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Organization and Evolution of the Rat Tyrosine Hydroxylase Gene†

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ABSTRACT: This report describes the organization of the rat tyrosine hydroxylase (TH) gene and compares its structure with the human phenylalanine hydroxylase gene. Both genes are single copy and contain 13 exons separated by 12 introns. Remarkably, the positions of 10 out of 12 intron/exon boundaries are identical for the two genes. These results support the idea that these hydroxylase genes are members of a gene family which has a common evolutionary origin. We predict that this ancestral gene would have encoded exons similar to those of TH prior to evolutionary drift to other members of this gene family.

Tyrosine hydroxylase (TH),[1] phenylalanine hydroxylase (PH), and tryptophan hydroxylase comprise a family of enzymes known as the aromatic amino acid hydroxylases. These three mammalian enzymes are iron-containing mixed-function oxidases which require a reduced pterin cofactor and molecular oxygen. All three exhibit cross-specificity for their aromatic amino acid derivatives. Their hydroxylation reactions occur by a similar mechanism which, in all cases, can be inhibited by *p*-chlorophenylalanine (Gal et al., 1970; Kaufman & Fisher, 1974). Along with similar biochemical properties, these enzymes also share immunological characteristics, indicating a common antigenic determinant (Friedman et al., 1972; Kaufman & Fisher, 1974; Chikaraishi et al., 1983).

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[1] Abbreviations: TH, tyrosine hydroxylase; PH, phenylalanine hydroxylase; kb, kilobase(s); bp, base pair(s).

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Due to their functional similarities, it was not surprising that comparisons of the rat TH cDNA (Grima et al., 1985) with the rat (Dahl & Mercer, 1986) and human PH cDNAs (Ledley et al., 1985), and a partial rat tryptophan hydroxylase cDNA (Darmon et al., 1986), revealed striking similarities between their predicted amino acid sequences. The comparison between the TH and PH cDNA sequences revealed two distinct regions: one homologous domain perhaps encoding the catalytic site, and another nonhomologous domain putatively encoding tissue- and enzyme-specific regulatory sites (Ledley et al., 1985; Dahl & Mercer, 1986). The human PH gene has an intron/exon junction at the boundary of the nonhomologous and homologous domains (DiLella et al., 1986). Recently we reported an intron/exon junction at the analogous position in the rat TH gene (Brown et al., 1986). Major changes have occurred in the 5′ region of the expressed portions of these genes which result in the diversity seen in the amino terminus of these proteins. Major changes probably have occurred in the intervening sequences of the 5′ regions of these genes as well.

Clearly, further characterization of the TH gene structure would answer this question and provide important information as to the functional and evolutionary relationship between these enzymes. To address these issues, and as the first step in developing experimental systems for studying the regulation of TH gene expression during development and differentiation, we have isolated and characterized the rat TH gene. Compared to the human PH gene, the rat TH gene is small, 7 vs. 90 kb (DiLella et al., 1986). Despite this large difference in size, both genes contain 13 exons separated by 12 introns. Notably, 10 out of 12 intron/exon junctions have been conserved.

Materials and Methods

Materials

Enzymes were purchased from International Biotechnologies, Inc., New England Biolabs, Inc., Boehringer Mannheim Biochemicals, and Promega Biotec. Ribonucleotide triphosphates, buffers optimized for transcription reactions, and deoxynucleotide triphosphates were obtained from Promega Biotec. All radionucleotides were purchased from Amersham Corp. Nitrocellulose filters were from Schleicher & Schuell. The plasmid Bluescribe was obtained from Stratagene Cloning Systems.

Methods

Isolation of Recombinant Bacteriophage Encoding Rat Tyrosine Hydroxylase Gene. Approximately 6×10^5 bacteriophage plaques from a rat genomic library (Sargent et al., 1979) were screened by established protocols (Benton & Davis, 1977) using a radiolabeled rat TH cRNA (O'Malley, 1986) as a hybridization probe. Six hybridization-positive plaques were purified according to Maniatis et al. (1978), and restriction enzyme digests showed them to be the same clone (λTH-1). This recombinant clone was further mapped by using single and double digests, Southern blotting (Southern, 1975), and hybridization to ^{32}P-labeled rat TH cDNA probes.

DNA Sequence Analysis. Subclones containing the entire TH gene were prepared in either Bluescribe or pUC19 vectors for further restriction mapping (Smith & Birnstiel, 1975). Exon and intron boundaries were sequenced by nonrandom analysis using either unidirectional digestion with exonuclease III (Henikoff, 1984) or specific oligonucleotide probes derived from the rat TH cDNA sequence (Grima et al., 1985; K. L. O'Malley, unpublished results). Sequencing was performed directly by using either strand of denatured double-stranded DNA as a template for primer-extended synthesis (Chen & Seeburg, 1985). All boundaries were sequenced several times; 60% of the intron sequences were also sequenced on both strands. DNA and protein structure analysis was facilitated by computer programs created by Bionet TM.

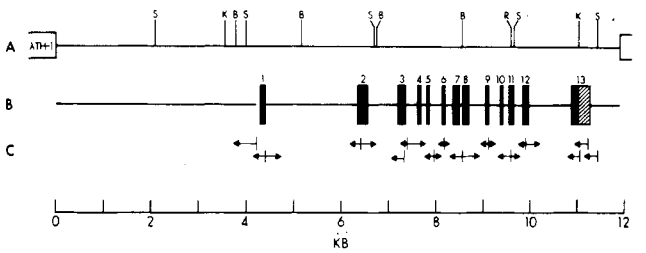

FIGURE 1: Structure of the rat TH gene. (A) Restriction map of the rat TH locus. Restriction enzyme abbreviations: B, *Bam*HI; K, *Kpn*I; R, *Eco*RI; S, *Sac*I. (B) Expressed exons are depicted by solid boxes, nonexpressed exons by hatched boxes, and introns by straight lines. (C) DNA sequencing strategy. Arrows indicate the direction of sequencing for each exon. Each intron/exon boundary was sequenced several times.

Results

Characterization of the Rat TH Locus. In order to assess the size and copy number of the rat TH gene, we hybridized rat genomic Southern blots with a full-length radiolabeled TH cDNA (O'Malley, 1986; K. L. O'Malley, unpublished results). The TH cDNA hybridized to a minimum of one to two bands of genomic DNA when restricted with *Hin*dIII, *Kpn*I, or *Eco*RI (results not shown). Since both the latter restriction endonuclease sites are present once in the cDNA and *Hin*dIII is absent (Grima et al., 1985; O'Malley, 1986), these results suggest, and subsequent analysis of the recombinant clone confirmed, that rat TH is a single-copy gene.

We used detailed restriction endonuclease mapping (Figure 1A), Southern blotting, and DNA sequencing to characterize the unique recombinant bacteriophage containing the rat TH gene (λTH-1). This 12 kb clone hybridized with oligonucleotide probes corresponding to the 5′ and 3′ ends of the cDNA (Grima et al., 1985; O'Malley, 1986), indicating that the entire TH coding region is contained in this isolate. Therefore, the rat TH gene spans 7.3 kb, which in λTH-1 is preceded by 4.1 kb of 5′-flanking sequence and followed by 630 bp of 3′-flanking sequence.

Nucleotide Sequence and Detailed Analysis of the Rat TH Gene. All exons and intron/exon splice junctions of the rat TH gene (Figure 1B) were sequenced and aligned with the rat TH cDNA sequence (Grima et al., 1985; K. L. O'Malley, unpublished results). The sequencing strategy used for determining splice regions is shown Figure 1C). The TH gene contains 13 exons ranging from 51 to 421 bp in length. Precise characterization of the transcription start site is in progress. A putative "TATA" box is located about 30 nucleotides upstream from the initiation site and is preceded 15 nucleotides upstream by a cyclic AMP responsive elemer' (Breathnach & Chambon, 1981; Comb et al., 1986). This is consistent with reports indicating that transcription of TH mRNA is regulated by cAMP derivatives (Lewis et al., 1983).

All splice regions (Figure 2) contain canonical consensus sequences (Mount, 1982). Remarkably, 10 out of 12 splice sites are conserved between the rat TH and the human PH genes (Figure 3). This conservation extends to whether the intron falls after the first, second, or third nucleotide of a codon. Of the nonconserved intron sites, one occurs in the 5′ nonhomologous domain; the other two, found in the conserved 3′ domain, represent an addition and a deletion of an intron

Exon #	Size(bp)	Intron #	Size(bp)	Exon	Intron	Exon
1	101	1	~2000	GTC ACG Val Thr 29 30	gtgaggag.....actcttag	TCC CCC Ser Pro 31 32
2	225	2	~640	TTT GAG Phe Glu 104 105	gtaggcct.....ttcccaaag	ACA TTT Thr Phe 106 107
3	175	3	261	AAG G Lys Val 163 164	gtgaggatg.....ttgtgaag	TC CCC Val Pro 164 165
4	90	4	115	CAC CCG His Pro 192 193	gtgagcttg.....cccttggccatag	GGC TTC Gly Phe 194 195
5	68	5	274	AAG CA Lys His 215 216	gtaaggagcc.....cccctccacag	C GGT His Gly 216 217
6	51	6	~160	ACC TG Thr Trp 232 233	gtaaggagcct.....ttagcctacag	G AAG Trp Lys 233 234
7	146	7	70	AAG G Lys Glu 281 282	gtgcggatgcaga.....ccccttacag	AG CGG Glu Arg 282 283
8	136	8	~350	GAG CC Glu Pro 326 327	gtgagtatg.....cctcacatag	G GAC Pro Asp 327 328
9	70	9	~250	TCC CAG Ser Gln 349 350	gtatgtcta.....cccacag	GAC ATT Asp Ile 351 352
10	57	10	139	TCC ACG Ser Thr 368 369	gttggtttt.....tctggcag	GTG TAC Val Tyr 370 371
11	96	11	~200	CTC CTG Leu Leu 400 401	gtgagattacc.....cactttctag	CAC TCC His Ser 402 403
12	134	12	~890	CTC AG Leu Arg 445 445	gtgcgtgaaccc.....ccctccacag	G AAC Arg Asn 446 447
13	421			AGC TAA Ser Stop	..aataaaggaaggaaagatctccagggctgcctctggtcactg..-3′	

FIGURE 2: Exon/intron junctions of the rat TH gene. All exons and splice sites were sequenced according to the strategy shown in Figure 1. Exon sequences are in upper case letters, and the intron sequences are in lower case letters. Corresponding amino acids are below the exon sequences (Grima et al., 1985; K. L. O'Malley, unpublished results). Consensus polyadenylation signals are underlined. An arrowhead indicates the polyadenylation site.

compared to the PH gene structure. The additional splice site precedes TH exon 7 and lies in a homologous amino acid block. The last intron that is present in PH is absent in TH and resides in an area of nonhomology between these two genes.

TH exon 13, the most 3′ exon, contains 157 bp of coding and 264 bp of 3′ noncoding region. Conserved sequences determining the site of 3′ cleavage include the ubiquitous AAUAAA, located 15 bp upstream from the poly(A) addition site (CA), followed by a CACTG motif (Birnstiel et al., 1985). The order of these consensus sequences is similar to that found in the human PH gene (DiLella et al., 1986). This sequence differs from that reported by Grima et al. (1985) but matches the sequence derived from our TH cDNA (K. L. O'Malley, unpublished results).

Discussion

In this paper, we report the organization of the rat TH gene and demonstrate its structural similarity with that of human PH. The rat gene contains several striking features, the most prominent being the extensive sequence and structural homology with the human gene. The rat TH gene is small; its 13 exons span only 7.3 kb and are separated by intervening sequences no greater than 2.0 kb. The human PH gene, however, spans over 90 kb with introns as large as 23 kb (DiLella

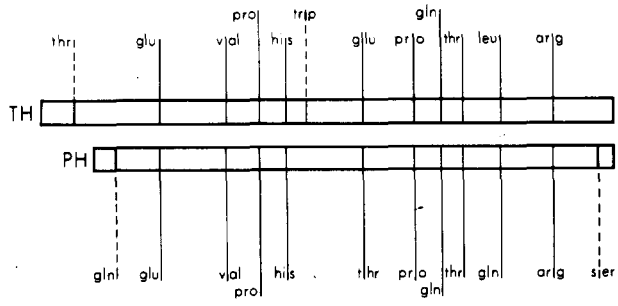

FIGURE 3: Conservation of splice sites between rat TH and human PH genes. Horizontal bars represent rat TH and human PH proteins, aligned to maximize amino acid homology (Ledley et al., 1985). The solid vertical lines indicate the intron positions shared in these two genes. Dashed lines represent nonconserved splice sites.

et al., 1986). This size differential is entirely due to the intervening sequences, as the 13 PH exons themselves are aproximately the same size as those of TH. In fact, in the conserved C-terminal portions of these genes, 7 out of 10 exons are of identical size (Figure 3). Whether the large intron size of the human PH gene represents a species bias or whether it is due to other genetic influences, such as intron sliding (Craik et al., 1984) or transposition into an existing intron, is unclear at present. It would be interesting to know if the rat PH gene has as high a ratio of noncoding to coding sequences as does the human gene. Arguing against species bias, we have preliminary evidence that the human TH gene spans only 8 kb of genomic DNA and thus would reflect the organization of the rat gene (K. L. O'Malley and E. I. Ginns, unpublished results).

The demonstration that 10 out of 12 splice sites present in the human PH gene are in homologous positions in the rat TH gene confirms and extends previous proposals that these enzymes are derived from the same ancestral gene (Ledley et al., 1985; Dahl & Mercer, 1986). In the human PH gene, DiLella et al. (1986) showed that exons 6 through 13 were clustered in 16 kb of genome, whereas exons 1 through 5 spread across 75 kb of sequence. Since the former exons encode the most significant homology between these two enzymes, DiLella et al. (1986) suggested that the 5′ ends of PH and TH may have arisen via the recruitment of exons from unrelated genes, subsequently creating new proteins with unique specificities and responses. Our study documents splice site homology not only in the 3′ homologous domain of these genes but also in the 5′ nonhomologous region as well. Specifically, splice sites following exons 2, 3, and 4 are conserved between the two genes, despite the lack of significant amino acid identity (less than 20%) in this region (Ledley et al., 1985; Dahl & Mercer, 1986). It would be unlikely that these intervening sequences appeared by chance precisely in the same location in both genes. Therefore, our data imply that the point of divergence between the TH and PH genes is upstream from exon 3.

Two sequences that have diverged from a common ancestral gene might acquire differences in a number of ways: via exon recruitment, deletions, insertions, transpositions, etc. (Boguski et al., 1986). As an aid in identifying possible exon donors, we have searched NIH and EMBL gene banks for proteins which might share some homology with the TH coding sequence, particularly with exons 1 through 4. No significant homologies were found in the current data base. It is certainly conceivable, then, that the stringency of selective pressures varied in the 5′ and 3′ regions of TH and PH genes such that the 5′ exons underwent more rapid change. Examples of this mechanism are the apolipoprotein and globin gene families (Boguski et al., 1986; Dickerson & Geis, 1983).

It is interesting to speculate on the lineage of these genes when considering the three differences that do exist in their intron locations. The first and most notable difference is that the splice site between exons 1 and 2 has not been conserved and that the rat TH gene encodes 48 additional amino acids. The conservation of splice sites following exons 2 through 4, a nonhomologous region, suggests that PH may have undergone a deletion in its 5′ end. Additional differences between these two genes suggest both an intron addition and an intron deletion. The most 3′ intron found in PH is not present in TH. Although this PH intron occurs in the conserved domain of the gene, the identity is lower in this region, 37% (Dahl & Mercer, 1986). Surprisingly, the additional intron in TH lies between exons 6 and 7, positioned within a highly conserved amino acid block. It has been suggested that new insertions tend to occur near the middle of the exon, creating a uniformity of size (Swift et al., 1984; Lonberg & Gilbert, 1985). The extra intron in TH creates exons of 51 and 146 bp, equivalent to the 197 bp PH analogue. This additional intron, therefore, does not fall into this pattern.

A parsimonious model to explain these differences is that the ancestral hydroxylase gene encoded a minimum of exons 3 through 13 of PH and TH. Differential rates of mutation and recombination created less homologous 5′ domains attached to the conserved 3′ region. Therefore, we would also predict that the ancestral gene, separated by possibly 14 introns, contained TH-like exons 1 and 2. Following duplication, TH and PH each lost two introns, and PH deleted a portion of its 5′ end. Continued mutation and independent accumulation of changes lead to the current, apparently nonhomologous, domains of these genes. The number of introns proposed for the ancestral hydroxylase would be 14 based on the model of Gilbert et al. (1986). This theory proposes that the maximum number of introns were present in the primordial gene and that with evolution introns were lost differentially. However, we cannot eliminate the possibility that selective insertion of introns into interdomain regions has occurred (Rogers, 1986). Such a mechanism could also explain the TH intron between exons 6 and 7 and the PH intron between exons 12 and 13. Both intron insertion and deletion might have operated on this primordial gene.

It is interesting to note that pterin-dependent hydroxylases specific for phenylalanine and tryptophan have been characterized from bacteria (Letendre et al., 1975, 1984). While exhibiting many of the properties of the vertebrate enzymes, a notable difference is that the bacterial PH from *Chromobacterium violaceum* is a copper-containing protein (Pember et al., 1986). Since dopamine β-hydroxylase is a copper-containing enzyme and a putative member of the hydroxylase gene family as well (Joh et al., 1983), it is interesting to speculate that the metal binding site in these genes is different; i.e., the specificity for iron or copper evolved subsequent to a functional pterin-dependent hydroxylase. What the degree of identity is and where such regions are located in these bacterial hydroxylases would aid in assigning functional correlates to the vertebrate hydroxylase domains. Recently, the *Drosophila* TH cDNA has been cloned and reportedly shares 72% identity with the C-terminal rat sequence (Neckameyer et al., 1986). Comparisons of this gene as well as those for tryptophan hydroxylase and dopamine β-hydroxylase could elucidate the evolutionary history of this gene family.

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