene

Putative binding sites for transcription factors are underlined in bold, the sequence of exon 1 started at +1 is underlined, the transcription start site (−15) identified by Sreedharan et al. [17] is indicated by the arrow and the translation initiation codon in exon 1 is boxed.

Figure 2 Expression of the VPAC1 receptor promoter/luciferase reporter gene in transfected HT-29 cells

Schematic diagrams represent the 5'-flanking region of the VPAC1 receptor gene (p4100Luc and p1424Luc) and deletion constructs fused into upstream regions of the luciferase reporter gene. Each construction was transiently transfected into HT-29 cells. Luciferase activities (firefly and *Renilla*) were measured in cell homogenates and results were normalized to *Renilla* luciferase activity (see Materials and methods section for details). They are expressed as a percentage of the activity obtained with the p1424Luc construct. pSNeoLuc represents the vector alone. Results are the means $\pm$ S.E.M. of 3–5 experiments.

Figure 3 Schematic representation of the VPAC1 receptor promoter

Hatched boxes indicate regions involved in the transcription of the VPAC1 receptor gene (silencer region, CCAAT box sequence and GC-rich region). Open and black boxes represent non-coding and coding regions of the first exon of the VPAC1 receptor gene, respectively. The start of exon 1 was set at +1. ATG and the arrow indicate the translation and transcription start sites, respectively.

the p1424Luc construct. Deletions of the sequences −1348/−933 (p1008Luc vector), −1348/−747 (p822Luc vector), −1348/−255 (p330Luc vector) and −1348/−220 (p295Luc vector) resulted in luciferase activities (approx. 50-fold higher than the background level) which were significantly higher than that observed with the −1348/+76 sequence (p1424Luc vector) (Figure 2). These data supported the theory that the sequence −1348/−933 is endowed with a silencer element. Construction of a vector in which the upstream sequence −1348/−1124 was linked to the −255/+76 sequence (p224Δ330Luc construct) confirmed this view. Indeed, luciferase activity was identical to that observed with the p1424Luc vector but significantly lower than that observed with the p330Luc vector (Figure 2), indicating the presence of silencer element(s) in the −1348/−1124 sequence. Further shorter deletions in the p1424Luc construct were analysed in the context of the presence of some putative regulatory elements such as a TATA box (−216/−211), two CCAAT boxes (−182/−178 and −107/−103) and a GC-rich region (−68/−38). Deletion of the sequence −1348/−206 (p281Luc vector) resulted in a luciferase activity identical to that obtained for the p330Luc vector (Figure 2), supporting the idea that the TATA box is not important for promoter activity. This was confirmed by the fact that mutating this TATA sequence into ATTT in the p330Luc vector did not result in any change in luciferase activity (results not shown). A further deletion between −1348 and −174 (p249Luc vector) resulted in a dramatic decrease in luciferase activity (Figure 2), indicating that the minimal promoter region encompasses the −205/+76 sequence. These data also suggested that the CCAAT box sequence −182/−178 is crucial for promoter activity. This was confirmed by the fact that mutating this CCAAT sequence into GGAAT in the p330Luc vector resulted in an almost complete absence of luciferase activity (results not shown). In this context, we also mutated the other CCAAT box (−107/−103) into GGAAT in the p330Luc vector. This mutation did not change the promoter activity (results not shown), supporting the theory that this second CCAAT box is not functional in the receptor promoter, a conclusion which is in good agreement with electrophoretic mobility-shift experiments (see below). Finally, partial deletion of the GC-rich region by deleting the sequence −54/+76 in the p330Luc vector (corresponding to the p330Δ130Luc vector in Figure 2) which contains the crucial CCAAT box (see above) abolished luciferase activity, supporting the idea that the GC-rich region is instrumental in promoter activity. Taken together these data showed that a minimal 281 bp sequence containing a crucial CCAAT box and a GC-rich sequence upstream of the initiation site of the human VPAC1 receptor is required for promoter activity (Figure 3). They also indicated the presence of silencer element(s) between positions −1348 and −1124 upstream of this minimal promoter (Figure 3).

Figure 4 Gel-retardation experiments using CCAAT-box oligonucleotides and protein extract from HT-29 cells

Electrophoretic mobility-shift assays were performed using nuclear extract (NE) from HT-29 cells ($5 \mu\text{g}$ of protein) incubated with labelled double-stranded wtC1 oligo (**A** and **B**, see Table 1) or wtC2 oligo (**C**, see Table 1) in the presence of $2 \mu\text{g}$ of poly(dI-dC) as described in the Materials and methods section. Binding specificity was established using increasing amounts (10, 25, 50 and 100 times molar excess) of unlabelled competitor oligonucleotide representing CCAAT box 1 (wtC1) (**A**, see Table 1), muC1 (**B**, see Table 1) and CCAAT box 2 (wtC2) (**C**, see Table 1). The incubation media were electrophoresed on a 6% polyacrylamide non-denaturing gel and bands were visualized by autoradiography. Specific complexes are indicated by arrows. Controls without nuclear extract are shown (—NE).

Band-shift analysis of CCAAT box sequences

In order to confirm the functional property of the CCAAT box sequence (−182/−178), an electrophoretic mobility-shift experiment was carried out using sequences between −191 and −91 as probes and HT-29 cell nuclear extracts. As shown in Figure 4(A), three major complexes appeared on the gel when a ^{32}P -oligonucleotide corresponding to sequence −191/−166 (Table 1) was used. The labelling of these three bands could be competitively prevented by preincubation with increasing concentrations of unlabelled oligonucleotide probe, indicating that interactions occurred between nuclear proteins and this promoter region. When the same experiment (Figure 4B) was carried out in the presence of increasing concentrations of unlabelled oligonucleotide mutated at the CCAAT locus (sequence −191/−166, Table 1) the labelling of the three bands was not prevented, demonstrating that nuclear proteins specifically bind to the CCAAT box sequence (−182/−178). In contrast, when a ^{32}P -oligonucleotide corresponding to sequence −116/−91 containing the second CCAAT box sequence (−107/−103, Figure 1) was incubated in the presence of HT-29 cell nuclear extracts, no specific band was detected (Figure 4C), further indicating that this CCAAT box is inactive in the VPAC1 receptor promoter.

Differentiation-dependent regulation of VPAC1 receptor mRNA levels in Caco-2 cells

The human colon adenocarcinoma cell line Caco-2 in culture spontaneously differentiates into enterocytes which exhibit apical brush borders, tight junctions and express brush border hydrolases [26,32]. Table 2 shows some characteristics of differentiation of the Caco-2 cell clone used in this study, which is referred to as Caco-2Cl.20. At day 25 of culture, Caco-2Cl.20 cells, grown on Tranwell clear membrane, exhibited high cell-layer resistivity indicating the establishment of tight junctions between polarized epithelial cells. Moreover, the activities of sucrase isomaltase

Table 2 Measurement of enzyme activities [sucrase isomaltase (SI) and dipeptidylpeptidase IV (DPPIV)], cell-layer resistivity and VPAC1 receptor concentration in Caco-2Cl.20 cells at day 5 and day 25 of culture

* VPAC1 receptor concentration is expressed as B_{max} . The dissociation constant of the VPAC1 receptor was identical at day 5 and day 25 of cell culture, i.e. 0.1 nM.

Day	SI (m-units/mg of protein)	DPPIV (m-units/mg of protein)	Resistivity (Ω/cm^2)	VPAC1 receptor concn. (fmol/mg of protein)*
5	4 ± 1	35 ± 3	111 ± 9	15 ± 2
25	53 ± 4	259 ± 6	739 ± 12	128 ± 11

Figure 5 Indirect immunofluorescence micrograph of apically expressed sucrase isomaltase or dipeptidyl peptidase IV in Caco-2Cl.20 cells at day 25 of culture

Cells were grown on Tranwell clear membrane, incubated with polyclonal anti-sucrase isomaltase antibody or monoclonal anti-dipeptidyl peptidase IV antibody (HBB/3/775/42) followed by incubation with a second antibody conjugated to fluorescein isothiocyanate. Cells were then fixed with 2% (w/v) paraformaldehyde and examined with an epifluorescence or confocal microscope. (**A** and **C**) Micrographs of epifluorescence detection of dipeptidyl peptidase IV (DPPIV) and sucrase isomaltase (SI). (**B** and **D**) Confocal laser scanning microscopic detection of SI or DPPIV after monolayer reconstruction in the xz (vertical) plane.

and dipeptidyl peptidase IV, two enzyme markers of intestinal differentiation [33], increased markedly from day 5 of culture to day 25 of culture. Immunofluorescence detection of sucrase isomaltase and dipeptidyl peptidase IV at day 25 of culture showed expression of both enzymes at the cell surface (Figure 5). For both enzymes, no immunofluorescence staining could be

Figure 6 VPAC1 receptor mRNA level in undifferentiated and differentiated Caco-2Cl.20 cells

VPAC1 receptor mRNA was characterized by RNase protection assays (upper panel), Northern-blot analysis (middle panel) and RT-PCR assays (bottom panel) in Caco-2Cl.20 cells at day 5 (D5) and day 25 (D25) of culture. (Upper panel) Samples (10 μ g) of total cellular RNA isolated from Caco-2Cl.20 cells were hybridized to VPAC1 receptor cRNA (5×10^5 c.p.m.) or β -actin cRNA for 12 h at 55 °C. After digestion and extraction, protected fragments were resolved on a 6% denaturated polyacrylamide gel. Controls were performed using labelled sense probe or tRNA instead of cRNA in the hybridization reactions (no labelled bands were detected). (Middle panel) 50 μ g of total RNA were applied to each lane. The blot was hybridized at high stringency with 32 P-labelled VPAC1 receptor cDNA. (Bottom panel) Total RNA (10 μ g) was reverse-transcribed and resulting cDNAs were used as templates for PCR reactions as described in [30]. After electrophoresis and transfer, the blot was hybridized with a 32 P-labelled VPAC1 receptor probe or a 32 P-labelled $G_{\alpha s}$ subunit probe.

observed at day 5 of culture (results not shown). Laser-scanning confocal microscopy of sections optically generated in a plane perpendicular to that of the polarized cell monolayer at day 25 of culture showed exclusive expression of both sucrase isomaltase and dipeptidyl peptidase IV at the apical membrane of polarized cells (Figure 5). Altogether, these observations indicate that Caco-2Cl.20 cells undergo typical enterocytic differentiation.

In a previous report we have demonstrated that in Caco-2 cells the expression of the VPAC1 receptor is strictly dependent upon enterocytic differentiation [25]. Scatchard analysis of competitive inhibition of 125 I-VIP binding to Caco-2Cl.20 cell membranes by unlabelled VIP (results not shown) revealed a single class of VPAC1 receptor, the concentration of which increased markedly from undifferentiated cells at day 5 of culture to differentiated

Figure 7 Estimation of half-lives of VPAC1 receptor mRNA and relative transcription rates of the VPAC1 receptor gene in Caco-2Cl.20 cells at day 5 and day 25 of culture

(A) Actinomycin D (10 μ g/ml) was added to the culture medium of Caco-2Cl.20 cells at day 5 (○) or day 25 (●) of culture in order to block transcription. Total RNA was subsequently extracted after various times of incubation. The decay of VPAC1 receptor mRNA was characterized using the RNase protection assay (see the Materials and methods section for details). The autoradiograms were scanned and the data were plotted as a percentage of the VPAC1 receptor mRNA detected at time zero. Results are the mean $\pm$ S.E.M. of three experiments. (B) Cells at day 5 or day 25 of culture were harvested, nuclei were isolated and the transcription rate was determined by run-on assays. After incubation of nuclei in the presence of [32 P]UTP, labelled RNA was purified and hybridized to dot-blots containing 10 μ g of VPAC1 receptor cDNA (see the Materials and methods section). Quantification of signals was performed by densitometric analysis. Results are expressed as AU. They are the mean $\pm$ S.E.M. of three experiments.

cells at day 25 of culture (Table 2). In order to determine the amount of VPAC1 receptor mRNA in differentiated and undifferentiated cells, RNase protection assays were performed. Figure 6 shows the results of a representative experiment. A protected fragment of appropriate size (354 bp) was detected. At

Figure 8 Expression of VPAC1 receptor binding site versus VPAC1 receptor promoter/luciferase reporter gene during enterocytic differentiation of Caco-2Cl.20 cells

(A) Growth curve of Caco-2Cl.20 cells (▲) was obtained by cell counting at different days of culture. ¹²⁵I-VIP specific binding (●) was measured on homogenates prepared from Caco-2Cl.20 cells at different days of culture (see Materials and methods section). Hatched bars; Caco-2Cl.20 cells were stably transfected with p1424Luc construct and luciferase activity was evaluated in cell lysates prepared from Caco-2Cl.20 cells at different days of culture. All data in this figure are the mean ± S.E.M. of three experiments. (B) Correlation between specific ¹²⁵I-VIP binding to cell homogenates and luciferase activity of the VPAC1 receptor promoter at different days of cell culture. rlu, rough luminescence unit.

Figure 9 Activity of the VPAC1 receptor promoter in undifferentiated and differentiated Caco-2Cl.20 cells

Caco-2Cl.20 cells were stably transfected with the indicated VPAC1 receptor promoter constructs, and reporter gene activities were measured in cell lysates prepared from cells at day 5 or day 20 of culture. Luciferase activity was normalized as described in the Materials and methods section. A control was determined by stable transfection of the pRL plasmid (luciferase reporter gene driven by the CMV promoter) into Caco-2Cl.20 cells. Results are the mean ± S.E.M. of at least three experiments. rlu, rough luminescence unit.

day 5 of culture (undifferentiated cells) a low level of VPAC1 receptor mRNA was detected (Figure 6). In contrast, at day 25 of culture (differentiated cells) a high amount of VPAC1 receptor mRNA was detected (Figure 6). Specificity of mRNA detection was determined in the presence of a sense probe or tRNA and no

protected fragment was observed (results not shown). The use of human β -actin as a control probe showed that a protected fragment of appropriate size (123 bp) was detected at a similar level in differentiated and undifferentiated Caco-2Cl.20 cells (Figure 6). Very similar results were obtained by RT-PCR experiments using specific VPAC1 receptor primers, since a low amount of amplified DNA was observed when cells were undifferentiated but a high level when cells were differentiated (Figure 6). This contrasted with the data obtained using primers of the $G_{s\alpha}$ subunit. Indeed, identical amounts of amplified DNA were observed regardless of the state of cell differentiation (Figure 6). Finally, Northern-blot analysis of VPAC1 receptor mRNA indicated the presence of a single transcript species of approx. 3 kb in differentiated cells (Figure 6). No receptor mRNA could be detected in undifferentiated cells by this technique, probably due to a lack of sensitivity (Figure 6). These results strongly suggested that the dramatic increase of VPAC1 receptor expression during enterocytic differentiation of Caco-2Cl.20 cells is regulated at the mRNA level.

Stability and transcription rate studies of the VPAC1 receptor mRNA

The increase of VPAC1 receptor mRNA during differentiation of Caco-2Cl.20 cells could be due to a modification of the stability of transcripts and/or to a change in the transcription rate of the VPAC1 receptor gene. To investigate the two mechanisms, transcript stability and run-on transcription assays were performed. The half-life of the VPAC1 receptor mRNA was determined after inhibition of mRNA transcription with actinomycin D in Caco-2Cl.20 cells at day 5 and day 25 of culture. The disappearance of VPAC1 receptor mRNA with time was then measured by RNase protection assays (Figure 7). The stability of VPAC1 receptor mRNA was very similar in undifferentiated (day 5) and differentiated (day 25) cells with half-lives of 9.0 ± 0.8 h and 9.2 ± 0.5 h, respectively. The run-on assay indicated that the rate of receptor gene transcription was 5-fold higher in nuclei from differentiated cells than in nuclei from undifferentiated cells (Figure 7). Taken together these data indicated that the expression of the VPAC1 receptor during enterocytic differentiation of Caco-2Cl.20 cells is transcriptionally regulated.

Role of the promoter in the transcriptional regulation of the expression of the VPAC1 receptor in Caco-2Cl.20 cells

In order to determine the regulatory sequence(s) of the VPAC1 receptor promoter involved in this transcriptional regulation, the p1424Luc vector (see Figure 2) containing the -1348/+76 sequence was stably transfected into Caco-2Cl.20 cells, and luciferase activity was measured during the enterocytic differentiation process in culture. Stable transfections were necessary since differentiation took place over 25 days of culture. As shown in Figure 8, a very low luciferase activity was measured in undifferentiated cells (day 5 and day 7 of culture). When cells reached confluency, they started differentiating into enterocytes (from day 10 of culture and onward) and luciferase activity increased markedly to reach a maximum at day 20 of culture when cells were fully differentiated (Figure 8). The increase in luciferase activity during Caco-2Cl.20 cell culture correlated well with the increase in specific ¹²⁵I-VIP binding (Figure 8B). Notably the ratios of activities in differentiated cells (day 25) and undifferentiated cells (day 5) were identical for luciferase activity in Caco-2Cl.20 cell homogenates and ¹²⁵I-VIP binding to Caco-2Cl.20 cell membranes, i.e. approx. 8–9 (Figure 8). These results

strongly suggested that the $-1348/+76$ 5'-flanking sequence contains regulatory sequence(s) involved in the transcriptional control of the VPAC1 receptor during differentiation of Caco-2Cl.20 cells.

In order to identify the region(s) of the promoter responsible for this transcriptional control, a set of 5' deletions of the p1424Luc construct were stably transfected into Caco-2Cl.20 cells and luciferase activity was measured at day 5 and day 20 of culture (Figure 9). Deletion of the $-1348/-254$ sequence did not modify the pattern of expression of luciferase activity during differentiation of Caco-2Cl.20 cells (Figure 9). Indeed, transfection of p1008Luc, p822Luc and p330Luc vectors resulted in low luciferase activity at day 5 of culture and high activity at day 20 of culture. In contrast, a control construction containing the CMV promoter upstream of the luciferase reporter gene resulted in high luciferase activities in both undifferentiated and differentiated Caco-2Cl.20 cells (Figure 9). Furthermore, it is worth pointing out that when the p1008Luc, p822Luc or p330Luc vectors were transfected into Cos-7 cells, Hela cells or HT-29 cells, luciferase activities were independent of the culture conditions, i.e. exponential growth phase or at cell confluency (results not shown). These results strongly suggested that the $-254/+76$ 5'-flanking sequence contains the regulatory element(s) necessary for the transcriptional regulation of the expression of VPAC1 receptors during differentiation of Caco-2Cl.20 cells. They also indicated that the silencer region $-1348/-1124$ is not involved in the differentiation-dependent transcriptional control of the receptor.

DISCUSSION

In this study, we have delineated the basic organization of the human VPAC1 receptor gene promoter, and characterized its crucial role in transcriptional control of the receptor during differentiation of colon cancer Caco-2Cl.20 cells. Functional assays using various 5'-flanking sequences of the VPAC1 receptor promoter linked to a luciferase reporter gene showed that the minimal sequence having the maximal promoter activity is contained in the $-254/+76$ promoter region. Further analysis of this sequence revealed a housekeeping gene structure characterized by the absence of an active TATA box sequence but having two crucial elements for transactivation of the VPAC1 receptor gene, i.e. a CCAAT box ($-182/-178$) and a GC-rich region ($-68/-38$). There was also evidence for the existence of an upstream ($-1348/-1124$) silencer sequence. Finally, this study demonstrated that the expression of the VPAC1 receptor during enterocytic differentiation of the colon cancer cell clone Caco-2Cl.20 is under strict transcriptional control.

The 5'-flanking region of the human VPAC1 receptor gene contains many putative regulatory elements (see Figure 1). Its promoter activity was assessed after insertion upstream of the luciferase coding sequence in the pSNeoLuc vector and detection in the human colon cancer cell line HT-29, which expresses receptors regardless of the cell culture conditions [32]. Deletion studies allowed us to define the minimal promoter sequence ($-254/+76$), which has a housekeeping gene structure with the absence of a functional TATA box. Only a CCAAT box and a GC-rich sequence appeared to be mandatory for promoter activity (see Figure 3). This contrasts with the rat VPAC1 receptor promoter which does not contain a functional CCAAT box [34] even though it is devoid of a TATA box sequence like the human promoter. Another important difference between the human and rat VPAC1 receptor promoters is that the rat promoter contains a negative glucocorticoid response element

(GRE) between -21 and -36 relative to the transcription start site [35]. This GRE, that may act to regulate both basal and glucocorticoid-mediated expression of the rat VPAC1 receptor gene, is not present in the human VPAC1 receptor promoter (see Figure 1). A hallmark of class II GPCRs, including the human VPAC1 receptor [17], is a complex genomic organization with at least 12 exons and intron/exon boundaries generally well conserved (see [4] for reviews). It is therefore of interest to analyse the available information regarding promoters of class II GPCRs such as the mouse PTH/parathyroid hormone-related peptide (PTHrP) receptor [36], the mouse PACAP receptor [37], the mouse glucagon receptor [38], the human glucagon-like peptide-1 receptor [39] and the human growth hormone releasing factor receptor [40]. All promoters lack an apparent TATA box and are devoid of CCAAT boxes with the exception of the PACAP receptor promoter [37]. GC-rich sequences have been identified in the proximal promoter region of the mouse PACAP receptor [37] and PTH/PTHrP receptor [36]. Altogether, our results and others [36–40] support the idea that promoters of class II GPCRs are TATA-less, a property found primarily in housekeeping genes. This characteristic is not specific for class II receptors since TATA-less promoters are also found in many class I GPCRs, such as adrenergic receptors [41].

Construction of vectors in which progressively shorter 5' deletions of the 1.5 kb 5'-flanking region of the human VPAC1 receptor gene were cloned upstream of a luciferase reporter gene also indicated the presence of silencer element(s) in the $-1348/-1124$ sequence (see Figure 2). This was confirmed by constructing a vector in which the upstream sequence $-1348/-1124$ was linked directly to the minimal promoter sequence $-255/+76$. The existence of a transcriptional repressor sequence has also been reported in the rat VPAC1 receptor gene 5' region [34]. Recently, a novel transcriptional repressor protein known as VPAC1 receptor repressor (VIPR-RP) of the rat VPAC1 receptor gene was cloned and this repressor was shown to interact with a 42-bp element in the 5'-flanking region of the rat VPAC1 receptor gene [42]. Sequence analysis of the upstream $-1342/-1124$ sequence of the human VPAC1 receptor gene revealed no similarity with the rat 42-bp repressor element suggesting the existence of a specific human VIPR-RP and/or different specificity of VIPR-RP in human and rat.

Previous studies have shown that human colon cancer Caco-2 cells spontaneously differentiate in culture into mature enterocyte-like cells [26,33]. This differentiation is a growth-related process starting after confluency and is characterized by the presence of tight junctions, apical brush-border microvilli with associated hydrolases, formation of domes and epithelial electric properties unique to transporting epithelia (reviewed in [26]). In particular, confluent Caco-2 cells catalyse active vectorial secretion of fluid and electrolytes across the cell monolayer, a process stimulated by VIP [43]. We recognized early on that Caco-2 cells were equipped with VIP receptors [44] and subsequently demonstrated that the expression of VIP binding sites and VIP-sensitive adenylate cyclase was strictly dependent upon differentiation of Caco-2 cells at cell confluency [25]. This Caco-2 cell model is invaluable insofar as it is a unique example of almost all-or-nothing expression of VIP receptors in a biological system [25,44], i.e. the VPAC1 receptor is the sole VIP receptor subtype expressed in intestinal epithelium (J.-J. Maoret, unpublished work). In the present work, we demonstrated that expression of VPAC1 receptors upon differentiation of a Caco-2 cell clone is under strict transcriptional control. Several lines of evidence support this view. (1) Depending on the technique used, VPAC1 mRNAs are either not (Northern-blot) or barely (RNase protection assay or RT-PCR) detectable in undifferentiated

Caco-2Cl.20 cells. In contrast, when cells are differentiated a high amount of VPAC1 receptor mRNA was detected regardless of the technique used (see Figure 6). (2) The stability of receptor transcripts is identical in undifferentiated and differentiated cells with a half-life of approx. 9 h (see Figure 7). (3) The transcription rate of the receptor gene is markedly increased in nuclei of differentiated cells as compared to undifferentiated cells, as assessed by run-on assays (see Figure 7). (4) The p1424Luc vector consisting of the 1.5 kb 5'-flanking region of the human VPAC1 receptor gene cloned upstream of a luciferase reporter gene was stably transfected into Caco-2Cl.20 cells. Measurement of the reporter gene activity during cell differentiation revealed a very low activity in undifferentiated cells and a high activity in differentiated cells, an increase which correlated remarkably well with the increase in specific VIP binding (see Figure 8). Deletions of the p1424Luc construct followed by stable transfection into Caco-2Cl.20 cells indicated that the -254/+56 genomic 5'-flanking sequence contains the regulatory elements sufficient to recapitulate the expression pattern of VPAC1 receptors during Caco-2Cl.20 cell differentiation.

Differentiation of small intestinal epithelial cells [45] or human colon cancer cell lines such as Caco-2 [26] is associated with gene expression of various proteins, which are regulated at the level of mRNA abundance. The level of mRNA of the enzyme dipeptidyl peptidase IV in Caco-2 cells [27] is not controlled at the transcriptional level but by an increased stability of mRNA in differentiated cells (D. Darmoul, personal communication). In contrast, most other proteins are regulated at the transcriptional level during the differentiation of Caco-2 cells as shown above for the VPAC1 receptor and described for sucrase isomaltase [27], villin [46], H-ferritin [47], carcinoembryonic antigen [48] and apolipoprotein B [49]. The regulatory elements responsible for transcriptional control of gene expression upon terminal differentiation of Caco-2 cells have been described in some genes. A critical factor in regulating the sucrase-isomaltase promoter is *Cdx2*, an intestine-specific homeobox gene related to caudal [45]. Similarly, members of the caudal family of homeodomain proteins repress transcription of the apolipoprotein B promoter in Caco-2 cells [49]. Other regulatory nuclear factors, such as Sp1, and upstream stimulatory factors are involved in the transcriptional regulation of the carcinoembryonic antigen gene in differentiating Caco-2 cells [48]. The up-regulation of H-ferritin gene transcription in differentiating Caco-2 cells is accompanied by a modification in the activity of the transcription B-box binding factor [47]. Sequence comparison of the VPAC1 receptor promoter with promoters of these genes revealed very low similarity. In particular no consensus regulatory element sequence for the *Cdx2* factor was found in the VPAC1 receptor promoter suggesting that regulation of the expression of the receptor during the enterocytic differentiation process involved other nuclear factor(s). This is in line with the fact that in contrast to sucrase isomaltase, which is selectively expressed in intestinal epithelium and uses *Cdx2* as an intestine-specific transcription factor [45], the VPAC1 receptor has a widespread distribution in mammalian tissues [4]. Besides the differentiation-dependent transcriptional control of the VPAC1 receptor in human colon cancer Caco-2Cl.20 cells, it may be mentioned that glucocorticoid-mediated downregulation of transcription of the rat VPAC1 receptor in lung cells has been described [35]. As mentioned above, no GRE is present in the 5'-flanking region of the human VPAC1 receptor promoter (see Figure 1). Finally, retinoic acid has been shown to down-regulate VPAC1 receptor mRNA levels in human breast carcinoma cell lines, probably through inhibition of receptor gene transcription, since no change in mRNA stability was observed upon retinoic acid treatment

[50]. Since a retinoic acid responsive element is present in the 5'-flanking region of the human VPAC1 receptor promoter (see Figure 1), human colon cancer HT-29 cells transfected with the p1424Luc vector were incubated with retinoic acid. No change in reporter luciferase activity was observed (J.-J. Maoret, unpublished work), suggesting that retinoic acid does not control VPAC1 receptor gene transcription in human colon cancer cells, at least in HT-29 cells.

In conclusion, the present study provides a detailed analysis of the basic organization of the human VPAC1 receptor gene promoter, and characterizes the crucial role of the promoter in the transcriptional control of the VPAC1 receptor during enterocytic differentiation of human colon cancer Caco-2Cl.20 cells. Further efforts will be directed towards identifying the role of the VPAC1 receptor promoter in the regulation of human VPAC1 receptor expression in tumour biology and development.

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