Isolation of a rat pineal gland cDNA clone homologous to tyrosine and phenylalanine hydroxylases

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A rat pineal gland cDNA expression library has been probed with an antiserum raised against rat tryptophan hydroxylase. A clone has been isolated and its sequence reveals a high degree of homology with those of tyrosine and phenylalanine hydroxylases.

Tryptophan hydroxylase *(Pineal gland)* *Tyrosine hydroxylase* *Phenylalanine hydroxylase*

1. INTRODUCTION

The three aromatic amino acid hydroxylases, phenylalanine hydroxylase (PAH), tyrosine hydroxylase (TH) and tryptophan hydroxylase (TPH), share many common features; they are iron enzymes and mixed-function oxidases requiring molecular oxygen, they use the electron donor tetrahydropterin as a cofactor [1], their mechanisms of reaction are similar [2], each enzyme can use another aromatic amino acid as substrate and *p*-chlorophenylalanine is an equally good inhibitor of all three hydroxylases [3]. TH and PAH have recently been cloned and sequenced [4–7]. As expected, comparison of the amino acid sequences of TH and PAH reveals a strong homology between these proteins which, in the central portion, reaches over 75% identity with no gap [8,9]. TPH is the rate-limiting enzyme in the biosynthesis of serotonin in the central nervous system and catalyzes the first step of the synthesis of melatonin in the pineal gland [10]. Here, a rat pineal gland cDNA expression library was generated and screened with an antiserum raised against TPH [11]. A clone has been isolated whose sequence reveals extensive homology with both TH and PAH.

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2. MATERIALS AND METHODS

2.1. *Preparation of the antiserum*

TPH was prepared from whole rat brain as described by Cash et al. [11]. An antiserum was raised in sheep against this purified TPH, and subjected to immunoadsorption to beef liver catalase as in [11].

2.2. *Construction of a rat pineal cDNA library and immunoscreening*

Pineal glands were dissected from 600 adult male rats (Wistar), during daytime. Polyadenylated mRNA was used to synthesize double-strand cDNA [12] which was inserted in the *Eco*RI site of the expression vector λgt11 (Amersham) [13]. Approx. 250000 recombinant phages were obtained from 2.5 μg mRNA. The recombinant phages were screened essentially as described by

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Huynh et al. [14], except that the second antibody was conjugated to horseradish peroxidase. Chimaeric proteins of induced recombinant lysogens [15] were analysed by immunoblotting [16].

2.3. *Nucleotide sequence and Northern blot analysis*

The DNA sequence was determined by the chain-termination method of Sanger et al. [17], using the M13 mp8 and mp18 vectors (Pharmacia). Polyadenylated RNA from rat pineal gland, brain stem, cerebellum, liver, and the muscular layers of the duodenum was analysed by Northern blotting according to Faucon Biguet et al. [18].

3. RESULTS

The antiserum was first tested by Western blotting on pineal tissue, where it recognized a protein of 55 kDa. Approx. 40000 recombinant phages were screened with the TPH antiserum. Eleven plaques were labelled, with two remaining positive after two additional rounds of screening. One clone (P7) that yielded a chimaeric protein strongly recognized by TPH antiserum (fig.1) was chosen for further analysis. The cDNA insert size of this clone was 1.2 kb; its *Eco*RI-*Bam*HI fragment of clone P7 was sequenced on both strands and translated into protein (fig.2). This amino acid sequence was compared with those of rat and human PAH and TH [5–8] (fig.3). These sequences can be aligned with no gap. Homologies of 71.2 and 69.1% were found with rat and human PAH respectively, within amino acids 180–274. Similar homologies of 68.1 and 66% were found with rat and human TH within amino acids 226–320. Most of the amino acid differences correspond to changes that are conservative with respect to the charge. Although at position 16 an asparagine is found instead of a lysine, this charge deficiency is compensated by a lysine at position 17. Of the 94 amino acids that we have analyzed 51 occur identically at the same place in all five sequences.

Northern blot analysis of mRNA from pineal gland shows a major and a minor band corresponding to a 2.1 and a 4.5 kb mRNA species, respectively. In the muscular layers of the duodenum, the 4.5 kb band is only detected after longer exposure of the blot with the 2.1 kb band

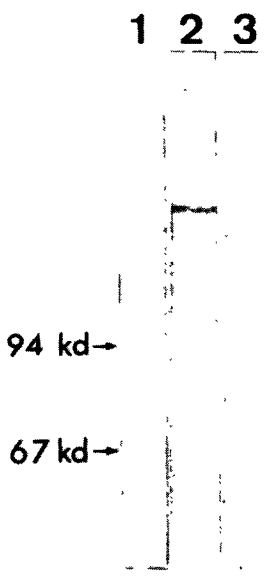

Fig.1. Immunoblot of IPTG (isopropyl-β-D-thiogalactopyranoside) induced lysogen extracts from λgt11 (1), clone P7 (2), and a clone derived from a pheochromocytoma library synthesizing a chimaeric protein which was used as control (3). The sheep antiserum against TPH was preadsorbed on λgt11 to reduce the background and was used at a dilution of 1:250 [13].

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1                  16                  31                  46
ACG GAA GAG GAG ATT AAG ACC TGG GGG ACC ATC TTC CGA GAG CTG AAC
THR GLU GLU GLU ILE LYS THR TRP GLY THR ILE PHE ARG GLU LEU ASN

49                 64                  79                  94
AAA CTC TAC CCA ACC CAC GCC TGC AGG GAG TAC CTC AGA AAC CTT CCT
LYS LEU TYR PRO THR HIS ALA CYS ARG GLU TYR LEU ARG ASN LEU PRO

97                 112                 127                 142
CTG CTC TCA AAA TAC TGT GGC TAT CGG GAA GAC AAC GTC CCG CAA CTG
LEU LEU SER LYS TYR CYS GLY TYR ARG GLU ASP ASN VAL PRO GLN LEU

145                160                 175                 190
GAA GAT GTC TCC AAC TTT TTA AAA GAA CGC ACA GGG TTT TCC ATC CGT
GLU ASP VAL SER ASN PHE LEU LYS GLU ARG THR GLY PHE SER ILE ARG

193                208                 223                 238
CCT GTG GCT GGT TAC CTC TCA CCG AGA GAT TTC CTG TCA GGG TTA GCC
PRO VAL ALA GLY TYR LEU SER PRO ARG ASP PHE LEU SER GLY LEU ALA

241                256                 271                 285
TTT CGA GTC TTT CAC TGC ACT CAG TAT GTG AGA CAC AGT TCG GAT
PHE ARG VAL PHE HIS CYS THR GLN TYR VAL ARG HIS SER SER ASP
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Fig.2. Nucleotide sequence of the *Eco*RI-*Bam*HI fragment of the clone P7 and its corresponding polypeptide using the three-letter code.

Fig.3. Alignment of the protein sequences of rat phenylalanine hydroxylase (RAT PAH), human phenylalanine hydroxylase (HU PAH), clone P7, rat tyrosine hydroxylase (RAT TH) and human tyrosine hydroxylase (HU TH). The amino acid numbers of the portion of human and rat PAH are above and for the corresponding portion of rat and human TH below the sequences (amino acids are numbered relative to the methionine corresponding to the initiation codon). The amino acids homologous to those derived from clone P7 are enclosed in black boxes.

barely detectable. No hybridization signal is detected with liver, brain stem and cerebellum mRNAs.

4. DISCUSSION

The present study allowed the isolation of a cDNA clone (P7) which encodes a polypeptide recognized by a specific TPH antiserum and which shares strong homology with both TH and PAH hydroxylases.

Rat pineal gland was chosen to generate an expression library because this tissue is homogeneous and also contains high TPH activity. In the brain this enzyme plays a crucial role in the physiology of serotoninergic neurones which are restricted to clusters of cells lying in the brain stem. It is also present in mast cells and in myenteric plexus. A number of studies conducted mostly with pineal and brain stem TPH have revealed that this enzyme has different substrate specificities in various tissues, suggesting that TPH activity may correspond to different molecular entities in these tissues [19].

The antiserum has been raised against TPH purified from whole rat brain including pineal gland and its specificity was established by two criteria. Firstly, it neutralizes the complete enzymatic activity from a brain extract at 1:500 dilution and secondly, it specifically stains putative serotoninergic neurones from raphe nuclei tissue sections (Belin, M.F., personal communication).

The immunoblotting experiments shown in fig.1 provided strong evidence that P7 encodes a TPH antigen. These data are further supported by amino acid sequence comparisons. The homology between clone P7 and either TH or PAH lies between 66 and 71%. Furthermore, most of the mismatches correspond to conservative changes. The proposal that P7 corresponds to the TPH enzyme is quite consistent with the broad similarity in structure and function of the three aromatic amino acid hydroxylases. It is probable that these three enzymes have evolved from a common ancestor gene.

In others series of experiments, when 1000 pineal cDNA clones were screened by differential hybridization, using cDNA probes derived from pineal gland, liver and cerebellum, 30 clones, including P7, were shown to be expressed specifically in the pineal gland but not in liver or cerebellum (not shown). The Northern blot experiment shown in fig.4 corroborates this finding. In this first mRNA analysis, P7 hybridizes in the pineal tissue with two bands of different intensities. Two similar bands with quite different relative intensities are also obtained using the muscular layers of the duodenum. Therefore, it is attractive to hypothesize that these two bands reveal an alternative splicing of a precursor mRNA which results

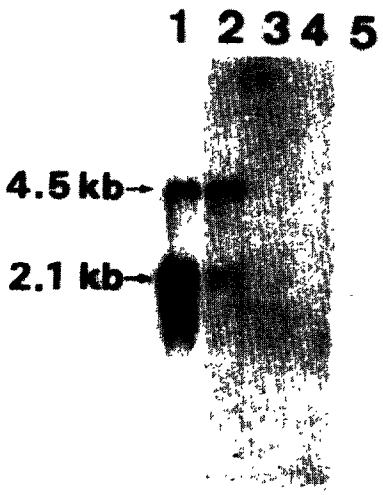

Fig.4. Northern blot analysis of 1 μg mRNA from pineal gland (1), muscular layers of the duodenum (2), liver (3), cerebellum (4) and brain stem (5) using the clone P7 as probe. The exposure time of the blot in 2–4, is 10-fold longer than in 1.

in two different TPH enzymes. In this experiment no hybridization was observed with tissue from brain stem. This lack of signal could be attributed either to the low level of TPH mRNA in this tissue or to a difference in the structure of brain TPH. In support of this latter hypothesis TPH antiserum recognized bands of slightly different molecular masses when comparing brain stem and pineal gland tissues by Western blotting (not shown).

Further work is in progress to establish whether P7 corresponds to an mRNA which codes for an active TPH enzyme and to resolve the molecular basis for the apparent diversity of TPH in various tissues.

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