Characterization of the rat vasoactive intestinal polypeptide receptor gene 5′ region

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The broad spectrum of vasoactive intestinal polypeptide (VIP) cellular functions are mediated by high-affinity binding sites. To determine regulation of the VIP receptor gene expression, we have isolated and characterized two genomic clones that contain the first three exons and the 5′ flanking region of the VIP receptor gene. Using RNase protection assays, receptor gene expression was detected in adult rat lung, liver and intestine, but not in fetal lung, indicating that VIP receptor is expressed in diverse tissues, and its expression is differentially regulated during lung development. The transcription start site of the gene was mapped to a cytosine residue, 76 bp upstream from the ATG initiation codon. Transfection into rat lung cell lines shows that although 126 bp of the VIP receptor 5′ DNA sequences are capable of activating VIP receptor gene basal transcription 30-fold over a promoterless control, 488 bp of the 5′ sequences further induce this activation to 97-fold over control. However, inclusion of up to 859 bp 5′ sequences results in a decrease in basal promoter activity (31-fold over control), indicating that while sequences between −126 and −488 bp contain potential enhancer sequences, sequences between −488 and −859 bp may include a transcriptional repressor sequence. Deletion analysis shows that transcription factor Sp1 plays an important role in activating basal promoter of the VIP receptor gene. DNase I footprinting and gel-mobility-shift assays show that Sp1 binds to its consensus binding sites in the VIP receptor promoter, suggesting that interaction of Sp1 with VIP receptor promoter transactivates this gene.

INTRODUCTION

Vasoactive intestinal polypeptide (VIP), a 28-amino-acid polypeptide, is widely distributed in many tissues and has a broad spectrum of biological functions [1,2]. VIP has been localized by immunohistochemistry to several tissues including lung, liver, brain and genital tracts [3,4]. In lung, VIP acts as a potent smooth-muscle relaxant and induces bronchodilation and vasodilation [2,5]. It also exerts anti-inflammatory effects by inhibiting T-lymphocytes [6] and alveolar macrophages [7]. The biological functions of VIP are mediated by high-affinity binding sites, which have been identified in several tissues including lung and liver [8–11]. High-affinity binding sites for VIP have also been found in lung cancer [12,13] and neuroblastoma cells [14,15]. Recently, cDNAs encoding rat [16] and human [17] VIP receptors have been cloned. The rat VIP receptor consists of 459 amino acids with a predicted molecular mass of 52 kDa. It contains seven transmembrane domains and mediates VIP-induced cellular accumulation of cyclic AMP. It is structurally related to the secretin, calcitonin and parathyroid hormone receptors, a subfamily of G_s protein (stimulatory subunit of G-protein)-coupled receptors. Expression of the rat VIP receptor mRNA was detected in liver, lung, intestines and brain [16]. To study the mechanisms that regulate VIP receptor expression during development and in disease, we have focused on characterizing the rat VIP receptor gene. We report here the initial isolation and structural characterization of the 5′ region of this gene. We show that transcription factor Sp1 is important in basal transcriptional activation of the VIP receptor gene in rat lung cells, and that sequences between −488 and −859 bp may include a transcriptional repressor.

MATERIALS AND METHODS

Isolation of the rat VIP receptor gene

A rat genomic library (using genomic DNA from Sprague–Dawley rat testis) in λDASH vector (Stratagene, La Jolla, CA, U.S.A.) was screened using the rat VIP receptor cDNA [16] as a probe. Southern-blot analysis identified two *Eco*RI fragments that were subcloned into PGEM3Z (Promega, Madison, WI, U.S.A.) for further characterization. Dideoxy-DNA sequencing was performed using a Sequenase kit (USB, Cleveland, OH, U.S.A.). Both strands of DNA were sequenced using either internal primers or sequencing subcloned fragments. DNA restriction and modification enzymes were purchased from BRL (Gaithersburg, MD, U.S.A.).

RNase protection assays

Total RNA was isolated from Sprague–Dawley rat tissues using TRIzol reagent (BRL, Gaithersburg, MD, U.S.A.), following manufacturer's instructions. A partially digested 490 bp *Ava*I–*Sma*I fragment spanning +177 to −313 bp was subcloned into PGEM3Z, and the antisense probe was synthesized from an Sp6 promoter using the MAXIscript kit (Ambion, Austin, TX, U.S.A.). RNA protection assays were performed with 20 μg of total RNA and 10^5 c.p.m. of the probe, using the RPA II ribonuclease protection assay kit (Ambion). As an internal control, a 316 bp fragment of the rat glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was transcribed from T7 promoter and included in the protection assays.

Plasmid constructions

To construct promoter and reporter fusion constructs, a 1 kb

Abbreviations used: VIP, vasoactive intestinal polypeptide; CAT, chloramphenicol acetyltransferase; DTT, dithiothreitol; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; AP2, activation protein 2; WT1, Wilm's tumour protein.

The nucleotide sequence reported in this paper has been submitted to GenBank with accession number U10635.

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*Sst*I VIP receptor subclone was either completely or partially digested with *Sma*I, resulting in two fragments containing 308 bp and 859 bp of the VIP receptor gene respectively. A *Hin*dIII linker was attached to their ends, and these fragments were cloned in front of the firefly luciferase reporter gene [18,19]. The resulting fusion constructs were termed VIPR308LUC and VIPR859LUC. Other fusion constructs were constructed by PCR using VIPR859LUC as the template. The 3′ primer used in PCR reactions is derived from luciferase gene: 5′-GCCTTTC-TTTATGTTTTTGGCG-3′. 5′ primers include: (1) 5′-TACCT-GAGCCGAGGCACCGCCTCC-3′, (2) 5′-GGGACTCCCTT-GTCGCAGACGGTT-3′, (3) 5′-AGAACGGATCTGTGGGC-AGTTTGG-3′, resulting in fusion constructs VIPR126LUC, VIPR488LUC and VIPR649LUC respectively. VIPR488ΔLUC was constructed by deletion of 48 bp from the 5′ end of the VIPR488LUC.

Cell cultures and transfections

Rat lung cell lines L2 and RFL6 were obtained from the American Type Culture Collection (A.T.C.C., Rockville, MD, U.S.A.). Cells were grown in F12K medium with 10% (L2) or 20% (RFL6) fetal bovine serum and were transfected by calcium phosphate precipitation [20]. For each plate, 350 μl of solution A (50 μl of 2 M $CaCl_2$, 300 μl of water) was added dropwise to 450 μl of solution B (400 μl of 2 × HeBS, pH 7.03, plus 5 μg of DNA in 50 μl of water). 2 × HeBS contains 42 mM Hepes, 270 mM NaCl, 9.9 mM KCl, 1.4 mM Na_2HPO_4 and 11 mM glucose. The DNA–calcium precipitate was incubated at room temperature for 20 min, and was added to cells in 8 ml of growth medium. After a 16 h incubation, DNA–calcium precipitates were aspirated, cells were washed twice with phosphate-buffered saline (PBS), and 10 ml of fresh growth medium was added to cells. After 24 h of incubation, cells were assayed for luciferase and chloramphenicol acetyltransferase (CAT) activities. Each construct was transfected using triplicate plates for each experiment, and each construct was tested in at least two independent experiments. In all experiments, cells were co-transfected with 1 μg of TKCAT (thymidine kinase promoter and CAT fusion gene) to monitor transfection efficiency. A negative control plasmid containing a promoterless luciferase gene and a positive control plasmid containing a Rous sarcoma virus (RSV) 3′ long terminal repeat (LTR) promoter fused to luciferase were included in all experiments.

Luciferase assays and CAT assays

Cell lysates were prepared by freezing and thawing cells in 0.25 M Tris (pH 7.8). Protein concentration was determined using a dye-binding assay (Bio-Rad, Richmond, CA, U.S.A.). Luciferase activity were determined by adding 100 μl of 1 mM luciferin (Analytical Luminescence Laboratory, San Diego, CA, U.S.A.) to the cell lysate (50 μg of protein) in assay buffer (4 mM ATP, 0.25 M Tris, pH 7.8, 15 mM $MgSO_4$, 1 μg/ml BSA). Light emission was integrated over 15 s using a luminometer (Autolumat LB953, EG&G Berthold, Germany). For CAT assays, 50 μg of cell lysate was incubated with 20 μl of 4 mM acetyl-CoA, and 1 μCi of [^{14}C]chloramphenicol in a total volume of 100 μl at 37 °C for 90 min. CAT activity was determined by ascending chromatography on TLC plates, and quantified using an Ambis Radioanalytic Imaging System (Ambis, San Diego, CA, U.S.A.).

DNase I footprinting and gel-shift assays

L2 and RFL6 cells were trypsinized and collected by centrifugation. The cell pellet was frozen in liquid nitrogen, then resuspended in 5 vol. of lysis buffer containing 10 mM Hepes (pH 7.9), 400 mM NaCl, 0.1 mM EGTA, 0.5 mM dithiothreitol (DTT), 5% (v/v) glycerol and 0.5 mM PMSF. This whole-cell homogenate was centrifuged at 4 °C at 54000 rev./min in a Beckman TLS-55 rotor, and the supernatant was divided into aliquots and stored at −80 °C until required for use. For subsequent binding reactions, 10 μg of protein was used. The probe used in the gel-shift assays is a double-stranded 30-mer from −97 to −67 bp of the VIP receptor promoter containing one consensus binding site for Sp1. The oligonucleotide was end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase. Binding reactions were performed at room temperature for 30 min in 10 mM Hepes, pH 7.9, 1 mM spermidine, 5 mM $MgCl_2$, 50 mM KCl, 0.5 mM DTT, 9% glycerol. Each reaction included 1 μg of poly d(I-C) and poly d(A-T) as non-specific competitor. Specific competition was performed using a 100-fold molar excess of the unlabelled oligonucleotide. Anti-Sp1 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.) was added during the last 10 min of incubation. The reactions were analysed on 4% non-denaturing polyacrylamide gels.

To generate probe for footprinting, primer 5′-GCCTTTCT-TTATGTTTTTGGCG-3′ was end-labelled using [γ-^{32}P]ATP and T4 polynucleotide kinase, and was then used in PCR with upstream primer 5′-GGGACTCCCTTGTCGCAGACGGTT-3′ and VIPR859LUC as template. The end-labelled probe contains sequences between −11 and −488 bp of the VIP receptor gene. 10000 c.p.m. of the probe was used in each reaction. Binding reactions were performed as described above, except that two footprint units of recombinant Sp1 (Promega, Madison, WI, U.S.A.) and 100 ng of poly d(I-C) were used. DNase I digestion was carried out at room temperature for 1 min using 0.2 unit of DNase I (Ambion, Austin, TX, U.S.A.). The reactions were terminated by adding 30 μl of 2× stop buffer containing 15 mM EDTA, 0.2% SDS and 40 μg/ml salmon sperm DNA. The reactions were analysed on a 6% polyacrylamide sequencing gel.

RESULTS

Isolation of the 5′ region of the rat VIP receptor gene

Genomic clones were isolated by screening a rat genomic library using the rat VIP receptor cDNA [16] as a probe. Two *Eco*RI fragments of approximately 8 and 12 kb were isolated and subcloned into PGEM3Z vector for further analysis. Restriction mapping and DNA sequencing indicate that these two *Eco*RI subclones contain the first three coding exons of the VIP receptor gene (Figure 1). The first exon includes amino acids 1 to 26, the

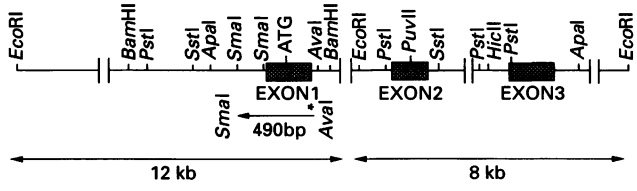

Figure 1 Schematic structure of the rat VIP receptor gene

The VIP receptor gene is represented by a line, with exons indicated as boxes. Several restriction sites are depicted. The long vertical lines indicate unsequenced gaps in the introns and in the 5′ region. The 490 bp *Aval–Sma*I fragment was subcloned for riboprobe synthesis and used in the RNA protection assay. The two arrows represent the two 12 and 8 kb subclones that were characterized.

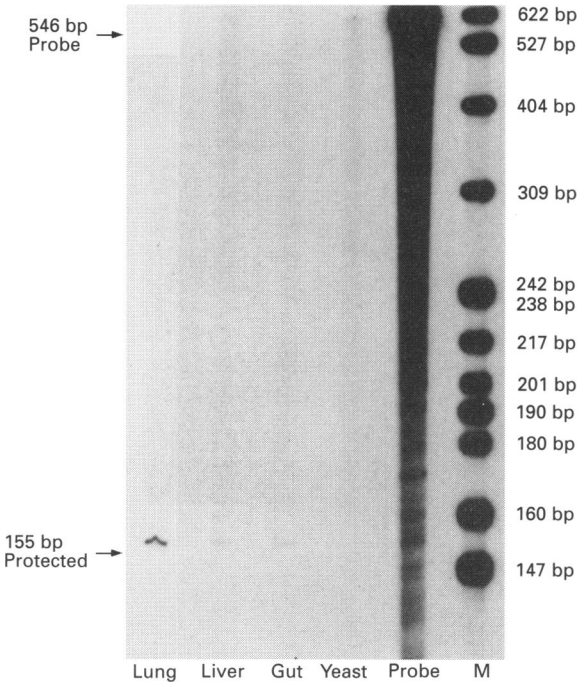

Figure 2 Transcription initiation site of the rat VIP receptor gene

RNase protection assay: ^{32}P-labelled riboprobe was hybridized to 20 μg of total RNA derived from intestine, liver and lung (lung expression is from an independent experiment). Yeast RNA was used as a negative control. Arrows indicate protected band, and the probe. Sizes were determined by comparison with molecular-mass markers.

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-2040  CCCGAGAAACAACCAGAAAGAACCAAGAGTAGCTTTCACAGCAGAGAACTGGAAGAAAAG
-1980  CTATTCTCCCTGAGCCACCTTCCTCTCTTGGGGGTTGGGCCAGGAAGACACCAGGGGGTC
-1920  CTGCCTATGCTCTCAGCTGTAGCCTAGCACCAGCCTTCCAACCTCCAGTGGAAGGAGGAT
-1860  CAGAGTAAACTGGGCAGTCATCCCCACGGGCCACCCTCTCACCGTGTATTTACTTCTCCT
-1800  ATCTGGTGAGGAGCAGAGCAAACGTGGAGTCTGGCTTCCCACTCCCTTCCGTGTACCCGA
-1740  AAGCCCCCCAAGATCTCTGTCCTCAGAGACAAGAAAATGCTAACAGGCCCCGTAAGGTGAA
-1680  GCACCAGAGCATTTCGCTCAGGCTGGGCTGAAATCGCCAGGAAATCGCCTCCCTCCCCCT
-1620  CCAGAGCAGTGTGATCACAGGCACATGCCACCATGCCCCACTTGCTGATGGGATATCAGC
-1560  AGTCTGCAGCAGCCTAGGTGACATATGGGGGTGGCAGGGTTGAGAGCCAAAGCTCACATC
-1500  TGGAACTTGAGCATGCCACCTCAAGAGCTGATAGGACTCCACTGCCCCAGTAGAGGACTA
-1440  AGGCCTGAGAGGAGGGTGCCCATGACCACAAGGGAGGCAAACCCCTGATTCCTATGCTCA
-1380  CAGTGGCTTCAGAAATCCTAGCCCTGAAGTGCTGAATGGCAACTACTAAACAGCTCCCTC
-1320  TTCTGAAATCCTGTATCTGGAGAAAGGTCTGTGCCAACTAGCCCATTCTTTATAGGAAGC
-1260  AGACATTGCAGGGAGTGCCTCTTACCTCTATGCCCTGATGTGTAGCAGAGGACTGGATCA
-1200  GGATCAGAGGGAGAGAGAACAAGGGAGGTGGCGGTAGACTAGGAAGGAGAGGACCGGGGA
-1140  AGCCAGGGGTCGCTTCCAGACTTTTCTGTAACCGACCTCAAGGCCACGCCCACAGCCCTA
-1080  GTTTCCCACGTTGCAGGTTGGGAAGCCTGGAGAGTCCTCGGGAAATTCCCGCGCCCCTGC
-1020  TGGCCAGAAAGTGCCTGGAGACCGACTTCCTTCAAGCGGATCGCATACCCTACTTCGCGT
 -960  AGGGACACGCGGCCCTCTCCACCGAACTCCGCCCCTTCCTCCCACCTTCTATCCCACCGCG
 -900  CCATCAGCCCCCTACATCCCCTTCTCACCATGACCACTCGAGCTCTCGGAAAGCCATCTG
 -840  CACGGGTTCCCTGAAAAGTCGCGCGCAAAGGGCTACGAGTCGGTGACCCCGCGCCGCACG
 -780  CTAGCACCGAATAATCCGAGCCCTAAGGACAAGCGTCGGAGGGAGATCAGCCTCAGATCC
 -720  CCCGCTGCCAGCCGCGCCCAATCCTGGAGACACAGCGCGGGACACTGGCACTAGCTCCTGG
 -660  GGACTCCGGGAGAACGGATCTGTGGGCAGTTTGGGCCCGCGAAAGGGTACAGGCTTCCGT
 -600  CCCCGCGCGGGGGCTGAAACATTGACTTCGCCTCCCTCACGCAGTGCGCGGGCCGAGACT
 -540  GAGTGCTCTGAGTGCACCCTGCCCTGGGAGCGGCCTGGAGGAACCCCAAGTCGGGACTCC
 -480  CTTGTCGCAGACGGTTCCAGCAGCGTGATTCGCGGACCCCATCGGAGCCCAGCCTGCAGC
 -420  CCCGTAAAACTGCAGCCCTTCTCGTTCAGGAATCTGGGTTGCAAGCTGGCGTGGGAACCA
 -360  GGGAGCGGGCCGCTGGACCCCCTGGAGCCGACGACTAGAGGAACTCAGGCCCGGGGACCG
 -300  AGGGTGCGGCCGAGCTTGGAAAACGAGGTGCAGGCGCGGGGAACTCTGGGCGGTGTCGAG
 -240  CCCAGCGTCCCTGCAAGGAGCCGAGAGAACGCGTGTTAGCGAGCAGCTCCAAACGACGCG
 -180  CACCTCTAGCGGGACTTTGCGCTCACGCCCCCAGGGCGCCCAGGCTCCACCCCCTACCTG
 -120  AGCCGAGGCACCGCCTCCGCCGCGCCATTGGCTAACCTCTCCGCCCCGTCAGACGAGCCCC
  -60  CGCCGCCCCCCCCCCCCCAGGGCCTGGACGTTCGCCTCTTAGCGCCCGGGCTGGCCCG
   +1  CGCCACTCTCCCGCGCTGCACCAGCCACAGACCCCGTTGCCGCTCCCGGCCGCTTGCCGC
  +60  CGGCTCAGGGCGGACCATGCGCCCTCCGAGCCCACCGCATGTCCGCTGGCTCTGCGTGCT
 +120  GGCAGGAGCCCTTGCCTGCGCCCTCAGACCCGCGgtgagtgtgcgcccctccacccagat
 +180  cccgaggatgggggaatt
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Figure 3 Nuclear sequence of the 5′ flanking region of the rat VIP receptor gene

The transcription start site is designated +1, and is boldly italicized and double underlined. The ATG initiation codon is boldly italicized and underlined. Binding sites for several DNA-binding proteins are bold and underlined with a dotted line. These include consensus binding sites for Sp1, GGGCGG or CCGCCC; WT1, GCGGGGGCG; and AP2, CCCCAGGC.

second exon contains amino acids 27 to 61 and the third exon includes amino acids 62 to 97. The sequences of the exon–intron junctions were determined by DNA sequencing. To map the transcription start site of the VIP receptor gene, a 490 bp *Ava*I–*Sma*I fragment was subcloned into PGEM3Z (Figure 1) and transcribed from the Sp6 promoter to generate an antisense probe of 546 bp. As shown in Figure 2, hybridization of this probe to RNA derived from rat gut, liver and lung results in a protected band of 155 bp in size, whereas no protected band was apparent when the probe was hybridized to yeast RNA. When the protection assay was performed alongside a sequencing ladder, the transcription start site was mapped to a cytosine residue 76 bp upstream from the initiation codon (Figure 3).

To identify potential *cis*-acting regulatory sequences in the VIP receptor gene, about 2 kb of DNA sequences upstream from the transcription start site were sequenced. Both strands of DNA were sequenced using internal primers and by sequencing of subcloned fragments. The sequences immediately upstream from the transcription start site contain no TATA consensus sequence, but they are extremely GC-rich (Figure 3). Five consensus binding sites for transcription factor Sp1 are apparent in this region. A perfect binding site for Wilm's tumour protein (WT1) and a consensus binding site for activation protein 2 (AP2) were also identified in this region (Figure 3).

Expression of VIP receptor mRNA during lung development

Since VIP receptor was found to be strongly expressed in the lung and since VIP is involved in regulating cell differentiation [21], we sought to determine whether VIP receptor expression is regulated during lung development. Total RNA was isolated from rats of different ages, and RNA protection assays were performed using the 490 bp *Ava*I VIP receptor fragment as a probe. As shown in Figure 4, VIP receptor mRNA was not expressed in the 18-day-gestation fetal lung, but high levels of receptor expression were detected by week 2. These results indicate that VIP receptor expression is differentially regulated during the different stages of lung development.

Regulation of VIP receptor gene promoter in rat lung cells

To determine whether the 5′ region of the VIP receptor gene contains a functional promoter, different portions of this region were cloned in front of the luciferase reporter gene. These fusion genes were transfected into two rat cell lines, L2 and RFL6. The expression of endogenous VIP receptor mRNA in these cell lines was confirmed by reverse-transcription PCR (data not shown). Figure 5 shows the average of two independent experiments, with triplicate transfections for each fusion construct. The construct containing 126 bp of the VIP receptor 5′ region induces luciferase activity 32- and 15-fold over a promoterless luciferase construct in L2 cells and RFL6 cells respectively. The construct containing 308 bp 5′ sequences further increases luciferase activity (54- and 24-fold in L2 and RFL6 cells respectively). Inclusion of 488 bp of the 5′ flanking sequences results in further induction of basal promoter activity to 97- and 38-fold over control. However, when additional 5′ flanking sequences were added to the fusion

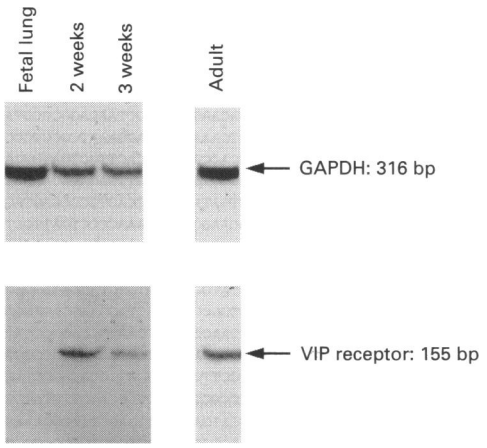

Figure 4 Regulation of the VIP receptor gene expression during lung development

RNase protection assay: ^{32}P-labelled riboprobes were hybridized to 20 μg of total RNA isolated from fetal rat lung (18-day gestation), from rat lungs 2 or 3 weeks after birth, and from adult lung. GAPDH, a widely expressed gene, was used as an internal control. Arrows indicate protected bands.

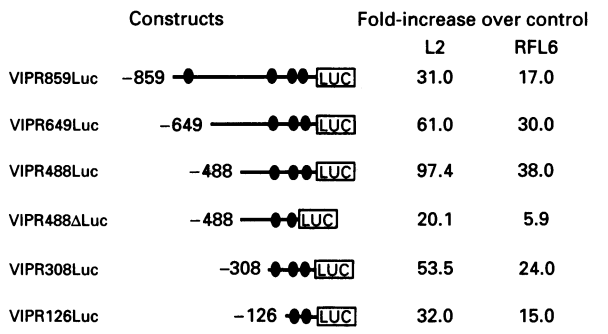

Figure 5 Expression of the VIP receptor promoter in transfected lung cells

Cells were transiently transfected with the indicated DNAs and assayed for luciferase reporter activity, represented as fold-increase over a promoterless luciferase control (activity of the control is taken as 1) after normalizing to internal control CAT activity. DNA constructs: control, a luciferase lacking a promoter; VIPR126LUC, VIPR308LUC, VIPR488LUC, VIPR488ΔLUC, VIPR649LUC, and VIPR859LUC. L2, a rat adult lung cell line; RFL6, a rat fetal cell line. Ovals represent Sp1-binding sites.

construct, a decrease in basal promoter activity was observed. As shown in Figure 5, the construct containing 859 bp of the VIP receptor 5′ sequences induces luciferase activity 31- and 17-fold in L2 and RFL6 cells respectively. This transcriptional activity is 3-fold lower than that of VIPR488LUC, indicating that the sequences between −488 and −859 bp may include a transcriptional repressor.

Sp1, a zinc-finger-containing transcription factor, activates transcription of many cellular and viral genes containing GC-rich promoters [22,23]. Since the VIP receptor 5′ flanking region contains multiple binding sites for Sp1, we sought to determine whether Sp1 is important for transcriptional activation of the VIP receptor gene. As shown in Figure 5, deletion of the most proximal Sp1 site (at −53 bp) from the transcription start site reduces the promoter activity about 5-fold (compare VIPR488LUC with VIPR488ΔLUC); deletion of the Sp1 site at −248 bp results in a 1.7-fold reduction of the promoter activity

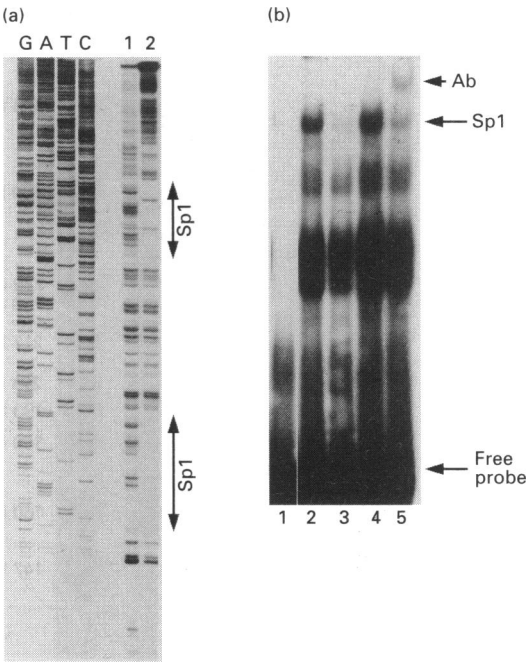

Figure 6 Interaction of Sp1 protein with VIP receptor promoter

DNase I footprinting and gel-mobility-shift assay: probes and binding reactions are described in the Materials and methods section. (**a**) DNase I footprinting assay. Lanes G, A, T, C are sequencing ladders; lane 1, probe; lane 2, with recombinant Sp1 protein. Protected areas are indicated by arrows. (**b**) Gel-mobility-shift assay. Lane 1, probe alone; lane 2, with L2 cell extract; lane 3, with L2 cell extract plus 100× unlabelled Sp1 oligonucleotide; lane 4, with L2 cell extracts plus 100× unlabelled, unrelated oligonucleotide; lane 5, with anti-Sp1 antibody. The specifically shifted band is indicated by an arrow, and the supershift is indicated by an arrowhead.

(compare VIPR308LUC with VIPR126LUC). These data suggest that although all three Sp1 sites in the proximal promoter region are important for VIP receptor gene transactivation in lung cells, the most 5′ Sp1 site may play a larger role.

Interaction of Sp1 protein with VIP receptor promoter

To determine whether Sp1 protein is capable of recognizing its consensus binding site in VIP receptor promoter, DNase I footprinting was performed using recombinant Sp1 protein. When an end-labelled fragment containing −11 to −488 bp of the VIP receptor 5′ region was used for DNase I footprinting, the recombinant Sp1 protected several sequences that correspond well to the Sp1 consensus sequences in this region (Figure 6a).

To determine whether Sp1 in lung cells interacts with the VIP receptor promoter, whole-cell extracts were prepared from lung cell lines and used in a gel-mobility-shift assay with a 30 bp oligonucleotide probe from the VIP receptor gene containing a consensus binding site for Sp1. When lung cell extracts were incubated with the Sp1-binding-site-containing probe, several mobility-shifted bands were observed (Figure 6b, lane 2). The band that has the lowest mobility was competed with by 100-fold excess Sp1-specific oligonucleotide (Figure 6b, lane 3), but not by unrelated oligonucleotide (Figure 6b, lane 4). Furthermore, when antibody against Sp1 was added to the reaction, this band was supershifted (Figure 6b, lane 5), indicating that this band was generated by specific interaction between the Sp1-containing oligonucleotide and Sp1 protein present in the lung cell extracts. These results suggest that Sp1 protein interacts with the VIP receptor promoter to activate transcription of this gene.

DISCUSSION

In this study, we have isolated and characterized the rat VIP receptor gene 5′ region. This initial report on the genomic organization of the VIP receptor shows that the first three exons and the 5′ region of the gene is spread over more than 20 kb of DNA sequences. The transcription start site is mapped to a cytosine, 76 bp upstream from the initiation codon. DNA sequences 5′ of the transcription start site lack a TATA box or CCAAT motif and the region is extremely GC-rich, indicating that GC-boxes may participate in transactivation of the VIP receptor gene. We have sequenced 2 kb of the 5′ flanking DNA of the VIP receptor gene and identified potential binding sites for several transacting factors. Five consensus binding sites for Sp1 are present in this region. Sp1 is a zinc-finger-containing transcription factor which activates transcription by interacting with GC-rich sequences present in a wide variety of cellular and viral promoters [22,23]. A perfect binding site for WT1 was also found in the VIP receptor gene 5′ region. WT1 is zinc-finger-containing transcription factor that binds to GCGGGGGCG DNA element [24–27], and represses transcription of the insulin-like growth factor II [28], the platelet-derived growth factor A-chain [29], and colony stimulating factor-1 [30] genes. Other potential binding sites in the VIP receptor 5′ region include a binding site for AP2 which mediates both cyclic AMP and phorbol ester induction of gene expression in genes containing its binding site [31,32].

Physiological and biochemical studies have shown that VIP plays important roles in the lung as an airway and vessel smooth-muscle relaxant and as an anti-inflammatory modulator [33]. We therefore examined VIP receptor mRNA expression during lung development. Our results show that the VIP receptor is not expressed in the fetal lung, but is expressed at high levels 2 weeks after birth, indicating that the onset of VIP receptor expression may be coordinate with post-natal lung maturation.

To determine the DNA sequences necessary for VIP receptor transcription, we transiently expressed VIP receptor promoter–luciferase fusion genes in lung cells. Our results show that 488 bp DNA sequences in the 5′ region of the VIP receptor gene are sufficient for transcriptional activation of the VIP receptor gene in lung cells. Three consensus binding sites for transcription factor Sp1 are present in this region. Deletion analyses show Sp1-binding sites are important for transactivation of the VIP receptor gene, as deletion of the most proximal Sp1-binding site resulted in a 5-fold reduction of the promoter activity. A fusion construct containing 859 bp of the 5′ region shows decreased activity in these cells, suggesting that the sequences located between −859 and −488 bp may contain a potential transcriptional repressor sequence. We are currently investigating this repressor sequence, and our preliminary data show that a nuclear protein from lung cells interacts with an oligonucleotide containing −819 to −779 bp of the VIP receptor gene (L. Pei and S. Melmed, unpublished work).

Because Sp1 plays an important role in transactivating the VIP receptor gene, we have examined whether Sp1 protein is capable of recognizing its consensus binding site in VIP receptor promoter. DNase I footprinting experiments show that recombinant Sp1 protects DNA sequences containing Sp1-binding sites in the VIP receptor from DNase I digestion. Since Sp1 is a widely expressed protein, it is probably present in lung cells. To verify this, an oligonucleotide from the VIP receptor promoter containing a consensus Sp1-binding site was used in gel-mobility-shift assays. Our results indicate that lung cells have a protein with the expected characteristics of Sp1; it specifically binds to an Sp1-binding site, and is recognized by an antibody against Sp1.

In summary, isolation and characterization of the VIP receptor gene provides a useful molecular tool to study the regulation of VIP receptor expression, which will enable us to understand cellular mechanisms for multiple VIP functions both in normal development and in disease states.

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