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Molecular cloning and structural analysis of human norepinephrine transporter gene(NETHG)[1]

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ABSTRACT

A cDNA molecule encoding a major part of the human Norepinephrine transporter(hNET) was synthesized by means of Polymerase Chain Reaction(PCR) technique and used as a probe for selecting the human genomic NET gene. A positive clone harbouring the whole gene was obtained from a human lymphocyte genomic library through utilizing the "genomic walking" technique. The clone, designated as phNET, harbours a DNA fragment of about 59 kb in length inserted into BamH I site in cosmid pWE15. The genomic clone contains 14 exons encoding all amino acid residues in the protein. A single exon encodes a distinct transmembrane domain, except for transmembrane domain 10 and 11, which are encoded by part of two exons respectively, and exon 12, which encodes part of domain 11 and all of domain 12. These results imply that there is a close relationship between exon splicing of a gene and structural domains of the protein, as is the case for the human γ-aminobutyric acid transporter(hGAT) and a number of other membrane proteins.

Key words: *Human norepinephrine transporter gene, neurotransmitter uptake, cloning.*

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INTRODUCTION

Norepinephrine(NE) is a major catecholamine neurotransmitter in the peripheral and central nervous systems[1]. In noradrenergic neurons, synaptic transmisslon includes three steps: release of NE into the synaptic cleft, interaction with a postsynaptic receptor, and subsequent removal of NE from the cleft into the presynaptic terminals or surrounding glial cells. This uptake process is carried out via a sodium-dependent NE transporter(NET). The NET is also apparently the initial site of action for therapeutic antidepressants and drugs such as cocaine and the amphetamines[2, 3].

Over the past five years, the cDNAs for many of the known neurotransmitter transporters have been elucidated using PCR, homology screening and expression cloning techniques[3-30]. According to their dependence on Na^+/K^+ or Na^+/Cl^-, these transporters can be divided into two families. Family I, with 6, 8 or 10 transmembrane domains, includes transporters for glutamate / aspartate[4], glutamate[5], certain neutral amino acids[6, 7] and excitatory amino-acid carrier 1[8]. Family II, based on the homology and other characteristics of the transport systems, can be further divided into three subfamilies: i) transporters for ɣ-aminobutyric acid(GABA) [9-13], taurine[14, 15] and choline[16] (enzymaticly dissolved from acetylcholine), but betaine transporter[17] and creatine transporter[18] can also be ineluded here, although betaine is an osmolyte and creatine is a component involved in energy exchange; ii) transporters for glycine[19-21] and proline[22]; and iii) transporters for norepinephrine[3], dopamine[23-26] and serotonin[27-291. All members belonging to family II have 12 constant transmembrane domains. It is noteworthy that transporters are expressed from prokaryote ie. E.coli.[31] to eukaryote ie. yeast[32], Drosophila[29] and mammalian systems, and that the expressions of transporters during development in one species appear to be tightly programmed[13].

Since neurotransmitter transporters are involved in diseases of the nervous system, drug addiction and synaptic plasticity[33, 34, 35], they had been studied pharmacologically and biochemically for a long time before. In contrast, studies on the genomic structures of these transporters have been much more limited[36, 37]. To learn the structure, function and regulation of neurotransmitter transporters in the nervous system, we cloned and analyzed genomic NET gene. The result of this study indicated the unique characters of the biogenic amine transporters' subfamily.

MATERIALS AND METHODS

1. Molecular cloning of the human NET gene

To clone and analyze NET gene, a human brain stem cDNA library cloned in Lambda ZAPII(Stratagene) was amplified and its DNA molecules were extracted according to published procedures. A pair of primers were designed for PCR amplification. The sense primer was corresponding to bases 1111-1137 of NET eDNA molecule with the sequence 5'GAACACAAG*GTCAAC*ATTGAGGATGTG 3', the antisense primer was corresponding to bases 1911-1892 with the sequence 5'CGG-*AAGCTT*GTGACCTGGACATTGGCATGG 3', HinclI and HindIII were underlined. The ampli-

fication protocol consisted of a 1 min denaturation at 94℃, a 1.5 min annealing at 55℃, and a 2 min extension at 70℃ for 30 cycles on DNA Amplifier. A 801 bp DNA fragment was generated by PCR. The 801 bp fragment was cloned into pTZ19u between SmaI and HindIII. The recombinant plasmid was called pNET1. Identification was done by sequencing using the method of Guo and Wu[38].

Using pNET1 insert as a probe to isolate human NET gene from a human genomic library cloned in Cosmid pWE15 (Stratagene), one positive clone harbouring 29.7 kb fragment was obtained and mapped. Far 5'terminal of the insert was used as a probe for chromosome walking. Together this technique was proceeded three times. Then the clone named as phNET contains the entire open reading frame(ORF) of NET.

2. Structural analyzing of the human NET gene

Standard protocols were used for restriction mapping, Southern hybridization, subclone and sequencing[39].

RESULTS AND DISCUSSION

A 801 bp fragment corresponding to 1111-1911 of NET cDNA molecular was obtained from a human brain stem cDNA libray (where hNET is highly expressed) by using PCR technique. After cloning and identification, it was used as a probe to isolate the human NET gene. One positive clone harbouring a 29.7 kb fragment was obtained and mapped. Southern hybridization with γ-^{32}P-dATP labelled oligonucleotides corresponding to 5' terminal in NET cDNA showed that the clone contains only part of the gene. Far 5' terminal of the insert was used as a probe for chromosome walking. Altogether this technique was proceeded for three times, giving 11 kb, 9.5 kb and 8.6 kb extensions towards 5' direction of the gene respectively. Then the clone named as phNET with the entire open reading frame(ORF) of NET was obtained. Fourteen exons encoding all amino acids of NET were determined on ~ 59kb genomic DNA fragment by restriction mapping, Southern hybridization, subclone, and sequencing. Exon 1 to exon 6 were determined by restriction sites in NET cDNA sequence and synthesized oligonucleotides.

The human NE transporter gene is much larger than the human GABA transporter gene[36]. In the NET genomic DNA, intron 1 and 2 exceed 10 kb in length, and exons are more concentrated on the 3' terminal (Fig 1). The deduced amino acid sequence of this gene is identical to that deduced from published NET cDNA molecule.

The intron-exon junctions of this gene are shown in Tab 1. The statistics of all splice donor and acceptor sequences is.....$g_{100}t_{100}a_{54}a_{46}g_{70}t_{61}$.....$c_{93}a_{100}g_{100}$. The twelve putative transmembrane regions of the human NET are encoded by exons 1-12. In general, each transmembrane domain is encoded by a single different exon, with the exception of transmembrane domain 10 and 11, which are encoded by parts of exon 10 and 11, and exon 11 and 12, respectively. Furthermore, exon 12 encodes part of domain 11 as well as the entire domain 12. Compared with the human GABA transporter[36], the organization of these two proteins is rather similar. There are, however, three distinct differences: i) while the human GABA transporter gene is

Tab 1. Exon-intron organization of the human norepinephrine transporter gene(NETHG)

Exon	Nucleotide No.	Size (bp)	3' Acceptor	5' Doner	3'Intron Size(kb)
1	-52 - 274	336	aagc*ag*AGCCTC	GCGGCG*gt*agcg	13.2
2	275 - 406	132	ctcc*ag*GTGCCT	TCAAAG*gt*aaag	2.0
3	407 - 644	238	tgcc*ag*GCGTTG	TTATGA*gt*aagt	12.0
4	645 - 783	139	gggc*ag*GCGTGG	GGAAAG*gt*aata	6.7
5	784 - 918	135	gccc*ag*GTGGTG	GCCACG*gt*cagt	2.1
6	919 - 1022	104	tttc*ag*GTATGG	TTACAG*gt*aaga	1.2
7	1023 - 1147	125	cccc*ag*GGATGC	CAGAAG*gt*gggt	0.9
8	1148 - 1260	113	ttac*ag*GAGCTG	AGCTCA*gt*gagt	1.3
9	1261 - 1389	129	ctcc*ag*ATGGGA	ACCAAG*gt*gcag	0.4
10	1390 - 1489	100	tgcc*ag*GGTGGA	TTTATG*gt*atgt	1.1
11	1490 - 1590	101	gcac*ag*GAGTGG	CTCCTG*gt*gtgt	0.9
12	1591 - 1758	168	ctct*ag*TTCGTG	TGGGAG*gt*agct	2.3
13	1759 - 1830	72	ttac*ag*AGACTG	TTCCAG*gt*gggt	0.3
14	1831 -	>93	ctgc*ag*TTGCAA		

a

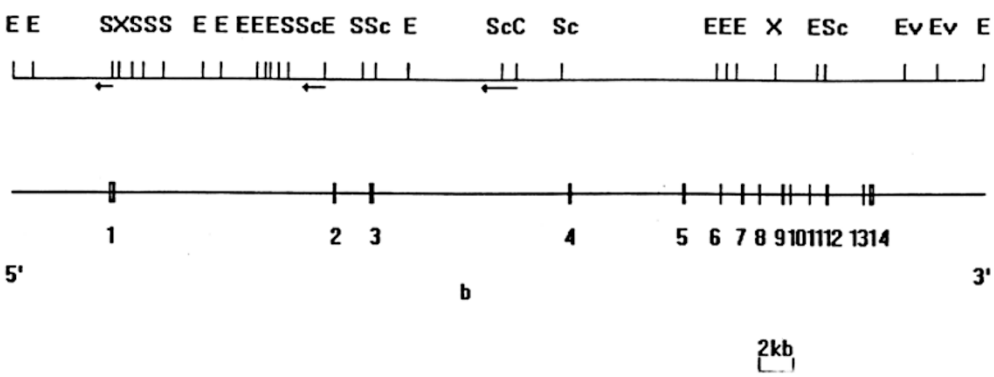

Fig 1. (a) Map of the human genomic DNA coding for norepinephrine transporter (about 59 kb in length). The DNA molecule cloned in cosmid pWE15 was selected by means of genomic walking technique, the probes used in "walking" are indicated by arrows. E: EcorRI; Ev: EcoRV; Sc: ScaI; X: XhoI; C: claI; S: SmaI.
(b) Location of 14 exons in the human norepinephrine transporter gene.

about 25 kb, consists of 16 exons, the human NE transporter is about 46 kb, consists of 14 exons; ii) in the human GABA transporter gene, the largest outside loop is encoded by a separate exon---exon 6 (the translation starting site is in exon 3), while in NE transporter gene, this loop is encoded by 3' terminal of exon 3 and 5' terminal of exon 4; iii) in the human GABA transporter, most of the amino acid residues of the cytoplasmic carboxyl terminus are encoded by exon 16, whereas in the human NE transporter, the C terminal in cytoplasm was encoded by three exons: part of exon 12, and all of exon 13 and 14 (Fig 2).

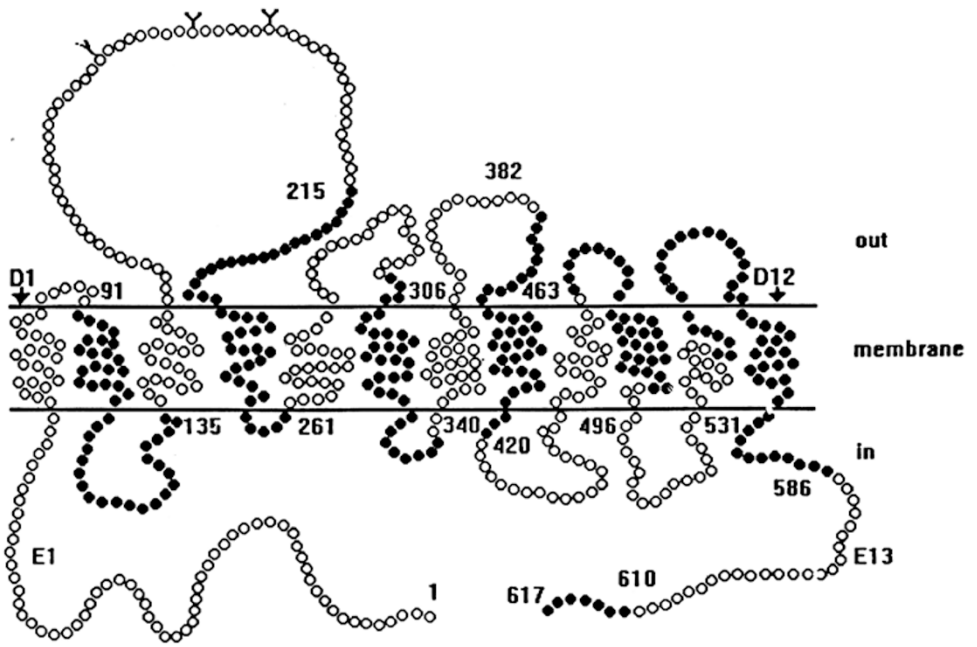

Fig 2. Schematic representation of the human NE transporter protein. The different domains encoded by the various exons within the gene and the proposed orientation in the plasma membrane are shown. Both the N and C termini of the NE transporter protein are positioned inside of the membrane. Open and closed circles represent amino acid residues. A stretch of sequence consisting of the same circles (open or closed) represents an exon encoding regiou(domain) in the transporter protein. Amino acid residue number is indicated. There are three potential glycosylation sites present in the largest loop outside marked by "Y" . D1 and D12 represent transnmmbrane domain 1 and 12. E1 and E13 represent exon 1 and exon 13, respectively.

Tab 2. Amino acid sequences at the domain boundaries in the human norepinephrine transporter protein

Exon	AA. in the Junction	Exon	AA. in the Junction
1	/Met	8	Glu/ Gly
2	Gly /Gly	9	Ser/ Met gly
3	Lys/ Gly	10	Lys/ Gly
4	Glu/Arg	11	Tyr/ Gly
5	GlyLys/ Val	12	Leu/ Phe
6	Thr/ Val	13	Glu/ Arg
7	Arg/ Asp	14	Gln/ Leu

Amino acid residues at domain boundaries are listed in Tab 2. It is noted that glycine or polar amino acid residues always occur there, except for amino acid residues between exon 11 and 12, which are inside transmembrane domain 11. This is possibly a result of evolution, since glycine, an amino acid without any branches in the residue, has the greatest flexibility.

The human NET gene(NETHG) reported here is the first human catecholamine transporter gene that has been cloned and analyzed. From the relationship between exons and domains of NETHG, we predict that for this gene, 14 exons encode 13 functional domains in the NET protein. Exon 14 only encodes seven amino acids which may not comprise an independent domain. Additionally, with the exception of domain 10 and 11, the exon-intron junctions are all located at the border of, or outside, the membrane (Fig 2). The significance of these observations is not known.

Future studies will be focused on the molecular, developmental and pharmacological properties of this protein as well as the regulation of the NET gene. Such analyses should enhance our understanding of the molecular mechanisms of neurotransmitter transporters, and their relationships with synaptic plasticity, learning and certain neurological disorders such as drug addiction and depression.

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