Molecular Biology of Human and Rat Genomic DNAs for Epinephrine Synthesizing Enzyme

Yoo Hun Suh, Hun Sik Kim, Il Sun Park, Sung Soo Kim, Yang Sook Chun and Chan Woong Park
Department of Pharmacology, Seoul National University)

Norepinephrine is N-methylated by the enzyme phenly ethanolamine N-metyltransferase(PNMT) to form epinephrine. This enzyme is largely restricted to the adrenal medulla where epinephrine serves as a hormone although low levels of activity have been reported in mammalian brain where epinephrine function as a neurotransmitter. It seems clear that central epinephrine is involved in the regulation of cardiovascular function and in several forms of hypertension. However, information about the structure of mammalian epinephrine forming enzyme has been limited until now. But recently we isolate bovine and human PNMT cDNA clone using gtil expression library and sequenced total nucleotide composition.

To obtain information about the structure of the human and rat PNMT proteins and genes and to further define the extent of the evolutionary relationships among the PNMT molecules of these species, human and rat genomic DNA clones to PNMT were sequentially isolated and characterized.

INTRODUCTION

The catecholamine biosynthetic pathway consists of three neural-

* Address Correspondence and reprint requests to Dr. Y.H. Suh.
specific enzymes: tyrosine hydroxylase (TH), which catalyzes the conversion of tyrosine to L-dopa, the first and rate-limiting step in the pathway: dopamine β-hydroxylase (DBH), which catalyzes norepinephrine synthesis from dopamine; and phenylethanolamine N-methyltransferase (PNMT), which mediates the conversion of norepinephrine to epinephrine (Fig. 1).

CATECHOLAMINE BIOSYNTHESIS

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\begin{align*}
\text{HO-} & \quad \text{CH}_2-\text{CH} & \quad \text{NH}_2 & \quad \text{COOH} & \quad \text{L-tyrosine} \\
\text{tyrosine hydroxylase} & \quad (\text{TH}) & \quad \downarrow & \quad \text{oxygen tetrahydrobiopterin} & \\
\text{HO-} & \quad \text{CH}_2-\text{CH} & \quad \text{NH}_2 & \quad \text{COOH} & \quad \text{L-dopa} \\
\text{HO} & \quad \downarrow & \quad \text{pyridoxal 5-phosphate} & \quad \text{aromatic 1-amino acid decarboxylase (AADC)} & \\
\text{HO-} & \quad \text{CH}_2-\text{CH}_2 & \quad \text{NH}_2 & \quad \text{dopamine} \\
\text{HO} & \quad \downarrow & \quad \text{OH} & \quad \text{L-norepinephrine} \\
\text{HO} & \quad \downarrow & \quad \text{OH} & \quad \text{S-adenosyl-L-methionine} & \quad \text{phenylethanolamine N-methyltransferase (PNMT)} \\
\text{HO-} & \quad \text{CH-CH}_2 & \quad \text{NH}_2 & \quad \text{L-epinephrine} \\
\text{HO} & \quad \downarrow & \quad \text{OH} & \quad \text{S-adenosyl-L-methionine} & \quad \text{phenylethanolamine N-methyltransferase (PNMT)}
\end{align*}
\]

Fig. 1. Catecholamine Biosynthetic Pathway.

Phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28, S-adenosyl-L-methionine: phenylethanolamine N-methyltransferase), the terminal enzyme in the catecholamine biosynthetic pathway, catalyzes the S-adenosylmethionine-dependent methylation of norepinephrine to form epinephrine (Axelrod, 1962). The expression of PNMT defines, in
part, the adrenergic cell phenotype. The enzyme is expressed at high levels in chromaffin cells of the adrenal medulla where epinephrine functions as a hormone (Axelrod, 1962) and is transiently expressed during the development of sympathetic ganglia and extraadrenal chromaffin tissue (Ciaranello et al., 1973; Bohn et al., 1982). In the central nervous system, where epinephrine may function as a neurotransmitter, PNMT has been localized to cell bodies within the medulla oblongata (Hökfelt et al., 1974), hypothalamus (Foster et al., 1985), and sensory nuclei of the vagus nerve (Pickel et al., 1986). Centrally, adrenergic neurons are believed to participate in the regulation of reproduction, temperature, cardiovascular function, and food and water intake.

There is substantial evidence indicating that high concentration of glucocorticoid are required for the ontogeny and maintenance of adult levels of adrenal PNMT (Bohn, 1983). In contrast to peripheral PNMT expression, however, neither adrenalectomy nor hypophysectomy affects PNMT in central adrenergic neurons (Wurtman and Axelrod, 1965, Bohn et al., 1986). This finding suggests that gene expression in the same neurotransmitter phenotype may be differentially regulated in central and peripheral systems. The mechanism of the glucocorticoid effects on adrenal PNMT are still controversial (Ciaranello, 1978; Wurtman and Axelrod, 1966; Sabban et al., 1982, Burke et al., 1983).

The complete sequence of a full-length cDNA encoding bovine PNMT has recently been reported (Suh et al., 1989) and a cDNA clone encoding human PNMT was isolated (Suh et al. 1988).

To better define the control of the expression of the adrenergic phenotype and to obtain information about the structure of the human PNMT and to further define the extent of the evolutionary relationships among PNMT molecules of several species, we isolated and characterized the genomic clone encoding human and rat PNMT.
MATERIALS AND METHODS

Reagents
Enzymes and chemicals were supplied by: Life Sciences (restriction enzymes and likers); Boehringer Mannheim Biochemicals (DNA polymerase and deoxynucleotides); BRL (T4 DNA ligase, restriction enzymes and RNase H); PL Biochemicals [terminal deoxynucleotidyl transferase and oligo (dT 12-18)].

Construction of DNA library and screening for PNMT genes
The DNA gene library of human leukocyte cells and rat brain was constructed by the method of Maniatis et al., 1982 (Fig. 3).

The genomic library of $10^8$ phage plaques was screened by the procedure described by Benton and Davis (1977). The probe used for screening was the full length 1.05-Kb fragment of bovine cDNA (Fig. 2) and was labeled with [$\alpha^{-32}$P]dCTP (5,000 Ci/mmol, New England Nuclear, Boston, MA, U.S.A.) by nick translation (Rigby et al., 1977).

Isolation of inserted phage DNA
Candidate positive signals obtained in the first high density screen were taken through three successive rounds of screening at progressively lower plaque densities. The resulting repeatedly positive, and well isolated phage plaques were picked, amplified to yield high titer plate stocks and used for the large-scale preparation of phage (Yamamoto et al., 1970; Maniatis et al., 1978). Isolated DNA was subjected to EcoRI endonuclease digestion to produce insert fragments free of flanking phage sequences. Fragments were fractionated by electrophoresis, and eluted.
**Fig. 2. Nucleotide sequence and deduced amino acid sequence of cDNA clone encoding bovine adrenal medullary PNMT ase. (Upper) Diagram of the restriction map and strategy used for sequencing the bovine cDNA clone PNMTase-17. (Lower) Complete nucleotide sequence of PNMTase-17. Initiator methionine is underlined at position 1. An open reading frame extends from nucleotide 40 to 882 where translation is terminated at the underlined TGA stop codon. The poly(A) addition recognition sequence "AATAAA" is underlined and located 15 nucleotides on the 5' side of the poly (A) start site. The predicted amino acid sequence is depicted below the nucleotide sequence (Lee et al., Proc. Natl. Acad. Sci. U.S.A. 1986).**
Fig. 3. Construction of genomic library in bacteriophage λ replacement vector.
Subcloning of inserted PNMT gene and detection of exons

Since the DNA inserted into Charon 4A and EMBL phage is long and has two more EcoRI sites in it, it was first divided into three subclones. Each of the three EcoRI-digested fragments are ligated into the EcoRI site of PBR322 and M13mp18 (Fig. 6).

Restriction mapping

Plague purified clones were digested with several restriction enzymes and fractionated by electrophoresis through agarose gels for size determination and tentative restriction mapping were deduced primarily from single and double restriction enzyme digests.

RESULTS

Isolation of human PNMT gene

Radiolabeled bovine PNMT cDNA was employed to screen a human

![Image: Identification of cloned insert by Eco RI digestion]

Fig. 4. Identification of cloned inserts from positive clones. Phage DNA (5 μg) was digested with EcoRI and electrophoresed on a 0.7% agarose gel. Left lane is Hind III digest of Lambda DNA (0.8 μg).
genomic library for clones containing PNMT gene-coding sequences. Initially, the screening of $1.5 \times 10^6$ pfu yielded one, repeatedly positive and well isolated clone. This phage contained 13.1Kb of human genomic DNA in which two EcoRI sites existed, generating 7.5Kb, 5.0Kb and 0.6Kb subfragments (Fig. 4).

**Identification of the human PNMT gene.**

In Southern and dot blot experiments, a 5.0Kb and a 0.6Kb EcoRI fragments strongly hybridized to the bovine cDNA probe but 7.5Kb showed background signal, indicating that PNMT gene coding sequences were localized to within 5.0Kb and 0.6Kb fragments flanked by a 7.5 Kb fragment (data not shown.)

**Fig. 5.** BamHI digestion of (+) clone recombinant phage DNA.
Fig. 6. Subcloning strategy of cloned insert subfragments.
Fig. 7. Tentative restriction map of human and rat genomic DNA for PNMT.
The 7.5Kb and 5.0Kb EcoRI fragments were subcloned into PBR 322 (called pSH 100; pSH 200, respectively) to facilitate further analysis and 0.6Kb fragment was also subcloned into M 13mp18 for subsequent dideoxy sequencing (called pSH300) (Fig. 6).

**Isolation and Identification of Rat Genomic DNA**

Three successive rounds of screening yielded one, repeatedly positive clone. This clone contained 13.5Kb of rat genomic DNA in which two BamHI sites existed, generating 7.0Kb, 5.0Kb and 1.5Kb subfragments (Fig. 5). Southern blot experiments of this gel showed that only a 7.0 Kb fragment strongly hybridized to the bovine cDNA probe, indicating that rat PNMT gene-coding sequences were localized to within a 7.0Kb fragment flanked by 5.0Kb and 1.5Kb fragments (data not shown).

Tentative Restriction Mapping of Human and Rat Genomic DNAs Restriction analysis showed that human PNMT genomic sequence had a striking homology with rat genomic sequence but slight difference existed between human and rat sequences (Fig. 7).

**DISCUSSION**

Phenylethanolamine N-methyltransferase (PNMT) is essential for the conversion of norepinephrine to epinephrine and its expression defines the adrenergic cell phenotype. It has been postulated that the genes for the catecholamine biosynthetic enzymes contain similar coding sequences and may have evolved through duplication of a common ancestral precursor (Joh et al., 1983, 1985).

To obtain information about the structures of the human PNMT and to further define the extent of the evolutionary relationships among PNMT molecules of several species, we have described the isolation,
identification of the gene encoding human PNMT. The size of isolate human genomic clone was 13.1Kb and it contained two EcoRI sites, generating 7.5Kb, 5.0Kb and 0.6Kb fragments.

In Southern and dot blot experiments, a 5.0Kb and a 0.6Kb EcoRI fragments strongly hybridized to the bovine DNA probe, indicating that PNMT gene coding sequences (exons) were localized to within 5.0 Kb and 0.6Kb fragments flanked by a 7.5Kb.

The clone contained 13.5Kb of rat genomic DNA in which two BamHI sites existed, generating 7.0Kb 5.0Kb and 1.5Kb subfragments. Southern blot experiments of this gel showed that only a 7.0Kb fragment strongly hybridized to the bovine cDNA probe, indicating that rat PNMT gene-coding sequences were localized to within a 7.0Kb fragment flanked by 5.0Kb and 1.5Kb fragments.

Restriction mapping shows that human PNMT genomic DNA has striking homology with rat genomic DNA, indicating that both genes may evolved from a common ancestral-primitive gene.

Isolation and characterization of the human and Rat PNMT gene will greatly facilitate studies of the molecular mechanisms underlying the expression and development of the adrenergic phenotype. These studies should greatly augment our understanding of the regulation of catecholamine biosynthesis.

In conclusion, the results of this investigation directly confirm the isolation and identification of a genomic DNA clone for human and Rat. DNA sequencing of this clone will clarify the complete structure of human and rat PNMT and evolutionary relationships among PNMT genes of several species.
References


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요 약

사람과 쥐의 에피네프린 합성효소의 계놈 DNA에 대한 분자생물학

_Yoo Hun Suh, Hun Sik Kim, Il Sun Park, Sung Soo Kim, Yang Sook Chun, and Chan Woong Park_
Department of Pharmacoloty, Medical College & Department of Molecular Biology, Neuroscience Research Institute, Seoul National University
Seoul, 110-744, Korea

카테콜아민 생합성에 관여하는 마지막 효소인 phenylethanolamine N-methyltransferase는 norepinephrine을 epinephrine으로 전환시키는 중요한 효소이다. PNMT 효소의 발현은 epinephrine 신경체포의 발현에 필수적이다. 따라서 PNMT 유전자를 코로닝하여 그 구조를 결정하고, 유전자 발현유전자 하는 것은 상당히 중요한 일이다. 그러나 최근에 저자가 bovine 및 human cDNA를 처음으로 분리하여 그 구조를 보고한 것 외에는 아직까지 인간과 백서 전체 genomic DNA의 분리 보고는 없다. 이에 저자들은 인간과 백서 PNMT 유전자와 전체구조와 여러종(species)사이의 전화적인 관계를 규명하기 위해서 human과 Rat genomic library를 만들고, 이 library를 이용하여 bovine cDNA를 probe로 13.1kb와 13.2kb 길이의 인간과 백서의 genomic clone을 분리 크리닝하는데 성공하여 유전자의 구조적 특성을 규명하였다.