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**Short Sequence-Paper**

# Molecular cloning of substance P receptor cDNA from guinea-pig uterus

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A cDNA encoding guinea-pig uterine substance P (SP) receptor has been isolated using the homology screening approach. Northern blot analysis reveals that the corresponding mRNA, of approx. 4.8 kb, is expressed in all tissues tested, but predominantly in the uteri of non-pregnant animals; during pregnancy its expression is reduced. The guinea-pig SP receptor was expressed in COS-7 cells and demonstrated relative ligand affinity in the order: SP ≫ neurokinin A > neurokinin B.

Substance P (SP) belongs to the tachykinin family of peptides which includes, in addition to SP, the peptides substance K (neurokinin A) and neuromedin K (neurokinin B). The tachykinins share a common carboxyl-terminal sequence and give rise to similar spectra of biological activity. SP was the first of these neuropeptides to be discovered, and is the best characterized. It causes smooth muscle contraction and vasodilation, and plays a role in neuron excitation, pain transmission and nerve regeneration [1,2]. An important target organ for SP is the uterus. Immunocytochemical studies have shown that numerous SP-positive neurons innervate the myometrium and uterine arteries [3–5]. SP, in combination with other neuropeptides, plays an essential role in the regulation of myometrial activity and, consequently, in the maintenance of pregnancy and parturition.

SP exerts its effect via the SP receptor (so called NK-1 receptor), a G-protein-coupled receptor which utilises the phosphatidyl inositol second messenger system.

cDNAs corresponding to the rat [6,7] and human [8] SP receptors have recently been cloned and characterized. Here we report the molecular cloning of the guinea-pig SP receptor cDNA and its expression in COS cells.

The nucleotide sequence reported in this paper has been deposited in the EMBL Data Bank under the accession No. X64323.

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The aim of our study was the isolation of cDNAs corresponding to neuropeptide receptors expressed in the guinea-pig uterus. These are G-protein-coupled receptors that share common structural features and have conserved amino acid sequences, particularly within the transmembrane domains. Therefore, we chose the homology screening approach to analyse guinea-pig uterine cDNA library constructed in λgt10 bacteriophage. A degenerate oligonucleotide (5′-GG$^{G}_{C}$A$^{A}_{G}$CCAGCAGA$^{GA}_{TG}$CG$^{G}_{A}$AA-3′) was designed to be complementary to one of the most conserved parts of the G-protein coupled receptors – a region within the sixth transmembrane domain. Duplicate filters were hybridized in 6 × SSC, 1 × Denhardt's solution, 0.05% sodium pyrophosphate at 40°C and washed in 6 × SSC, 0.05% sodium pyrophosphate at 52°C. After subjecting the λgt10 cDNA library (approx. 200 000 pfu) to two screening rounds, two independent positive plaques were identified with inserts of 1.5 kb (clone PU11) and 2 kb (clone PU61), respectively. These inserts did not show cross-hybridization and seemed to represent distinct mRNA species. The inserts were recloned into pUC19 and sequenced by the dideoxy chain termination method using a Sequenase version 2.0 kit (United States Biochemicals) and utilising the exonuclease III/mung bean nuclease approach of Henikoff [9].

The complete cDNA sequence from clone PU11 is shown in Fig. 1. (The studying of clone PU61 will be published elsewhere.) It comprises 1506 bp and has an open reading frame corresponding to 407 amino acids, representing a relative molecular mass of 46 257. Nucleotide sequence comparison analysis revealed that PU11 clone contains cDNA which represents the

guinea-pig SP receptor. Fig. 2 shows the alignment of the amino acid sequence encoded by PU11 with that of rat [6] and human [8] SP receptors. Rat and guinea-pig SP receptors show 94.6% overall homology and human and guinea-pig SP receptors 96.6%. The nucleotide sequence homology between the cDNAs encoding the various SP receptors is, as expected, lower than the homology found at the amino acid level, i.e., 89% for rat and guinea-pig cDNAs and 93% for human and guinea-pig cDNAs. This degree of identity is restricted to the coding region, the 5′ and 3′ untranslated regions differing considerably.

In order to examine the expression of the SP receptor mRNA in several guinea-pig tissues, Northern blot analysis was performed using PU11 cDNA as hybridization probe. Approx. 7.5 $\mu$g of each RNA sample was resolved on a formaldehyde-agarose gel, transferred to Hybond-N membrane (Amersham) and fixed by UV-crosslinking (UV Stratalinker, Stratagene). Hybridization was carried out at 42°C in 50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0.5% SDS using a $^{32}$P-labelled PU11 cDNA insert. The filters were washed to a final stringency of 0.25 × SSPE, 0.1% SDS at 65°C. The SP receptor mRNA was shown to be

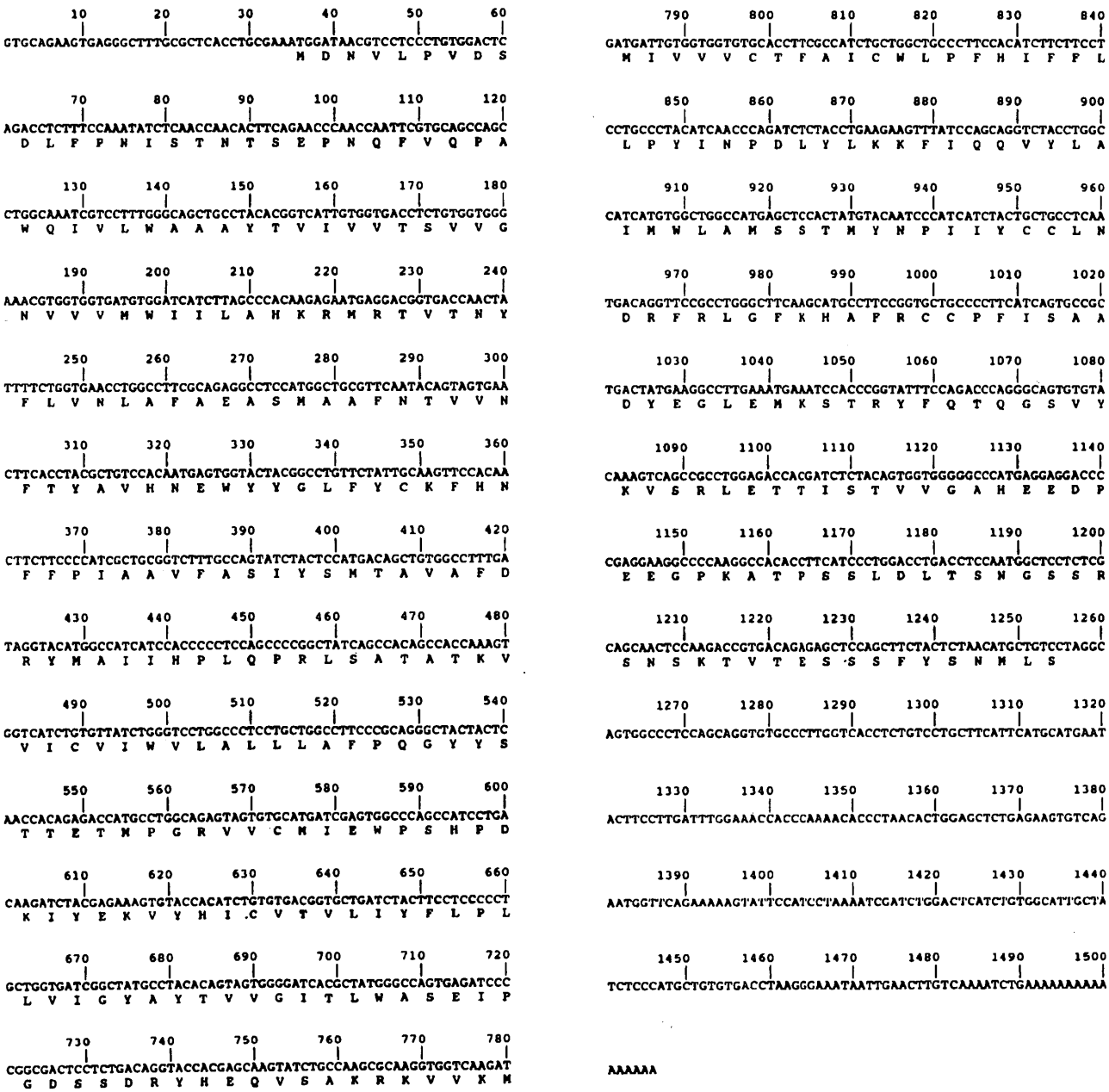


Fig. 1. Nucleotide and deduced amino acid sequence of PU11 cDNA.

present in all mRNA preparations tested, being present in by far the greatest amount in uterus from non-pregnant guinea-pig (Fig. 3). Surprisingly, hybridization occurred to a transcript of approx. 4.8 kb, while PU11 cDNA has a length of 1.5 kb and contains a 5′ untranslated region and an obvious poly(A) tail, with a typical polyadenylation signal, AATAAT [10], 24 bp upstream of the poly(A) tail. There are two possible explanations for this observation: (1) the guinea-pig SP receptor cDNA has an unusually long 5′ untranslated region and/or (2) the poly(A) stretch in PU11 cDNA represents an internal long poly(A) sequence within the 3′ noncoding region of guinea-pig SP receptor mRNA. These possibilities are not mutually exclusive.

In order to demonstrate that PU11 cDNA encodes a functionally active SP receptor, we recloned it into *Bst* XI sites in the eukaryotic expression vector pCDM8, which contains a cytomegalovirus promoter [11]. COS-7 cells were transfected with such a recombinant plasmid by the DEAE-dextran method [12] and, after 72 h, examined for $^{3}$H-labelled SP-binding. Binding assays were performed essentially as described in Ref. 13.

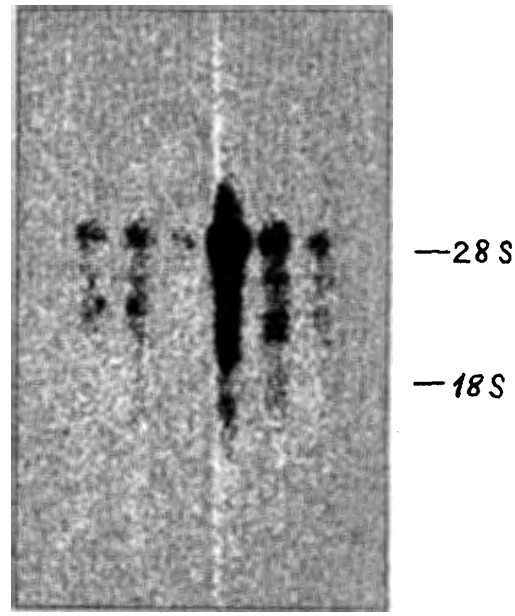


Fig. 3. RNA blot analysis of PU11 guinea-pig mRNA expression. Poly(A)$^{+}$ RNA isolated from pregnant uterus, 50 and 57 days after conception (lanes 1 and 2, respectively), lactating mammary gland (lane 3), nonpregnant uterus, 10 days post partum (lane 4), brain (lane 5) and kidney (lane 6) was run on a formaldehyde-agarose gel, transferred to Hybond-N, and hybridized to $^{32}$P-labelled PU11 cDNA. The positions of 18S and 28S ribosomal RNAs are indicated.

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                                                                1
PU11     - MDNVLPVDSDLFPNISTNTSEPNQFVQPAWQIVLWAAAYTVIVVTSVVGN -50
           |||||| |||| |||||||| |||||| ||||||||||||||||||||||
RNSPR    - MDNVLPMDSDLFPNISTNTSESNQFVQPTWQIVLWAAAYTVIVVTSVVGN
           |||||| |||| |||||||| |||||| ||||||||||||||||||||||
HSSPR    - MDNVLPVDSDLSPNISTNTSEPNQFVQPAWQIVLWAAAYTVIVVTSVVGN
                                                    2
PU11     - VVVMWIILAHKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVHNEWYY -100
           ||| |||||||||||||||||||||||||||| |||||||||||||||||
RNSPR    - VVVIWIILAHKRMRTVTNYFLVNLAFAEACMAAFNTVVNFTYAVHNVWYY
           ||| |||||||||||||||||||||||||||| |||||||||||||||||
HSSPR    - VVVMWIILAHKRMRTVTNYFLVNLAFAEASMAAFNTVVNFTYAVHNEWYY
                                  3
PU11     - GLFYCKFHNFFPIAAVFASIYSMTAVAFDRYMAIIHPLQPRLSATATKVV -150
           |||||||||||||| |||||||||||||||||||||||||||||||||||
RNSPR    - GLFYCKFHNFFPIAALFASIYSMTAVAFDRYMAIIHPLQPRLSATATKVV
           |||||||||||||| |||||||||||||||||||||||||||||||||||
HSSPR    - GLFYCKFHNFFPIAAVFASIYSMTAVAFDRYMAIIHPLQPRLSATATKVV
                                4
PU11     - ICVIWVLALLLAFPQGYYSTTETMPGRVVCMIEWPEHPNRTYEKAYHICV -200
           | |||||||||||||||||||||| |||||||| ||   ||| |||||
RNSPR    - IPVIWVLALLLAFPQGYYSTTETMPSRVVCMIEWPEHPNRTYEKAYHICV
           | |||||||||||||||||||||| |||||||| ||   ||| |||||
HSSPR    - ICVIWVLALLLAFPQGYYSTTETMPSRVVCMIEWPEHPNKIYEKVYHICV
                                5                           6
PU11     - TVLIYFLPLLVIGYAYTVVGITLWASEIPGDSSDRYHEQVSAKRKVVKMM -250
           |||||||||||||||||||||||||||||||||||||||||||||||||||
RNSPR    - TVLIYFLPLLVIGYAYTVVGITLWASEIPGDSSDRYHEQVSAKRKVVKQM
           |||||||||||||||||||||||||||||||||||||||||||||||||||
HSSPR    - TVLIYFLPLLVIGYAYTVVGITLWASEIPGDSSDRYHEQVSAKRKVVKMM
                          6                                 7
PU11     - IVVVCTFAICWLPFHIFFLLPYINPDLYLKKFIQQVYLAIMWLAMSSTMY -300
           |||||||||||||| |||||||||||||||||||||| ||||||||||||
RNSPR    - IVVVCTFAICWLPFHVFFLLPYINPDLYLKKFIQQVYLASMWLAMSSTMY
           |||||||||||||| |||||||||||||||||||||| ||||||||||||
HSSPR    - IVVVCTFAICWLPFHIFFLLPYINPDLYLKKFIQQVYLAIMWLAMSSTMY
PU11     - NPIIYCCLNDRFRLGFKHAFRCCPFISAADYEGLEMKSTRYFQTQGSVYK -350
           |||||||||||||||||||||||||||| |||||||||| ||| ||||
RNSPR    - NPIIYCCLNDRFRLGFKHAFRCCPFISAGDYEGLEMKSTRYLQTQSSVYK
           |||||||||||||||||||||||||||| |||||||||| ||| ||||
HSSPR    - NPIIYCCLNDRFRLGFKHAFRCCPFISAGDYEGLEMKSTRYLQTQGSVYK
PU11     - VSRLETTISTVVGAHEEDPEEGPKATPSSLDLTSNGSSRSNSKTVTESSS -400
           |||||||||||||||| || |||||||||||| |||| |||| ||| |||
RNSPR    - VSRLETTISTVVGAHEEEPEEGPKATPSSLDLTSNGSSRSNSKTMTESSS
           |||||||||||||||| || |||||||||||| |||| |||| ||| |||
HSSPR    - VSRLETTISTVVGAHEEEPEDGPKATPSSLDLTSNCSSRSDSKTMTESFS
PU11     - FYSNMLS -407
           | || |
RNSPR    - FYSNMLA
           | || |
HSSPR    - FSSNVLS
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Fig. 2. Comparison of the amino acid sequences of the guinea-pig, rat and human substance P receptors. Identical residues are indicated by the vertical lines. Putative transmembrane domains (1–7) are shown by the horizontal line.

Cells transfected with the recombinant plasmid carrying PU11 cDNA in the correct orientation showed a transient SP receptor expression, at approx. 260 fmol/$10^{6}$ cells (or 220 fmol/mg protein), whereas nontransfected cells and cells transfected with pCDM8 without insert or containing PU11 cDNA in the reverse orientation demonstrated no specific [$^{3}$H]SP-binding. The expressed receptor was further characterized by comparing the ability of SP and two other members of the tachykinin family, neurokinin A and neurokinin B, to inhibit the specific binding of [$^{3}$H]SP to PU11 cDNA-transfected cells. The cell suspension was incubated with 10 nM [*prolyl*$^{2,4}$-3,4(n)-$^{3}$H]substance P (Amersham, 50 Ci/mmol) in the presence or absence of nonradioactive substance P, a neurokinin A analog (Nle$^{10}$-neurokinin A (4–10), Bachem) [14] or a neurokinin B analog (Pro$^{7}$-neurokinin B, Bachem) [15]. Fig. 4 shows that SP competes most potently for [$^{3}$H]SP-binding in displacement experiments, the neurokinin A and B analogs displacing [$^{3}$H]SP much less effectively. The apparent $IC_{50}$ values for SP, neurokinin A and neurokinin B are 28.6 ± 5.7 nM, 1.2 ± 0.37 $\mu$M and 5.3 ± 2.5 $\mu$M, respectively. Thus, ligand-

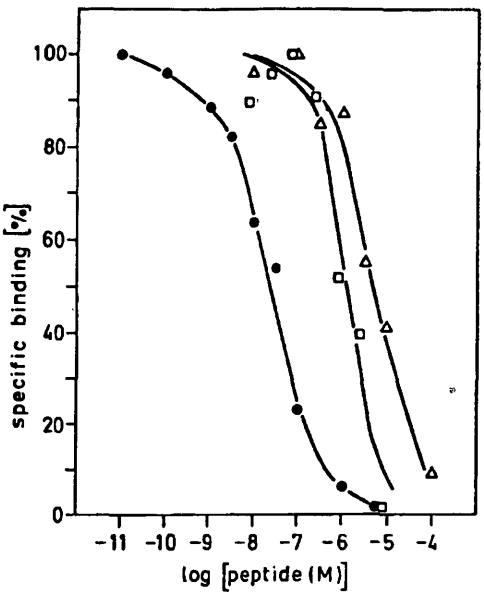


Fig. 4. Displacement of $^{3}$H-labelled substance P binding by tachykinin peptides. The binding data were analysed using the LIGAND program [16]. The unlabelled ligands added to [$^{3}$H]SP are as follows: ●, Substance P; □, neurokinin A analog; △, neurokinin B analog.

binding tests further confirm that the cloned cDNA represents guinea-pig SP receptor cDNA.

The finding that guinea-pig SP receptor expr is reduced during pregnancy is of interest and it would certainly be pertinent to study the regulation of the uterine SP receptor during pregnancy. The cloned SP receptor cDNA is a good tool for a more detailed investigation of this nature.

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