A Previously Undescribed Intron and Extensive 5′ Upstream Sequence, but Not Phox2a-mediated Transactivation, Are Necessary for High Level Cell Type-specific Expression of the Human Norepinephrine Transporter Gene*

(Received for publication, November 6, 1998)

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The synaptic action of norepinephrine is terminated by NaCl-dependent uptake into presynaptic noradrenergic nerve endings, mediated by the norepinephrine transporter (NET). NET is expressed only in neuronal tissues that synthesize and secrete norepinephrine and in most cases is co-expressed with the norepinephrine-synthetic enzyme dopamine β-hydroxylase (DBH). To understand the molecular mechanisms regulating human NET (hNET) gene expression, we isolated and characterized an hNET genomic clone encompassing approximately 9.5 kilobase pairs of the 5′ upstream promoter region. Here we demonstrate that the hNET gene contains an as-yet-unidentified intron of 476 base pairs within the 5′-untranslated region. Furthermore, both primer extension and 5′-rapid amplification of cDNA ends analyses identified multiple transcription start sites from mRNAs expressed only in NET-expressing cell lines. The start sites clustered in two subdomains, each preceded by a TATA-like sequence motif. As expected for mature mRNAs, transcripts from most of these sites each contained an additional G residue at the 5′ position. Together, the data strongly support the authenticity of these sites as the transcriptional start sites of hNET. We assembled hNET-chloramphenicol acetyltransferase reporter constructs containing different lengths of hNET 5′ sequence in the presence or the absence of the first intron. Transient transfection assays indicated that the combination of the 5′ upstream sequence and the first intron supported the highest level of noradrenergic cell-specific transcription. Forced expression of the paired-like homeodomain transcription factor Phox2a did not affect hNET promoter activity in NET-negative cell lines, in marked contrast to its effect on a DBH-chloramphenicol acetyltransferase reporter construct. Together with our previous studies suggesting a critical role of Phox2a for noradrenergic-specific expression of the DBH gene, these data support a model in which distinct, or partially distinct, molecular mechanisms regulate cell-specific expression of the NET and DBH genes.

* This work was supported by National Institutes of Health Grant MH48866 (to K. S. K.) and by the Department of Veterans Affairs Grant DA 00167 (to J. F. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The synaptic action of norepinephrine (NE)1 is terminated primarily by rapid, NaCl-dependent re-uptake into presynaptic nerve terminals. NE reuptake is mediated by the norepinephrine transporter (NET) located in the plasma membrane of noradrenergic neurons (1). The NET is a major target for tricyclic antidepressant drugs and for illicit drugs such as cocaine and amphetamine. These agents block NE transport by the NET, resulting in enhancement of the synaptic activity of NE. Blockade of NET is an important mechanism of therapeutic action of tricyclic antidepressants, and this observation provided support for the catecholamine hypothesis of depression (2). Recently, Klimek et al. (3) reported that the level of NET is significantly reduced in the locus coeruleus from major depressive subjects. These observations suggest that regulation of NET expression could play an important role in human mental illnesses such as major depression and schizophrenia (3, 4). In rodent studies, the mouse NET (mNET) locus, Slc6a5, overlapped with a quantitative trait locus (Lore4) for differential ethanol sensitivity in long-sleep and short-sleep mice (5–7). Together with previous studies indicating ethanol-induced changes of noradrenergic neurotransmission (8, 9), these findings suggest the additional possibility that regulation of NET expression may contribute to ethanol tolerance and/or addiction.

A cDNA encoding human NET (hNET) was isolated by an expression cloning approach (10), and subsequently, cDNAs for the bovine and the rat NET genes were cloned on the basis of their sequence homologies (11, 12). NET belongs to a superfamily of Na⁺- and Cl⁻-dependent transporters that includes transporters for dopamine, serotonin, γ-aminobutyric acid, betaine, glycine, proline, and taurine (13–16). Recently, genomic clones of the hNET and mouse placental NET (mNET) have been isolated and characterized. The hNET gene, spanning approximately 45 kb, consists of 14 exons separated by 13 introns and is located on chromosome 16q12.2 (17). The structure of the mNET gene was found to be similar to that of the hNET gene. The mNET gene is composed of 14 exons spanning approximately 36 kb and is located on chromosome 8 which is homologous with 16q12.2 of the human genome (5).

In the brain, NET is specifically expressed in noradrenergic neurons but is not expressed either in dopaminergic neurons (e.g. substantia nigra) or in adrenergic neurons (e.g. C1 and C2 cell groups), demonstrating that NET is a hallmark protein of

1 The abbreviations used are: NE, norepinephrine; NET, norepinephrine transporter; DBH, dopamine β-hydroxylase; CAT, chloramphenicol acetyltransferase; 5′-RACE, 5′-rapid amplification of cDNA ends; h, human; m, mouse; PCR, polymerase chain reaction; kb, kilobase pair; bp, base pair; RSV, Rous sarcoma virus; TH, tyrosine hydroxylase.
noradrenergic cells (18). Another protein selectively expressed in noradrenergic (and adrenergic) neurons is dopamine β-hydroxylase (DBH) which catalyzes the conversion of dopamine to noradrenaline (19, 20). Collectively, NET and DBH are tightly co-expressed in noradrenergic neurons but differentially in adrenergic neurons, such as those in the C1 and C2 cell groups (18). These findings suggest that NET gene expression is subject to stringent cell-type-specific control mechanisms that overlap with, but are not identical to, those regulating DBH gene expression.

Levels of NET mRNA in noradrenergic neurons and neurosecretory cells are acutely or chronically regulated in response to various physiological and pharmacological signals including reserpine (21), glucocorticoids (22), leukemia inhibitory factor, and ciliary neurotrophic factor (23), angiotensin II (24), retinoic acid (23), nerve growth factor (22), and protein kinase C (25). The foregoing observations suggest that changes in NET gene expression induced by physiological or pharmacological stimuli may alter noradrenergic transmission depending on physiological demand.

Despite the clinical and physiological significance of NET gene regulation, little is known about the transcriptional control mechanisms governing its expression. To investigate transcriptional regulation of the human NET gene, we isolated and analyzed genomic clones that encompass the 5′-flanking promoter region of the human NET gene. Here we report that the human NET gene contains an as-yet-unidentified intron of 476 bp in the middle of the 5′-untranslated region. Multiple transcription start sites of the hNET gene were detected by both primer extension analysis and the 5′-RACE technique. On the basis of this information, we constructed several hNET-chloramphenicol acetyltransferase reporter constructs. Transient transfection assays of these reporter constructs in both NET-positive and NET-negative cell lines show that combination of the 5′ upstream sequences and the first intron is critical for high level noradrenergic cell type-specific promoter activity of the hNET gene. However, the paired-like homeodomain transcription factor Phox2a, which is critical for noradrenergic cell type-specific DBH promoter activity (26, 27), appears not to regulate the promoter activity of the hNET gene.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of hNET Genomic Clones—A 0.5-kb rat NET cDNA fragment corresponding to the base pairs 990–1512 of the coding sequence (21) was radiolabeled by the random primer method and used as the probe to screen a λEMBL3 human genomic library derived from placenta chromosomal DNA (CLONTECH, Mountain View, CA). Positive clones were isolated and characterized by restriction mapping, using the same rat NET cDNA probe. Then these clones were retested by genomic Southern blot analyses. An oligonucleotide primer 8A, 5′-GGAGACTCGAGCATGCA(T)17-3′, which encompasses the start codon of hNET cDNA (see Ref. 10; Fig. 6), was radiolabeled by T4 polynucleotide kinase. Approximately 1 × 10^6 cpm of 32P-labeled oligonucleotide was mixed with 5 μg of poly(A)^+ RNA isolated from SK-N-BE/2M17, SK-N-BE/2C, and HeLa cells in 10 μl hybridization solution containing 40 μg/ml Tris-HCl, pH 8.3, 150 μM NaCl, and 1 μM EDTA. Five μg of yeast tRNA served as a negative control. The oligonucleotide was annealed to RNA by heating the mixture to 100 °C for 5 min followed by incubation at 42 °C for 16 h. The annealed primer was extended by incubating with SUPERSKRIPT II RNase H- Reverse Transcriptase (Life Technologies, Inc.) in a 20-μl reaction containing 50 μl Tris-HCl, pH 8.3, 75 μM NaCl, 3 mM MgCl2, 10 mM dithiothreitol, 40 units of RNasin, and 1 μM dNTPs (1 μM 7-deaza-dGTP, 1 μM dCTP, 1 μM dATP, and 1 μM dTTP) at 42 °C for 1 h. The reaction products were analyzed on a 6% denaturing polyacrylamide gel. The sizes of the extension products were determined by counting the number of nucleotides in a concurrently run sequencing ladder using a control template (the bacteriophage M13mp18) and a control primer with the nucleotide sequence, 5′-GCATGGATGCGGCT-CACGAC-3′. In an additional experiment, another oligonucleotide primer 7A, 5′-TCCCGCGTGGTG CGTTCGGGGGCGGCGGAGT-CACGAC-3′, complementary to nucleotides from +47 to +84 bp of the hNET gene (Fig. 6) was used as the primer.

5′-Rapid Amplification of cDNA Ends (5′-RACE)—To identify the 5′-end of the hNET mRNA, 5′-RACE was carried out as described (33) with the following modifications (Fig. 4A). Poly(A)^+ RNA from SK-N-BE/2M17 cells was reverse-transcribed with SUPERSKRIPT II RNase H- Reverse Transcriptase by priming with the oligonucleotide HN3-1. Because the 5′-flanking region of hNET gene is highly GC-rich, 7-deaza-dGTP was used along with the other 3 dNTPs for synthesizing cDNA. A 3′ poly(A) tail was added to the cDNA by terminal deoxynucleotidyldUTransferase (Life Technologies, Inc.). The product was subjected to the first PCR using an adaptor-d(T), primer HN6A, 5′-GGAGACTCAGTCGCAATC(T)17-3′, and hNET gene-specific inner primer HN5-3′. For the second PCR, primer HN5-3′, 5′-GGAGACTCAGTCGCAATC(T)17-3′, and hNET gene-specific inner primer HN5-3′ were used (Fig. 6). The second PCR was run for 25 cycles of the same temperature profile as that of the first PCR. To improve the specificity of DNA amplification, Masteramp™ PCR Optimization Kit (Epitomé Technologies) was used according to the manufacturer’s protocol for both the first and second rounds of PCR. The final PCR products were directly cloned into pCR II (Invitrogen). Insert DNA was purified from individual clones and sequenced.

Northern Blot Analysis—Two micrograms of poly(A)^+ RNA prepared from each cell line were fractionated on a formaldehyde agarose gel, transferred to a nylon membrane, and hybridized with radiolabeled probes. To detect DBH messages, cDNA probes for the rat (35) and human (29) DBH genes were labeled and used as a mixture. To detect NET messages, the rat NET cDNA fragment (21) was radiolabeled and used as a probe. In addition, a cDNA fragment of 700 bp, encompassing the mouse NET cDNA from middle of exon 1 to exon 6 (a kind gift from Dr. Randy Blakely, Vanderbilt University), was also used as a probe. A cDNA fragment for the mouse glyceraldehyde-3-phosphate dehydrogenase was used as a positive control. These probes were labeled with [32P]dATP using Klenow fragment and random hexamer priming. Blots were autoradiographed on an intensifying screen for 6 to 24 h at 70 °C according to the relative strength of signals. For rebiozization, previous signals were removed by thoroughly washing the membrane at 80 °C in 1.5 mM NaCl, 0.15 mM sodium citrate, pH 7, 0.01% SDS until no signal was detected after overnight exposure.

Reporter Gene Constructs and Transient Transfection Assay—pBLCAT3-1 is a derivative of pBLCAT3 (26) which was constructed by deleting the CRE-like sequence and TATA-like sequence upstream of the multiple cloning site (26). Several NET-CAT reporter constructs were prepared using pBLCAT3-1 as follows. A 14-kb SalI fragment of hNET clone 3 (Fig. 1) was cloned into pBLCAT3-1 that was cut with SalI. The resulting plasmid was digested with SalI, and the resulting fragment was ligated into pCMV-BamHI, ligated, and named as pNET900CAT. For the construct containing promoter plus the first intron, intronic sequence was amplified by PCR using sense...
Fig. 1. Southern blot analysis and organization of the hNET genomic clones. A, the structure of the hNET genomic clones #3 and #7. Black boxes denote the first three exons as described by Porzgen et al. (17). The relative position of the translation start codon is indicated by the arrow. Clones 3 and 7 contained substantially overlapping insert DNAs of 14 and 12 kb. Locations of restriction enzyme sites are shown as follows: S, SalI; E, EcoR I; B, BamHI; C, SacI. B, Southern blot analysis of restriction fragment. The genomic DNA was cut with SalI (lane 1), SalI and EcoRI (lane 2), SalI and BamHI (lane 3), or SacI (lane 4) and was probed with a radiolabeled oligonucleotide HN3-1 that encompasses the start codon. Positions of size markers are indicated on the left. C, comparison of nucleotide sequences of the cDNA and genomic clones of hNET gene immediately 5' to the start codon. The nucleotide sequence of the corresponding region of the mouse NET genomic clone (5) is also shown. The translation start codon (ATG) is boxed. The nucleotide sequences of the cDNA and genomic clones of hNET diverge after a perfect match of 51 bases. In contrast, the sequence homology continues beyond this junction between the genomic sequences of human and mouse NET genomic clones. At the junction of divergence, a consensus splicing acceptor sequence, 5'-AG-3', is found and denoted by a circle.

RESULTS

Isolation and Sequencing of hNET Genomic Clones That Contain the 5'-Flanking Sequences—Eight positive clones, containing insert DNAs of 6–14 kb length, were isolated by screening approximately 1.5 × 10^6 plaque-forming units of a AEMBL3 human genomic library with a 0.5-kb cDNA probe corresponding to the N-terminal 167 amino acids of the rat NET gene. On subsequent Southern blot analysis, clones 3 and 7 hybridized to an oligonucleotide probe encompassing the translational start codon of hNET. Clones 3 and 7 contained insert DNAs of 14 and 12 kb, respectively, of overlapping sequence (Fig. 1A). Further restriction mapping and Southern blot analyses indicated that the BamHI-SacI fragment residing at the 5' side of both clones contained the start codon of hNET (Fig. 1, A and B). Clones 3 and 7 contained approximately 9.5- and 7.5-kb sequence upstream of the start codon of hNET, respectively.

Sequence analyses of the BamHI-SacI fragment of both clones 3 and 7 demonstrated that it includes the start codon, untranslated leader sequence, and an exon described as the first exon in previous reports (10, 17), and a part of an intron (Fig. 1A) previously described as the first intron (17). Surprisingly, however, the nucleotide sequences of the genomic clone diverged from the published untranslated leader sequence starting at the base 52 bp upstream of the start codon, following perfect matches of 51 bases (Fig. 1C). Both clones 3 and 7 contained the same nucleotide sequences in this area, indicating that these are not cloning artifacts. In addition, the corresponding nucleotide sequence of the mouse genomic NET clone shows significant homology with that of hNET clone (Fig. 1C; see Ref. 5). The presence of a consensus splice acceptor site (5'-AG-3') in the genomic sequence at the junction of divergence suggested the possibility that the human NET gene contains an as-yet-unidentified intron(s) in the middle of the leader sequence. To investigate these findings in detail, the...
are denoted by

represents an as-yet-unidentified intron. In support of this, the conserved GT/AG splice donor/acceptor motifs were found at these junctions and the sequence of hNET is observed in the upstream region of the genomic sequence after the intervening region of 476 bases, suggesting that this region resides in the middle of the untranslated leader sequence of hNET. Primer extension and 5'-RACE analyses further substantiated this conclusion and identified the full untranslated leader sequences of hNET.

An Intron of 476 bp Resides in the 5'-Untranslated Leader Sequence of hNET—To assess whether the hNET gene contained an additional intron(s) in the leader sequence, we determined additional nucleotide sequences upstream of the start codon and compared this sequence to the remaining 9 bases of the previously described leader sequence of hNET (see Ref. 10; Fig. 1C). As shown in Fig. 2 (also see Fig. 6), the sequence of clone 3, corresponding to 528–536 bp upstream of the start codon, perfectly matched the remaining leader sequence, 5'-GCCGGACAC-3' (Fig. 1C). This observation strongly suggests that the intervening 476-bp region represents an as-yet-unidentified intron of the hNET gene. Also consistent with this hypothesis, the putative intervening sequence contains a consensus splicing donor site (5'-GT-3') at its 5' end in addition to an acceptor site (5'-AG-3') at the 3' end (Fig. 2). Furthermore, eight out of nine nucleotides at the potential exon/intron junction area match the consensus sequence (5'-[A/C]AG/GT(A/G)-3') at the 3' end (Fig. 2). This observation strongly suggests that the first intron of 476 bp length resides in the middle of the untranslated leader sequence of hNET.

Sequence of hNET—To identify the transcription start site(s) of hNET mRNA, primer extension analysis was performed using poly(A)+ RNAs prepared from SK-N-BE(2)M17, SK-N-BE(2)C, and HeLa cell lines. When the mRNAs from SK-N-BE(2)M17 and SK-N-BE(2)C were primed with the primer 8A, extended cDNA products appeared at multiple locations, clustered in two areas. The first of these clusters occurred between 176 and 184 bp and the second between 208 and 212 bp, upstream of the 3' most base of the primer 8A, respectively (Fig. 3). In a separate experiment using the distinct primer 7A, two clusters of multiple bands were similarly detected at corresponding locations (data not shown). In contrast, no such clusters of extension products were detected when mRNA from HeLa cells was used as the template, suggesting that these bands represent catecholamine cell-specific cDNA products of NET mRNA.

PCR was performed using the adaptor primer HN5A and the antisense primer HN3-1. This first PCR generated two major products of approximately 310- and 280-bp lengths (data not shown), which were used as the template in the second PCR with another adaptor primer HN5 and an inner hNET primer HN3-2 which partially overlaps with HN3-1 (Figs. 4A and 6).
Two major products of approximately 300- and 270-bp lengths were prominently detected by gel analysis (Fig. 4B). Assuming that the potential intron of 476-bp length is spliced out, the sizes of the PCR products match well with the putative two clustered transcription start sites identified in the above primer extension analyses.

We isolated and subcloned the second PCR products of 5'-RACE into pCRII vector. Sequence analysis of 32 individual clones showed that all these clones contained the previously identified leader sequence of hNET, indicating that they were derived from the intact hNET cDNAs (see below). The putative first intron which represents the genomic sequences at 527 to 52 bp upstream of the start codon were missing in all RACE products, demonstrating that this sequence indeed represent the first intron of the hNET gene. Along with the above gel analysis of PCR products (Fig. 4B), our sequencing analysis of 5'-RACE products indicates that there is no alternate splicing of this newly identified first intron in hNET mRNAs of the SK-N-BE(2)M17 cell line. Among 22 individual isolates that were cloned from the smaller molecular weight (270 bp) products, 5 transcription start sites were found to be clustered at 233 to 227 bp upstream of the start codon in hNET mRNAs (Fig. 6; it is to be noted that transcription start sites are shown to be at 709 to 703 bp upstream of the start codon since it includes the intronic sequence); 1 clone contained an apparent initiation site at the A located 233 bp upstream of the ATG codon, 10 clones at the G 231 bp upstream, 6 clones at the G 230 bp upstream, 2 clones at the C 228 bp upstream, and 3 clones at the A 227 bp upstream. Remarkably, all these 22 clones were found to contain an additional G residue expected for intact mRNA capped at the 5' end (Fig. 4C and data not shown; see Ref. 40). These results strongly suggest that the foregoing nucleotides represent authentic start sites of mature hNET transcripts. We next isolated 10 individual clones from the larger product (300 bp) and compared their nucleotide sequences. All 10 clones included the same nucleotide sequences with the 5' termini residing at the C residue 259 bp upstream of the start codon, suggesting that this site is another major transcription start site of the hNET gene (Fig. 4D). However, in contrast to the smaller RACE products, these clones did not contain the additional G residue. Therefore, the authenticity of this C residue as the transcription start site remains open to question. Given that all 10 clones isolated from the larger product exhibited the same sequence without the additional G residue and that the size matches well with the primer extension result, we favor the possibility that the larger form(s) of

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**Fig. 4.** Determination of the hNET gene transcription start sites by 5'-RACE. A, a diagram showing the procedure of 5'-RACE analysis of hNET mRNA. 1, single-stranded cDNA was synthesized from SK-N-BE(2)M17 mRNA by priming with the oligonucleotide HN3-1 and subsequently digested with RNase H. 2, the cDNA was then tailed with poly(A) at its 3' side and used as template in two consecutive nested PCR using adaptor primers and hNET-specific inner primers as indicated (steps 3 and 4). 3, ethidium bromide staining of 5'-RACE products. Two RACE products (300 and 270 bp in size) were generated as denoted by two arrows. A 1-kb DNA ladder (Life Technologies, Inc.) was run in parallel as a size marker. C, sequence analysis of 22 independent subclones of the smaller product (~270 bp) identified 5 different transcription start sites and all contained additional G residue, indicating that they represent mature hNET RNA molecules. Nucleotide sequences around the transcription start site of the 5'-RACE products are shown at the right side of each sequence panel. Next to these sequences are also shown the nucleotide sequences of the corresponding genomic sequences of the hNET gene. The additional G residue is indicated by an asterisk. D, in contrast, all 10 subclones of the larger product (~300 bp) included the same sequence and did not contain an additional G residue.
hNET mRNA are generated without capping at the 5’ site. Taken together, we conclude that hNET transcription starts at multiple sites clustered at two areas approximately 30 bases apart from each other. The extra G residues at the 5’ positions of most RACE products further supports the authenticity of these transcription start sites. Among these, we arbitrarily designated the G residue 231 bp upstream of the translation start site as +1 because both primer extension analysis and RACE indicated this site is the most common apparent initiation site that contains a capping G residue.

**NET Is Co-expressed with DBH in Several Catecholaminergic Cell Lines**—As a first step toward investigating transcriptional regulation of the hNET gene, we tested several cell lines for significant homology to restrictive element/neuron-restrictive silencer element that appears to regulate a large number of neuronal genes (45–47).

**Cell-specific Promoter Activity of the hNET Gene Requires Both the 5′ Upstream Sequences and the First Intron**—Based on the structural organization of the hNET genomic clone, we wished to identify the promoter/enhancer region(s) containing critical regulatory function for cell-specific transcriptional activity. For this purpose, we assembled four reporter gene constructs, containing hNET 5′ sequences, with or without the putative first intron. NET9000CAT and NET1400CAT plasmids contained 9.0- and 1.4-kb nucleotide sequences upstream of the transcription start site, respectively, fused to the reporter gene, chloramphenicol acetyltransferase (CAT). NET9000(i)CAT and NET1400(i)CAT plasmids, in addition to those upstream sequences, included the newly identified first intron as well (Fig. 7A). The transcriptional activities of these constructs were examined by transient transfection assay in four different cell lines. As shown in Fig. 7B, NET9000CAT-driven reporter gene expression was approximately 4–7-fold higher in NET-expressing cell lines compared with that in NET-negative cell lines. In NET/DBH-positive cell lines, the relative CAT activity driven by this construct was approximately 50% that driven by the DBH-CAT construct (DBH978CAT) containing the 978-bp upstream sequence of the human DBH gene (29, 38). In negative cell lines, the CAT activities driven by NET9000CAT and DBH978CAT are com-

**Sequence Analysis of the 5′-Flanking Promoter Region of the hNET Gene**—We determined the nucleotide sequence up to 4.8-kb upstream of the start codon (GenBank™ accession number AF061198). Nucleotide sequences including the first intron and the 5′ proximal 554-bp region are presented in Fig. 6. We numbered the position of each nucleotide based on the major transcription start site which is arbitrarily designated as +1 site (denoted by the bent arrow in Fig. 6). Two areas encompassing two clustered transcription start sites centered at –28 and +1, respectively, and individual nucleotides identified as transcription start sites in primer extension and 5′-RACE analyses are indicated by asterisks above each base. A TATA-like sequence (TTAAT) is found at –64/–60, which may control transcription initiation at around –28 bp. Intriguingly, a second TATA-like sequence (TACATTA) is found at –30/–24 bp, that presumably controls transcription initiation around +1. This suggests the interesting possibility that the second TATA sequence area may work both as the TATA box and as a transcription initiation site. These analyses show that mature hNET mRNAs, once the first 476-bp intron is spliced out, contain untranslated leader sequences of 227–259 bp (Fig. 6). Like other neurotransmitter transporter genes (41–44), the proximal promoter region of hNET is highly GC-rich. The leader sequence contains 77% GC residues and the first 200-bp upstream region contains 72% GC residues.

The location and nucleotide sequence of potential cis-regulatory elements are summarized in Table I, and those residing in the proximal promoter area are shown in Fig. 6. The potential cis-regulatory elements residing in the upstream sequence include four AP1-binding sites, seven CCAAT boxes, two C/EBP motifs, a cAMP response element (CRE)-like motif, a Oct1-binding site, seven Sp1-binding sites, and a sterol response element. Notably, the first intron area also contains multiple cis-regulatory elements such as sterol response element, C/EBP, E-box, CRE, and Sp1-binding sites. Our sequence search, however, did not identify sequence motif(s) with significant homology to restrictive element/neuron-restrictive silencer element that appears to regulate a large number of neuronal genes (45–47).

**Structure and Function of the Human NET Gene Promoter**
parable. These results strongly suggest that the upstream 9.0-kb sequence contains important regulatory information for the cell-specific expression of the hNET gene. CAT activity driven by NET1400<i>CAT</i> was approximately 20% that by NET9000<i>CAT</i> in NET-positive cell lines, indicating that the upstream region between 29.0 and 21.4 kb may contain positive regulatory sequence element(s) required for the full promoter activity of the hNET gene. In contrast the CAT activity driven by NET1400<i>CAT</i> is not significantly higher (less than 2-fold) in NET-positive cell lines compared with that in NET-negative cell lines, suggesting that the proximal 1.4-kb flanking region may have some, but not sufficient, information for driving cell-specific NET gene expression (Fig. 7, B and C). This is in sharp contrast to the case of hDBH gene regulation, in which a relatively short upstream sequence (1.1 kb) contains sufficient information for the cell-specific expression both in transgenic mice and transient transfection experiments (26, 29, 37, 38). Taken together, it appears that the upstream region at 29.0 to 21.4 kb imparts substantial cell-type specificity to hNET expression, and the proximal 1.4-kb sequence may have general promoter function (Fig. 7C).

The First Intron of the hNET Gene Exerts Important Transcription Enhancing Activity—Inclusion of the first intron in NET9000<i>CAT</i> reporter constructs increased transcriptional activity 30–40-fold in NET-positive cell lines (Fig. 7B). It also increased the CAT activity by approximately 3–15-fold in NET-negative cell lines. When the first intron was included in the shorter NET1400<i>CAT</i> construct, it increased the transcriptional activity by approximately 15- and 10-fold, respectively, in NET-positive and NET-negative cell lines. Collectively, our transient transfection assay indicates that the fullest cell-specific transcriptional activity of the hNET gene so far examined requires both the 5′ upstream region up to 9.0 kb and the first intron residing in the untranslated leader sequence.

The combination of the 9.0-kb upstream sequence and the first intron drove a remarkably high level of noradrenergic cell-specific expression of the reporter gene, comparable to 30–40% that driven by the intact RSV promoter/enhancer (Fig. 7B). At present, it is not clear why NET9000<i>(i)</i>CAT drove much higher CAT expression in SK-N-BE(2)C and SK-N-BE(2)M17 cell lines, given that the steady state mRNA levels of DBH and NET are comparable in these cells (Fig. 5).

**FIG. 6.** Nucleotide sequences of the 5′-flanking region of the hNET gene. The first 18 amino acids of hNET are shown under a single-letter code. The nucleotide sequences of the newly identified first intron are indicated by lowercase letters. Numbers at the right and left side are the nucleotide positions relative to one of the major transcription initiation sites designated as +1 and is indicated by a bent arrow. All nucleotide bases identified as transcriptional start sites are denoted by asterisks. Two TATA-like sequences, residing approximately 30 bp upstream of the transcription start sites, are indicated by boxes and designated as TATA1 and TATA2. Potential transcription factor binding sites such as Sp1-binding site (GC box), E-box, C/EBP-binding site, CRE-like sequence, and CCAAT box are indicated by boxes. The nucleotide sequences of oligonucleotides used as primers in the primer extension and 5′-RACE analyses are underlined. The GenBank™ accession number for this proximal and additional upstream sequences is AF061198.
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The location of each potential cis-element is indicated relative to the transcription start site. (c) at the right side of the location shows that the cis-element is found in the complementary sequence. We included two consensus Sp1 sites here because both motifs are shown to be authentic Sp1-binding sites.

| Element         | Sequence           | Consensus | Location | Ref. |
|-----------------|--------------------|-----------|----------|------|
| AP1             | TGAATTA            | TGANTMA   | −623     | 67   |
|                 | TTAATCA            |           | −946     |      |
|                 | TGATTCA            |           | −1126    |      |
|                 | TGAATTA            |           | −1379    |      |
|                 | TGTTGAAA           |           | −1765    | 67   |
|                 | TGTTGAAAA          |           | −2343    |      |
|                 | TGTTGTTTA          |           | −2469    |      |
|                 | AAACCACA           |           | −3435    |      |
|                 | CCAAT              |           | −3403    |      |
|                 | CCAAT              |           | −3425(c) |      |
|                 | CCAAT              |           | −3865(c) |      |
| CCAAT BOX       | CCAAT              |           | −502     | 67   |
|                 | CCAAT              |           | −1798    |      |
|                 | CCAAT              |           | −1912    |      |
|                 | CCAAT              |           | −2909    |      |
|                 | CCAAT              |           | −2925    |      |
|                 | CCAAT              |           | −3303    |      |
|                 | ATTGG              |           | −3425(c) |      |
|                 | ATTGG              |           | −3865(c) |      |
| C/EBP           | TGCTGCAAG          | TKNNNGA   | +420     | 67   |
|                 | TTTTGGAA           |           | −2528    |      |
|                 | TGAGGAA            |           | −2994    |      |
|                 | TGACGTA            |           | −274     | 68, 71|
| C/EBP           | TGACGTC           | TGACGTC   | −734     |      |
| CRE             | GcACATTCCC         | GGGAMTN   | −424(c)  | 67   |
|                 | aGGAATCTCC         |           | −727     |      |
| NF-KB           | ATGCAAT            | ATGCAAT   | +32(c)   | 67   |
|                 | GGGCGG             |           | +62(c)   |      |
|                 |                  |           | +437(c)  |      |
|                 |                  |           | −144(c)  |      |
|                 |                  |           | −3187    |      |
| SRE (Sterol response element) | CCGGCC             | KRGGCKRRK | +86(c)   | 67   |
|                 | CTCAGCCTC          |           | +355(c)  |      |
|                 | TGGGCTGTG          |           | −595(c)  |      |
|                 | TGGGCTGTG          |           | −1209    |      |
|                 | TGGGCTGTG          |           | −1509    |      |
|                 | TGGGCTGTG          |           | −3496(c) |      |
|                 | TGGGCTGTG          |           | −3795(c) |      |
|                 | TGGGCTGTG          |           | −3865(c) |      |
|                 | TGGGCTGTG          |           | −2148    |      |

* The abbreviations used are: N, any base; M, A or C; R, A or G; S, C or G; Y, C or T; K, G or T; W, A or T.

drenergic neurons (18), we hypothesized that Phox2a may coordinate DBH and NET transcription. To test this hypothesis, we examined the effect of forced expression of Phox2a on NET promoter function. In agreement with previous studies (26, 27), cotransfection of Phox2a significantly increased (approximately 10-fold) the transcriptional activity of DBH/NET-negative HeLa and C6 cell lines (Fig. 8; data not shown). In contrast, forced expression of Phox2a in these cell lines did not affect hNET promoter activity, in either NET-expressing or non-expressing cells, regardless of the presence or absence of the first intron in the reporter construct (Fig. 8; data not shown). These data suggest that in contrast to the DBH gene, the Phox2a does not exert a strong influence on cell-specific expression of the NET gene.

**DISCUSSION**

To understand molecular mechanisms regulating transcription of the hNET gene, we isolated a genomic clone containing approximately 9.5 kb of the 5’ upstream region of the hNET gene. The experiments presented here have defined critical aspects of the promoter structure and function of hNET gene, including the following: (i) the location of transcription start site(s); (ii) the sequence of the full 5’-untranslated leader sequence; (iii) the sequence of approximately 5 kb of the 5’-flanking promoter region, including a search for potential cis-regulatory motifs; (iv) most unexpected, an as-yet-unidentified region of the hNET gene that appears by multiple criteria to be the first intron and which resides in the middle of the 5’-untranslated leader sequence; and (v) that the combination of the upstream 9-kb sequence and the first intron is critical for the high level, noradrenergic-specific transcriptional activity of the hNET gene. Finally, we tested, and rejected, the hypothesis that Phox-2a transactivation regulates hNET expression, as it does DBH expression.

**Promoter Structure of the hNET Gene**—Data from previous studies have shown that the coding region of the human NET gene consists of 14 exons disrupted by 13 introns (10, 17), with the entire 5’-untranslated leader sequence present in the first exon, and the intron immediately 3’ to that first exon designated intron 1 (17). In contrast to these earlier findings, the studies described here demonstrate that the hNET gene contains an additional 476-bp-long intron in the middle of the 5’-untranslated leader sequence. Several pieces of evidence support this conclusion as follows: (i) the nucleotide sequences of two independent genomic clones (clones 3 and 7) diverge from that of the 5’ leader sequence previously identified (10), following a perfect match of 51 bases immediately upstream of the start codon; (ii) the remaining 9 bases of the leader sequence appear at the 5’ end of the putative intron of 476 bp; (iii) the intervening sequence contains the consensus splicing donor and acceptor sites at both junctions; and (iv) this 476-bp se-
sequence is missing in all RACE products isolated and cloned in the present studies. We therefore conclude that the hNET gene contains an additional exon at the 5'9most area and that the first intron of 476 bp resides in the middle of the 5'9untranslated leader sequence. In addition, Porzgen et al. (49) recently reported that the hNET mRNA is alternatively spliced at its 3' region, in agreement with the Northern blot experiment showing more than one transcript (Fig. 5), and thus contains an additional exon at its 3' side of the gene.

The region both upstream and downstream from the transcriptional start sites is rich in GC sequence, having an average GC content of 77 and 72% in the leader sequence and the first 200-bp upstream region, respectively. Previous studies likewise reported that the proximal upstream regions of other transporter gene family contain high GC content (41–44). It seems likely that secondary structure in these GC-rich regions might explain why previous analyses did not identify the putative first intron described here (17). In support of this possibility, successful determination of the transcription start sites by 5'-RACE and primer extension analyses described in this study was possible only after extensive optimization of these procedures, including substitution of 7-deaza-dGTP in the synthesis of cDNA:RNA template from poly(A)1RNA in the primer extension and 5'-RACE experiments. In addition, specific PCR-enhancing agents included in the Masteramp™ PCR Optimization Kit (Epicentre Technologies) were necessary to amplify

![Diagram of the human NET gene promoter](image-url)

**Fig. 7.** Promoter activities of the 5'-flanking and upstream regions of the hNET gene. A, diagrams showing the structures of hNET-CAT reporter constructs. The bent arrow represents the major transcription start site of hNET, designated as + 1 in this study. The bold line denotes the 5'-untranslated leader sequence, and the thin line denotes the 5' upstream sequence of the hNET gene. The shaded box represents the first intron of hNET, identified in this study, which resides in the leader sequence. B, promoter activities of hNET-CAT constructs in NET-positive SK-N-BE(2)/C (BE) and SK-N-BE(2)/M17 (M17) and NET-negative C6 and HeLa cell lines. Levels of CAT reporter gene activity were determined by transient transfection assays and expressed relative to that of pRSV-CAT plasmid which shows universally high promoter activity in all cell lines used. Due to its strong promoter activity, only one-tenth the relative amount of the pRSV-CAT construct was used in the transient transfection assays (see “Experimental Procedures”). The normalized CAT activity driven by pRSV-CAT in each cell line was set to 100 to compare the relative strength of each reporter construct. The numbers shown in the table are the average of triplicate samples, with variation less than 20%. This experiment has been repeated twice more in triplicate using independently prepared plasmid DNAs, resulting in similar patterns. Each sample was assayed in different dilutions so that the final cpm were in the linear range. C, schematic diagrams showing the location of potential regulatory elements in the 5' upstream region and the first intron of the human NET gene. The relative transcription-promoting/enhancing activities in NET-positive (NET1) and NET-negative (NET2) cell lines are indicated as very strong (+ + + + ), strong (+ + + ), moderate (+ + ), and weak (+ + ). As indicated in this diagram, the 1.4-kb proximal sequence was not sufficient for driving cell-specific NET gene expression. Instead, the upstream region at −9.0 to −1.4 kb of the hNET gene appears to have cell type-specific promoter activity, whereas the first intron exhibits a strong transcription-enhancing activity.
specific products of the correct size. Furthermore, we found that both the sequence and the length of the primer were crucial for generating the specific products in primer extension analysis (data not shown). Data from our primer extension analysis and 5'-RACE experiments strongly suggest that transcription of the hNET gene initiates at multiple sites clustered at two locations, one at 703–711 bp and the other at 735–739 bp upstream of the start codon in the genomic sequence. Given that the first intron is missing in all cDNA isolates from 5'-RACE, this indicates that the 5' leader sequences of hNET mRNAs are of approximately 230- and 260-bp lengths. In support of this, PCR analysis of the 5'-RACE generated two major products of corresponding lengths (Fig. 3). Two TATA-like sequence motifs reside approximately 30 bp upstream of each location, respectively, suggesting that the hNET gene contains two alternate promoters. The locations of transcription start sites are further confirmed by sequence analyses of individual clones of 5'-RACE and PCR products, which all mapped to the above two locations. Most of these clones (22 out of 32 isolates) contained an extra G residue at their 5' ends, which was not present in the hNET genomic sequence (Fig. 3). Given that most mature eukaryotic mRNAs are capped at the 5' end (40), these findings strongly support these locations as authentic transcription start sites of hNET mRNAs. Among these multiple sites, we designated the G residue which is 707 bp upstream of the start codon as +1 (Fig. 6). It is worth noting that the 5' leader sequences do not contain any ATG codons, indicating that the ATG codon previously identified by Pacholczyk et al. (10) is the genuine start codon of hNET.

Largely Overlapping but Also Distinct Expression Pattern of NET and DBH—DBH and NET are both hallmark proteins of noradrenergic cell types because they are responsible for biosynthesis and reuptake of noradrenaline, respectively, in the nervous system. In contrast to NET, DBH is also expressed in cells that synthesize and release epinephrine. An exhaustive in situ hybridization study showed that the majority (>90%) of noradrenaline-containing cell bodies in the brainstem express NET mRNA, whereas it is not detected at all in dopaminergic or adrenergic cell bodies (18). Outside the nervous system, some non-neuronal tissues, such as the placental syncytiotrophoblast (50), also express NET mRNA. Most, but not all, norepinephrine-synthesizing cells in the adult nervous system co-express DBH and NET. The vast majority of cells that express NET, with only rare exceptions, also express DBH. Understanding similarities and differences in the molecular mechanisms governing cell type-specific expression of DBH and NET promises to shed light on how different classes of neurons express overlapping but distinct biochemical phenotypes.

Various cell lines derived from catecholaminergic cell types are available and have been used in different laboratories to study catecholamine gene regulation and neuronal differentiation. Among these, PC12, from the rat pheochromocytoma (51), is the prototype cell line and has been extensively used in different laboratories. Several human neuroblastoma cell lines, e.g. SK-N-BE(2)C and SK-N-BE(2)M17, are of neural crest origin and express catecholamine-synthesizing enzyme genes (29, 52). CATH.a and PATH.2 cells derived from brain and adrenal tumors, respectively, in transgenic mice carrying the SV40 T antigen gene driven by a portion of the TH promoter were also shown to express catecholamine-synthesizing enzyme genes (30). To assess whether NET is co-expressed in these catecholaminergic cell lines along with catecholamine synthetic enzyme genes, Northern blot analysis was performed using NET and DBH cDNAs as the probe. As expected, all five noncatecholaminergic cell lines tested in this study showed no detectable levels of TH, DBH, or NET, suggesting that expression of these genes is strongly suppressed in these cell lines. Also as expected, all five catecholaminergic cell lines abundantly expressed DBH (Fig. 3) and TH message (27). In contrast, and to our surprise, NET message, although easily detected in PC12, SK-N-BE(2)C, and SK-N-BE(2)M17, was not detected in CATH.a and PATH.2 cells. Thus, most, but not all, of the catecholaminergic cell lines examined expressed NET. The CATH.a cell line, derived from a central noradrenergic neuronal population, did not express any detectable level of NET. It is possible that the CATH.a cell line has lost its capability to express NET while it continues to express synthesizing enzymes, TH and DBH, or that NET mRNA expression...
occurs at a low level in this cell line, below the limits of detection of Northern blot analysis. Alternatively, based on the in vivo observation that small portions (approximately 10% or less) of noradrenergic cells of the brainstem express DBH but not NET (18), the CATH.a cell line may have originated from these DBH ⁄ NET - cell populations. Likewise, PATH.2 cells did not express detectable levels of NET mRNA.

Cell Type-specific Expression of NET, Potential Role of the 5’ Upstream Sequence and the First Intron—To define promoter regions important for driving cell-specific transcriptional activity of the hNET gene, transient transfection assays using multiple cell line systems were performed. The results are summarized schematically in Fig. 7. 5’ sequence up to 9.0 kb appears to contribute to cell-specific promoter activity of the hNET gene. In addition, the newly described first intron contains information that appears necessary for full high level expression of the reporter gene in all cell lines examined. Interestingly, the 1.4-kb upstream sequence of the hNET gene was not sufficient at all for the high level, noradrenergic-specific expression of the reporter gene. This is in contrast to that of the hDBH gene, in which a relatively short upstream sequence (<1.1 kb) is necessary and sufficient for cell-specific expression of the reporter gene both in transgenic mouse experiments (37, 53–56) and in transient transfection assays using cultured cell lines (26, 29, 38). Similarly, a relatively short upstream sequence of the rat DBH gene was shown to drive the cell-specific expression of the reporter gene (57). In addition, there is no evidence available to suggest a role of the intronic sequence for cell-specific transcription of the DBH gene. In this regard, it is worth noting that reporter gene expression driven by NET9000(i)CAT was much higher than that driven by DBH978CAT or other longer DBH-CAT constructs (Fig. 7B; data not shown), despite the fact that steady state mRNA levels of DBH and NET were comparable in the cell lines used in this assay (Fig. 5). Thus, one interesting possibility to explain this discrepancy is that full expression of the DBH gene may also require as yet undiscovered sequence information residing in areas other than the 5’ upstream region, e.g. intron(s). Alternatively, the enhancer activity of the first intron of the hNET gene may be modest in the intact chromatin and may have(s) been exaggerated in our transient transfection assay. Finally, it is possible that NET mRNA is less stable than DBH mRNA and therefore requires higher levels of transcription for maintenance of similar steady state levels. Further work is necessary to address these issues. In other eukaryotic genes, ever increasing evidence suggests that the intronic sequences play a crucial role in cell type-specific regulation of a variety of genes (58, 59).

In summary, our study defined the structural organization of the 5’-flanking promoter of the hNET gene. (i) We identified a new intron of 476 bp that resides in the middle of the 5’-untranslated leader sequence; (ii) we determined multiple transcription start sites, clustered at two loci, by primer extension and 5’-RACE analyses; and (iii) we determined the nucleotide sequence of the approximate 5-kb upstream sequence. We have shown that the combination of the upstream sequence and the first intron is required for driving the cell type-specific transcriptional activity of the hNET gene. Furthermore, despite their striking co-expression in most noradrenergic neurons, our results suggest that differential control mechanisms may underlie the cell type-specific expression of the DBH and NET genes. The present study will serve as a basis for future studies of NET gene regulation and for studies of coordinate regulation of catecholamine-specific neuronal phenotypes.

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