

# Molecular Cloning and Characterization of an L-Epinephrine Transporter from Sympathetic Ganglia of the Bullfrog, *Rana catesbiana*

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Chemical signaling by dopamine (DA) and L-norepinephrine (L-NE) at synapses is terminated by uptake via specialized presynaptic transport proteins encoded by the DA transporter (DAT) and L-NE transporter (NET) genes, respectively. In some vertebrate neurons, particularly the sympathetic neurons of amphibians, L-NE is converted to L-epinephrine (L-Epi, adrenaline) and released as the primary neurotransmitter. Although evidence exists for a molecularly distinct L-Epi transporter (ET) in the vertebrate brain and peripheral nervous system, a transporter specialized for extracellular L-Epi clearance has yet to be identified. To pursue this issue, we cloned transporter cDNAs from bullfrog (*Rana catesbiana*) paravertebral sympathetic ganglia and characterized functional properties via heterologous expression in non-neuronal cells. A cDNA of 2514 bp (fET) was identified for which the cognate 3.1 kb mRNA is highly enriched in frog sympathetic ganglia. Sequence analysis of the fET cDNA reveals an open reading frame coding for a protein of 630 amino acids. Inferred fET protein sequence bears 75, 66, and 48% amino acid identity with human NET, DAT, and the

5-hydroxytryptamine transporter (SERT), respectively. Transfection of fET confers Na<sup>+</sup>- and Cl<sup>-</sup>-dependent catecholamine uptake in HeLa cells. Uptake of [<sup>3</sup>H]-L-NE by fET is inhibited by catecholamines in a stereospecific manner. L-Epi and DA inhibit fET-mediated [<sup>3</sup>H]-L-NE uptake more potently than they inhibit [<sup>3</sup>H]-L-NE uptake by human NET (hNET), whereas L-NE exhibits equivalent potency between the two carriers. Moreover, fET exhibits a greater maximal velocity ( $V_{max}$ ) for the terminal products of catecholamine biosynthesis (L-Epi > L-NE >> DA), unlike hNET, in which a  $V_{max}$  rank order of L-NE > DA > L-Epi is observed. fET-mediated transport of catecholamines is sensitive to cocaine and tricyclic antidepressants, with antagonist potencies significantly correlated with hNET inhibitor sensitivity. Amino acid conservation and divergence of fET with mammalian catecholamine transporters help define residues likely to be involved in catecholamine recognition and translocation as well as blockade by selective reuptake inhibitors.

**Key words:** frog; catecholamine; epinephrine; neurotransmitter; antidepressant; transporter

In both the CNS and peripheral nervous system of mammals, L-norepinephrine (L-NE) is the principal catecholamine released from adrenergic neurons (Axelrod and Kopin, 1969; von Euler, 1972). L-Epinephrine (L-Epi), synthesized from L-NE by phenylethanolamine *N*-methyltransferase (PNMT) (Axelrod, 1962, 1966), is released from the adrenal gland as an endocrine hormone, triggering coordinated metabolic and physiological process in response to stress (Lewis, 1975). Although generally treated as an endocrine hormone, L-Epi also seems to be a neurotransmitter for central and peripheral adrenergic neurons (Loewi, 1936; Jarrott, 1970; Fuller, 1982; Holmgren and Nilsson, 1982; Laurent et al., 1983; Hökfelt et al., 1984). Indeed, the classical experiments of Loewi (1921) that demonstrated the presence of “aceleranstoff” released from sympathetic neurons innervating the frog heart later were found to reflect the synthesis, storage, and release of

L-Epi (Loewi, 1921, 1936; Azuma et al., 1965; Norberg and MacIssac, 1967; Woods, 1977). The presence of L-Epi in the mammalian CNS initially was recognized in biochemical and neuroanatomical studies (von Euler, 1946; McGeer and McGeer, 1964; Ciaranello et al., 1969; Hökfelt et al., 1974; Moore and Bloom, 1979; Howe et al., 1980). Axelrod and coworkers’ (Axelrod et al., 1959; Weil-Malherbe et al., 1959) description of a blood–brain barrier for L-Epi in mammals also revealed that the catecholamine is synthesized in the brain as well as in the adrenal gland. Subsequently, neuroanatomical studies using PNMT-directed antibodies established the presence of L-Epi-synthesizing neurons in the C1, C2, and C3 cell groups of the medulla oblongata and the nucleus tractus solitarius in multiple species, including humans (Goodchild et al., 1984; Hökfelt et al., 1984; Burke et al., 1986; Carlton et al., 1987; Halliday et al., 1988; Kitahama et al., 1988; Carlton et al., 1991; Gai et al., 1993). Although the existence and function of L-Epi-synthesizing neurons are not without controversy (Mefford, 1987), studies have implicated brainstem L-Epi-synthesizing neurons in cardiovascular homeostasis and nociception (Fuller, 1982; Reis et al., 1984; Ross et al., 1984; Tucker et al., 1987; Carlton et al., 1991). Indeed, loss of PNMT-positive neurons in the human medulla oblongata has been implicated in cardiovascular dysfunction occurring in Parkinson’s disease, Alzheimer’s disease, and Sudden Infant Death Syndrome (Burke et

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al., 1987, 1988, 1994; Kopp et al., 1992; Gai et al., 1993). Additional molecular probes would be useful to clarify the roles played by these neurons in normal and pathological conditions.

A hallmark of aminergic neurons is their use of presynaptic transporters to clear released neurotransmitter (Iversen, 1967; Axelrod and Kopin, 1969; Graefe and Bönisch, 1988; Barker and Blakely, 1995). Dopamine (DA) and L-NE are cleared from extracellular spaces after vesicular release by specialized transporters localized in the plasma membrane of neuronal terminals, varicosities, and dendrites (von Euler, 1972; Trendelenburg, 1991; Boja et al., 1994; Ciliax et al., 1995; Nirenberg et al., 1996). DA transporters (DATs) and L-NE transporters (NETs) exhibit overlapping, yet distinct, substrate selectivity, translocation efficiency, and antagonist sensitivity (Gu et al., 1993; Buck and Amara, 1994; Giros et al., 1994; Pifl et al., 1996). Comparative data incorporating L-Epi as a substrate have not been reported in cloned NETs and DATs. However, uptake studies reveal lower flux rates for L-Epi relative to L-NE in the rat heart where NETs are expressed (Iversen, 1967), and voltametric studies reveal lower flux rates for L-Epi relative to DA in striatal synaptosomes, a preparation dominated by DATs (Meiergerd and Schenk, 1994). To date, a transporter specifically associated with L-Epi clearance suitable for kinetic, pharmacological, and anatomic studies has yet to be identified. Further progress in the elucidation of critical transport domains and residues would benefit from the identification of divergent genes that share overlapping substrate selectivity, translocation efficiency, and antagonist sensitivity with DAT and NET.

PNMT-positive brainstem neurons in the rat seem to express little or no DAT or NET mRNA (Lorang et al., 1993). Although it is possible that these neurons may lack a reuptake system altogether, these findings also may reflect the existence of a unique carrier specialized for L-Epi clearance at CNS adrenergic terminals. However, we reasoned that the small number of L-Epi neurons in mammals might hinder a direct attempt to determine the structural basis for mammalian L-Epi transport by direct cDNA cloning. In contrast, L-Epi-synthesizing neurons are abundant in sympathetic ganglia of lower vertebrates, particularly amphibians (Azuma et al., 1965; Jarrott, 1970; Holmgren and Nilsson, 1982; Laurent et al., 1983). Cocaine and antidepressants inhibit catecholamine uptake in the innervated frog heart, consistent with the presence of a carrier structurally related to DAT and NET in frog sympathetic neurons (Seirra and Velazquez, 1973; Stene-Larsen and Helle, 1978). Interestingly, cocaine potentiates L-Epi responses in the frog more than it does L-NE responses, suggesting a greater physiological requirement for L-Epi clearance at amphibian sympathetic synapses (Stene-Larsen and Helle, 1978). In addition, radioligand-binding experiments have documented tricyclic antidepressant binding sites in frog heart membranes with pharmacological properties similar to, but not identical to, mammalian NETs (Pimoule et al., 1987; Schoemaker et al., 1988). These findings and the relative ease of obtaining significant quantities of frog sympathetic ganglia mRNA led us to target this tissue as a suitable source to clone catecholamine transporters responsible for L-Epi clearance *in vivo*.

In the present study we have cloned and characterized a NET-related catecholamine transporter from bullfrog (*Rana catesbiana*) paravertebral sympathetic ganglia mRNA. We determined the regional distribution of transporter RNA expression and compared the kinetic and pharmacological properties of the identified clone with human NET (hNET), transfected in parallel cultures, to search for functional distinctions that might correspond to structural divergence. Our results suggest that the novel catechol-

amine transporter we have identified has evolved for clearance of L-Epi at frog sympathetic synapses. L-Epi transport capacity is enhanced significantly (and DA transport efficiency diminished greatly) relative to the substrate capacities exhibited by mammalian NETs and DATs. We discuss the structure of fET and the significance of our kinetic findings of fET-mediated transport with regard to present concepts for substrate and antagonist contact sites.

## MATERIALS AND METHODS

**RT-PCR of bullfrog RNA.** Poly(A<sup>+</sup>) RNA (1 μg) isolated from bullfrog (*Rana catesbiana*, West Jersey Biologicals) paravertebral sympathetic ganglia was converted to single-stranded cDNA (Superscript Reverse Transcriptase, Life Technologies, Gaithersburg, MD) with random hexamer primers (2.5 μM) and subjected to PCR with degenerate oligonucleotides designed to encode two highly conserved amino acid sequences NVWRFPY (5'-CCGCTCGAGAA(C/T)GT(G/C)TGGCGG(C)TT(C/T)CC(A/G/C/T)TA-3') and WIDAATQ (5'-GCTCTAGAGCTG(A/G)GTIGC(A/G)GC(A/G)TC(A/G)A(T/G)CCA-3') near the first and sixth transmembrane domains of the Na<sup>+</sup>- and Cl<sup>-</sup>-dependent cotransporter gene family (Amara and Kuhar, 1993; Shafiqat et al., 1993; Uhl and Johnson, 1994). Underlined sequences reflect added nucleotides to provide restriction sites for subcloning PCR fragments. Oligonucleotides (1 μM) were combined with single-stranded cDNAs and dNTPs (0.2 μM) and subjected to PCR with *Taq* polymerase (Promega, Madison, WI) for 30 cycles at 94°C for 1 min, 50°C for 1 min, and 72°C for 2 min in a Coy Instruments Thermocycler (Grass Lake, MI). PCR fragments of ~700 bp were isolated and cloned in pBluescript SKII<sup>-</sup> after *Xba*I/*Xho*I digestion. Dideoxynucleotide sequencing of plasmids (Sequenase, Upstate Biotechnology, Lake Placid, NY) prepared from individual transformants was performed to identify potential NET homologs. A directional, oligo(dT)-primed bullfrog sympathetic ganglia cDNA library was prepared in λZapII (Stratagene, La Jolla, CA) after size selection of cDNAs by gel filtration. An oligonucleotide (5'-GGGTCATACCTGACCATAGA-3') was synthesized to match a unique region of one hNET-related PCR fragment, labeled with [<sup>32</sup>P]ATP by T4 polynucleotide kinase (New England Biolabs, Beverly, MA), and used to screen the library. Approximately 100 positive plaques were found in a screen of 100,000 clones, and 17 of these were plaque-purified and rescued as pBluescript plasmids by *in vivo* excision according to manufacturer recommendations. Restriction digests and partial sequencing suggested that all clones were fragments of the same gene product. We then fully sequenced the largest cDNA and characterized its functional properties in transfected mammalian cells. Analysis of cDNA and protein sequences was performed with Geneworks (IntelliGenetics, Mountainview, CA) and Lasergene (DNASTAR, Madison, WI) software.

**Transient expression of catecholamine transporters in HeLa cells.** One 2514 bp cDNA containing an hNET-related coding sequence (fET-GenBank Accession number U72877) was found to be oriented for sense transcription by T7 RNA polymerase in pBluescript SK<sup>-</sup> after *in vivo* excision. The vaccinia-T7 transient expression system was used (Fuerst et al., 1987; Blakely et al., 1991) to characterize the functional properties induced by the cloned cDNA. HeLa cells (10<sup>5</sup> cells/well of 24-well plate) were infected with recombinant virus encoding T7 RNA polymerase (10 pfu/cell). fET, hNET in pBluescript SKII<sup>-</sup> (Pacholczyk et al., 1991), or pBluescript SKII<sup>-</sup> DNA (0.1 μg/well for fET and 0.5 μg/well for hNET) was transfected into virus-infected HeLa cells by liposome-mediated transfection with Lipofectin (Life Technologies) at a 3:1 lipid/DNA ratio. Uptake measurements were performed in triplicate 6 hr after transfection by incubating fET, hNET, or pBluescript SKII<sup>-</sup> transfected cells for 10 min at 37°C, pH 7.4, with [<sup>3</sup>H]-L-Epi or [<sup>3</sup>H]-L-NE or [<sup>3</sup>H]-DA in 0.5 ml of Krebs-Ringer's-HEPES buffer (KRH) containing (in mM): 120 NaCl, 10 HEPES, 4.7 KCl, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 1.2 MgSO<sub>4</sub>, 2.2 CaCl<sub>2</sub>, 10 D-glucose, 0.1 ascorbate, 0.1 pargyline, and 0.01 U-0521 (Upjohn Laboratories, Kalamazoo, MI). Assays were terminated with 1 ml of ice-cold KRH buffer and the cells washed twice with 2 ml of ice-cold KRH buffer. Cells were solubilized with 1 ml of Optiphase scintillant (Wallac, Gaithersburg, MD) and accumulated radioactivity quantified by direct scintillation spectrometry with a Microbeta microplate scintillation counter (Wallac). Inhibitors were added 10 min before the addition of labeled substrates, whereas unlabeled substrates were added simultaneously with labeled substrates. In experiments examining the Na<sup>+</sup> and Cl<sup>-</sup> dependence of catecholamine transport, Na<sup>+</sup> was replaced with either choline<sup>+</sup> or Li<sup>+</sup> on an equimolar



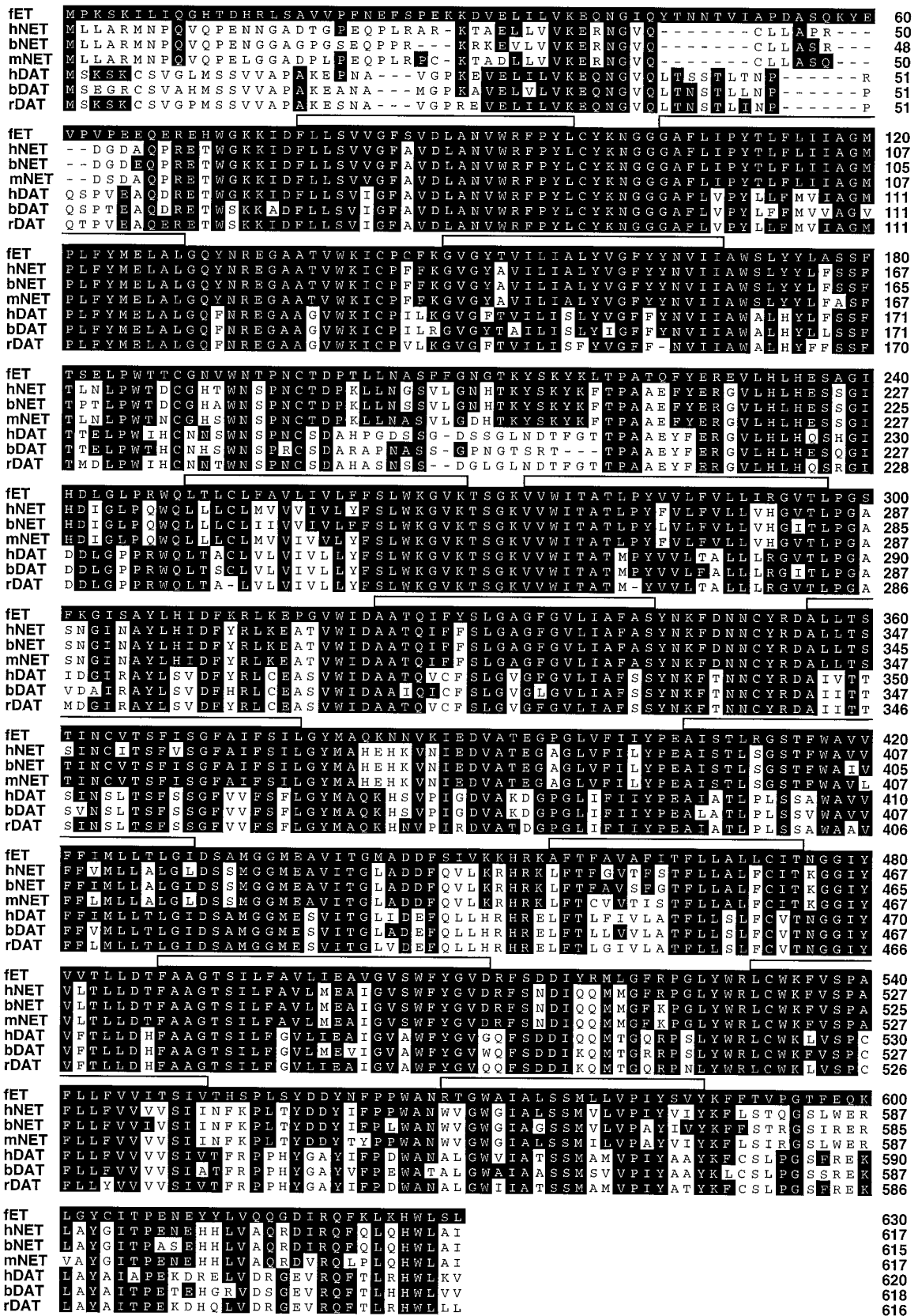
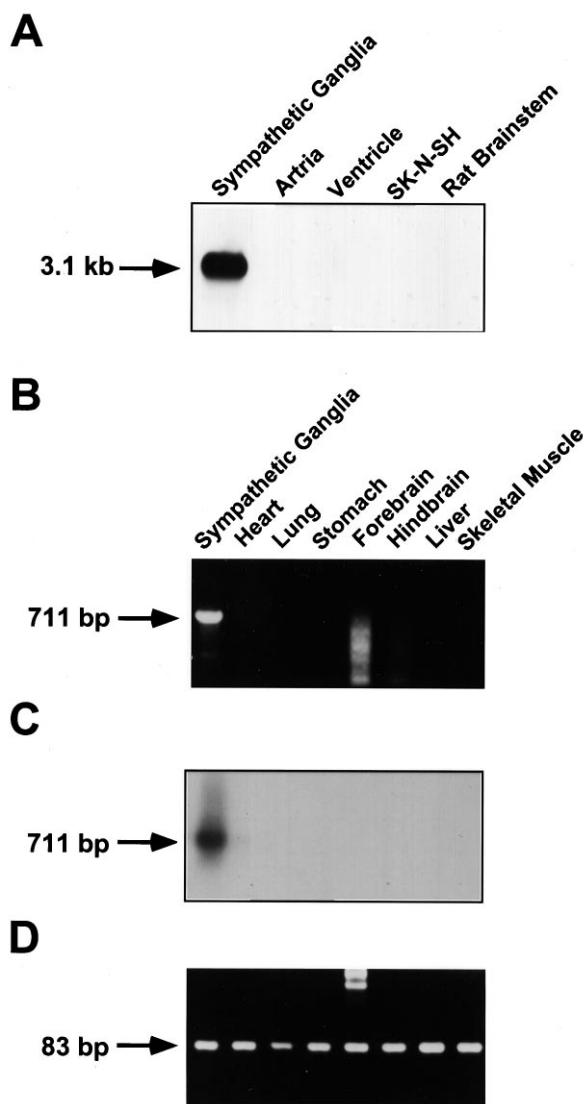


Figure 2. Alignment of amino acid sequences encoding vertebrate catecholamine transporters. Alignment was performed by using amino acid sequence of the cloned *fET* (this study), human NET (*hNET*; Pacholczyk et al., 1991), bovine NET (*bNET*; Lingen et al., 1994), mouse NET (*mNET*; L. D. Jayanthi, J. D. Fritz, M. A. Thoreson, R. D. Blakely, unpublished data), human DAT (*hDAT*; Giros et al., 1992), bovine DAT (*bDAT*; Usdin et al., 1991) and rat DAT (*rDAT*; Shimada et al., 1991) proteins. Residues matching *fET* sequences are blackened. Residues spanning putative TMDs inferred from hydrophobicity analysis are represented by a rectangular box drawn above the sequences.

hybridizing bacteriophage plaques and subsequent restriction and sequence analysis (data not shown) yielded data consistent with the presence of a single parental RNA. The largest cDNA of 2514 bp (fET) was found to contain a single open reading frame of 1890 bp encoding a protein of 630 amino acids (Fig. 1). As with hNET (Pacholczyk et al., 1991), the sequence surrounding the presumptive start codon of hNET does not compare well with optimal initiation sites (Kozak, 1987, 1991). However, multiple in-frame stop codons are located upstream of the predicted start site, and no additional in-frame methionine residues are predicted in the presumptive NH<sub>2</sub> terminus, suggesting that the ATG beginning at base 230 initiates translation. The fET cDNA ends with a short poly(A<sup>+</sup>) tail, although a canonical polyadenylation signal (AATAAA) is absent in the 3' noncoding region. Thus an additional 3' noncoding sequence (as well as 5' material) may account for the difference in size between the cloned cDNA and the endogenous fET mRNA sized at 3.1 kb (see below). However, the sequence ATTAAA, located 128 nucleotides upstream of poly(A<sup>+</sup>) tail, might be the polyadenylation signal for the fET mRNA, because previous studies have indicated that this sequence, in a similar position relative to the poly(A<sup>+</sup>) tail, may serve as the polyadenylation signal for transcription factor IIIA cloned from *Bufo americanus*, *Rana pipiens*, and *Xenopus laevis* (Joho et al., 1990; Gaskins et al., 1992).

As with other members of the GAT/NET gene family, hydrophobicity analysis (Kyte and Doolittle, 1982) of fET amino acid sequence (data not shown) and the absence of an N-terminal signal sequence (von Heijne, 1983) suggest a topology of 12 transmembrane domains (TMDs) with cytoplasmic amino and carboxy tails (Fig. 1). Three canonical N-glycosylation sites are located in the large extracellular loop between TMD3 and TMD4 at the same positions found for mammalian NETs (Fig. 1), suggesting that, like hNET, the encoded protein may be N-glycosylated (Melikian et al., 1994, 1996). Additionally, fET has N-glycosylation sites in the N terminus and the putative extracellular loop between TMD11 and TMD12 (Fig. 1) not conserved in other catecholamine transporters. A leucine zipper-type repeat observed in hNET (Pacholczyk et al., 1991) and other gene family members (Liu et al., 1993; Shafiqat et al., 1993) is present from AA107 to AA129 (LIPYTLFLIIAGMPLFYMELAL). Canonical sites for phosphorylation (Pearson and Kemp, 1991) are present on the N (PKA, PKC, and PKG, S17; PKC, S56) and C termini (tyrosine kinase, Y611; PKG, S629). Putative cytoplasmic PKC and PKG sites also are predicted at T271, between TMD4 and TMD5, and at a site between TMD10 and TMD11 (S515); a PKG site is predicted on domains separating TMDs at S272 between TMD4 and TMD5 (Fig. 1).

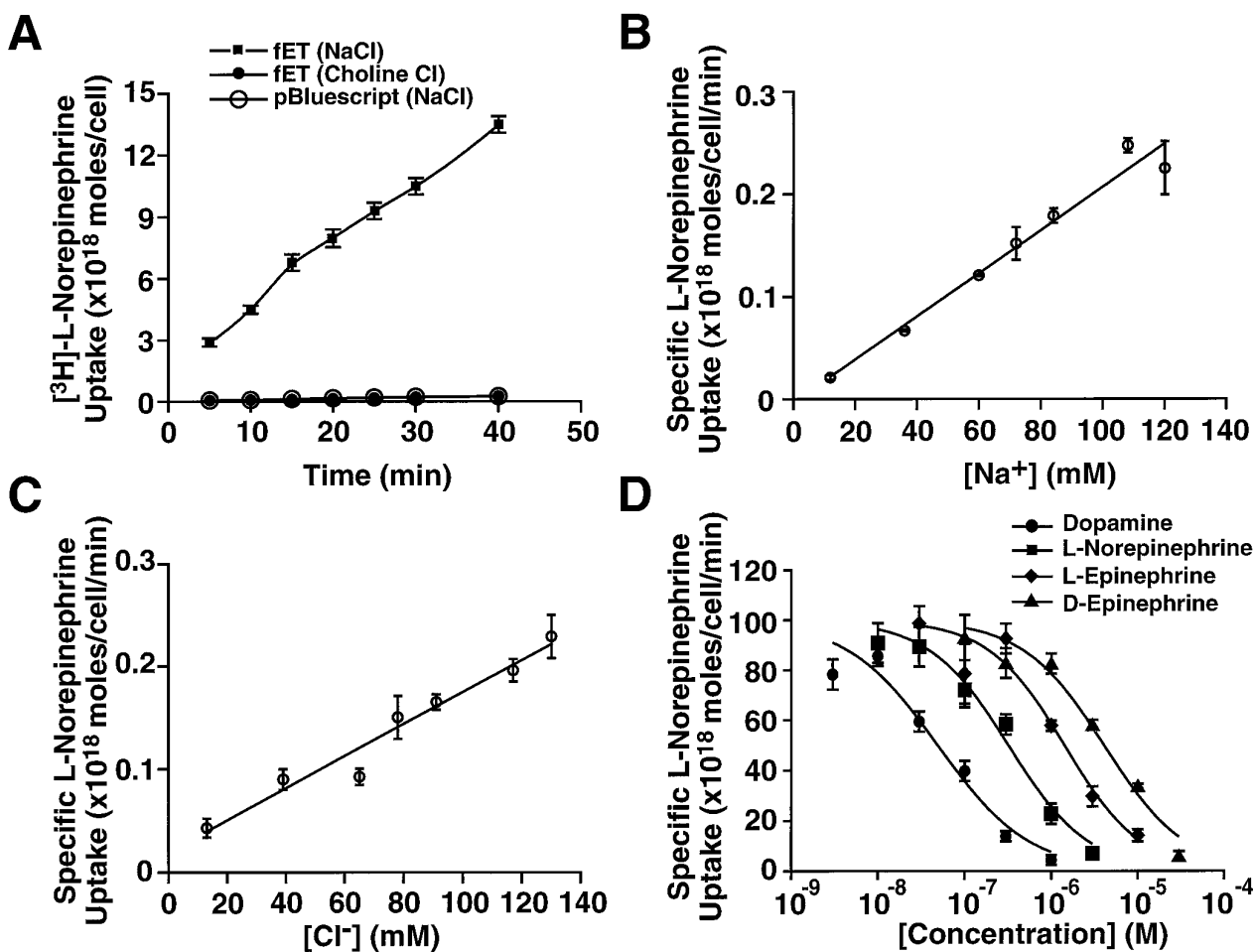
In a comparison of fET amino acid sequence with other members of the Na<sup>+</sup>- and Cl<sup>-</sup>-coupled transporter gene family, fET bears highest identity to the biogenic amine transporters, NET and DAT (Fig. 2). Overall, fET displays 75% amino acid identity with hNET (Pacholczyk et al., 1991), 66% identity with human DAT (hDAT; Giros et al., 1992) and 48% identity with the human 5-HT transporter (hSERT; Ramamoorthy et al., 1993). fET exhibits 35–39% amino acid identity with other members of the Na<sup>+</sup>/Cl<sup>-</sup> neurotransmitter transporter gene family. The regions comprising the fET TMDs and several intervening loops are highly conserved with those of other catecholamine transporters, whereas greater divergence is seen in the N and C termini (Fig. 2). Surprisingly, although fET bears the greatest overall similarity to hNET, the N terminus of fET displays 50% amino acid identity with the N terminus of hDAT, yet only a 26% identity with hNET



**Figure 3.** Distribution of fET mRNA in bullfrog tissues revealed by Northern and RT-PCR analyses. *A*, Northern analysis of bullfrog sympathetic ganglia, heart atrium, heart ventricle, human SK-N-SH neuroblastoma cells, and rat brainstem probed with the 711 bp fET RT-PCR product. Each lane contained 2  $\mu$ g of poly(A<sup>+</sup>) RNA. Ethidium bromide staining and hybridizations with a  $\beta$ -actin (data not shown) cDNA probe revealed even loading and transfer across lanes. *B*, RT-PCR analysis of fET expression in bullfrog tissues. RT-PCR was performed as described in Materials and Methods with 2  $\mu$ g of total RNA and oligonucleotides originally used to identify fET cDNA. *C*, Southern analysis of RT-PCR-derived products from *B* probed with fET 711 bp cDNA obtained by RT-PCR. Blotting and hybridization were performed as described in Materials and Methods. Note that amplification products seen in brain RNA did not hybridize with fET cDNA probes, even with long exposure. *D*, RT-PCR products obtained from RNAs used in *C* amplified with oligonucleotides encoding highly conserved sequences of 28S ribosomal RNA.

across the same region. This is accounted for mainly by the striking conservation in fET of the DAT-specific sequence VE-LILVKEQNG in this otherwise highly divergent domain. In contrast to the DAT-like nature of the fET N terminus, the C terminus after TMD12 showed relatively similar conservation to both NETs and DATs.

Tissue distribution of fET mRNA was evaluated by Northern analysis and RT-PCR. Northern analysis that used the partial fET

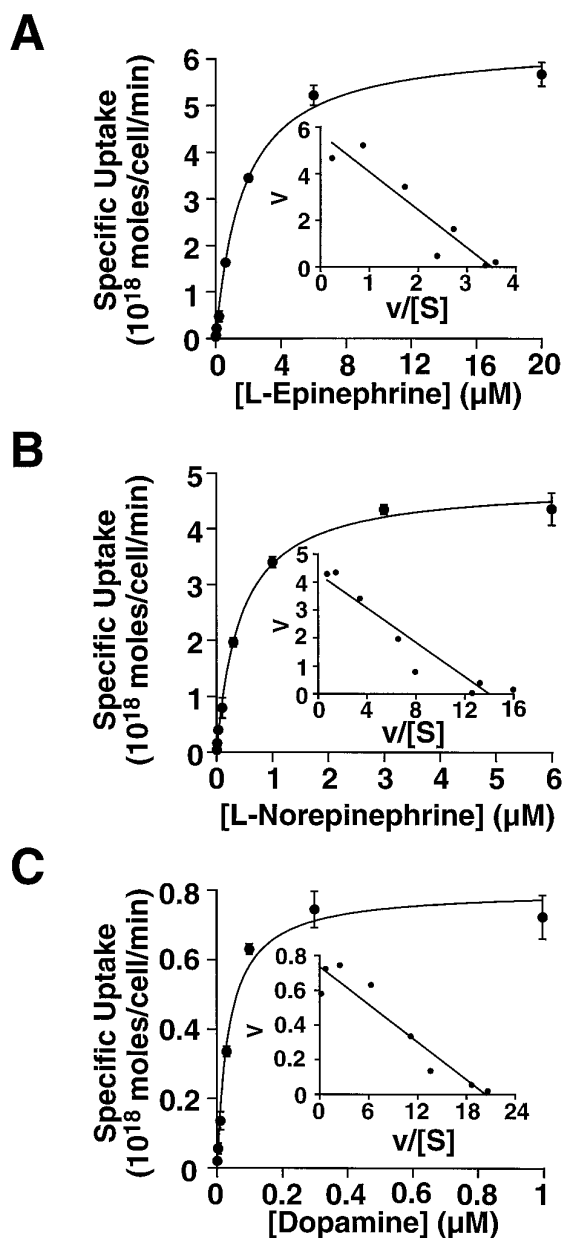


**Figure 4.** fET-induced catecholamine transport in transfected HeLa cells. *A*, Time-dependent accumulation of [<sup>3</sup>H]-L-NE uptake. Cells were transfected either with pBluescript SKII<sup>-</sup> vector containing the fET cDNA or pBluescript vector and assayed in normal buffer (squares) or buffer with complete isotonic substitution of Na<sup>+</sup> by choline (filled circles). *B*, Sodium dependence of [<sup>3</sup>H]-L-NE uptake in HeLa cells expressing fET cDNA, assessed in the presence of different concentrations of extracellular Na<sup>+</sup> with isotonicity maintained with LiCl. *C*, Chloride dependence of [<sup>3</sup>H]-L-NE uptake in HeLa cells expressing fET cDNA assessed in the presence of different concentrations of extracellular Cl<sup>-</sup>. Isotonicity was maintained with sodium gluconate. *D*, Catecholamine inhibition of [<sup>3</sup>H]-L-NE uptake in transfected HeLa cells. Nonspecific uptake was determined by parallel transfections with pBluescript SKII<sup>-</sup>. [<sup>3</sup>H]-L-NE uptake assays were performed as described in Materials and Methods, with increasing concentrations of L-NE, L-Epi, D-Epi, or DA added simultaneously with 20 nM [<sup>3</sup>H]-L-NE. Data are expressed as a percentage of specific L-NE uptake and presented as mean ± SEM of three to five separate experiments.

cDNA as a probe (Fig. 1 sequence between arrows) revealed the presence of a single 3.1 kb RNA in bullfrog sympathetic ganglia, but not in the sympathetic targets, heart atria, and ventricle (Fig. 3A). Probe specificity was confirmed by an absence of NET detection in SK-N-SH and rat brainstem. To evaluate the tissue distribution of fET more extensively, we performed RT-PCR on RNA isolated from multiple bullfrog tissues, including sympathetic ganglia, heart, lung, stomach, forebrain, hindbrain, liver, and skeletal muscle (Fig. 3B), and hybridized blotted PCR products with the same fET cDNA probe used in Northern blots. These blots again showed fET mRNA expression restricted mainly to sympathetic ganglia (Fig. 3C). We failed to detect fET mRNA in the brain, although RNAs seemed to be intact, as revealed by ethidium staining (data not shown) and equivalent amplification of amphibian ribosomal RNA (Fig. 3D). After prolonged exposure a very faint hybridizing band of 3.1 kb was observed in the liver (data not shown).

To evaluate functional properties of the cloned cDNA, we expressed fET in HeLa cells with the vaccinia-T7 expression

system (Fuerst et al., 1987; Blakely et al., 1991). Because of the sequence similarity to hNET, our initial tests for uptake activity used [<sup>3</sup>H]-L-NE as a test substrate. HeLa cells transfected with fET cDNA in pBluescript SK<sup>-</sup> rapidly accumulate L-NE in a time-dependent manner, with transport abolished by choline substitution for Na<sup>+</sup> (Fig. 4A). Transfection of HeLa cells with plasmid vector lacking the cDNA yielded no specific L-NE uptake. The specific uptake of L-NE was found to be linearly dependent on extracellular concentrations of both Na<sup>+</sup> and Cl<sup>-</sup>, with saturation not achieved in the isotonic range when Li<sup>+</sup> was substituted for Na<sup>+</sup> or gluconate for Cl<sup>-</sup> (Fig. 4B,C). Unlabeled DA, L-NE, L-Epi, and D-Epi produced concentration-dependent reduction of [<sup>3</sup>H]-L-NE accumulation in fET transfected HeLa cells (Fig. 4D). The rank order of apparent K<sub>i</sub> values for these potential substrates at fET was DA < L-NE < L-Epi < D-Epi. Remarkably, DA was almost as potent (K<sub>i</sub> = 34 ± 6.7 nM) as a number of tricyclic antidepressants in inhibiting [<sup>3</sup>H]-L-NE uptake. The K<sub>i</sub> values for L-Epi and DA at fET were significantly lower (approximately fivefold) relative to their potencies at hNET (L-Epi 998 ± 167 nM



**Figure 5.** Concentration dependence of L-Epi (A), L-NE (B), and DA (C) transport into HeLa cells transiently transfected with fET cDNA. [ $^3\text{H}$ ]-L-Epi, [ $^3\text{H}$ ]-L-NE, and [ $^3\text{H}$ ]-DA uptake assays were performed on separate cultures transfected in parallel as described in Materials and Methods. Data are presented as mean  $\pm$  SEM of four separate experiments. Insets are Eadie-Hofstee linear transformations of saturation data.

vs  $4424 \pm 752$  nM; DA  $34 \pm 6.7$  nM vs  $180 \pm 46$  nM). To assess directly the efficiencies of substrate transport at fET versus hNET, we performed uptake assays with radiolabeled L-Epi, L-NE, and DA in fET and hNET transfected cells. These experiments confirmed saturability of substrate transport with apparent single-site kinetics (Fig. 5) for all substrates.  $K_m$  values at fET (L-Epi  $1520 \pm 152$  nM, L-NE  $560 \pm 92$  nM, DA  $47 \pm 9$  nM) bore the same rank order relationship as observed for hNET (L-Epi  $2872 \pm 24$  nM, L-NE  $737 \pm 77$  nM, DA  $91 \pm 6$  nM). As noted for their  $K_i$  values for uptake inhibition,  $K_m$  values for L-Epi and DA transport by fET were significantly lower than their  $K_m$  for transport by hNET ( $p < 0.05$ , Student's unpaired  $t$  test). Moreover, we

observed clear differences between fET and hNET in rank order maximal velocities ( $V_{\max}$ ) for different catecholamine substrates (Table 1), particularly evident as (1) an enhanced capacity for L-Epi transport and (2) a markedly reduced efficiency for DA transport by fET. The rank order of  $V_{\max}$  for catecholamine transport by fET was L-Epi  $>$  L-NE  $\gg$  DA (7.7:7.2:1.0 relative to DA), whereas the rank order of  $V_{\max}$  values for hNET determined in parallel was L-NE  $>$  DA  $>$  L-Epi (1.7:1.0:0.9 relative to DA). The ratio  $V_{\max}/K_m$ , a measure of the catalytic efficiency of translocation, was found to be shifted in fET to enhance L-Epi flux and diminish DA transfer (Segel, 1975).

Finally, we compared antagonist sensitivities of catecholamine transport between fET and the other biogenic amine transporters. For direct comparisons between fET and hNET, we performed parallel transfections with fET and hNET cDNAs and tested antagonists against the single substrate [ $^3\text{H}$ ]-L-NE because L-NE is a relatively equivalent substrate for both carriers. Other comparisons were afforded by published potency values of antagonists at cloned human DAT and SERT, respectively (Giros et al., 1991; Barker et al., 1994). Absolute  $K_i$  values of all agents at fET were consistent with potencies observed for hNET (Table 2). Thus, the NET-selective antagonist desipramine, but not the DAT-selective antagonist GBR 12909 and SERT-selective antagonist paroxetine, potently inhibit L-NE transport in fET transfected HeLa cells. Indeed, correlation analyses of antagonist  $K_i$  values at all three cloned human biogenic amine transporters demonstrate significant relationships only between antagonist  $K_i$  values for fET and hNET (hNET vs fET,  $r^2 = 0.96$ ; hDAT vs fET  $r^2 = 0.20$ ; hSERT vs fET,  $r^2 = 0.02$ ). Cocaine exhibited submicromolar potency for blockade of uptake mediated by fET, in keeping with the activity of this nonspecific amine uptake inhibitor in the frog sympathetic nervous system (Seirra and Velazquez, 1973).

## DISCUSSION

Catecholamine uptake in the innervated frog heart is sensitive to antidepressants and cocaine (Seirra and Velazquez, 1972, 1973). Unlike mammalian NETs (Iversen, 1967; Trendelenburg, 1991), the cocaine-sensitive catecholamine uptake process in the innervated frog heart seems to accumulate L-Epi preferentially over L-NE (Stene-Larsen and Helle, 1978). Furthermore, radioligand-binding studies have identified the presence of high-affinity desipramine binding sites in frog heart (Pimoule et al., 1987). The radioligand-binding analyses of Schoemaker et al. (1988) also indicate that catecholamine uptake sites in the frog heart are more sensitive than those in rat heart to certain NET antagonists, including imipramine, iprindole, and mianserin. On the basis of these observations, we reasoned that a distinct cocaine- and antidepressant-sensitive catecholamine transporter, specialized for the transport of L-Epi, is expressed in frog sympathetic axons and terminals and considered that its identification might reveal evolutionary constraints and variations used to achieve shared and unique ligand interactions.

We screened a cDNA library prepared from bullfrog sympathetic ganglia with an oligonucleotide derived from a sympathetic ganglia RT-PCR product and identified a novel cDNA for which the distribution, structure, and properties suggest it to be the carrier for catecholamines in frog sympathetic neurons. fET mRNA is expressed abundantly in frog sympathetic ganglia, as compared to brain and peripheral tissues. The large number of positive clones identified in our cDNA library screen certainly suggests a significant representation of fET mRNA in sympathetic ganglia. Furthermore, RT-PCR revealed detectable quan-

**Table 1. Comparison of catecholamine transport kinetics for frog catecholamine (fET) and human norepinephrine transporter (hNET)**

Substrate	fET			hNET		
	$K_m$ (nM)	$V_{max}$ ( $\times 10^{18}$ mol/cell per min)	$V_{max}/K_m$ ( $\times 10^7$ l/min)	$K_m$ (nM)	$V_{max}$ ( $\times 10^{18}$ mol/cell per min)	$V_{max}/K_m$ ( $\times 10^7$ l/min)
L-Epinephrine	1527 $\pm$ 152 <sup>a</sup>	5.94 $\pm$ 0.97	3.87 $\pm$ 0.51 <sup>c</sup>	2872 $\pm$ 24	3.16 $\pm$ 0.08 <sup>d</sup>	0.90 $\pm$ 0.1
L-Norepinephrine	560 $\pm$ 92	5.45 $\pm$ 0.69	10.63 $\pm$ 2.2	737 $\pm$ 77	6.01 $\pm$ 0.23	8.4 $\pm$ 0.8
Dopamine	47 $\pm$ 9 <sup>a</sup>	0.74 $\pm$ 0.07 <sup>b</sup>	17.38 $\pm$ 3.3 <sup>c</sup>	91 $\pm$ 6	3.55 $\pm$ 0.61 <sup>d</sup>	38.9 $\pm$ 6.8
		L-Epi > L-NE $\gg$ DA			L-NE > L-Epi > DA	

Transport of [<sup>3</sup>H]-L-epinephrine, [<sup>3</sup>H]-L-norepinephrine, and [<sup>3</sup>H]-dopamine were determined in HeLa cells transfected with expression vector containing either the fET or hNET cDNA, as described in Materials and Methods. Data are presented as mean  $\pm$  SEM of three to four separate experiments. Statistical comparisons were performed using Student's unpaired *t* test.

<sup>a</sup>*p* < 0.05 as compared with  $K_m$  of substrates tested on hNET.

<sup>b</sup>*p* < 0.05 as compared with  $V_{max}$  of L-Epi and L-NE in fET.

<sup>c</sup>*p* < 0.05 as compared with  $V_{max}/K_m$  of substrates tested on hNET.

<sup>d</sup>*p* < 0.05 as compared with  $V_{max}$  of L-NE in hNET.

tities of fET mRNA in the sympathetic ganglia, but not in sympathetically innervated target tissues, consistent with a presynaptic localization of fET protein in adrenergic terminals. Additional studies with fET-specific antibodies are needed to validate the precise localization of fET protein in sympathetic neurons and terminals *in vivo*. Interestingly, frog brain contains predominantly noradrenergic i.e., NE-synthesizing neurons (Gonzalez and Smeets, 1993; Gonzalez et al., 1993). Although low abundance of fET mRNA could account for our inability to amplify fET cDNAs from frog brain, these data also may indicate the presence of a distinct catecholamine transporter responsible for the clearance of L-NE in the bullfrog CNS.

fET possesses structural features common to members of the

Na<sup>+</sup>/Cl<sup>-</sup>-dependent neurotransmitter transporter family. Transfection of fET conferred Na<sup>+</sup>/Cl<sup>-</sup>-dependent uptake of catecholamines on transfected HeLa cells. In preliminary studies we also have found L-NE- and L-Epi-induced currents in *Xenopus laevis* oocytes injected with fET cRNA (R. D. Blakely, unpublished data). The presence of multiple in-frame translation termination codons before the predicted translation start site suggests the presence of a complete N terminus in the sequence predicted by fET cDNA. Like most members of Na<sup>+</sup>/Cl<sup>-</sup> cotransporter gene family, fET bears 12 prominent hydrophobic regions likely to constitute TMDs as well as a large putative extracellular loop that bears multiple (3) canonical N-glycosylation sites. N-glycosylation sites in the large extracellular loop have been shown to be important for transporter protein stability, surface trafficking, and transport activity of other members of Na<sup>+</sup>/Cl<sup>-</sup> cotransporter gene family, including hNET (Melikian et al., 1994, 1996; Tate and Blakely, 1994). The three sites described are located exactly in register with those of hNET, but, unlike hNET, additional N-glycosylation sites also were found in the fET N terminus and in the extracellular loop between TMD11 and TMD12, although their use remains unknown. Immunofluorescence and electron microscopy studies support an intracellular localization of the N terminus of NETs (Bruss et al., 1995) and DATs (Ciliax et al., 1995; Nirenberg et al., 1996), respectively, and thus it is unlikely that the N terminus glycosylation site is used. Like hNET, the presence of multiple sites for protein phosphorylation in putative cytoplasmic domains of fET suggests that these transporters may be regulated acutely by post-translational modification. fET also possesses a leucine zipper motif associated with TMD1 and TMD2. Leucine repeat motifs have been implicated in protein-protein interactions for soluble proteins and recently have been implicated in trafficking efficiency of glucose transporters (Asano et al., 1992).

Comparison of functional characteristics of DATs, NETs, and fET suggests that specificity for physiologically relevant substrates may be revealed best by relative translocation capacity rather than by the  $K_m$  of substrates. Indeed DATs, NETs, and fET have identical rank order of  $K_m$  values for catecholamine substrates (DA < L-NE < L-Epi) (Giros et al., 1992; Buck and Amara, 1994). Our studies indicate that fET transports L-Epi with a greater maximal velocity than L-NE and DA. On the other hand, DATs and NETs display enhanced translocation efficiencies for DA and L-NE, respectively (Buck and Amara, 1994; Giros et al., 1994; Piff et al., 1996), and L-Epi is a less efficient substrate for either carrier

**Table 2. Inhibitor and substrate sensitivity of frog catecholamine (fET) and human norepinephrine (hNET) transporter**

Agent	$K_i$ or $K_m$ (nM)	
	fET	hNET
Mazindol	0.22 $\pm$ 0.02	0.20 $\pm$ 0.03
Desipramine	0.82 $\pm$ 1.5	0.53 $\pm$ 0.06
Imipramine	2.2 $\pm$ 0.9	8.7 $\pm$ 3.5
Mianserin	2.5 $\pm$ 0.03	2.3 $\pm$ 0.3
RTI-55	6.7 $\pm$ 1.3	4.4 $\pm$ 0.9
Nomifensine	6.8 $\pm$ 1	3.3 $\pm$ 0.4
Nortriptyline	11.4 $\pm$ 3	7.0 $\pm$ 2.0
Clomipramine	14 $\pm$ 2	27 $\pm$ 4
Amytriptyline	22 $\pm$ 4	44 $\pm$ 7
Dopamine	34 $\pm$ 7	180 $\pm$ 46*
D-Amphetamine	42 $\pm$ 8	57 $\pm$ 1
Paroxetine	71 $\pm$ 10	153 $\pm$ 23
L-Amphetamine	85 $\pm$ 12	65 $\pm$ 9
Cocaine	160 $\pm$ 24	76 $\pm$ 11
N-Methyl-		
pamine	301 $\pm$ 48	320 $\pm$ 22
GBR 12909	320 $\pm$ 48	320 $\pm$ 22
L-Norepinephrine	460 $\pm$ 71	686 $\pm$ 50
Iprindole	832 $\pm$ 90	1313 $\pm$ 110
L-Epinephrine	998 $\pm$ 167	4424 $\pm$ 752*
5-HT	20,000	10,000

$K_i$  or  $K_m$  values for different agents were determined from inhibition curves obtained by [<sup>3</sup>H]-L-norepinephrine uptake assays performed on transfected cells in parallel. Data are presented as mean  $\pm$  SEM of three separate experiments. Asterisks indicate statistically different potency for hNET as compared with fET (*p* < 0.05; Student's unpaired *t* test).

(Iversen, 1975; Meiergerd and Schenk, 1994). We also demonstrate that DA blocks L-NE uptake by fET with high affinity although its translocation efficiency is reduced. These data are consistent with the idea that increased affinity for substrates may be detrimental to the evolution of increased substrate flux rates. Frog sympathetic neurons synthesize, store, and release L-Epi (Angelakos et al., 1965; Woods, 1977) and probably release little, if any, DA. Levels of amine transmitters may reach micromolar concentrations at synapses (Johansson et al., 1972; Stjärne et al., 1990), and thus transport capacities, as described by the L-Epi  $V_{max}$ , may reflect evolutionary pressure to achieve optimal transfer of L-Epi at these synapses. The ratio  $V_{max}/K_m$  provides one index of catalytic efficiency for a given substrate (Segel, 1975). Relative to hNET, fET displays enhanced efficiency of transport for L-Epi and reduced efficiency for DA. Because at low concentrations DA is still a superior substrate for fET, our data suggest that a rate-limiting step in DA transport is blocked once substrate concentrations are elevated. DA seems to bind very tightly to fET; thus DA may dissociate poorly to the cytoplasm (or rebind rapidly after dissociation) and thus promote inefficient cycling. Conversely, L-Epi may gain high rates of transfer by minimally impacting this step in the cycle (Rudnick and Clark, 1993).

Availability of sequence for multiple cloned catecholamine transporter species variants should allow for increased utility of sequenced-based mutagenesis efforts to define sites responsible for substrate recognition, translocation, and antagonist sensitivity. In addition to fET, we recently have cloned the murine NET (L. D. Jayanthi, J. D. Fritz, M. A. Thoreson, R. D. Blakely, unpublished data) and have included this sequence in our alignment in Figure 2. Overall, we find 32 residues that are conserved selectively in catecholamine transporters (fET = NETs = DATs  $\neq$  gene family). These residues may define the ability of fET, NETs, and DATs to recognize and transport catecholamines, interact with nonselective catecholamine transporter antagonists, or undergo common modes of trafficking and regulation. With regard to substrate recognition, functional studies with chimeras of hNET and hDAT have focused attention on the regions spanning TMD1–TMD3 and TMD9–TMD12 as contributing to substrate  $K_m$  (Buck and Amara, 1994). Of the catecholamine transporter-specific residues listed above, six are found in TMD1–TMD3 (fET A90, N133, A137, A138, I155, F163) and four in TMD9–TMD12 (fET T466, C473, F488, V546) and thus may be involved in catecholamine binding and transport. Catecholamine transporters also may use residues for substrate recognition that are common to homologous 5-HT transporters. For example, point mutation studies of Kitayama et al. (1992) first demonstrated that mutation of TMD1 aspartate residue (hDAT<sub>D79</sub>) and serine residues in TMD7 (hDAT<sub>S356</sub> and hDAT<sub>S359</sub>) can affect DA affinity and DA uptake (Kitayama et al., 1992). By analogy with models for the interaction of catecholamines with adrenoceptors (Strader et al., 1988, 1989), negatively charged hDAT<sub>D79</sub> in TMD1 has been suggested to interact with the amine group of catecholamine substrates, whereas hDAT<sub>S356</sub> and hDAT<sub>S359</sub> in TMD7 have been proposed to interact with hydroxyl moieties of catecholamines (Kitayama et al., 1992). Although other models to explain these data are tenable, these residues also are conserved in SERTs, NETs, and fET (fET<sub>D88</sub>, fET<sub>S367</sub>, and fET<sub>S370</sub>), suggesting a common contribution in organization of the amine binding pocket.

We have found fET to differ significantly from NET and DAT in L-Epi and DA translocation capacity ( $V_{max}$ ) at saturating substrate concentrations. Which residues have diverged to account

for these functional distinctions? Regions comprising the N terminus and TMD5–TMD8 have been implicated in catecholamine transport capacity (Buck and Amara, 1994). Therefore, residues in this regions shared by all DAT and NET species variants, but not by fET, might contribute to differential catecholamine transport capacity. Overall, 24 amino acids are conserved in NETs and DATs, but not in fET (fET  $\neq$  NETs = DATs), with four occurring in the TMD5–TMD8 region (fET S300, P318, Y330, N385). We presently are exploring these sites by site-directed mutagenesis. The N terminus of fET is distinguished by the presence of a DAT-specific domain VELILVKEQNG in an otherwise highly divergent region. A BLASTP search of the GenBank database (Release 90.0) failed to identify the presence of VELILVKEQNG or significantly similar sequences in other mammalian proteins, including members of the GAT/NET gene family. In chimera studies the DAT N terminus when substituted for the NET N terminus was found to confer *increased* capacity for DA and 1-methyl-4-phenylpyridinium (MPP<sup>+</sup>) transport relative to L-NE. A reciprocal swap placing the hNET N terminus (and part of TMD1) on rDAT *reduced* DA and MPP<sup>+</sup> transport capacity relative to NE. The striking DAT-like sequence of the fET N terminus warrants further consideration of this region for functional contributions to differential substrate translocation efficiencies as well as transporter localization and regulation mediated by intramolecular or intermolecular interactions. Although we find no reason on the basis of homology to implicate the fET C terminus in *differential* substrate translocation, short stretches of the DAT C terminus have been suggested to play a role in DA recognition via an as yet unknown mechanism (Lee et al., 1996), and multiple residues are conserved throughout this domain in all catecholamine transporters.

fET shares with NETs, but not DATs, high affinity for tricyclic antidepressants and low affinity for DAT-selective inhibitors. In addition, amphetamine displays stereoselective inhibition of DATs, whereas both stereoisomers have comparable affinity for NETs (Coyle and Snyder, 1969; Giros et al., 1992, 1994) and fET (this study). We did find small differences in potency of antagonists when comparing fET and hNET, although not to the degree reported previously (Pimoule et al., 1987; Schoemaker et al., 1988). This may reflect the use of other amphibian species, dependence on radioligand-binding analysis, or both. With the use of chimeras of hDAT and hNET, regions spanning TMD1–TMD3 and TMD5–TMD8 have been implicated in antagonist sensitivity (Buck and Amara, 1994, 1995; Giros et al., 1994). fET and NETs share 58 residues not conserved in DATs. Of these, nine are located in TMD1–TMD3 (fET V83, I109, T112, L115, I116, Y132, T139, A158, Y164), and 19 residues are located in TMD5–TMD8 (fET L282, V289, H309, I310, K316, F329, A334, A343, D349, L357, S360, C364, A373, I374, I377, E395, v400, S409, T415). As above, further studies are warranted to determine the degree to which NET-specific antagonist recognition displayed by fET are determined by any of these sites.

Finally, our identification of an amphibian catecholamine transporter with enhanced efficiency for L-Epi transport raises the question of whether the two transporters coexist in a single species. PNMT and L-Epi are found in the mammalian brain. Administration of 6-hydroxydopamine (6-OHDA), which must be concentrated by catecholamine transporters for its neuronal toxicity, produces depletion of L-Epi in the hypothalamus (Tessel et al., 1978). 6-OHDA-induced loss of L-Epi can be abolished by previous administration of tricyclic antidepressants (Tessel et al., 1978). Using  $\alpha$ -methyl-*m*-tyrosine, Fuller (1982) made similar

observations in rat hypothalamic L-Epi-containing neurons. However, 6-OHDA lesions did not reduce PNMT activity (Fety and Renaud, 1983), raising the possibility that L-Epi is synthesized by target cells or glia rather than by L-Epi-synthesizing terminals. On the other hand, clear evidence for PNMT-positive terminals has been found by immunoelectron microscopy in the brainstem and spinal cord (Milner et al., 1987; Chung et al., 1989; Alonso, 1993). Recent *in situ* hybridization studies indicate the absence of both DAT and NET mRNA in L-Epi-synthesizing neurons in the brainstem (Lorang et al., 1993). Thus, if L-Epi is cleared at these sites by reuptake, these terminals might elaborate a catecholamine transporter distinct from DAT and NET. The fact that L-Epi-synthesizing neurons are involved in cardiovascular homeostasis in both physiological and pathophysiological conditions and nociception (Moore and Bloom, 1979; Fuller, 1982; Goodchild et al., 1984; Ross et al., 1984; Tucker et al., 1987; Carlton et al., 1991; Kopp et al., 1992; Gai et al., 1993; Burke et al., 1994) suggests that identification of a molecularly distinct catecholamine transporter at these sites would provide an important new drug target and clarify physiological effects of catecholamine transport antagonists presently attributed to NET blockade. The close relationship between fET and hNET antagonist sensitivities suggests that, if such a carrier exists in mammals, it will be difficult to discriminate from NETs with present pharmacological agents, but it could be identified from enriched preparations by homology-based approaches. Such efforts are presently underway in our laboratory.

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