Cell Research (1998),**8**,143-149

Analysis of the 5' flanking sequence of the human norepinephrine transporter gene

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ABSTRACT

The human norepinephrine transporter(NET) gene was cloned and structurally analyzed. The far 5' fragment containing exon 1 (a non-coding exon) and exon 2 was sequenced. The transcription start site of the gene in human brain stem tissue was determined by primer extension analysis. It was found that the gene could be transcribed from multiple starting points. The 5' flanking sequence contains a proximal G-C rich region, one possible GSG element and several SP1 sites. However it does not contain TATA box and CAAT box motifs. Gel shift analysis with nuclear extracts from different tissues of mouse shows that the G-C rich region may be involved in tissue specific expression of the gene.

Key words: *Norepinephrine transporter gene, primer extension, transcription regulation.*

INTRODUCTION

Norepinephrine (NE) is an important neurotransmitter both in embryonic and adult nervous system. Its function in nervous system is dependent on two kinds of important membrane proteins: the receptors on the postsynaptic neurons or effector cells that are responsible for the NE signal transduction, and the transporters on the presynaptic neurons, which are responsible for the termination of NE signaling. The deficiencies of NE transduction are involved in many nervous disorders,

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drug addiction, and other diseases. Numbers of neurotransmitter transporters have been cloned and studied since 1990[1-3]. Norepinephrine transporter(NET) is a major one of catecholamine transporters that belong to sodium-, chloride-dependent neurotransmitter transporter subfamily. Although much work has been done on the NET, little is known about its regulation at the transcription level. We had reported the cloning and structure analysis of human NET gene previously[4]. Here the 5'-proximal DNA sequence of the gene was sequenced and the transcription start sites were determined by primer extension. In addition, preliminary analysis of protein-DNA interaction in the 5' flanking region of NET gene was studied.

MATERIALS AND METHODS

Subcloning and sequencing

The 5' part of the human NET gene, about 5.2 kb in length,was mapped, subcloned, and sequenced by using standard methods[5]. Restriction endonucleases were purchased from Promega and Boehringer, ^{32}P-dATP and ^{35}S-dATP from Amersham, and T7 sequencing Kit from Pharmacia.

Primer extension analysis[6]

Determination of the transcription start sites was carried out by primer extension analysis using the antisense oligonucleotide 5' GATGCGGCTGGCGAGAGG 3', located at -2 ~ -19 of the NET cDNA sequence. Human brain stem total RNA and poly(A)+RNA were prepared with Tripure^{TM} Isolation Reagent (Boehringer), and PolyATract mRNA isolation system (Promega) respectively. Three hundred fmol of 5'-labeled oligonucleotide was hybridized to 30 μg total RNA or 2 μg poly(A)$^+$ RNA at 65℃ for 10 min. in a total volume of 20 μl, then immediately cooled on ice. Reverse transcription was carried out using Expand^{TM} Reverse Transcriptase (Boehringer) according to the instruction of the manufacturer. The product was precipitated with ethanol and separated on sequencing gel.

Nuclear extracts and gel shift assay

Nuclear extracts from human brain, mouse brain, liver and kidney were prepared according to Dignam et al[7]. Protein concentration was quantified with Lowry's method. A fragment, 577bp in length, 506bp upstream from the initial codon (ATG), was used as a probe in gel shift assay. 2-4ng ^{32}P end-labeled DNA fragment, 2 μg poly (dI).(dC) and 7 μg nuclear protein were mixed and incubated at room temperature for 20 min. in binding buffer (10 m*M* hepes, 50 m*M* KC1, 5 m*M* EDTA, 5mM $MgC1_2$, 10% glycerol, pH 7.9). The binding complex was separated by 4% polyacrylamide gel at 10V/cm in buffer (50 m*M* Tris, 380 m*M* glycine, 2 m*M* EDTA) at 4 ℃.

RESULTS AND DISCUSSION

We previously reported the isolation and characterization of a 59 kb of DNA fragment containing the whole ORF of NET. The far 5' fragment of the gene, about 5.2 kb in length, was subcloned and sequenced, the part of the sequence was listed in Fig 1. Exons were determined in the sequence according to the NET cDNA sequence published. The initial translation codon ATG is in the exon 2, and the 9 bp of DNA fragment in 5' terminal of published NET cDNA sequence belongs to another separate exon, the exon 1. Between exon 1 and exon 2 there is the intron 1 of 439 bp with typical gt/ag intron splicing sites.

Fig 1. 5' proximal sequence of human NET gene.
The translation starting codon(ATG) is located in exon 2, with A as position +1. Exon region, Sp1 site, TATA boX sequence, CREs site, GSG site and some restriction sites are marked in the sequence. The G+C rich regions are shown in bold italic characters. transcription starting sites are indicated by star.

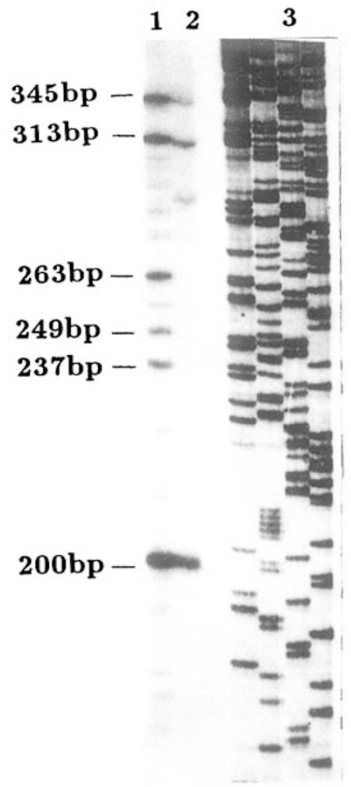

Fig 2. Primer extension mapping of the transcription start site of the human NET gene.
The major transcription start sites are marked by arrow.
1. 2 μg of human brain stem poly $(A)^+$ RNA was used for reverse transcription
2. 30 μg of human brain stem total RNA was used for reverse transcription
3. A DNA sequence ladder was used for DNA length marker.

The sites of transcription initiation were determined by primer extension analysis using human brain stem total RNA and poly$(A)^+$ RNA. It was found that the gene was transcribed from multiple start sites. Six major reverse transcription products with length of 198bp, 235bp, 247bp, 261bp, 311bp, 343bp (Fig 2 lane 1) were determined using poly$(A)^+$ RNA, and if the total RNA was used, the major products were 198bp, 311bp and 343bp (Fig 2 lane 2). Put these together and If there is no additional exon upstream of the exon 1, the major transcription start sites could be located at -638, -751, -783 respectively (Fig 1). There is no appropriate TATA box and CCAAT site that is usually at -25 ~ -30bp upstream from the transcription start site according to typical gene promoter structure. Instead, there are several short GC rich region before the transcription start sites that can also be seen in the gene of mouse GABA transporter (GAT1). Besides,the organization of 5' flanking region of NET is similar to that of N-methyl-D-aspartate receptor 1 (NMDAR1)[8, 9] and ASCT (neutral amino acid transporter)[10]. It is common to some transporters that they don't have TATA and /or CAAT box, which are characters of housekeeping genes. These characters may indicate some common regulation mechanisms in nervous system. Genes with less TATA-box may use GC-rich or AT-rich region as their promoter and tend to be transcribed from multiple starting points[11]. Several Sp1,

CREs (cAMP responsive elements) consensus sequences, remote TATA structures and GSG motif, which may also function in NET gene expression regulation, are indicated in Fig 1. GSG motif that appears in promoter region of many CNS protein genes is recognized by zinc finger proteins. The existence of this site in NET gene may explain that the nerve growth factor acts as an inhibitory neuromodulator during NE transmission[12].

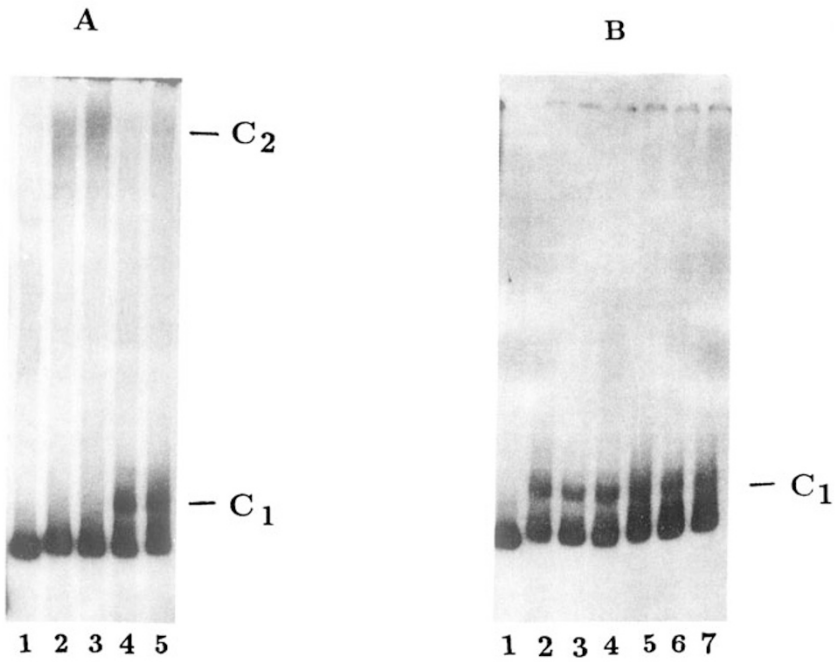

Fig 3. Gel mobility shift analysis of the interactions of NET 5' proximal sequence with nuclear factors from different tissues. the probe was the 577bp of Apa 1/BamH1 fragment in NET 5' proximal region. The double strand oligonucleotides used for competitor are:
S40: 5'GTTTGTCCTGAGGGTTCCATAAGTTTAGTTTTATGTTGTT 3'
S28: 5' TCCCTGTGTGGTGGCCACGTCTTTCCAG 3'
The shift band is marked by arrow.
A: 1. probe alone; 2. nuclear extract from human brain; 3. nuclear extract from mouse brain; 4. nuclear extract from mouse liver; 5. nuclear extract from mouse kidney.
B: 1. probe alone; 2. nuclear extract from mouse liver; 3. nuclear extract from mouse liver and 100 ng S40; 4. nuclear extract from mouse liver and 100 ng S28; 5. nuclear extract from mouse kidney; 6. nuclear extract from mouse kidney and 100 ng S40; 7. nuclear extract from kidney and 100 ng S28.

Analysis of 5' flanking sequence of the human NET

The NET gene has its own tissue specific expression patterns and is regulated subtly. Angiotensin II can stimulate synthesis of NET mRNA[13], but the insulin reduces the concentration of NET mRNA in rat brain[14]. Tricyclic drugs, such as despramine, generally used to treat depression, can increase mRNA level of NET in rat locus Ceruleus[15]. Evidence based on ligand binding study suggests that NET decreases in locus Ceruleus during aging and in Alzheimer's disease[16]. Although there is lots of work on NET, we almost know nothing up to now, about its gene expression regulation. In order to know if the 5'-proximal region of the gene contains gene regulation elements, we cloned the Apa I/BamH I fragment from the 5' sequence of NET gene (577bp, shown in Fig I). It contains main transcription start sites, high G+C region, GSG motif, CRE element, and SP1 sites that were mentioned above. This fragment was then used as a probe in gel shift assay. One major DNA-protein complex band was generated with nuclear proteins from both mouse liver and kidney (C1 in Fig 3A, lane 4, lane 5). However, similar shift band was not detected in case of brain (Fig 3A, lane 2, lane 3), but instead the band at the more up position may be contributed by the binding of factors that recognize the sites such as SP1 and GSG motif (C2 in Fig 3A, lane 2, lane 3). Considering the fact that NET is mainly expressed in the nerve system and adrenal[17], the binding event in the liver and kidney may contribute to the negative regulation of NET gene expression. So, this fragment may contain cis -element that functions in tissue expression pattern of NET. Using the synthesized oligonucleotides S40 and S28 as a competitor (S40, a potential silencer element shown to be conserved in mouse and human GAT1 gene, and S28, a DNA fragment adjacent to S40 in mouse GAT1 that contains eight bps palindrome sequence[18]), the shift band could not be competied (C1 in Fig 3B lane 2-7). It indicates that the protein factors taking part in the gene regulation of GAT1 and NET are different from each other. Further studied will be needed to elucidate the regulation mechanism of NET gene expression.

ACKNOWLEDGEMENTS

We thank Professor WANG Da, Dr. XIA Guo Hong and Dr. YIN Guo Yong for their kind suggestion and help. This work was supported by Foundation From Chinese Academy of Sciences and Qi Ming Xing Foundation from Shanghai Science and Technology Committee.

REFERENCES

[1] Amara S.G. and Kuhar M.J. Neurotransmitter transporters: recent progress. Annu Rev Neurosc 1993; **16**:73-93.
[2] Huang F, Fei J, Guo LH. Neurotransmitter transporter. Life Sciences 1994; **6**:5-8.
[3] Attwell D. and Mobbs P. Neurotransmitter transporters. Current Opinion in Neurobiology 1994; **4**:353-9.
[4] Guo LH, Zhu LH, Huang F, et al. Molecular cloning and structural analysis of human norepinephrine transporter gene (NETHG). Cell Research 1995; **5**:93-100.

[5] Sambrook J, Fritsch EF, Maniatis T (eds). Molecular cloning, second edition. Cold Spring Harbor Laboratory Press, 1989.
[6] McKnightSL, Kingsbury R. Transcriptional control signals of a eukaryotic protein coding gene. Science 1982; 217:316-24.
[7] John DD, Lebovitz RM, Roeder-Jones GJ. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. Nucleic Acids Research 1983; 11(5):1475-89.
[8] Bai G, Kusiak JW. Cloning and analysis of the 5' flanking sequence of the rat N- methyl-D-aspartate receptor I (NMDAR1) gene. Biochimica et Biophysica Acta 1993; 1152:197-200.
[9] Bai G, Kusiak JW. Functional analysis of the proximal 5'-flanking region of the N- methyl-D-aspartate receptor subunit gene, NMDAR1. J Biol Chem 1995; 270:7737-44.
[10] Hofmann K, Diker M, Fink T, et al. Human neutral amino acid transporter ASCTI: structure of the gene (SLC1A4) and localization to chromosome 2p13-p15. Genomics 1994; 24:20-6.
[11] Aso T, Conaway JW, Conaway RC. Role of core promoter structure in assembly of the RNA polymerase II preinitiation complex. J Biol Chem 1994; 269(42):26575-83.
[12] Ueyama T, Hano T, Hamada M, et al. 'New role of nerve grwoth factor-an inhibitory neuromodulator of adrenergic transmission. Brain Res 1991; 559:293-6.
[13] Raizada MK, Lu D, Yang H, Yu K. ATl-receptors and cellular actions of angiotensin II in neuronal cultures of stroke prone-spontaneously hypertensive rat brain. Adv Exp Med Biol 1996; 396:71-8.
[14] Figlewicz DP, Brot MD, McCall AL, Szot P. Diabetes causes differentia] changes in CNS noradrenergic and dopaminergic neurons in the rat: a molecular study. Brain Res 1996; 736(1-2):54-60.
[15] Shores MM, Szot P, Veith RC. Desipramine-induced increase in norepinephrine transporter mRNA is not mediated via alpha 2 receptors. Brain Res Mol Brain Res 1994; 27(2):337-41.
[16] Tejani Butt SM, Yang J, Zaffar H. Norepinephrine transporter sites are decreased in the locus coeruleus in Alzheimer's disease. Brain Res 1993; 631(1):147-50.
[17] Pacholczyk T, Blakely R, Amara S. Expression cloning of a cocaine- and antidepressant-sensitive human noradrenaline transporter. Nature 1991; 350:350-4.
[18] Fei J, Huang F, Ma YH, Guo LH. Characterization of 5'-proximal sequence of mouse GABA transporter gene (GAT-l). Cell Research 1997; 7:61-7.

Received March-31-1998. Revised May-22-1998. Accepted May-25-1998