The Human Neurokinin A (Substance K) Receptor

MOLECULAR CLONING OF THE GENE, CHROMOSOME LOCALIZATION, AND ISOLATION OF cDNA FROM TRACHEAL AND GASTRIC TISSUES*

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Neurokinin A (substance K) is a peptide neurotransmitter of the tachykinin family with potential as a major mediator in human airway and gastrointestinal tissues. Neurokinin A acts via a receptor (the NK-2 receptor) believed to be localized on smooth muscle cells and pharmacologically coupled to a GTP-binding protein. To characterize the human NK-2 receptor, we prepared a partial cDNA from human tracheal RNA using the polymerase chain reaction with oligonucleotide primers derived from the bovine NK-2 receptor cDNA sequence (Masu, Y., Nakayama, K., Tamaki, H., Harada, Y., Kuno, M., Nakannishi, S. (1987) Nature 329, 836-838). This partial human NK-2 receptor cDNA was used to screen a human genomic DNA library and yielded a clone, NGNK-2, of approximately 20 kilobases. Analysis of NGNK-2 indicates that it contains the entire coding sequence of the NK-2 receptor as well as 5'- and 3'-flanking sequences. The gene is organized with five exons interrupted by four introns. The complete sequence of the exons and the intron-exon junctions was determined, as were the transcription initiation site and the 3’-polyadenylation signal. Analysis of EcoRI digests of genomic DNA from human-mouse cell hybrids indicates a single gene for the human NK-2 receptor localized to chromosome 10. Sequence analysis of exons 1 and 5, where major differences occur between the human and animal species, provided information for polymerase chain reaction primers which allowed us to prepare full-length cDNA for the human NK-2 receptor. The protein predicted from the gene sequence is extended by 14 amino acids at the COOH terminus compared to the bovine and 9 residues compared to the rat molecules. The seven membrane-spanning regions are encoded by exons 1-4 and none is interrupted by introns. These regions are highly conserved among the species studied, suggesting stringent evolutionary control over these molecules.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) J06688.

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Neurokinin A (formerly known as substance K) is a member of a family of peptide neurotransmitters known as tachykinins (1). These peptides are associated with the central and peripheral nervous systems and display a wide tissue distribution (2-4). Tachykinins share the COOH-terminal structure Phe-X-Leu-Met-NH₂. The best known members of this family are substance P and neurokinin A or substance K. Tachykinins were first recognized for their spasmodogenic effect on smooth muscle-containing tissues (5); however, the full physiologic role for these peptides is still under active investigation. Substance P has been identified as a prominent candidate in sensory neurotransmission (6, 7), and additional evidence suggests effects on immune cells as well (8, 9). Neurokinin A is particularly associated with smooth muscle-containing tissues found in the gastrointestinal, respiratory, genitourinary, and vascular systems (10). The molecular characterization of the tachykinins reveals that they arise from common precursor molecules known as preprotachykinins, by proteolytic processing. Three forms of message (α, β, γ) arise by alternative splicing events (11-13). The β and γ forms of preprotachykinins encode both substance P and neurokinin A, while the α form contains only the substance P sequence. Additionally, an amino-terminally extended form of neurokinin A, termed neuropeptide K or NpK, is present in the β form.

Three classes of tachykinin receptors have been identified by bioassay and radioligand binding (14-16). While the COOH-terminal consensus sequence of the tachykinins controls biological activity, the divergent amino terminal sequences determine receptor affinity. Thus, each tachykinin recognizes the three receptor types, but with varying avidity. The NK-1 receptor preferentially binds substance P, the NK-2 receptor prefers neurokinin A and the NK-3 receptor recognizes neurokinin B. The latter is a tachykinin first found in the porcine spinal cord and brain, and arises from a gene distinct from that for substance P and neurokinin A (17). Synthetic tachykinin analogs act as competitive inhibitors with relative selectivity for each of the three neurokinin receptors (18-20).

Within the respiratory system, tachykinins have a number of important physiologic effects. These include bronchoconstriction of large airways, enhancement of vascular permeability, and stimulation of mucus secretion (21-24). Characterization of these responses using tachykinins and structural antagonist analogs have indicated that the NK-2 receptor, which is selective toward neurokinin A (substance K), predominates in animal and human airways (21, 24).

Masu et al. (25) and Sasai and Nakannishi (26) reported the cDNA and deduced protein sequences for the NK-2 receptors from bovine and rat stomach, respectively, showing the NK-
2 receptor to be a member of the rhodopsin superfamily. This multi-gene family is characterized by the presence of seven hydrophobic sequences which are believed to represent membrane-spanning regions, and are coupled to GTP-binding proteins as signal transducers (27, 28). Because of the potential importance of the neurokinin A receptor in human respiratory biology, a thorough understanding of the structure of these molecules and their regulation is indicated. In the present study we report the molecular organization of the gene for the human NK-2 receptor.

MATERIALS AND METHODS

Preparation of RNA—Human tracheal tissue was obtained at autopsy (approximately 4 h postmortem) from an individual with cystic fibrosis and stored at -80 °C. RNA was extracted from 3-4 g segments of trachea with guanidinium thiocyanate after pulverizing in liquid nitrogen (29, 30) and purified by centrifugation through cesium chloride as described elsewhere (31). Poly(A) RNA was then purified by passing the material over oligo(dT)-Sepharose (32) and transcribed into cDNA by the method of Gubler and Hoffman (33). Human gastrointestinal RNA from resected tissue was the generous gift of Dr. Chris Stevens, Beth Israel Hospital, Boston. Poly(A) RNA and cDNA were prepared from human stomach RNA as described above.

Polymerase Chain Reactions—Polymerase chain reaction was carried out using primers based on the cDNA sequence reported for the bovine NK-2 receptor (29). A sense primer from nucleotides 91-108, 5'-AATGAATTCTGGCAGCTGGCACTGTGG-3', and an antisense primer from nucleotides 538-555, 5'-AATGAATTCCCGGCCACCCGAGCT-3', with EcoRI restriction sites at their 5' ends, were synthesized using standard cyanoethyl phosphoramidite chemistry (Applied Biosystems model 313A DNA Synthesizer), and used with human tracheal cDNA and recombinant λ DNA polymerase (Perkin-Elmer) through 25 cycles consisting of 1 min at 95 °C, 2 min at 37 °C, and 3 min at 72 °C with a final extension period of 7 min at 72 °C. The PCR product was purified following agarose gel electrophoresis. GeneClean (Bio 101, LaJolla, CA), digested with EcoRI, repurified following a second electrophoresis, and ligated to pBluescript SK+ (Stratagene). Full-length cDNA for the human NK-2 receptor was prepared from human gastrointestinal cDNA using primers determined from the gene sequence, and corresponded to nucleotides 1-18 and 1173-1196. These primers also contained EcoRI restriction sites at their 5' ends. The PCR reactions utilized 5 cycles consisting of 1 min at 95 °C, 1.5 min at 45 °C, and 3 min at 72 °C, followed by 25 cycles of 1 min at 95 °C, 1.5 min at 55 °C, and 3 min at 72 °C and a final extension of 7 min at 72 °C. Ten percent of the reaction mixture was subjected to secondary PCR using the same primers and the same cycling conditions as were used for the initial reaction. The material obtained was purified, digested with EcoRI, repurified, and ligated to plasmid vector as described above for the partial cDNA.

Genomic Library Screening—A human placental genomic DNA library (generously provided by Dr. S. Orkin, Children's Hospital and Harvard Medical School, Boston) was screened with the 32P-labeled 465-bp fragment of the human NK-2 receptor cDNA generated by PCR. Bacteriophage DNA was transferred to nitrocellulose filters in duplicate and hybridized with the probe in 5 x SSC (0.75 M NaCl, 0.075 M sodium citrate), 50% formamide, containing 20 mM Tris-HCl, pH 7.5. 1 x Denhardt's solution, 1% dextran sulfate, and 0.1% SDS for 16 h at 65 °C. Filters were washed 10 min in 2 x SSC, 0.1% SDS at 22 °C with three changes followed by 30 min at 68 °C in 0.2 x SSC, 0.1% SDS, and exposed to x-ray film (Kodak, X-Omat) at -70 °C.

Restriction Mapping—Localization of exons within the genomic clone was determined by digesting with PstI or EcoRI, electrophoresing the products and blotting to nylon membranes (Genescreen, Du Pont) and probing with 32P-labeled cDNA corresponding to nucleotides 91-555 generated by PCR, or with synthetic oligonucleotides based on the bovine cDNA sequence. Partial digests were generated with PstI or EcoRI (34), blotted as above, and probed with the 32P-labeled PstI fragment containing exon 4, or a 32P-labeled oligonucleotide primer corresponding to nucleotides -191 to -159 in the 5' untranslated region. The restriction map was determined from the ladder of partial digestion products and confirmed by complete digestion with the same enzymes. All mapping distances are accurate to within 100 bp.

Sequence Analysis—PstI or EcoRI fragments hybridizing with the cDNA corresponding to nucleotides 91-555 or synthetic oligonucleotides based on the bovine cDNA sequence were isolated by agarose gel electrophoresis, purified with the Geneclean kit (Bio 101), dissolved in 5 mM Tris-HCl, pH 7.5, and 0.3 mM EDTA. The annealed RNA/primer mixture was precipitated with ethanol from 0.3 M sodium acetate, redissolved in RT 1 buffer (Boehringer Mannheim), containing 200 units of RNase A, 20 mM dNTPs and extended with 5 units of avian myeloblastosis virus reverse transcriptase (Boehringer Mannheim) for 90 min at 42 °C (36, 37). Remaining RNA was digested with DNase-free RNase A, extracted with phenol/chloroform (38), ethanol-precipitated, and redissolved in 5 mM Tris-HCl, pH 7.5, containing 48% formamide, 10 mM EDTA, 0.025% heparin, and 0.05% Tween 20. The reaction products were analyzed by electrophoresis on a polyacrylamide sequencing gel beside a sequencing reaction conducted with the same primer.

Chromosome Localization—Genomic DNA was prepared from mouse liver and from human leukocytes as previously described (39, 40). Aliquots of 7 µg each were digested with EcoRI, PstI or HindIII (Boehringer Mannheim), products separated on a 1% agarose TAE gel and the DNA blotted to a nylon membrane (Genescreen, Du Pont). The blots were hybridized with 32P-labeled NK-2 receptor cDNA 91-555 obtained by PCR for 16 h at 42 °C in 50% formamide, 1% SDS, 1 M NaCl, 10% dextran sulfate, containing 100 µg/ml salmon sperm DNA. Nonspecifically bound probe was washed 2 x 5 min in 2 x SSC at room temperature followed by 2 x 30 min at 65 °C in 2 x SSC, 1% SDS and the blots were exposed to x-ray film at -70 °C (Kodak, X-Omat). The restriction fragments hybridizing to the NK-2 receptor probe generated by EcoRI and PstI were digested with human and mouse DNA. Chromosome localization for the NK-2 receptor gene was accomplished using DNA from a 40 cell hybrids involving 18 unrelated human cell lines and 4 mouse cell lines (41-43). The hybrids were characterized by karyotypic analysis and by mapped enzyme markers (41, 43, 44). The human NK-2 receptor cDNA 91-555 was hybridized to Southern blots containing EcoRI-digested DNA from the human-mouse hybrids as described above. Scoring was determined by the presence or absence of human bands in the hybrids on the blots.

RESULTS

Isolation of a Partial cDNA for the Human NK-2 Receptor—Synthetic oligonucleotide primers were designed based on the sequence reported for the bovine cDNA (25) from regions of the molecule that contained sequences enriched in tryptophan or cysteine residues. One such pair encoded protein residues 30-35 and 190-195 of bovine sequence. When these primers were used in polymerase chain reaction with cDNA prepared from human trachea obtained at autopsy, they generated a 465-bp cDNA fragment, as shown in Fig. 1. The PCR product was subcloned into pBluescript for subsequent studies. Sequence analysis confirmed the identity of this cDNA as a portion of the human NK-2 receptor by homology with the bovine molecule (data not shown).

Isolation of a Genomic Fragment Encoding the Human NK-2 Receptor—A human genomic DNA library was probed with the 465-bp cDNA, encoding a fragment of the NK-2 receptor.
the NK-2 receptor coding sequence are indicated by the thick line.

...from 1 to 4 kilobases. Although the overall sequence identity is approximately 90%, the human coding sequence is 42 nucleotides longer than the bovine and 30 nucleotides longer than the rat (25, 26).

**Fig. 2. Restriction map of the human NK-2 receptor gene.** DNA from clone NGNK-2, isolated from a human genomic library in EMBL 3, was digested with EcoRI (E) or PstI (P) and analyzed by hybridization with appropriate cDNA or oligonucleotide probes as detailed under "Materials and Methods." The five exons encoding the NK-2 receptor coding sequence are indicated by the thick line.

obtained from PCR. Of approximately \(1 \times 10^6\) bacteriophage plaques screened, four positive clones were obtained, three of which yielded identical restriction patterns with PstI and EcoRI. One of the latter bacteriophage clones, NGNK-2, was subjected to extensive characterization. The restriction map of this clone is shown in Fig. 2 for PstI and EcoRI. Exon-containing fragments were identified by hybridization of Southern blots prepared following digestion with either of these restriction enzymes with cDNA generated by PCR or with oligonucleotide probes based on the bovine sequence. Fragments hybridizing with the tracheal cDNA probe identified exons 1 and 2. Exons 4 and 5 were identified using oligonucleotides encoding the cysteine-rich sequences (CCLNHR, bovine residues 308-313 for exon 4; CCPWVT, bovine residues 324-329 for exon 5). Exon 3 was identified using an oligonucleotide probe patterned after the M5 hydrophobic sequence, LIVIAL (bovine residues 199-205). Fragments identified by hybridization were then subcloned into pBluescript for double-stranded sequencing. The DNA and deduced amino acid sequences are shown in Fig. 3. The coding sequence is interrupted by four introns which vary in size from 1 to 4 kilobases. Although the overall sequence identity is approximately 90%, the human coding sequence is 42 nucleotides longer than the bovine and 30 nucleotides longer than the rat (25, 26).

**Location of the Transcription Initiation Site—**Analysis of exon 1 revealed a single open reading frame which initiated with a methionyl residue analogous to the bovine and rat sequences (25, 26). Inspection of \(\sim 500\) bp of sequence upstream from the methionine revealed a putative TATA box (nucleotide -307) and a GC-like box (nucleotides -356 to -366). The transcription initiation site was determined by primer extension using a synthetic oligonucleotide corresponding to nucleotides -191 to -159 in the 5'-untranslated region. This region was chosen because of the proximity to the putative TATA and GC boxes, within 200 bp upstream of the primer. No other characterized transcriptional control signals were observed within 500 bp of the presumed initiating methionine. The results of this experiment are shown in Fig. 4 and locate this position at nucleotide -282.

**Molecular Cloning of NK-2 Receptor cDNA Using PCR—** An oligonucleotide corresponding to the 5' end of the cDNA was prepared by analyzing the genomic sequence of exon 1 for ATG sites in the reading frame displaying some homology with the bovine sequence. An 18-nucleotide sense oligomer, containing a nested 5'-EcoRI site, was synthesized. At the 3' end, sequence analysis of exon 5 revealed extensive homology with the bovine sequence through the M7 membrane-spanning sequence. Sequences diverged at the genomic region corresponding to the 3'-end of the bovine coding sequence, and the stop codon occurred 42 bp downstream from the bovine homolog. An antisense oligomer, complementary to the genomic sequence proximal to the putative human stop codon, and containing a nested EcoRI site, was synthesized. As shown in Fig. 5, these primers amplified a message of approximately 1.2 kilobases from human stomach RNA, which hybridized specifically with the tracheal cDNA clone on Southern analysis (data not shown).

**Chromosome Assignment—** Preliminary data indicated that EcoRI and PstI restriction digests of mouse and human genomic DNA yielded different patterns of hybridization with the tracheal NK-2 receptor cDNA fragment (not shown). Southern blots of EcoRI-digested mouse-human hybrid cell DNAs probed with human NK-2 receptor cDNA 91-555 and analyzed by concordance/discordance ratios indicate that the receptor is encoded on human chromosome 10 (Table I). The hybrid XTR-3BSAGB with the 10q-, 10pter+10q23: (and no intact chromosome 10), would localize the NK-2 receptor to be on the pter→q23 region of human chromosome 10.

**DISCUSSION**

We generated a cDNA fragment of the human NK-2 receptor molecule by reverse transcriptase-polymerase chain reaction using RNA from human tracheal tissue and a primer pair derived from the bovine structure (25). Sequence analysis of this partial cDNA, which spans protein residues 31-155 of the bovine molecule, was remarkable for the high degree of sequence identity between the human and bovine structures over this region (Fig. 6). Subsequent attempts to generate full-length cDNA for the human molecule were unsuccessful when primers based on the bovine sequence were used. Retrospectively, this is not surprising, since the 5' and 3' ends of the molecule are quite divergent.

The preliminary Southern blot analyses with restriction digests of genomic DNA suggested a single copy gene for the human NK-2 receptor. DNA from human-mouse hybrid cells mapped the gene to the p23-pter region of chromosome 10 (Table I). No evidence was found for cross-hybridization with related genes or pseudogenes under conditions of medium stringency. The chromosomal localization of other members of the rhodopsin superfamily are largely unknown, although...
The tracheal NK-2 receptor cDNA was used to screen a human genomic DNA library, resulting in the isolation of several identical clones. One of these, NGNK-2, was characterized in detail. The partial cDNA provided the identification of exons 1 and 2, and allowed for preliminary mapping experiments. In order to determine the coding sequence for the receptor gene, several methods were used.
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Primer extension analysis. An antisense oligonucleotide corresponding to nucleotides -191 to -159 in the 5'-untranslated region was labeled with [32P]dCTP and polynucleotide kinase. The labeled primer was hybridized with ~2 μg of human stomach poly(A') RNA as described under "Materials and Methods," and extended with avian myeloblastic virus reverse transcriptase. A, dideoxy sequencing reaction, using the identical primer is shown for comparison. The results indicate a cytosine residue at position -282 as the transcription initiation site. B, diagramatic presentation of primer and assigned cap site, indicated by the triangle.

remainder of the molecule, we relied on the high degree of homology encountered in the initial experiments to design appropriate oligonucleotide probes for the remaining exons. Exons 4 and 5 were identified using oligonucleotides spanning regions containing Cys-Cys sequences in the bovine molecule (nucleotides 922-939 for exon 4 and nucleotides 970-987 for exon 5). We reasoned that these sequences might represent structurally important regions with potential for disulfide bond formation, and hence might be more likely to be conserved between species. Hybridization experiments with PstI- or EcoRI-digested NGNK-2 allowed for the isolation of plasmid subclones containing these exons. Exon 3 was identified using an oligonucleotide patterned after the M5 membrane-spanning sequence, because we had come to appreciate that these sequences are, in fact, the most conserved among species. Each exon was sequenced extensively on both strands, as were the intron-exon junction sequences. All intron-exon junctions are compatible with the reported consensus sequences (46).

Our finding of four introns in the human NK-2 receptor gene is in contrast with the organization of genes for other members of the G-protein coupled receptor family. To date, only bovine rhodopsin (47), and D2 dopamine receptor (48) and an unidentified rat molecule (49) contain introns; the coding sequences of the other genes in this family are generally intronless (49).

The sequence deduced from analysis of the genomic clone indicated that the human molecule would be longer than either the bovine or the rat sequences. In order to confirm this finding, we synthesized PCR primers encoding the predicted 5' and 3'-coding sequences and amplified message transcribed from human stomach RNA (Fig. 5). Hybridization and sequence analyses confirmed the identity of the PCR-generated cDNA as the NK-2 receptor.

In analyzing the sequences of the three species (Fig. 6), it is seen that significant variability occurs in the 5'- and 3'-regions of the coding sequences. This is of interest since the model for this class of receptors suggests that the amino-terminal region is part of the extracellular domain, and is potentially part of the ligand-binding site (50). A single site for N-glycosylation is preserved among the three species, at residue 19 of the predicted proteins. Human and bovine, but not rat, have an additional Asn-X-Ser sequence at residues 11-13. Also by analogy with the proposed model for adrenergic receptors, the COOH-terminal segment is the predicted cytoplasmic tail. Recent mutagenesis studies with the β-adrenergic receptor indicate that the proposed third cytoplasmic loop and the proximal portion of the COOH terminus form an interaction site with the Gs protein (50, 51). As previously mentioned, the least structural diversity is seen in the seven hydrophobic domains, which presumably form a pocket or pore within the membrane for ligand binding.

Analysis of the flanking sequences of the human neurokinin A receptor gene revealed a polyadenylation signal (AATTTA) at a position 329 nucleotides downstream from the TGA stop signal of the coding sequence. At the 5'-flanking end, a putative TATA box sequence (ATTTTATA) occurs at nucleotide -307 relative to the initiation methionyl codon. A possible modified GC box (GGGCTGGTCCCG) occurs in the 5'-flanking region at position -356 to -366. Inspection for
### Table I

**Segregation of neurokinin A receptor with human chromosomes in EcoRI-digested human-mouse cell hybrid DNAs**

The NK-2 receptor mapped to human chromosome 10 by somatic cell hybrids as described under "Materials and Methods." Scoring was determined by the presence (+) or absence (−) of human bands in the hybrids on the blots. Concordant hybrids have either retained or lost the human bands together with a specific human chromosome. Discordant hybrids have either retained the human bands, but not a specific chromosome or the reverse. Percent discordancy indicates the degree of discordant segregation for a marker and a chromosome. A 0% discordancy is the basis for chromosome assignment.

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<th>Translocation</th>
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<td>18p+</td>
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<td>−</td>
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<tr>
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<td>−</td>
<td>X/15, 15/X</td>
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<td>+</td>
<td>+</td>
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| Chromosome | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | X |
|------------|---|---|---|---|---|---|---|---|---|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Concordant No. of hybrids | 18 | 20 | 25 | 19 | 27 | 22 | 23 | 26 | 18 | 39 | 26 | 27 | 20 | 28 | 24 | 19 | 26 | 25 | 17 | 16 | 24 | 15 | 20 |
| Discordant No. of hybrids | 17 | 19 | 12 | 21 | 13 | 18 | 15 | 14 | 21 | 0  | 13 | 13 | 20 | 12 | 15 | 21 | 13 | 15 | 23 | 14 | 16 | 24 | 14 |
| Percent discordancy | 49 | 49 | 32 | 52 | 33 | 45 | 39 | 35 | 54 | 0  | 33 | 32 | 50 | 30 | 38 | 52 | 53 | 57 | 58 | 35 | 40 | 89 | 41 |
The technical assistance of Christine Squassoni is appreciated.

Acknowledgments—Drs. Stuart Orkin and Chris Stevens generously provided the genomic library and RNA samples essential to this study. The technical assistance of Christine Squassoni is appreciated.

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