Proc. Natl. Acad. Sci. USA
Vol. 92, pp. 2939–2943, March 1995
Biochemistry

Structure, expression, and chromosomal localization of the type I human vasoactive intestinal peptide receptor gene

(G protein-coupled receptors/type II pituitary adenylyl cyclase-activating peptide receptor/secretin receptor/chromosome 3p22)

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Communicated by Yuet Wai Kan, University of California, San Francisco, CA, December 21, 1994

ABSTRACT **Vasoactive intestinal peptide (VIP) and other members of the pituitary adenylyl cyclase-activating peptide (PACAP) and secretin neuroendocrine peptide family are recognized with specificity by related G protein-coupled receptors. We report here the cloning, characterization, and chromosomal location of the gene encoding the human type I VIP receptor (HVR1), also termed the type II PACAP receptor. The gene spans ≈22 kb and is composed of 13 exons ranging from 42 to 1400 bp and 12 introns ranging from 0.3 to 6.1 kb. Primer extension analysis with poly(A)$^+$ RNA from human HT29 colonic adenocarcinoma cells indicated that the transcription initiation site is located at position −110 upstream of the first nucleotide (+1) of the translation start codon, and 75 nt downstream of a consensus CCAAT-box motif. The G+C-rich 5′ flanking region contains potential binding sites for several nuclear factors, including Sp1, AP2, ATF, interferon regulatory factor 1, NF-IL6, acute-phase response factor, and NF-κB. The HVR1 gene is expressed selectively in human tissues with a relative prevalence of lung > prostate > peripheral blood leukocytes, liver, brain, small intestine > colon, heart, spleen > placenta, kidney, thymus, testis. Fluorescence *in situ* hybridization localized the HVR1 gene to the short arm of human chromosome 3 (3p22), in a region associated with small-cell lung cancer.**

The 28-aa vasoactive intestinal peptide (VIP) is structurally related to other members of a family of peptide neuroendocrine mediators that include pituitary adenylyl cyclase-activating peptide (PACAP), secretin, glucagon, calcitonin, and parathyroid hormone. VIP has potent relaxing effects on nonvascular and vascular smooth muscle, enhancing blood flow. It also regulates water and ion flux from lung and intestinal epithelia, promotes neuronal growth and survival, and modulates many immune functions (1–3).

Specific high-affinity receptors for VIP are found on distinct subsets of neural, respiratory, gastrointestinal, and immune cells (1–3). We previously cloned a cDNA encoding a human high-affinity type I VIP receptor (HVR1), also termed type II PACAP receptor, from HT29 colonic adenocarcinoma cells and human lung tissue (4). The 3-kb cDNA insert encoded a seven-transmembrane-domain HVR1 protein of 457 aa, with a deduced molecular mass of 52 kDa, that was most homologous to other members of the PACAP and secretin G protein-coupled receptor family and had a similar binding affinity for both VIP and PACAP (4). Two other structurally distinct subtypes of VIP/PACAP receptors have been identified, the type I PACAP receptor, which binds PACAP with a 1000-fold higher affinity than VIP, and a type II VIP receptor subtype, also termed $RVIP_2$/type III PACAP receptor, which binds VIP and PACAP with similar affinities (5–7). Protein sequence alignments have revealed that HVR1 has about 50% amino acid sequence identity with rat type I PACAP receptor and type II VIP receptor (7). We report here the isolation of the gene encoding HVR1 and the analysis of its structure, tissue expression, and chromosomal location.§

MATERIALS AND METHODS

Isolation and Characterization of the HVR1 Gene. Human placental genomic libraries in EMBL3 SP6/T7 λ-phage vector (Clontech) and pWE15 cosmid vector (Stratagene) were screened with a mixture of ^{32}P-labeled oligonucleotide probes derived from the HVR1 cDNA sequence. Approximately 3×10^6 phage clones or 2×10^6 cosmid clones were screened under standard conditions of plaque hybridization (8). Three positive clones were further characterized by restriction mapping and by Southern blot analyses with specific oligonucleotide probes corresponding to various regions of the HVR1 cDNA. Restriction fragments were subcloned into pGEM vectors (Promega), and DNA sequencing was performed by the dideoxy chain-termination method (Sequenase version 2.0; Amersham).

Analysis of Introns. The size of introns in the HVR1 gene was determined by polymerase chain reaction (9) of genomic clones with specific primers derived from exon sequences, for 35 cycles of 30 sec at 94°C, 2 min at 55°C, and 6 min at 72°C, using *Taq* polymerase (GIBCO/BRL). The reaction products were analyzed by electrophoresis in 1% agarose gels.

Primer Extension. Two micrograms of poly(A)$^+$ RNA from HT29 cells was hybridized overnight with 50,000 cpm of a ^{32}P-end-labeled primer (5′-ACTTGGCGGGCGCATGGTCT-3′) which spans the cDNA translation start codon (whose complement is underlined in the sequence), and primer extension was carried out as described (8). The ^{32}P-labeled primer was also used to generate a sequence ladder from a 7-kb *Sac* I fragment of the HVRG6.4 genomic cosmid clone. Samples were analyzed by electrophoresis in a 5% polyacrylamide/8 M urea denaturing gel.

Northern Blot Analysis of HVR1 Gene Expression. Northern blot membranes bearing poly(A)$^+$ RNA from multiple human tissues (Clontech) were hybridized to a ^{32}P-labeled HVR1 cDNA probe in Rapid-hyb buffer (Amersham) at 65°C for 2 hr in an Autoblot microhybridization oven (Bellco Glass). Membranes were washed for 20 min in 2× SSC (1× SSC is 0.15 M NaCl/0.015 M sodium citrate, pH 7.0)/0.1% SDS at room temperature and then twice for 15 min in 0.1× SSC/0.1% SDS at 65°C and autoradiographed.

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Abbreviations: VIP, vasoactive intestinal peptide; PACAP, pituitary adenylyl cyclase-activating peptide; HVR1, human type I VIP/type II PACAP receptor.

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§The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U11079–U11087).

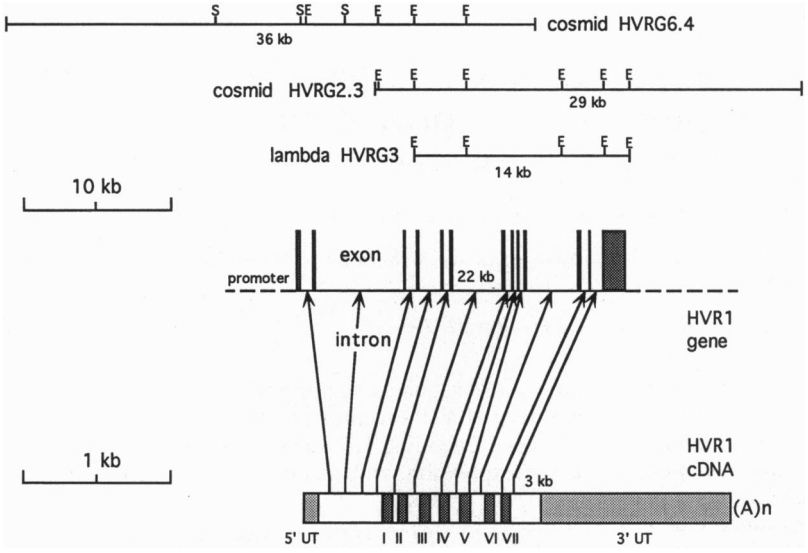

FIG. 1. Spatial organization of the HVR1 gene exons. The organization of the HVR1 gene exons (solid bars) with respect to the seven putative membrane-spanning segments (I–VII) of the cDNA (bottom), and the λ and cosmid genomic HVR1 clones (top), is depicted. *Eco*RI (E) and *Sac* I (S) restriction sites for the genomic HVR1 clones, utilized for subcloning and sequence analysis, are indicated, and the locations of introns interrupting the gene, relative to the cDNA structure, are marked (arrows).

Fluorescence *in Situ* Hybridization. Metaphase spreads prepared from short-term lymphocyte cultures of a normal donor were used for *in situ* hybridization (10). Chromosomal DNA was denatured at 70°C for 2 min in 70% (vol/vol) formamide/2× SSC and then dehydrated successively in 70%, 90%, and 100% ethanol before hybridization. The genomic phage clone HVRG3 was nick-translated with biotin-16-dCTP (Boehringer Mannheim), and 100–200 ng of the labeled DNA was denatured at 80°C for 5 min in hybridization buffer, composed of 50% (vol/vol) deionized formamide, 10% (wt/

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ccgccgcccgtcagacgagcccccgggcccgcccccagccctgacgtgcgcctcttagctctggggccacaggccagcgc - 95
Exon 1
CACTCTGCCAGGCTCCCGGCCATCGCCCGCCTGGTGCGCCGCCGCCGCCAGCTCTTTGCCCGCCGCGGGGCCGCCCGCCGCGG - 5
GCTCAGGGCAGACCATGCGCCCGCCAAGTCCGCTGCCCGCCCGCTGGCTATGCGTGCTGGCAGGCGCCCTCGCCTGGGCC 66
                                                    Exon 2
CTTGGGCCGGCGgtgagtgttcg.....(0.9 kb)....ctttgttctcagGGCGGCCAGGCGGCCAGGCTGCAGGAGG 106
AGTGTGACTATGTGCAGATGATCGAGGTGCAGCACAAGCAGTGCCTGGAGGAGGCCCAGCTGGAGAATGAGACAATAGgt 184
                                          Exon 3
gaggccccca....(6.1 kb)....ttaccccaatagGCTGCAGCAAGATGTGGGACAACCTCACCTGCTGGCCAGCCA 226
CCCCTCGGGGCCAGGTAGTTGTCTTGGCCTGTCCCCTCATCTTCAAGCTCTTCTCCTCCATTCAAGgtaagacccct... 292
                                    Exon 4
..(0.8 kb)....ccctccacccagGCCGCAATGTAAGCCGCAGCTGCACCGACGAAGGCTGGACGCACCTGGAGCCTG 346
GCCCGTACCCCATTGCCTGTGGTTTGGATGACAAGGCAGCGAGTTTGGATGAGgtgggtctca.(1.6 kb).cctccaa 399
  Exon 5                    TM I
cagCAGCAGACCATGTTCTACGGTTCTGTGAAGACCGGCTACACCATTGGCTACGGCCTGTCCCTCGCCACCCTTCTGGT 476
                                                    Exon 6
CGCCACAGCTATCCTGAGCCTGTTCAGgtgaggcccagc....(0.5 kb)....caccccacacagGAAGCTCCACTGC 516
    TM II
ACGCGGAACTACATCGACATGCACCTCTTCATATCCTTCATCCTGAGGGCTGCCGCTGTCTTCATCAAAGACTTGGCCCT 596
                                                    Exon 7
CTTCGACAGCGGGGAGTCGGACCAGTGCTCCGAGGGCTCGgtgaggatcc.(3.4 kb).ccccctgcagGTGGGCTGTA 646
  TM III
AGGCAGCCATGGTCTTTTTCGAATATTGTGTCATGGCTAACTTCTTCTGGCTGCTGGTGGAGGGCCTCTACCTGTACACC 726
                    TM IV
CTGCTTGCCGTCTCCTTCTTCTCTGAGCGGAAGTACTTCTGGGGGTACATACTCATCGGCTGGGgtatggtaccagg... 790
                  Exon 8
..(0.5 kb)...gggcctgacagGGGTACCCAGCACATTCACCATGGTGTGGACCATCGCCAGGATCCATTTTGAGGAT 846
                                  Exon 9.          TM V
TATGGgtgagctgctgcc...(0.3 kb).....ggtctgttcagGTGCTGGGACACCATCAACTCCTCACTGTGGTGGA 886
                                                  Exon 10
TCATAAAGGGCCGCATCCTCAGCTCGATCTTGgtaagatacctcc..(0.4 kb)..tccccacccactagGTAAACTT 926
CATCGTGTTTATTTGCATCATCCGAATCCTGCTTCAGAAACTGCGGCCCCCAGATATCAGGAAGAGTGACAGCAGTCCAT 1006
                              Exon 11      TM VI
ACTCgtgagtgtgg......(3.5 kb)......ctgtctccagAAGGCTAGCCAGGTGCAGACTCCTGCTGATCCCCCT 1046
                                                    TM VII
GTTTGGAGTACACTACATCATGTTCGGCGTTCTTTCCGGACAATTTTAAGCCTGAAGTGAAGATGGTCTTTGAGCTCGTCG 1126
                                          Exon 12
TGGGGTCTTTCCAGgtatgggctgt....(0.6 kb)....tcttccttctcagGGTTTTGTGGTGGCTATCCTCTACTG 1166
                                          Exon 13
CTTCCTCAATGGTGAGgtaagcccct......(0.9 kb)......cctctcctagGTGCAGGCGGAGGCTGAGGCGGAAG 1206
TGGCGGCGCTGGCACCTGCAGGGCGTCCTGGGCTGGAACCCCAAATACCGGCACCCGTCGGGAGGCAGCAACGGCGCCAC 1286
GTGCAGCACGCAGGTTTCCATGCTGACCCGCGTCAGCCCAGGTGCCCGCCGCCTCCTCCAGCTTCCAAGCCGAAGTCTCCC 1366
TGGTCTGACCACCAGGATCCCAGGGGCCCAAGGCGGCCCCTCCCGCCCCTTCCCACTCACCCCGGCAGACGCCGGGGACA 1446
GAGGCCTGCCCGGGCCGCGGCCAGCCCCGGCCCTGGGCTCGGAGGCTGCCCCCGGCCCCCTGGTCTCTGGTCCGGACACTC 1526
CTAGAGAACGCAGCCCTAGAGCCTGCCTGGAGCGTTTCTAGCAAGTGAGAGAGATGGGAGCTCCTCTCCTGGAGGATTGC 1606
AGGTGGAACTCAGTCATTAGACTCCTCCTCCAAAGGCCCCCTACGCCAATCAAGGGCAAAAAGTCTACATACTTTCATCC 1686
TGACTCTGCCCCCTGCTGGCTCTTCTGCCCAATTGGAGGAAAGCAACCGGTGGATCCTCAAACAACACTGGTGTGACCTG 1766
AGGGCAGAAAGGTTCTGCCCGGGGAAGGTCACCAGCACCAACACCACGGTAGTGCCTGAAATTTCACCATTGCTGTCAAG 1846
TTCCTTTGGGTTAAGCATTACCACTCAGGCATTTGACTGAAGATGCAGCTCACTACCCTATTCTCTCTTTACGCTTAGTT 1926
ATCAGCTTTTTAAAGTGGGTTATTCTGGAGTTTTTGTTTGGAGAGCACACCTATCTTAGTGGTTCCCCACCGAAGTGGAC 2006
GGCCCTCTGGGTCAGTCTGGTGGGAGGACGGTGCAACCCAAGGACTGAGGGACTCTGAAGCCTCTGGGAAATGAGAAGGC 2086
AGCCACCAGCGAATGCTAGGTCTCGGACTAAGCCTACCTGCTCTCCAAGTCTCAGTGGCTTCATCTGTCAAGTGGGATCT 2166
GTCACACCAGCCATACTTATCTCTCTGTGCTGTGGAAGCAACAGGAATCAAGAGCTGCCCTCCTTGTCCACCCACCTATG 2246
TGCCAACTGTTGTAACTAGGGTCAGAGATGTGCACCCATGGGCTCTGACAGAAAGCAGATACCTCACCCTGCTACACATA 2326
CAGGATTTGAACTCAGATCTGTCTGATAGGAATGTGAAAGCACGGACTCTTACTGCTAACTTTTGTGTATCGTAACCAGC 2406
CAGATCCTCTTGGTTATTTGTTTACCACTTGTATTATTAATGCCATTATCCTGAATTCCCCTTGCCACCCCACCCTCCCT 2486
GGCGTGTGGCTGAGGAGGCCTCCATCTCATGTATCATCTGGATAGGAGCCTGCTGGTCACAGCCTCCTCTGTCTGCCCTT 2566
CACCCCAGTGGCCACTCAGCTTCCTACCCACACCTCTGCCAGAAGATCCCCTCAGGACTGCAACAGGCTTGTGCAACAAT 2646
AAATGTTGGCTTGGAttggcttggttcccctggtggttgatattctcaatatggcctaaaatgggaccagcttccaactt 2726
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FIG. 2. Nucleotide sequence of the HVR1 gene exons. Nucleotide numbers are for the HVR1 cDNA sequence (uppercase), with the first nucleotide of the translation start codon (ATG) designated as +1. Introns and flanking gene sequences (lowercase) and intron/exon splice junctions (boldface) are indicated. Translation start and stop codons and the polyadenylylation site are underlined. Transmembrane segments I–VII for the cDNA coding region are underlined, and the sizes of the intron segments determined by PCR analysis are indicated in parentheses.

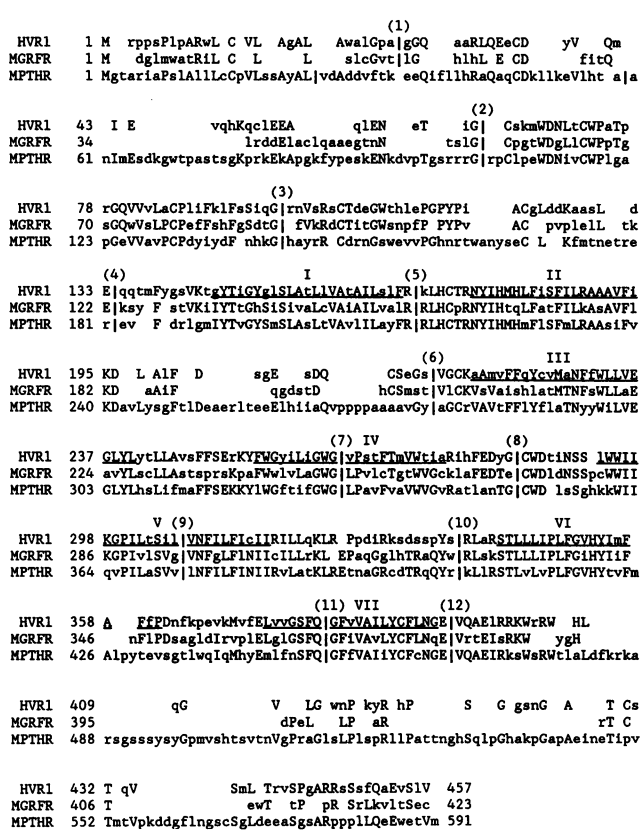

FIG. 3. Alignment of amino acid sequences of HVR1, murine growth hormone-releasing factor (MGRFR), and murine parathyroid hormone receptor (MPTHR) proteins and the location of intron/exon boundaries. Residues common to two or more receptors (uppercase), the locations of introns 1–12 (|), and transmembrane segments I–VII (underlined) for HVR1 are indicated. Gaps were introduced to maximize the alignment.

vol) dextran sulfate, 2× SSC, human competitor DNA (200 μg/ml), and sonicated salmon sperm DNA (1 mg/ml). Hybridization was carried out in a moist chamber overnight at 37°C. Posthybridization washes were performed sequentially in 50% formamide/2× SSC at 42°C and 0.2× SSC at 60°C, three times for 5 min each. Hybridized probes were detected by incubation with fluorescein isothiocyanate-conjugated avidin (5 mg/ml) in 4× SSC/1% (wt/vol) bovine serum albumin/0.1% (vol/vol) Tween 20 at 37°C for 30 min, followed by washing in 4× SSC/0.1% Tween-20 at 42°C for 15 min. The chromosomes were G-band labeled with 4′,6′-diamidino-2-phenylindole (DAPI) dihydrochloride (Sigma) and counterstained with propidium iodide (Sigma). The slides were examined under a Zeiss fluorescence photomicroscope and photographed with Kodak color 400 ASA film.

RESULTS AND DISCUSSION

Cloning and Characterization of the HVR1 Gene. Human placental genomic libraries in the EMBL3 SP6/T7 λ-phage vector or in the pWE15 cosmid vector were screened by hybridization with a mixture of ^{32}P-labeled oligonucleotide probes derived from the HVR1 cDNA sequence. After three sequential rounds of hybridization, one positive phage clone (HVRG3) and two cosmid clones (HVRG2.3 and HVRG6.4) were obtained and subjected to Southern blot restriction analysis with a series of ^{32}P-labeled oligonucleotide probes spanning the entire HVR1 cDNA sequence. The 14-kb λ-phage HVRG3 insert containing 3′ HVR1 sequence was completely contained within the 29-kb cosmid HVRG2.3 insert, while the 36-kb cosmid clone HVRG6.4 bearing 5′ HVR1 sequence partially overlapped with HVRG2.3 (Fig. 1). Fragments derived from *Eco*RI digestion of HVRG3 and HVRG2.3 and *Sac* I digestion of HVRG6.4 (Fig. 1) were subcloned for sequence analysis.

Southern blot analysis of restriction enzyme-digested human genomic DNA probed with a ^{32}P-labeled HVR1 cDNA fragment indicated the presence of a single-copy gene (data not shown). The HVR1 gene spanned ≈22 kb and consisted of at least 13 exons, ranging from 42 to 1479 bp in length (Figs. 1 and 2). The largest exon encoded the cytoplasmic tail and the entire 3′ untranslated region, including a single polyadenylylation site (Figs. 1 and 2). Several allelic point variations were observed, predominantly in the 3′ untranslated region. All of the 12 introns, ranging from 0.3 to 6.1 kb, were located within the coding region (Figs. 1 and 2). The extracellular amino terminus was interrupted by four introns, and introns were present in the loop regions between transmembrane segments I and II, II and III, IV and V, and V and VI, and after transmembrane segment VII. In addition, introns were located within transmembrane segments IV, V, and VII. All of the exon/intron splice junctions characterized for the HVR1 gene (Fig. 2) followed the consensus GT–AG rule (11).

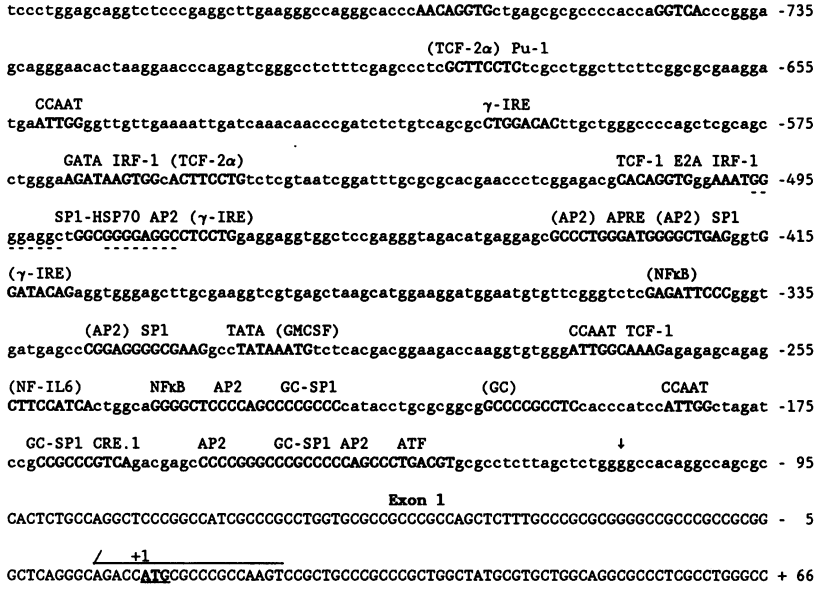

FIG. 4. Structure of the 5′ flanking region of the HVR1 gene. The first nucleotide of the ATG translation start codon (underlined) is designated as +1. The cDNA sequence and upstream consensus motifs for cis elements (uppercase) and cis elements observed in the reverse orientation (parentheses) are indicated (see text for explanation). The transcription start site obtained by primer extension analysis with a 20-nt primer (overlined) is marked (arrow). The locations of an 8-bp repeat are designated with dashed lines.

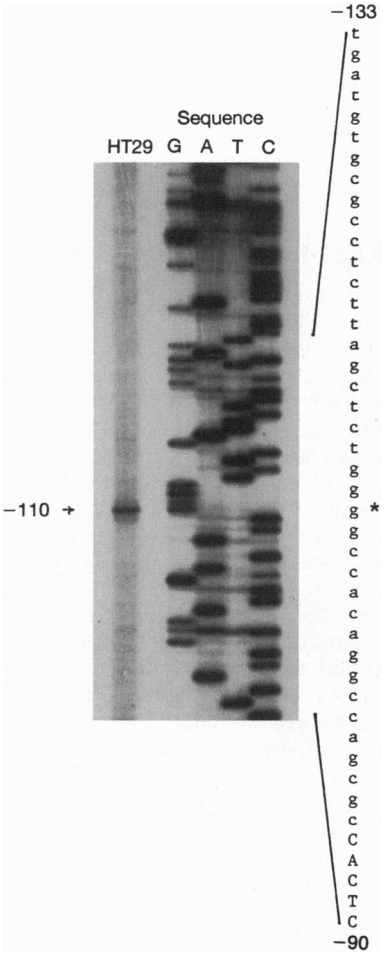

FIG. 5. Analysis of transcription initiation of the HVR1 gene by primer extension. An end-labeled primer traversing the ATG start codon for the HVR1 cDNA was hybridized to 2 μg of poly(A)$^+$ RNA from HT29 cells and extended with reverse transcriptase (Superscript II; GIBCO/BRL). Reaction products were separated in a 5% polyacrylamide/8 M urea sequencing gel. Marker lanes G, A, T, and C indicate the sequencing ladder for exon 1 of the HVRG1 gene. The nucleotide position for the transcription start site (arrow), relative to the ATG start codon (+1), is indicated along with flanking nucleotide sequence.

Both type I VIP and type I PACAP receptors can positively couple to concurrent increases in intracellular cAMP and free Ca^{2+}, presumably through association with different G proteins (12, 13). Five spliced variants of the type I PACAP receptor cDNA have been identified with insertions at the C terminus of the third cytoplasmic loop connecting transmembrane segments V and VI, and these variants appeared to affect receptor coupling to adenylyl cyclase and phospholipase C stimulation (13). A 3.5-kb intron was located in this region for the HVR1 gene (Fig. 2), leading to the possibility of additional exons within this region.

Recently, gene structures of murine receptors for growth hormone-releasing factor (MGRFR) and parathyroid hormone (MPTHR) were described (14–16). Remarkably, the location of intron/exon boundaries with respect to the primary amino acid sequences for HVR1, MGRFR, and MPTHR proteins are generally well conserved, indicating a common ancestory for this receptor gene family (Fig. 3). For the encoded extracellular N-terminal region, intron positions and numbers were similar for HVR1 and MGFR genes but differed for the MPTHR gene, which had an additional exon in this region (Fig. 3).

Analysis of the 5′ Regulatory Flanking Sequence of the HVR1 Gene. The start for the HVR1 cDNA sequence previously obtained was at nucleotide position −94 upstream of the first nucleotide (+1) for the ATG translation start codon (Fig. 2). Primer extension analysis with poly(A)$^+$ RNA from HT29 cells indicated that a prominent transcription initiation site was located at position −110 (Figs. 4 and 5). This finding was further confirmed with RNase protection assays (data not shown).

Transcription promoters for many eukaryotic genes consist of a TATA-box sequence and a CCAAT-box sequence, which are typically located 20–30 bp and 60–80 bp, respectively, upstream of the transcription start site. Although a TATA motif (TATAAA) was present at −310, it was unlikely to be functional in HT29 cells, because it was located too far upstream of the observed transcription start site (Fig. 5). Consensus inverse CCAAT motifs (ATTGG) were present at −185, −275, and −651. The proximal CCAAT box was located 75 nt upstream of the transcription start site, and the G+C-rich 5′ region in the vicinity contained four inverse GC-box motifs associated with binding sites for the Sp1 transcription factor, as well as three recognition sequences for the AP2 transcription factor (Fig. 4). Similar arrangements have been observed for several genes with TATA-less promoters (17). Other consensus cis-element sequences (18) observed upstream included interferon γ recognition elements (γ-IRE), interferon regulatory factor 1 (IRF-1) sites, NF-κB sites, cAMP response elements (CREs) (CRE-1 and ATF), an acute-phase response element (APRE), a nuclear factor-interleukin 6 (NF-IL6) recognition site, and a retinoic acid response element (RARE) (Fig. 4).

A role for HVR1 in acute-phase reactions and inflammatory responses may be inferred from the presence of IRF-1, γ-IRE, NF-IL6, and APRE motifs in the 5′ flanking sequence. Interferons, tumor necrosis factor, interleukins 1 and 6, and leukemia inhibitory factor induce expression of IRF-1 and activate NF-IL6 and APRE-binding factors, which can interact with their respective elements and stimulate transcription (18–21).

Tissue Expression. Human tissue Northern blots examined with a ^{32}P-labeled HVR1 cDNA probe revealed prominent expression of the HVR1 transcript in lung, with weaker expression in several tissues, attesting to the wide range of expression for HVR1 (Fig. 6). The relative intensity of expression for the 3-kb HVR1 transcript was lung > prostate > peripheral blood leukocyte, liver, brain, small intestine >

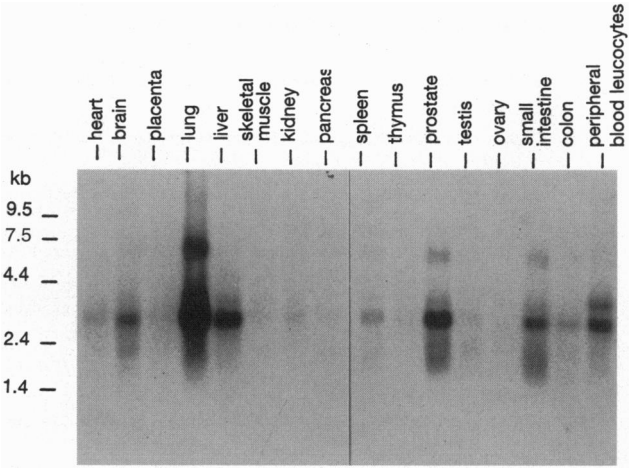

FIG. 6. Tissue expression of the HVR1 gene. Northern blot membranes containing poly(A)$^+$ RNA from the indicated human tissues were hybridized to a ^{32}P-labeled HVR1 cDNA insert and autoradiographed. The sizes and locations of RNA molecular weight markers (GIBCO/BRL) are also depicted.

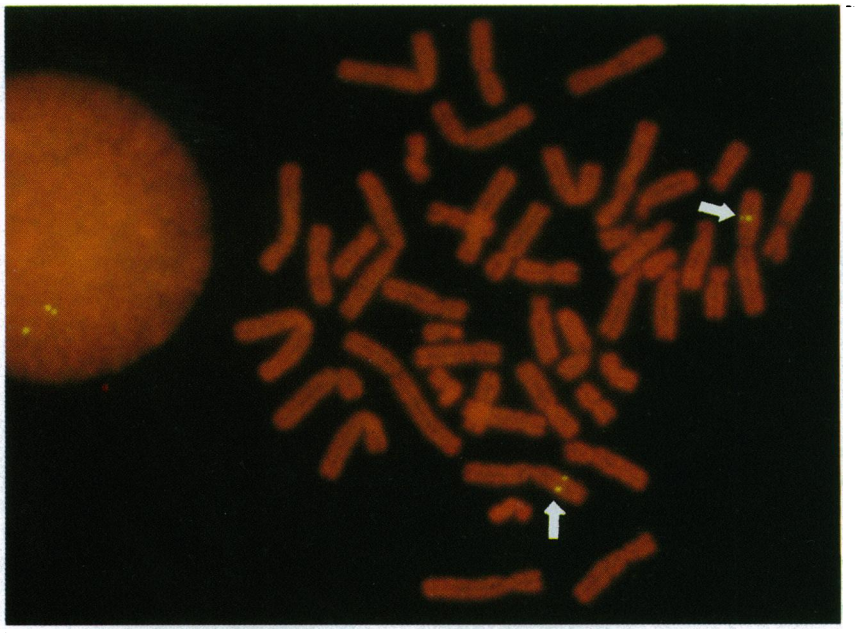

FIG. 7. Chromosome localization of the HVR1 gene shown by fluorescence *in situ* hybridization of a human metaphase chromosome spread with a biotin-labeled HVR1 gene probe. Arrows indicate the location of the HVR1 gene on the short arm of chromosome 3, in the region p22.

colon, heart, spleen > placenta, kidney, thymus, testis. In addition, 4- and 6-kb HVR1 transcripts with varying intensities of expression were observed for all of the above tissues (Fig. 6). These may represent splicing intermediates or spliced variants of the 22-kb HVR1 gene, the complex structure of which could result in diverse patterns of splicing of the primary transcript.

Chromosomal Localization. The chromosomal location of the HVR1 gene was determined by fluorescence *in situ* hybridization of about 200 metaphase chromosome spreads. Hybridization of the biotinylated HVRG3 phage probe to chromosome spreads was detected with fluorescein isothiocyanate-conjugated avidin, and the location of the spots was revealed by G-band labeling of the chromosomes. In 90% of these metaphase spreads, specific signals were detected on one or both sister chromatids of chromosome 3 around the region of p22, with no significant signal on any other chromosome (Fig. 7).

Allele loss in the region 3p23–p21 has been linked to many types of cancer, including small-cell lung carcinoma, possibly due to the presence of a functional tumor-suppressor gene in the region 3p22–p21 (22, 23). A high level of expression of HVR1 in lung tissue, coupled with the location of the gene in a region associated with small-cell lung cancer, may imply a role for the receptor in the etiology of tumor malignancy. Analysis of gene-associated polymorphisms could provide further information on the role of HVR1 in human disease.

We thank Tong-Hui Ma, Derek Patel, and Yvonne Kong for expert assistance. We are very grateful to Drs. Ying Su and Roger Lebo (University of California, San Francisco) for providing the genomic libraries. This work was supported in part by National Institutes of Health Grants DK44876, AI29912, and AI34570.

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