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Organization and chromosomal localization of the gene for the human bombesin receptor subtype expressed in pregnant uterus

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Abstract

The gene encoding the human homologue of the guinea pig uterine bombesin receptor [(1992) Eur. J. Biochem. 208, 405] was isolated from a genomic lambda library by the PCR/homology screening approach. The gene spans more than 4 kb and consists of 3 exons and 2 introns. The deduced amino acid sequence shows about 86% identity to that of guinea pig bombesin receptor. This subtype of bombesin receptor is expressed in the pregnant uterus and in two human tumour cell lines, T47D (ductal breast carcinoma) and A431 (epidermal carcinoma). PCR analysis of genomic DNA from human-mouse cell hybrids allows the cloned gene to be localized to the region q26–q28 on chromosome X.

Key words: Bombesin; Bombesin receptor; Chromosomal localization

1. Introduction

Bombesin isolated from frog skin in 1971 [1] gave the name to the large family of related regulatory peptides discovered later. More than 20 peptides of this family can be classified into three subfamilies according to their C-terminal tripeptide: bombesin, ranatensin/litorin and phyllolitorin subfamilies [2]. To date, only three bombesin-like peptides – gastrin-releasing peptide (GRP), its C-terminal decapeptide neuromedin C, and neuromedin B – were identified in mammals. The first two represent the bombesin subfamily, neuromedin B belongs to the ranatensin/litorin subfamily. No mammalian member of the phyllolitrin subfamily has been yet found.

Bombesin-like peptides demonstrate a wide spectrum of biological activities in mammals, such as secretion of gut hormones, the regulation of smooth-muscle contraction, the regulation of homeostasis and thermoregulation. They are involved in cell proliferation and act as autocrine growth factors for small-cell lung carcinoma [3].

Bombesin-like peptides exert their effects via receptors, that can be distinguished based on binding studies as GRP-preferring and neuromedin-B-preferring receptors [4]. Both types of human and rodent receptors have been cloned and characterized [5–7]. Recently we have cloned a new bombesin receptor subtype from guinea pig that is expressed in uterus during pregnancy [8]. The similarity between this receptor and the two other cloned rodent bombesin receptors, the GRP receptor and the neuromedin B receptor, is 52% and 47%, respectively, i.e. lies in the range usually observed for receptor subtypes within the same family. The new receptor subtype binds GRP better than neuromedin B, but the low affinity of the binding (IC_{50} = 300 nM) does not allow to attribute this receptor to the GRP-subtype.

In this paper we report the identification of the gene for a human homologue of this receptor, its molecular organization and chromosomal localization.

2. Materials and methods

Polymerase chain reaction (PCR) was carried out using human genomic DNA and degenerate oligonucleotides based on the bombesin receptor sequence from guinea pig. The PCR conditions were following: 30 cycles 94°C, 1 min; 50°C, 1.5 min; 72°C, 3 min. PCR products were resolved on agarose gel, transferred to Hybond-N and hybridized to the ^{32}P-labelled 1.5-kb *Eco*RI–*Pst*I fragment of PU61 clone [8]. Amplified DNA was cloned into pGEM-7Zf(+) (Promega) and partially sequenced. The DNA insert of the clone with high homology to the bombesin receptor from guinea pig was used to screen a human placenta genomic library (Stratagene, Heidelberg). Phage DNA from one positive clone, HSUBR, was isolated and subjected to restriction analysis. DNA subfragments were isolated, recloned into pGEM-7Zf(+) or into pUC19 and sequenced on both strands by the dideoxynucleotide chain-termination method using a Sequenase version 2.0 kit (United States Biochemicals) and utilizing universal and internal oligonucleotide primers.

Most techniques, including Southern blotting, screening of phage library, plasmid and phage DNA isolation, recloning, were done by standard protocols [9].

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The novel nucleotide sequence data presented here have been deposited in the EMBL sequence data bank under Accession No. X76498

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Human myometrium (3 probes at term, two from non-pregnant uterus) was obtained from surgical specimen with informed consent of the patients. For reverse transcription–polymerase chain reaction (RT-PCR) total RNA was isolated from frozen tissues or cells by the acid-guanidinium-isothiocyanate/phenol-chloroform method [10] and mRNA was prepared using the PolyATtract mRNA Isolation system (Promega). Single-stranded cDNA was synthesized with Superscript reverse transcriptase (Gibco BRL), the template RNA was hydrolysed with NaOH and the cDNA was subjected to 30 cycles PCR (94°C, 1 min; 44°C, 2 min; 72°C, 3 min) with primers HBR 3 (5'-ACTCAC-TACCTTGCAGAA-3') and HBR 4 (5'-TACATAGGTTTGAG-AAGT-3') in the presence of 5% DMSO. The PCR product was analysed by Southern blot hybridization with the ^{32}P-labelled 380-bp *XbaI–ScaI* fragment of HSUBR (exon 2). The PCR product was cloned into pGEM-T (Progema) and partially sequenced.

For chromosomal localization of the human bombesin gene, 200 ng DNA from various somatic cell hybrids was subjected to PCR (30 cycles 94°C, 1 min; 53°C, 1.5 min; 72°C, 3 min) with the primers HBR 1 (5'-CTGGGAGCATACAGATGTC-3') and HBR 2 (5'-CCATTT-GACTTCCCTTACG-3'). The experiments were done first on 14 human–rodent somatic cell hybrids and then on cells, carrying different parts of the human X chromosome. The origin of the cell hybrids used in this study and their chromosome content have been described previously [11,12].

3. Results and discussion

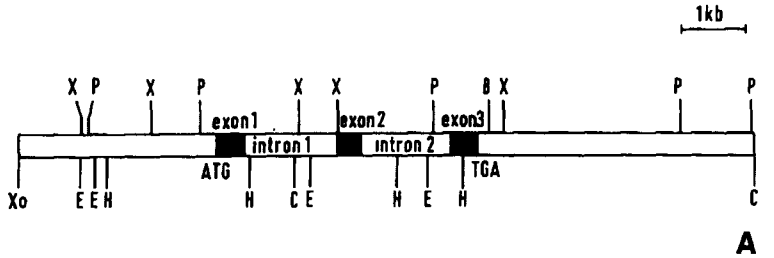
Because our first experiments showed that it would be difficult to find a human tissue expressing this new subtype of bombesin receptor, we decided to clone the genomic gene from a human genomic library. Southern blot analysis of guinea pig genomic DNA revealed that this bombesin receptor subtype like two others [7] contains introns (data not shown). We designed degenerate oligonucleotides for PCR based on the bombesin receptor cDNA sequence, taking into account that the amino acid sequence of the receptor in the position of the primers should be most divergent within the bombesin receptor subfamily and at the same time enough conserved to be present in human species. One pair of oligonucleotides 5'-GGAT$^{A}/_{C}$ATGTC$^{C}/_{T}$ATGAT$^{A}/_{C}$TT$^{T}/_{C}$GC-3' and 5'-GT$^{G}/_{C}$AC$^{G}/_{A}$ATGAAA$^{A}/_{G}$TGGATGGC-3' (transmembrane domains 4 and 7, respectively) gave after PCR on human genomic DNA a product that hybridized to a guinea pig bombesin receptor cDNA fragment. This PCR product (2.3 kb) was gel-purified and cloned into pGEM-7Zf(+). By partial sequencing a clone was identified that contained a coding region for a bombesin receptor similar to the guinea pig receptor but different from two other cloned human bombesin receptors. The two parts of the receptor coding region were separated by a large intron. Using the cloned PCR product as a ^{32}P-probe we screened 500,000 recombinant phage from a human placenta genomic library. One positive clone, HSUBR, was identified, its DNA was isolated and subjected to restriction analysis. The fragments of the large insert were recloned then into pGEM-7Zf(+) or pUC19 and sequenced on both strands (Fig. 1).

The comparison of the cloned human bombesin receptor with other bombesin receptors allowed to localize the start and end of the coding region and to suggest the position of exon–intron boundaries. The last could be proved only by cDNA analysis. For this purpose we applied RT-PCR approach with two HSUBR-specific oligonucleotides designed to the sequences within exons 1 and 3 (see Section 2). Using RT-PCR and Southern blotting of PCR products we observed the fragment of expected size (591 bp) in human pregnant myometrium as well as in two human cell lines, T47D (ductal breast carcinoma) and A431 (epidermal carcinoma) (Fig. 2). The cloning and partial sequencing of the PCR product confirmed that these cell lines and human pregnant myometrium do express bombesin receptor. The sequence data showed the exact position of introns in the bombesin receptor gene. Intron 1 interrupts the codon for Arg^{145}; intron 2 is located between codons for Gln^{262} and Ile^{263}. These positions coincide with that in the two other bombesin receptor subtypes [7]. Every intron has a size of about 1.5 kb and the exon–intron boundaries fit well to the consensus splicing signal [13].

The comparison of the predicted amino acid sequence of HSUBR with that of the guinea pig bombesin receptor (Fig. 3) shows 86% overall identity. Even N-terminal regions of these receptors have 89% identity. The most divergent parts are their C-terminal regions. Nevertheless, all potential phosphorylation sites present in the guinea pig bombesin receptor are conserved in the human homologue too.

Due to the low level of bombesin receptor expression in the tested cell lines and tissues we could not determine the transcription initiation site in this gene. We have sequenced more than 1 kb upstream from the first ATG codon in the coding region (Fig. 1) and tried to localize the promotor elements using computer analysis by the PC-gene program EUKPROM. Several TATA-boxes were identified, but none of them contained a paired CAAT-box element and a cap signal. Some other G-protein-coupled receptors also lack TATA-box sequences or paired TATA/CAAT elements in their promoter region [14,15]. But all these receptors have a high G + C content upstream from the translation initiation site and this feature resembles the promoters of 'housekeeping genes' [16]. In our case, however, the 5' flanking sequence of HSUBR is rather A/T rich, there are no remarkable SP_1-binding motifs [17], thus it is still unclear, how and where the HSUBR gene transcription is initiated.

The Southern blot analysis of human genomic DNA indicated that there is only one copy of this gene in the genome (data not shown). To localize the receptor gene on a chromosome we carried out the PCR analysis on chromosomal DNA from 14 human–rodent somatic cell hybrids [11] using two HSUBR-specific primers (see Section 2). The primers were designed to the C-terminal part of the receptor and to the 3' non-coding region, to regions where there is the highest difference between HSUBR and two other human bombesin receptor genes



A

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caactgcatttgaactgagaagaagaaatattaaagacacagtcttcagaagaaATGGCTCAAAGGCAGCCTCACTCACCTAATCAGACTTTAATTTCAA 1200
                                                                MetAlaGlnArgGlnProHisSerProAsnGlnThrLeuIleSerI   16

TCACAAATGACACAGAATCATCAAGCTCTGTCGTTTCTAACGATAACACAAATAAAGGATGGAGCGGGGACAACTCTCCAGGAATAGAAGCATTGTGTGC 1300
leThrAsnAspThrGluSerSerSerSerValValSerAsnAspAsnThrAsnLysGlyTrpSerGlyAspAsnSerProGlyIleGluAlaLeuCysAl   49

CATCTATATTACTTATGCTGTGATCATTTCAGTGGGTATCCTTGGAAATGCTATTCTCATCAAAGTCTTTTCAAGACCAAATCCATGCAAACAGTTCCA 1400
aIleTyrIleThrTyrAlaValIleIleSerValGlyIleLeuGlyAsnAlaIleLeuIleLysValPhePheLysThrLysSerMetGlnThrValPro   82

AATATTTTCATCACCAGCCTGGCTTTTGGAGATCTTTTACTTCTGCTAACTTGTGTGCCAGTGGATGCAACTCACTACCTTGCAGAAGGATGGCTGTTCG 1500
AsnIlePheIleThrSerLeuAlaPheGlyAspLeuLeuLeuLeuThrCysValProValAspAlaThrHisTyrLeuAlaGluGlyTrpLeuPheG   116

GAAGAATTGGTTGTAAGGTGCTCTCTTTCATCCGGCTCACTTCTGTTGGTGTGTCAGTGTTCACATTAACAATTCTCAGCGCTGACAGgtgagtttcttt 1600
lyArgIleGlyCysLysValLeuSerPheIleArgLeuThrSerValGlyValSerValPheThrLeuThrIleLeuSerAlaAspAr              145

                                                intron 1 ~ 1.1 kb
tctccattatatttgccaggatgtgaaattgggcaaaaagaaaggaaagctt-----tgaattactctcagtcacacataaagtcatgctgataaatgtt 2800
tatttgtcctaaatagagagtctatacataaaattgcaatctggaatatctcataggagtgaataatggaaaaggagttagcataaataaaaatattggg 2900
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                                                                        gTyrLysAlaValValLysProLeuGluA   155

GACAGCCCTCCAATGCCATCCTGAAGACTTGTGTAAAAGCTGGCTGCGTCTGGATCGTGTCTATGATATTTGCTCTACCTGAGGCTATATTTTCAAATGT 3200
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lTyrThrPheArgAspProAsnLysAsnMetThrPheGluSerCysThrSerTyrProValSerLysLysLeuGlnGluIleHisSerLeuCysa   221

TTCTTAGTGTTCTACATTATTCCACTCTCTATTATCTCTGTCTACTATTCCTTGATTGCTAGGACCCTTTATAAAAGCACCCTGAACATACCTACTGAGG 3400
PheLeuValPheTyrIleIleProLeuSerIleIleSerValTyrTyrSerLeuIleAlaArgThrLeuTyrLysSerThrLeuAsnIleProThrGluG   255

                                                intron 2 ~ 1.35 kb
AACAAAGCCATGCCCGTAAGCAGgtatgtattaatcagtactcatgcaaatctagtttgaac-----gctaaatgttttgtttttttttgttgttgttgtt 4850
luGlnSerHisAlaArgLysGln                                                                                 262

ttttgtgtttttttttttttttttttttttttttttgctatgtttcttcccccctatagATTGAATCCCGAAAGAGAATTGCCAGAACGGTATTGGTGTTG 4950
                                                                IleGluSerArgLysArgIleAlaArgThrValLeuValLeu   276

GTGGCTCTGTTTGCCCTCTGCTGGTTGCCAAATCACCTCCTGTACCTCTACCATTCATTCACTTCTCAAACCTATGTAGACCCCTCTGCCATGCATTTCA 5050
ValAlaLeuPheAlaLeuCysTrpLeuProAsnHisLeuLeuTyrLeuTyrHisSerPheThrSerGlnThrTyrValAspProSerAlaMetHisPheI   310

TTTTCACCATTTTCTCTCGGGTTTTGGCTTTCAGCAATTCTTGCGTAAACCCCTTTGCTCTCTACTGGCTGAGCAAAAGCTTCCAGAAGCATTTTAAAGC 5150
lePheThrIlePheSerArgValLeuAlaPheSerAsnSerCysValAsnProPheAlaLeuTyrTrpLeuSerLysSerPheGlnLysHisPheLysAl   343

TCAGTTGTTCTGTTGCAAGGCGGAGCGGCCTGAGCCTCCTGTTGCTGACACCTCTCTTACCACCCTGGCTGTGATGGGAACGGTCCCGGGCACTGGGAGC 5250
aGlnLeuPheCysCysLysAlaGluArgProGluProProValAlaAspThrSerLeuThrThrLeuAlaValMetGlyThrValProGlyThrGlySer   376

ATACAGATGTCTGAAATTAGTGTGACCTCGTTCACTGGGTGTAGTGTGAAGCAGGCAGAGGACAGATTCTAGctttttcaaggaaaaatgctgcttctcct 5350
IleGlnMetSerGluIleSerValThrSerPheThrGlyCysSerValLysGlnAlaGluAspArgPheEnd                               399

cccagcgtgtgtatccgactctaagctgtgtgcaggtgtatggtgtccagatttttgttgtttgaaaaqtgtgttgaaatctttaggagtgaaggatccct 5450
ataagtaagtaaaatacaaaccattacttttcttcaaagtacaaatagtaatgtcatcgggctttctaaataaaatgaagccccactaagtgcagaaagac 5550
aagtttatatatgccagtgaatcgtaagggaagtcaaatgggaaggaaggtgtaataaacaagatgaaacttaaaaatctcatttgttgtttaatcacat 5650
ctgtgatgcttctaactcctcatatactgtgatttgcataaaattgtgtttgtgtttactgtgtggtgtaaatctagagatactttgtatgtgaaaaggg 5750
ggatcgagtcgagtcgacgcc
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B

Fig. 1. Genomic organisation of the human bombesin receptor gene HSUBR. (A) Schematic represensation of the HSUBR gene. The coding region is shown by closed boxes. The sites for translation initiation (ATG) and termination (TAG) are indicted. Xo, *Xho*I; E, *Eco*RI; X, *Xba*I; P, *Pst*I; H, *Hin*dIII; C, *Cla*I; B, *Bam*HI. (B) Nucleotide and deduced amino acid sequence of HSUBR. 5'- and 3'-flanking regions and introns indicted by lower case letters. TATA-boxes in the 5'-flanking region found by computer analysis are underlined.

[7], from one side, and also between HSUBR and PU18 [8], from the other side. In control PCR experiments with chromosomal DNA from human cell lines a single 349 bp fragment was observed. The mouse and Chinese hamster cells produced also PCR products, but these fragments had another size (1,060 and 900 bp, respectively) and did not disturb the interpretation of results. In the panel the human specific band segregated with the human X chromosome. All other human chromosomes could be excluded by their segregation pattern. A number of the panel clones had been generated with cells from human X autosome translocation carriers that re-

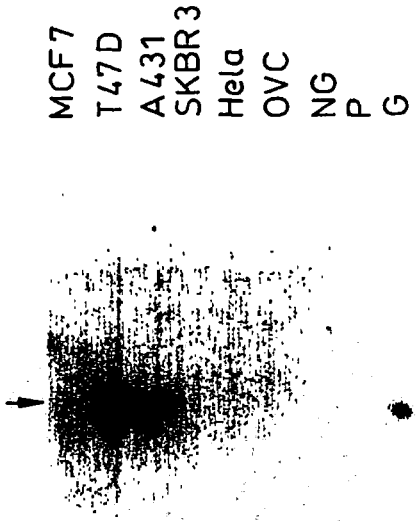

Fig. 2. Analysis of bombesin receptor mRNA expression. RT-PCR products were analysed by Southern blotting (see Section 2). mRNA was prepared from mammocarcinoma cell lines (MCF7, T47D, SKBR3), epidermal carcinoma (A431), ovary carcinoma OVCA3 (OVC), HeLa, from pregnant myometrium (G), non-pregnant myometrium (NG) and placenta (P). Arrow indicates the expected 591-bp fragment.

tained only part of this chromosome (Fig. 4). In these clones the smallest region of overlap (SRO) for the segregation of the human specific PCR fragment was the region Xq26–28. These data indicate that the bombesin receptor gene HSUBR is located in a well studied region of the human X chromosome hosting among other genes the γ-aminobutyric acid A receptor α 3 (GABRA 3), the arginine vasopressin V_2 receptor (AVPR2), a gene for spastic paraplegia 1 (SPG1), for myotubular myopathy

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HSUBR  - MAQRQPHSPNQTLISITNDTESSSSVVSNDNTNKGWSGDNSPGIEALCAI -50
PU18   - MSQKQPQSPNQTLISITNDTESSSSVVSNDTTNKGWTGDNSPGIEALCAI -50
HSUBR  - YITYAVIISVGILGNAILIKVFFKTKSMQTVPNIFITSLAFGDLLLLLTC -100
PU18   - YITYAVIISVGILGNAILIKVFFKTKSMQTVPNIFITSLALGDLLLLLTC -100
HSUBR  - VPVDATHYLAEGWLFGRIGCKVLSFIRLTSVGVSVFTLTILSADRYKAVV -150
PU18   - VPVDATHYLAEGWLFGRIGCKVLSFIRLTSVGVSVFTLTILSADRYKAVV -150
HSUBR  - KPLERQPSNAILKTCVKAGCVWIVSMIFALPEAIFSNVYTFRDPNKNMTF -200
PU18   - KPLERQPSNAILKTCAKAGCIWIMSMIFALPEAIFSNVHTLRDPNKNMTS -200
HSUBR  - ESCTSYPVSKKLLQEIHSLLCFLVFYIIPLSIISVYYSLIARTLYKSTLN -250
PU18   - EWCAFYPVSEKLLQEIHALLSFLVFYIIPLSIISVYYSLIARTLYKSTLN -250
HSUBR  - IPTEEQSHARKQIESRKRIARTVLVLVALFALCWLPNHLLYLYHSFTSQT -300
PU18   - IPTEEQSHARKQVESRKRIAKTVLVLVALFALCWLPNHILNLYHSFTHKA -300
HSUBR  - YVDPSAMHFIFTIFSRVLAFSNSCVNPFALYWLSKSFQKHFKAQLFCCKA -350
PU18   - YEDSSAIHFIVTIFSRVLAFSNSCVNPFALYWLSKTFQKQFKAQLFCCKG -350
HSUBR  - ERPEPPVADTSLTTLAVMGTVPGTGSIQMSEISVTSFTGCSVKQAEDRF -399
PU18   - ELPEPPLAATPLNSLAVMGRVSGTENTHISEIGVASFIGRPMKKEENRV -399
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Fig. 3. Amino acid sequence alignment of guinea pig uterine bombesin receptor (PU18) and HSUBR. Identical residues are shown by vertical lines. Putative transmembrane domains are overlined.

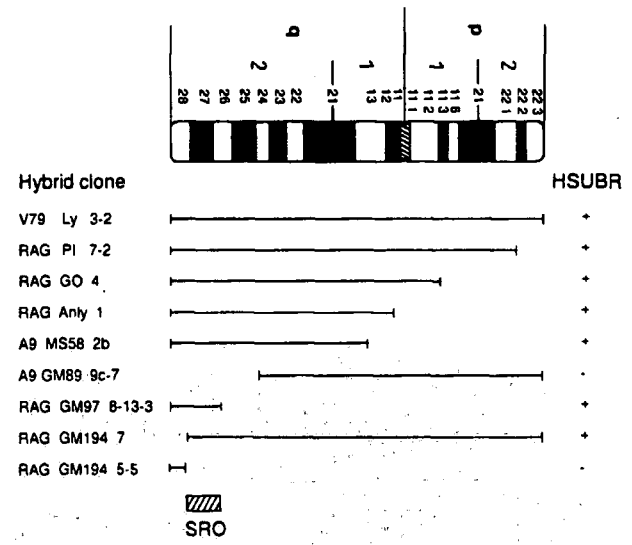

Fig. 4. PCR analysis of DNA from human–mouse somatic cell hybrids containing different fragments of the human X chromosome. The lines indicate which part of the X chromosome is retained in the individual clones used for PCR with primers HBR1 and HBR2. Presence or absence of the HSUBR-specific PCR product is indicated by + or −. SRO, the smallest region of overlap.

1 (MTM1), for Emery–Dreifuss muscular distrophy (EMD) and for Pettigrew syndrome [18].

In conclusion, we have cloned the gene for the human homologue of the guinea pig uterine bombesin receptor, determined its genomic organisation and localized this gene on the long arm of the human X chromosome.

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