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Molecular Cloning of Human Genomic DNA for Epinephrine Synthesizing Enzyme, Phenylethanolamine N-Methyltransferase

Yoo Hun Suh,* Sung Oh Huh, Yang Sook Chun, Hun Sik Kim, Jung Kyoo Lim and Chan Woong Park

Department of Pharmacology, College of Medicine and Neuroscience Reseach Institute, Seoul National University, Seoul 110-460, Korea

ABSTRACT

To obtain information about the structure of the human phenylethanolamine N-methyltransferase (PNMT) and to further define the extent of the evolutionary relationships among PNMT molecules of several spesies, a full length cDNA clone for bovine adrenal PNMT was used to screen a charon 4A genomic library. One phage was isolated and identified, which included the entire PNMT gene. The length of inserted genomic DNA was 13.1-Kilobase (Kb) containing two internal EcoRI sites. Construction of a restriction map and subsequent Southern and dot blot analysis with 5'-and3'-specific cDNA probes allowed the identification of exon-containing fragments. This is the first report of the cloning of gene for human epinephrine synthesizing enzyme.

Key Words: Human, PNMT, Genomic Cloning

INTRODUCTION

Phenylethanolamine N-methyltransferase (PNMT; EC 2.1.1.28, S-adenosyl-Lmethionine: 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.,* 1986).

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*To whom reprint requests should be addressed.

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 PNMT.

MATERIALS AND METHODS

Reagents

Enzymes and chemicals were supplied by: Life Scienccs (restriction enzymes and linkers); 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 human PNMT genes

The DNA gene library of human leukocyte cells was constructed by ligation of EcoRI-digested DNA to Charon 4A phage vector by the method of Maniatis *et al.,* 1982 (Fig. 1). The genomic library of 10^5 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 and was labeled with [α-^{32}P]dCTP (5,000 Ci/mmol, New England Nuclear, Boston, MA U.S.A.) by nick translation (Rigby *et al.,* 1977).

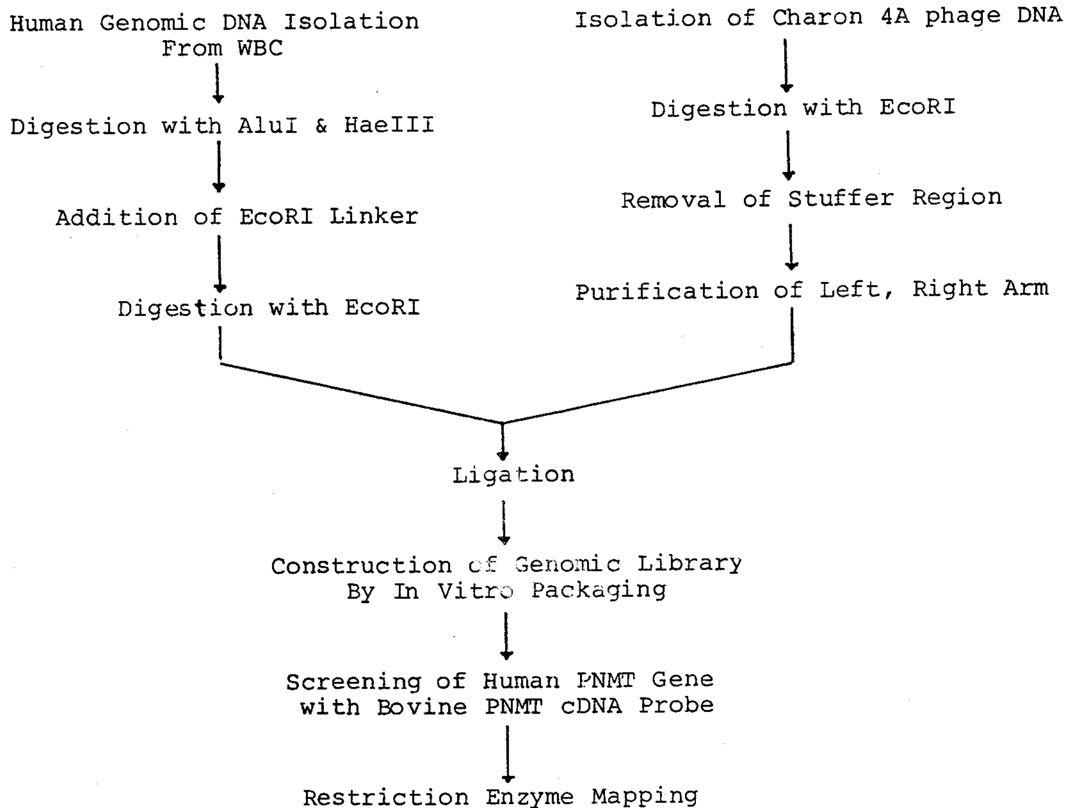


Fig. 1. Scheme for construction of human genomic library and cloning strategy in Charon 4A phage vector.

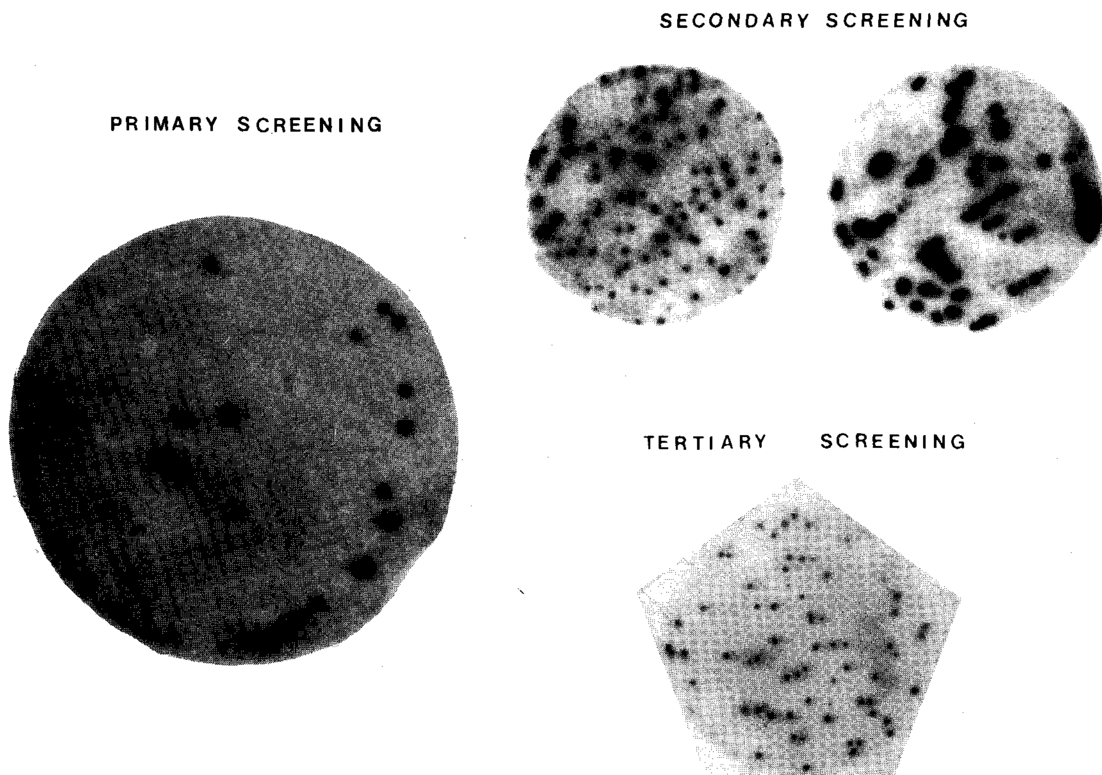


Fig. 2. Successive three rounds of screening for genomic clones containing PNMT sequences.
Genomic sequences in Charon 4A vector in situ were fixed onto nitrocellulose filter papers. Filters were incubated with a ^{32}P-labeled bovine cDNA probe. Positive clones were detected by autoradiography.

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. Fraqments were fractionated by electrophoresis, and eluted.

Subcloning of inserted PNMT gene and detection of exons

Since the DNA inserted into Charon 4A 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. 3).

Southern blot analysis

Restriction endonuclease digested DNA was subjected to electrophoresis in the appropriate percentage agarose gels, and transferred to Gene Screen Plus or Gene Screen (New England Nuclear, Boston, MA) as described by the manufacturer. Blots containing genomic DNA were prehybridized at 42℃ in 50% deionized formamide containing 25 mM Tris HCl (pH 7.5), 1 M NaCl, 0.2% BSA, 0.2% polyvinyl pyrrolidone, 0.2% ficoll, 0.1% sodium pyrophosphate, 1% SDS, 100 μg/ml sheared, denatured, E. coli DNA. After 16 hr, approximately 1×10^8 cpm/ml [^{32}P]labeled probe was added, and the blots were hybridized for 1 day

Identification of Cloned Insert by Eco RI Digestion

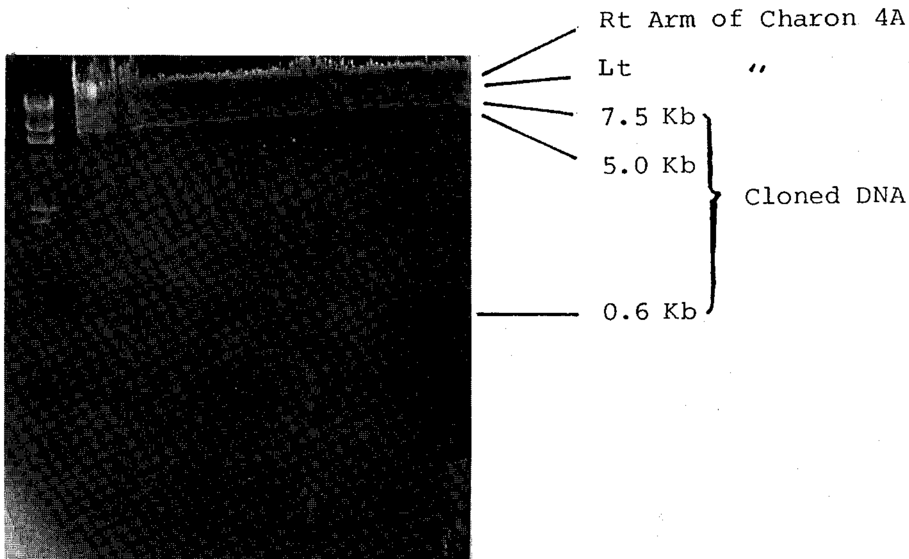


Fig. 3. Identification of cloned inserts from positive clones. phage DNA (5 μg) was digested with EcoRI and electrophoresed on a 0.7% agarose gel. Left lane is Hind III digest of Lambda DNA (0.8 μg).

at 42℃. The filters were washed twice in 2×SSC, 2 mM EDTA at 23℃ for 15 min, three times in 0.1×SSC, 2 mM EDTA, 1% SDS at 55℃ for 30 min, and twice in 0.1×SSC, 2 mM EDTA at 23℃ for 15 min. All filters were exposed to Kodak XAR-5 film for autoradiogrpahy (with intensifying screen for the genomic blots).

RNA analysis (Northern blot)

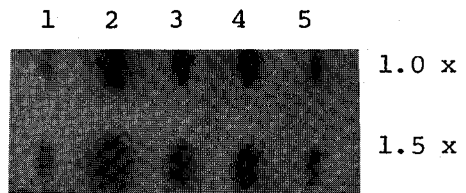
RNA was isolated from human adrenal using the guanidine isothiocyanate procedure of Chirgwin *et al.,* (1979). RNA was fractionated by electrophoresis through agarose gels containing formaldehyde transferred to nitrocellulose filters and hybridized with a radioactive cDNA probe.

Dot blot analysis

Either purified DNA or plasmid DNA was treated with 0.3 ml of 57 mM Tris (pH 7.0)-0.2 M NaOH-6×SSC at 80℃ for 10 min. The samples were placed on ice and neutralized with 2M HEPES (pH 7.5).

Denaturd human genomic DNA were spotted

Dot Blot Analysis of Human PNMT



Slot NO.

1. Lamda DNA (1.0 ug)
2. Bovine PNMT (0.5 ug)
3. pSH300 (0.6 Kb; 0.5 ug)
4. pSH200 (5.0 Kb; 0.5 ug)
5. pSH100 (7.5 Kb; 0.5 ug)

Fig. 4. Dot blot analysis of cloned inserts.

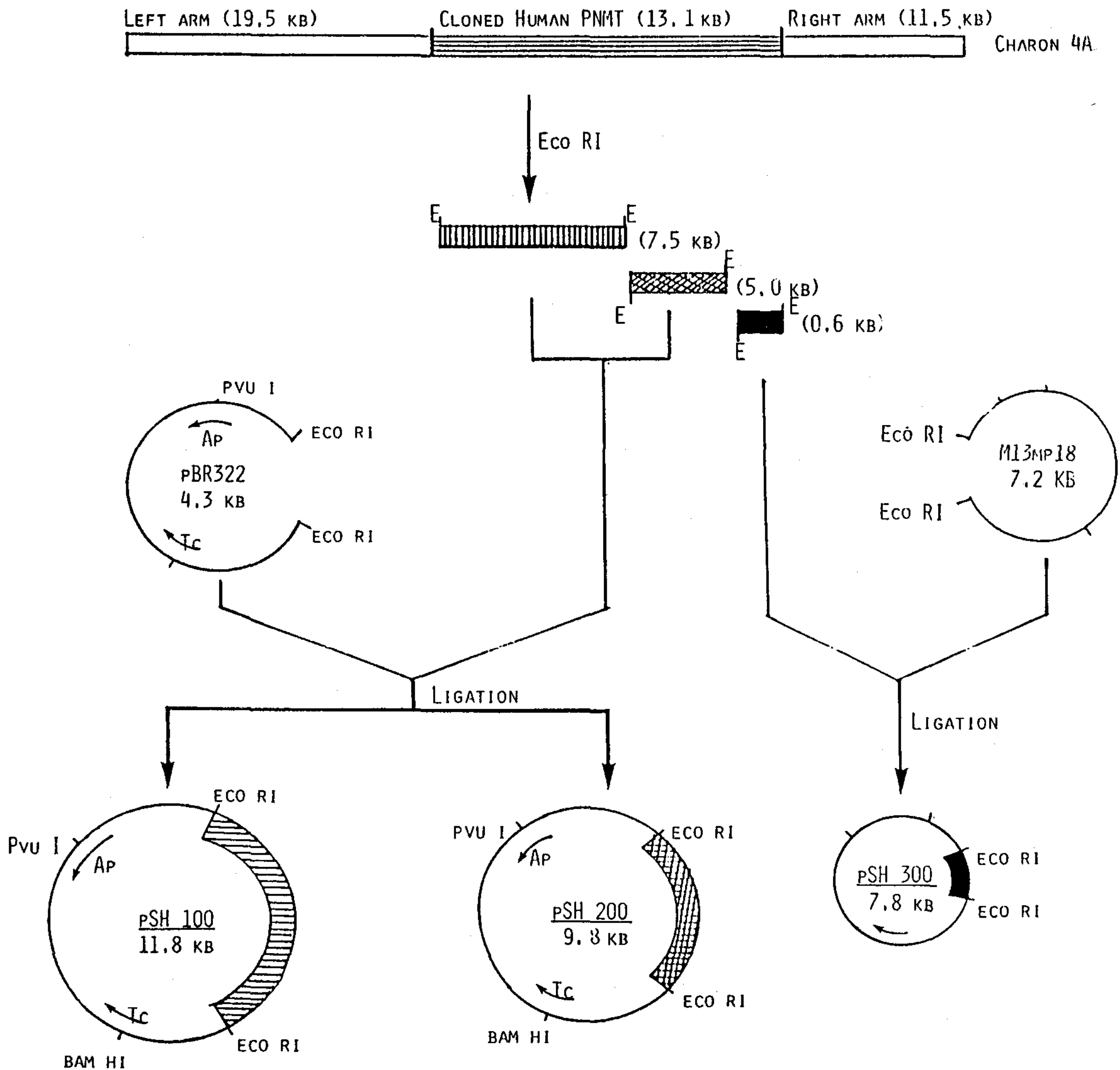


Fig. 5. Subcloning strategy of cloned insert suhfragments.

on nitrocellulose paper by using a microfold dot slot (BRL) and the filters were prehybridized and hybridized with nick-translated bovine probe.

Restriction mapping

Plague purified clones were digested with several restiction 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 a human PNMT gene

Radiolabeled bovine PNMT cDNA was em·

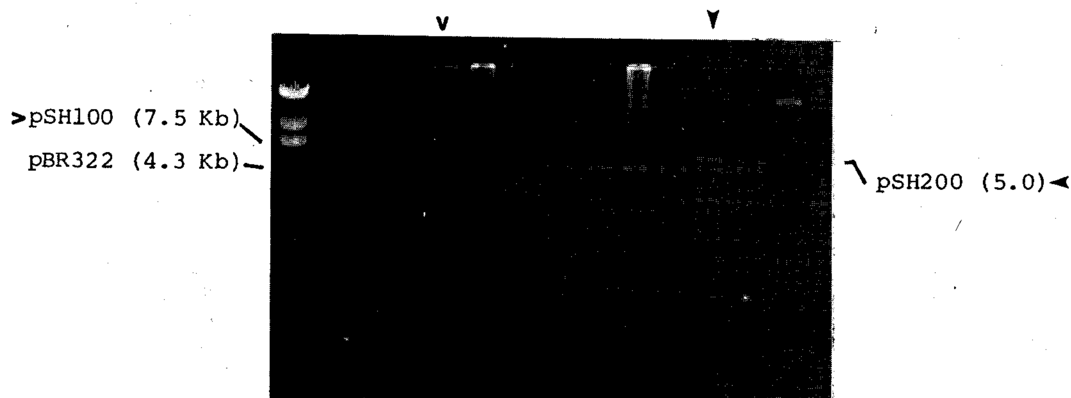
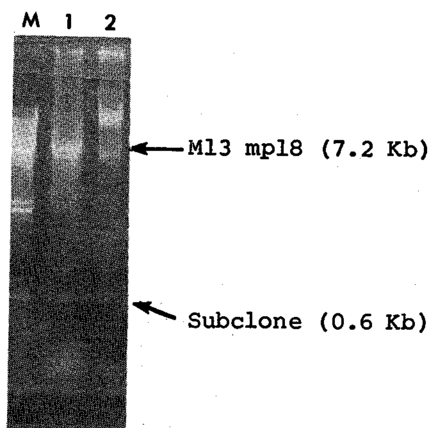


Fig. 6. Subcloning of pSH 100 (7.5 Kb insert) and pSH 200 (5.0 Kb insert).
Lt. most lane : Lambda/Hind III marker.



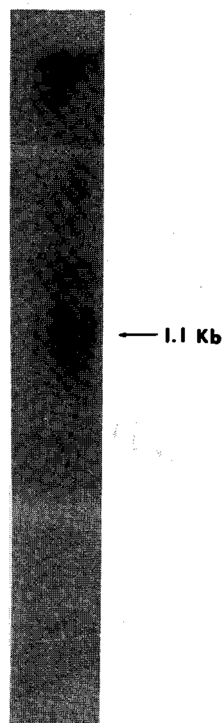
Lanes:

M. Lamda/ Hind III Marker

1. pSH300/Eco RI

2. pSH300 Intact

Fig. 7. Subcloning of pSH 300 (0.6 Kb insert).

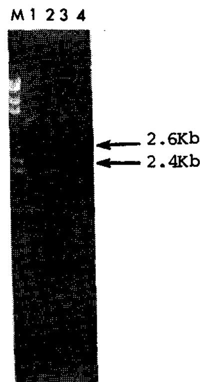


NOTHERN BLOT

Fig. 8. Northern blot dnalysis of mRNA encoding human PNMT.
Total RNA (10 μg) prepared from human adrenal tissiue was fractionated by agarose gel electrophoresis containing formaldehyde, transferred to nitrocellulose filter paper and hybridized with ^{32}P-labeled cDNA probe.

ployed to screen a human genomic library for clones containing PNMT gene-coding sequences. Initially, the screening of 1.5×10^6 pfu yielded one, repeatedly positive and well isolated clone (Fig.2). This phage contained 13.1 Kb of human genomic

RESTRICTION ENZYME ANALYSIS OF pSH200



Lanes:

M. Lamda DNA Size Marker
1. 5.0 Kb Cloned Insert
2. 5.0 Kb/ Hind III
3. 5.0 Kb/ Sac I
4. 5.0 Kb/ Sal I

Fig. 9. Restriction enzyme analysis of pSH 200 (5.0 Kb insert).

DNA in which two EcoRI sites existed, generating 7.5 Kb, 5.0 Kb and 0.6 Kb subfragments (Fig. 3).

Identification of the human PNMT gene.

In Southern and dot blot experiments, a 5.0 Kb and a 0.6 Kb EcoRI fragments strongly hybridized to the bovine cDNA probe but 7.5 Kb showed background signal, indicating that PNMT gene-coding seguences were localized to within 5.0 Kb and 0.6 Kb fragments flanked by a 7.5 Kb fragment (Fig. 4).

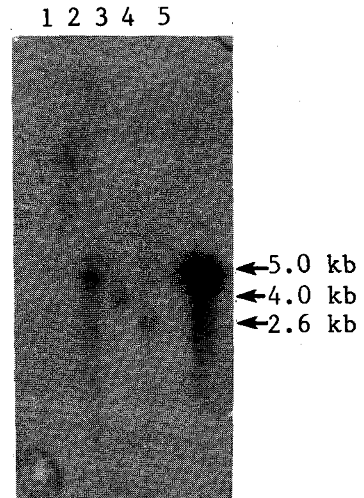
The 7.5 Kb and 5.0 Kb EcoRI fragments were subcloned into PBR 322 (called pSH 100, pSH 200, respectively) to facilitate further analysis and 0.6 Kb fragment was also subcloned into M13mp18 for subsequent dideoxy sequencing (called pSH 300) (Fig. 5, 6, 7).

The Northern blot analysis showed that bovine cDNA probe hybridized to an RNA species of approximately 1.1 Kb, a size appropriate for an mRNA coding for a 31,000 dalton protein (Fig. 8).

Tentative restriction mapping

A 5.0 Kb genomic DNA fregment was isolated and characterized by restriction mapping (Fig. 9,10).

SOUTHERN BLOT OF HUMAN PNMT



Lanes:

1. Lamda/Hind III
2. 5.0 kb (Intact)
3. 5.0/Pst I
4. 5.0/Pst I + Sal I
5. pSH200/ Eco RI

Fig. 10. Southern blot analysis of pSH 200 (5.0 Kb insert) digested by restriction enzymes.

A physical map of pSH 200 containing 5.0 Kb insert is shown in Figure. 11.

DISCUSSION

Phenylethanolamine N-methyltransferase (PNMT) is essential for the conversion of norepinephrine to epinephrine and its expression difines 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 isolated human geno-

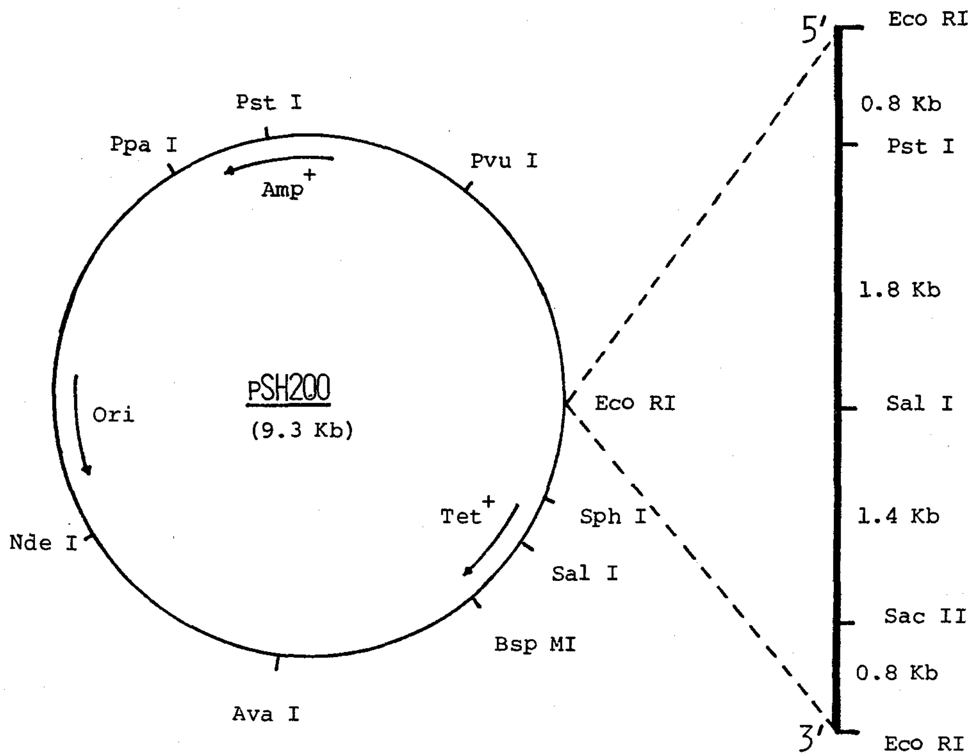


Fig. 11. Tentative restriction map of pSH 200 containing 5.0 Kb insert.

mic clone was 13.1 Kb and it contained two EcoRI sites, generating 7.5 Kb, 5.0 Kb and 0.6 Kb fragments.

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

Our identification was based on the following criteria; first, this clone contained internal fragments (5.0 Kb and 0.6 Kb) hybridizing to a 1050 bp cloned bovine PNMT cDNA; Second, one of the fragments (5.0 Kb) hybridized with a probe containing 100 bp of the 5' portion of the cDNA; third, this 5.0 Kb fragment contained SmaI site which was a unique near 5' end of the bovine cDNA; fourth, a 0.6 Kb fragment hybridized to a 400 bp of the 3' end of the cDNA (data not thown)

Isolation and characterization of the human 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 PNMT. Future DNA sequencing of this clone will clarify the complete structure of human PNMT gene and the evolutionary relationships among PNMT genes of several species.

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＝국문초록＝

Epinephrine 합성효소인 phenylethanolamine N-methyltransferase의 인간 genomic DNA의 유전자 크로닝

서유헌·허성오·전양숙·김헌식·임정규·박찬웅

서울대학교 의과대학 약리학교실 및 신경과학연구소 분자신경생물학 연구부

카테콜아민 생합성에 관여하는 마지막 효소인 phenylethanolamine N-methyltransferase는 Norepinephrine을 epinephrine으로 전환시키는 중요한 효소이다. PNMT 효소의 발현은 epinephrine 신경세포의 발현에 필수적이다. 따라서 PNMT 유전자를 크로닝하여 그 구조를 결정하고, 유전자 발현연구를 하는 것은 상당히 중요한 일이다. 그러나 최근에 저자가 bovine cDNA를 처음으로 분리하여 그 구조를 보고한 것 외에는 아직까지 인간 PNMT cDNA나, 전체 genomic DNA의 분리 보고는 없다. 이에 저자들은 인간 PNMT 유전자의 전체구조와 여러 종(species) 사이의 진화적인 관계를 규명하기 위해서 human genomic library(Charon 4A)를 만들고, 이 library를 이용하여 bovine cDNA를 probe로 13.1 Kb 길이의 genomic clone을 분리 크로닝하는데 성공하였다. 이 유전자는 두개의 EcoRI site가 포함되어 있어서, EcoRI 제한효소에 의해서 7.5 Kb, 5.0 Kb, 0.6 Kb로 분리되었으며, Southern과 dot blot 실험에서 보면 5.0 Kb와 0.6 Kb에 exon이 흩어져 존재하고 있으며, 7.5 Kb는 flanking sequence로 판명되었다.