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# Molecular Cloning of cDNA for Human Epinephrine Synthesizing Enzyme, Phenylethanolamine N-Methyltransferase(PNMT)

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= Abstract =An oligo(dT)-primed cDNA was constructed from poly(A)$^+$-containing RNA from human adrenal tissues. The cDNA was inserted into the EcoRI site of $\lambda$ gt10 phage and the human library was screened for clones containing cDNAs coding for human phenylethanolamine N-methyl transferase(PNMT).

Approximately $1.5 \times 10^6$ recombinant phage plaques were transferred to nitrocellulose filters and a cDNA clone was isolated and identified from the $\lambda$ gt10 DNA by digestion with EcoRI and was found to be approximately 1150 base pairs in length containing one internal EcoRI site. Northern blot analysis showed that the cDNA strongly hybridized to an RNA species of approximately 1.1 kb, a size appropriate for an mRNA coding for PNMT. The human adrenal mRNA selected by the cDNA codes for a 31k dalton protein.

This is the first report of the cloning of cDNA for human epinephrine synthesizing enzyme, PNMT.

Key words: ***$\lambda$ gt10, Human library, Molecular cloning, PNMT***

## INTRODUCTION

The catecholamine biosynthetic pathway consists of three neural-specific enzymes: tyrosine hydroxylase(TH), which catalyzes the conversion of tyrosine to L-dopa, the first and rate-limiting step in the pathway; dopamine $\beta$-hydroxylase(DBH), which catalyzes norepinephrine synthesis from dopamine; and phenylethanolamine N-methyltransferase(PNMT), which mediates the conversion of norepinephrine to epinephrine. On the basis of immunocrossreactivity and amino acid composition data, 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). In addition to these evolutionary considerations, the catecholamine biosynthetic pathway serves as an excellent model system in which to study the molecular genetic mechanisms controlling the expression of a specific neurotransmitter phenotype. 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.* 1978; 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

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neurons are believed to participate in the regulation of reproduction, temperature, cardiovascular function, and food and water intake.

The complete sequence of a full-length cDNA encoding bovine PNMT has recently been reported (Suh *et al.* 1986). But there is no available information about the primary structure and the gene encoding human PNMT.

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 human cDNA clone encoding human PNMT.

## MATERIALS AND METHODS

All enzymes and chemicals (molecular biology grade) were purchased from New England Biolabs Inc. and Boehringer Mannheim except where otherwise noted.

### Isolation of RNA

Human adrenal total cellular RNA was prepared essentially as described by Chirgwin *et al.* (1979).

Four grams(wet weight) of tissue were homogenized in 40 ml of 4 M guanidine thiocyanate, 0.025 M sodium citrate, pH 7.0, 0.5% Sarkosyl, 0.1 M 2 mercaptoethanol. After adding 0.4 g CsCl/ml homogenate, the homogenate was layered on a cushion of 6.2 M CsCl, 0.1 M EDTA, pH 7.0 and RNA was pelleted as described. RNA pellets were dissolved in 0.01 M Tris-HCl, pH 7.5, 0.001 M EDTA, extracted twice with phenol-chloroform, and precipitated with ethanol.

The poly(A)$^+$ RNA was selected by oligo(dT)-cellulose (Collaborative Research, type 3) chromatography.

### cDNA synthesis

Synthesis of first and second strands was carried out as described by Okayama and Berg (1982) and modified by Gubler and Hoffman (1983). Poly(A)$^+$RNA, 6.0 $\mu$g, was used as a template. cDNA was labelled by 10 Ci( $\alpha$-$^{32}$P) dTTP(3000 Ci/mmol; New England Nuclear). For second-strand synthesis 800 ng of the first streand was treated with E. coli polymerase I., RNase H, and $T_4$ DNA ligase. The average size of the cDNA was estimated to be 1000 base pairs.

### Addition of EcoRI linkers and ligation to λ gt 10 Arms

cDNA was methylated at internal EcoRI sites with EcoRI methylase (Maniatis *et al.*, 1975). An equal mass of EcoRI linker was added and ligated to the cDNA in 50 mM Tris HCl, pH 7.8, 5 mM $MgCl_2$, 1 mM ATP and 20 mM DTT, 0.25 u $T_4$ DNA ligase/ng cDNA at 14°C for 20 hr ligation. Linker oligomers were removed from the cDNA by digestion with 0.3 u EcoRI/ng linker.

$\lambda$ gt10 DNA was digeted with EcoRI and treated with 0025 U calf intestine alkaline phosphatase (Boehringer Mannheim)/$\mu$g DNA for 1 hr to decrese ligation of arms to each other and therefore reduce background nonrecombinant plaques. cDNA was ligated to $\lambda$ gt10 arms at a molar ratio of 1.5 and a DNA concentration of 1.7 $\mu$g/$\mu$l which was calculated as described by Dugaiczyk *et al.* (1975) and determined empirically to yield the maximum number of recombinants (Fig. 1).

Packaging extracts were prepared and recombinant DNA was packaged as described by Grosveld *et al.* (1981). Recombinant phage were adsorbed to bacteria (E. coli strain C600 hfl): infected cells were diluted with top agarose and poured onto LB-ampicillin plates. 89-90% of plaques were clear and therefore contained cDNA inserts. Recombinants were obtained at an efficiency of 3000/ng cDNA.

### Screening for human PNMT cDNA clones

The human adrenal cDNA library of $1.5 \times 10^6$ phage plaques were 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 (Suh *et al.* 1986) 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 phage DNA

Candidate positive signals obtained in the first high density screen were taken through three successive rounds of antibody screening at progressively lower plaque densities. The resulting, repeatedly positive, and well-isolated phage plaques were picked, amplified to yield high titer plate stock, 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

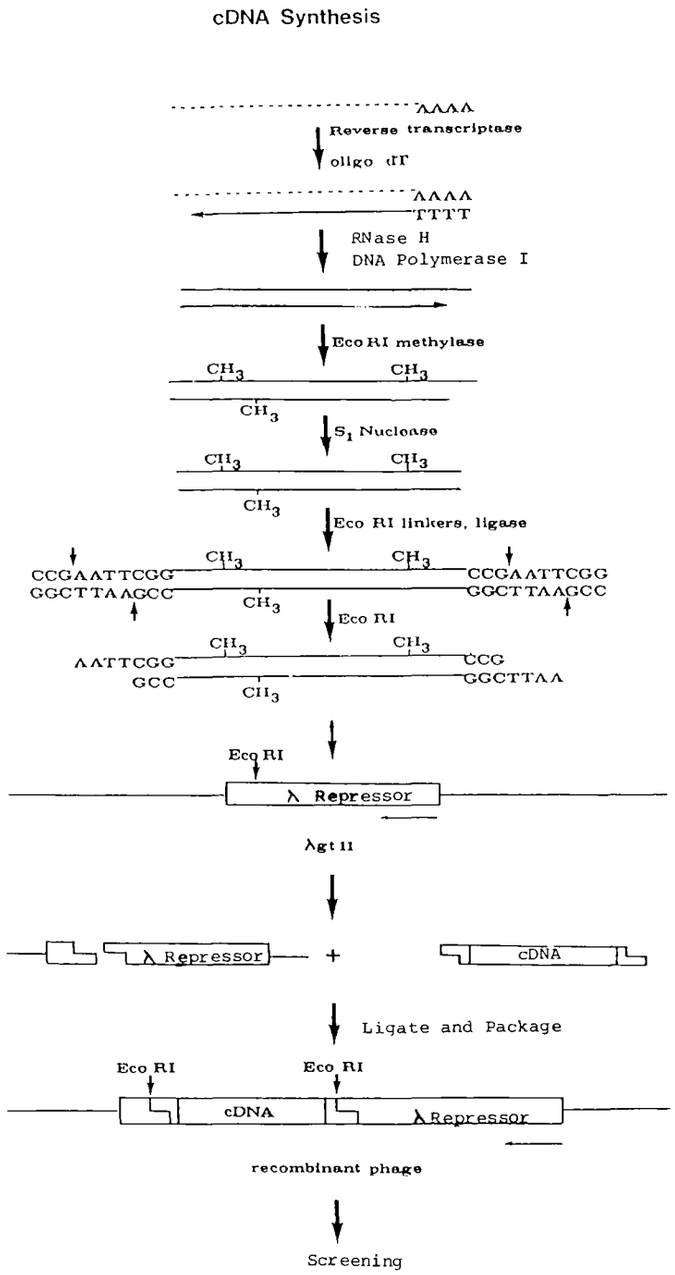


Fig. 1. Scheme for double-stranded cDNA synthesis and cloning in λ gt10 vector.
Details of these protocols are given in Materials and Methods.

fragments free of flanking phage sequences. Fragments were fractionated by electrophoresis and eluted.

### Southern blot analysis

Restriction endonuclease digested DNA was subjected to electrophoresis in the appropriate percentage agarose gels (0.7~1.0%), and transferred to Gene Screen Plus or Gene Screen (New England Nuclear, Boston, MA) as described by the manufacturer. Blots containing cDNA were prehybridized at 42°C in 50% deionized formamide containing 25 mM Tris HCl(pH 7.5), 1M 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 × $10^8$ cmp/ml [$^{32}$P] labeled probe was added, and the blots were hybridized for 1 days at 42°C. The filters were washed twice in 2 × SSC, 2 mM EDTA, 1% SDS at 55°C for 30 min, and twice in 0.1 × SSC, 2 mM EDTA at 60°C for 15 min. All filters were exposed to Kodak XAR-5 film for autoradiography (with intensifying screens for the cDNA blots).

### Dot blot analysis

Either purified DNA or phage DNA was treated with 0.3 ml of 57 mM Tris(pH 7.0), 0.2 N NaOH, 6 × SSC at 80°C for 10 min. The samples were placed on ice and neutralized with 2 M HEPES(pH 7.5). Denatured cDNA or phage DNA was spotted on nitrocellulose paper by using a microfold dot slot(BRL) and the filters were prehybridized and hybridized with nick translated bovine probe.

### RNA analysis (Northern blot)

RNA was isolated from human adrenal medulla 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 human cDNA probe.

### Hybridization-selected mRNA translation and Immunoprecipitation

The mRNAs corresponding to the cloned cDNA was analyzed to the hybridization selection procedure described previously (Ricciardi *et al.* 1979; Parnes *et al.* 1981). cDNA(2 μg-5 μg) was bound to 13 mm nitrocellulose filter disks. The filters were incubated with 10 μg of poly(A $^+$) mRNA and rinsed extensively prior to elution of the hybridized mRNA. The hybridization selected mRNA was translated in vitro using a RNA-dependent rabbit reticulocyte translation kit(New England Nuclear) and [$^{35}$S] methionine as the labeled aminoacid. After translation, the samples were incubated with RNase to remove charged tRNAs and the translation products were immunoprecipitated with rabbit anti PNMT antibody and immunoprecipitates were isolated with protein A Sepharose CL-4B(pharmacia).

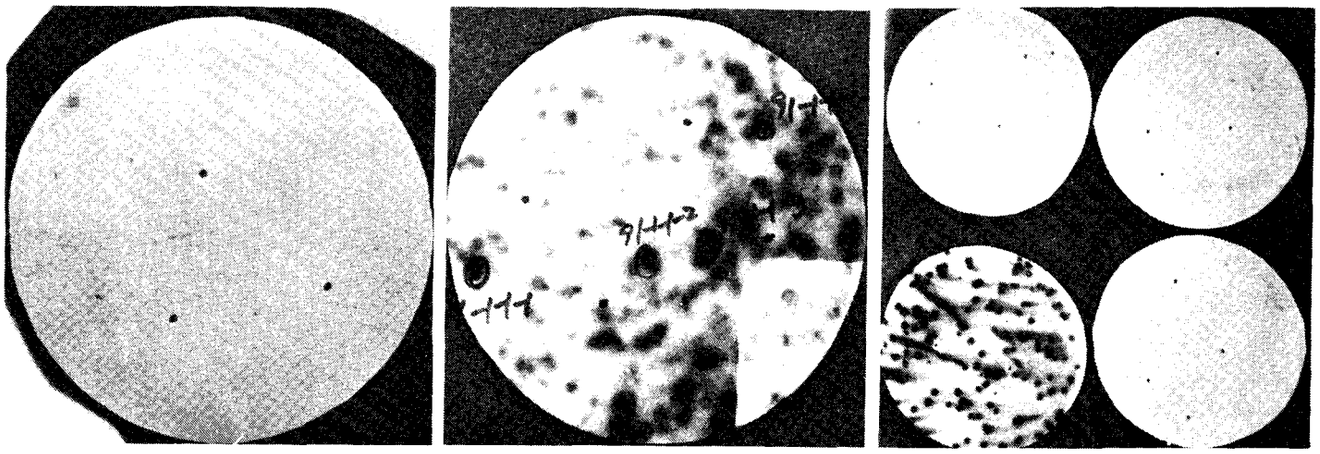


Fig. 2. Three successive screening for cDNA clones containing PNMT sequences. cDNAs in λgt10 clones in situ were fixed onto nitrocellulose filter papers. Filters were incubated with $1 \times 10^5$ cpm of nick-translated bovine cDNA. Positive clones were detected by autoradiography.

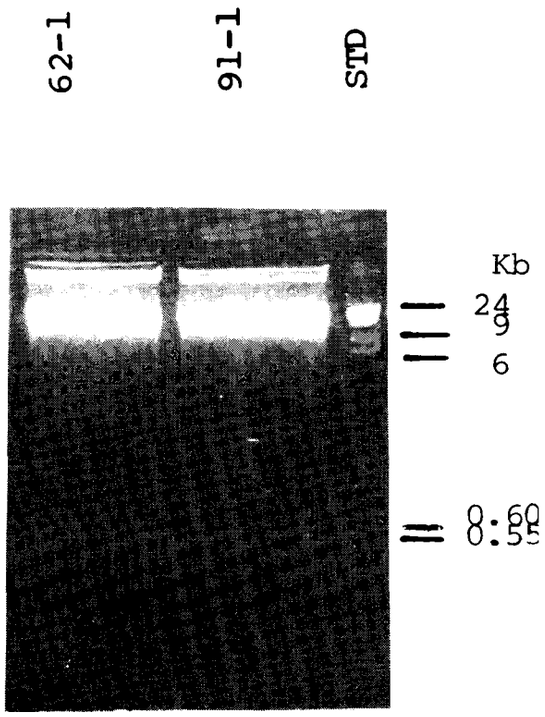


Fig. 3. Measurement of lengths of the cDNA inserts from positive clones. Phage DNA(5 μg) was digested with EcoRI and electrophoresed on a 1% agarose gel. Lanes: 1, Clone 62-1; 2, Clone 91-1; STD, Hind III diget of DNA(0.8 μg).

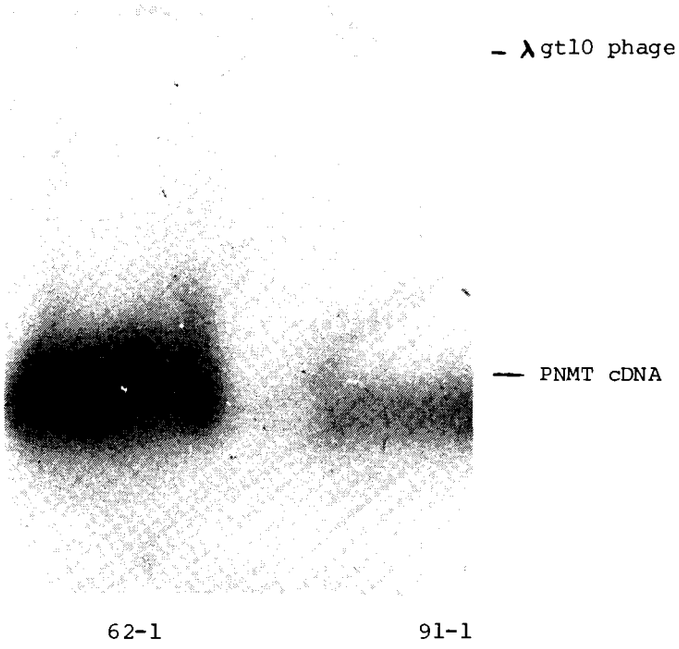


Fig. 4. Southern blot analysis of the cDNA inserts in agarose gel. Phage DNA(7 μg) was digested with EcoRI and electrophoresed on a 0.8% agarose gel and transferred to nitrocellulose filter paper and hybridized with $^{32}$P-labeled probe.

Washed immunoprecipitates were boiled in NaDodSO4 sample buffer and analyzed by SDS polyacrylamide slab gel electrophoresis as described by Laemmli (Laemmli 1970). Gels were fixed and impregnated with New England Nuclear enhance, dried, and exposed to Kodak XAR-X ray film.

## RESULTS

### Isolation of human PNMT cDNA clones

As shown in Fig. 2., candidate positive signals

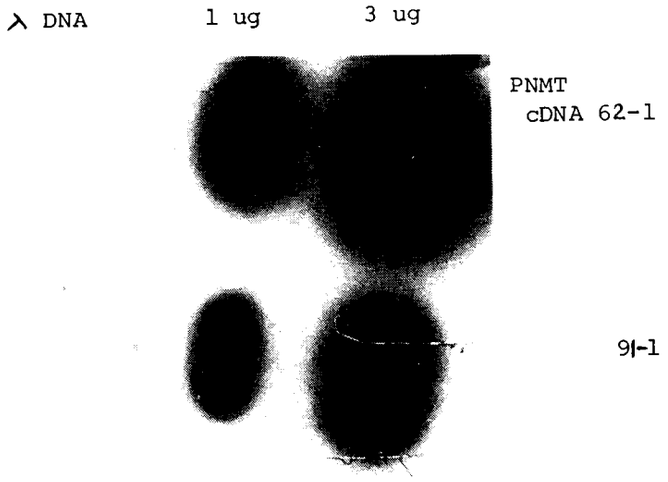


Fig. 5. Dot blot analysis of the cDNA inserts. cDNA inserts purified from clones 62-1 and 91-1 were denatured and spotted on nitrocellulose paper by using a microfold dot slot(BRL). The filters were hybridized with nick-translated bovine probe.

obtained in the first high density screen were taken through two successive rounds of screening at progressively lower plaque densities. The resulting, two repeatedly positive, and well isolated phage phaques, named 62-1 and 91-1 were isolated. Isolated cDNAs were subjected to EcoRI digestion to produce insert fragments, which were found to be 1.15 kb in length containing one internal EcoRI site (Fig. 3).

**Southern, Northern and dot blot analysis**

The southern transfer experiment of the inserted cDNAs, in the gel, shown showed that the cDNAs strongly hybridized with bovine cDNA probe (Fig. 4). Dot blot analysis of the cDNAs purified from clones 62-1 and 91-1 showed that these cDNAs hybridized to a bovine cDNA probe in a concentration dependent fashion (Fig. 5). The Northern blot analysis showed that isolated cDNA probes strongly hybridized to an RNA species of approximately 1.1 kb, a size appropriate for an mRNA coding for a 31,000 dalton protein (Fig. 6).

**Hybridization-Selection of PNMT mRNA with cloned cDNA**

To determine whether the positive cDNA

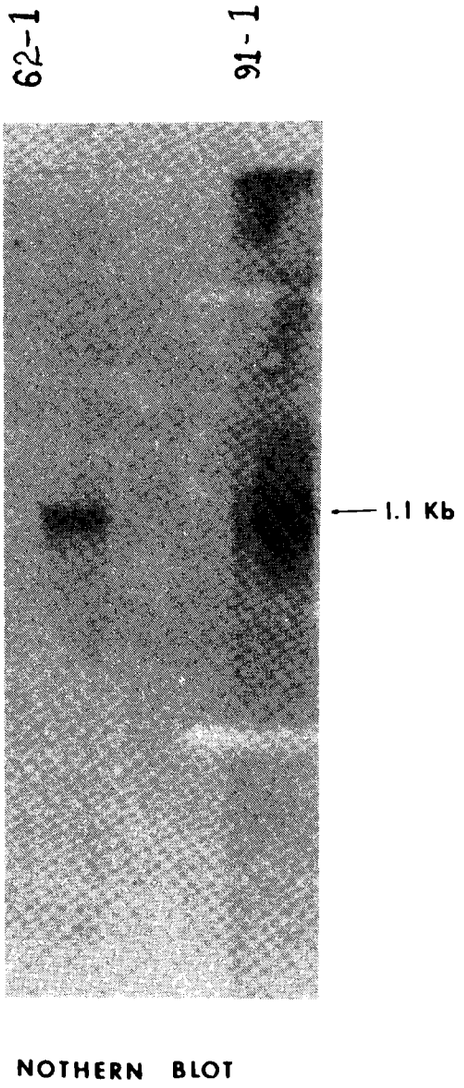


Fig. 6. Northern blot of mRNA encoding PNMT. Total RNA(10 $\mu$g) prepared from human adrenal medulla was fractionated by agarose gel elctrophoresis containing formaldehyde, transferred to nitrocellulose filter paper and hybridized with $^{32}$P-labeled cDNA probes.

clone contained human PNMT sequences, a hybridization-selection procedure was used. The positive cDNA(clone 62-1) was isolated and used to hybrid-select mRNAS with complementary sequences. Thee mRNAS were subsequently translated in vitro, immunoprecipitated, fractionated by gel electrophoresis and fluorography. As shown in Figure 7, the mRNA selected by the cDNA clone codes for an 31 k dalton protein(same M.W. for PNMT).

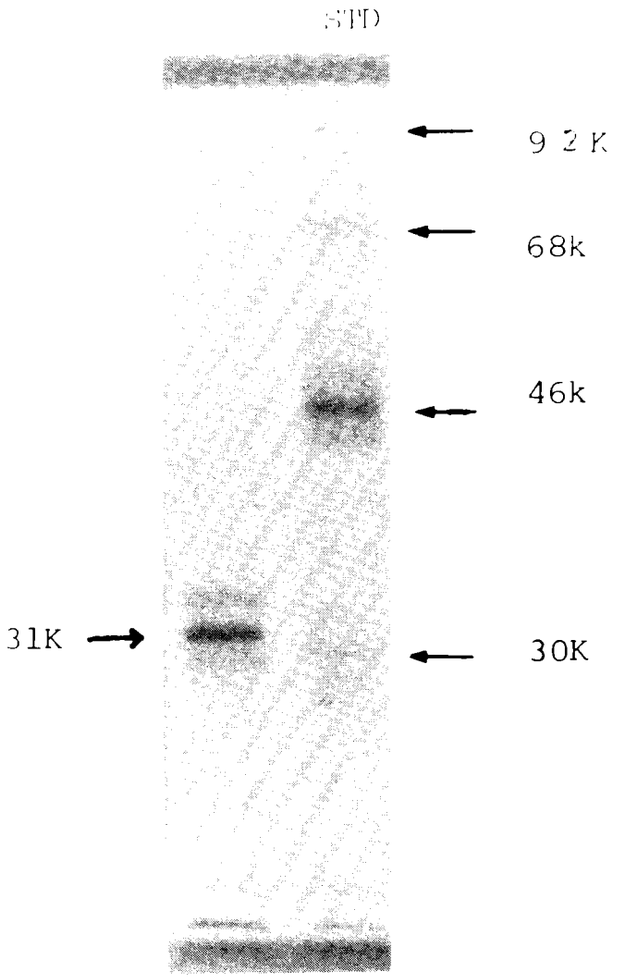


Fig. 7. Immunoblot analysis of In vitro translated products by hybridization-selected mRNA.
The mRNA selected by the cDNA clone was translated in vitro using rabbit reticulocyte translation system and the products were immunoprecipitated with anti bovine PNMT antibody and immunoprecipitates were isolated with protein A-Sepharose CL-4B and analyzed by SDS PAGE. The gels were fixed and impregnated with NEN Enhancer, dried and exposed to Kodak XAR-film.

## DISCUSSION

To initiate an investigation into the molecular mechanisms controlling the catecholamine neurotransmitter phenotype and to obtain information about the structure of human PNMT and to further define the extent of the evolutionary relationships among PNMT molecules of several species, we constructed a human adrenal cDNA library and screened $1.5 \times 10^6$ recombinant phage plaques with a bovine probe. In this study, we here report the isolation of a nearly full length cDNA clone encoding human PNMT.

Our identification of a cDNA clone encoding human PNMT was based on the following criteria; first, isolated cDNA clones strongly hybridized with a bovine cDNA probe inspite of high stringency washing conditions (0.1 × SSC, 60°C). Second, the isolated cDNAs hybridized to an RNA species of about 1.1 kb, a size appropriate for an mRNA coding PNMT. Third, the mRNA selected by this cDNA codes for an approximately 31 k dalton protein (same M.W. for human PNMT).

In conclusion, the results of this investigation directly confirm the isolation and identification of cDNA clones for human PNMT. DNA sequencing and restriction mapping for complete analysis of human PNMT cDNA structure are currently under way.

Isolation and characterization of the human PNMT cDNA will greatly facilitate studies of the molecular mechanisms underlying the expression and regulation of gene for PNMT.

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= 국문초록 =

# 인간 Epinephrine합성효소 Phenylethanolamine N-methyltransferase cDNA의 유전자 크로닝

서울대학교 의과대학 약리학교실 및 신경과학연구소 분자신경생물학연구부

서유현 · 전양숙 · 김성수 · 박찬웅

Phenylethanolamine N-methyltransferase(PNMT)는 카테콜아민 신경전달물질 생합성에 관계하는 마지막 효소로써, norepinephrine을 epinephrine으로 전환시킨다. 이 PNMT효소는 epinephrine 신경세포의 표지효소로써, 이 신경세포의 발현에 필수적인 효소이다.

또한 dopamine신경세포, norepinephrine 신경세포의 발현에 필수적인 tyrosine hydroxylase(TH), dopamine- β hydroxylase(DBH)와는 생합성단계에서 연속적으로 작용하지만, 어떻게 발현이 조절되므로써 각각 특이한 신경세포가 형성되는지는 전혀 알려진 바가 없다. 저자들은 최근에 bovine에서 epinephrine합성효소 cDNA를 처음으로 분리 보고하였으나 아직까지도 human cDNA의 크로닝 보고는 없다. 따라서 저자들은 human 유전자의 구조를 알기 위해서 인간 부신에서 mRNA를 분리하여 λgt10 library를 만들었으며, 여기에서 bovine cDNA probe를 이용하여 인간 cDNA clone을 분리하였다. 분리한 clone은 1.15kb 크기였으며 Southern, Northern, dot blot 등의 실험에서 인간 PNMT임이 증명되어서 이에 보고하는 바이다.