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Cloning, Expression and Partial Characterization of Tryptophan Hydroxylase in *Caenorhabditis elegans.*

by

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A Thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Master of Science

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ABSTRACT

In helminths, including the free-living nematode, *Caenorhabditis elegans*, serotonin (5-HT) acts as an important neuroactive agent and is associated with carbohydrate metabolism, glucouse utilization, motility, feeding and reproductive behaviour. In mammals and other organisms, 5-HT is synthesized through the action of tryptophan hydroxylase (TPH). TPH is the rate limiting enzyme in the biosynthesis of 5-HT, and as such sets the pace for the formation of 5-HT. TPH is a member of a family of enzymes that hydroxylate aromatic amino acids and have an absolute requirement for the pterin cofactor, tetrahydrobiopterin (BH_4). It is unknown if this same enzyme catalyzes the synthesis of 5-HT in *C. elegans* and other helminths.

Based on sequence information from the *C. elegans* Genome Data Base and RT-PCR, we have cloned a full-length *C. elegans* TPH cDNA (CeTPH) that shows high homology to mammalian TPH. The predicted coding sequence of CeTPH was subcloned into the prokaryotic expression vector, pET-15b, and the resulting construct was introduced into *E. coli* (BL21 DE_3 pLys strain) for IPTG-inducible expression of CeTPH protein. Results show that CeTPH expressed in *E. coli* has TPH activity and also shows an absolute requirement for the cofactor, BH_4, just as shown previously for the mammalian enzyme. It has been well established that 5-HT is present and is biologically active in the tissues of *C. elegans*. By way of characterizing furthur CeTPH, we examined the localization of TPH in whole mounts of *C. elegans* by immunofluoresence using a polyclonal antibody againgst TPH.

Taken together, the results of this thesis characterize at the structural, functional and *in situ* levels one of the most primitive forms of TPH enzyme ever cloned.

ABRÉGÉ

Chez les helminthes, incluant le nématode *Caenorhabditis élegans*, la sérotonine (5-HT) est un agent neuroactif important. Elle est associée au métabolisme des hydrates de carbone, à l'utilisation du glucose, à la mobilité ainsi qu'à l'alimentation et la reproduction. Chez les mammifères ainsi que chez d'autres organismes, la 5-HT est produit grâce à l'action de l'hydroxylase tryptophane (TPH). TPH est l'enzyme limitant lors de la biosynthèse de la 5-HT, contrôlant ainsi le taux de production de cette dernière. La TPH fait partie de la famille des enzymes qui hydoxylisent les acides aminés aromatiques et qui nécessitent obligatoirement le cofacteur pterin soit la tetrahydrobiopterine (BH_4). On ne sait pas si cet enzyme catalyse également la synthèse de la 5-HT chez le nématode *C. élegans* et dans les autres helminthes.

Grâce à l'information obtenue sur la séquence de *C. élegans* provenant d'une banque de données génomiques ainsi qu'à la technique RT-PCR, nous avons cloné la séquence de la TPH de *C. élegans* (CeTPH). Cette séquence démontre une homologie élevé avec la TPH des mammifères. Nous avons ensuite sous cloné la séquence codante de CeTPH dans un vecteur d'expression prokariotique pET-15b. Cette construction finale fut introduit dans la bactérie *E. coli* $BL21DE_3$ plys permettant ainsi l'expression de la protiene CeTPH induite par la présence de l'IPTG. L'expression de la CeTPH dans *E. coli* démontre la présence de l'activité enzymatique de la TPH. La présence du cofacteur BH_4 est absolument nécessaire, comme pour le système mammalien. La 5-HT est donc présente et biologiquement active dans les tissus de *C. élegans*. Pour une caractérisation plus poussée de CeTPH, nous avons localisé la TPH par immunofluorescence dans le nématode *C. èlegan* grâce à l'utilisation d'un anticorps polyclonal contre la TPH.

La résultats de cette thèse caractérisent une des formes clonées les plus primitives de l'enzyme TPH au niveau de sa structure, ses fonctions ainsi que sa localisation et son niveau d'abondance in-situ.

SUGGESTED SHORT TITLE:

Tryptophan hydroxylase in *Caenorhabditis elegans*.

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STATEMENT OF CONTRIBUTION

The experimental work reported herein was performed by Suzanne D. Hill.

This thesis was written by Suzanne D. Hill

Dr. P. Ribeiro acted as research/thesis supervisor.

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LIST OF ABBREVIATIONS

AAAH	Aromatic Amino Acid Hydroxylases
BH4	Tetrahydrobiopterin
CeTPH	*Caenorhabditis elegans* TPH
CGC	*Caenorhabditis* Genetics Center
CNS	Central Nervous System
PAH	Phenylalanine hydroxylase
PNS	Peripheral Nervous System
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SL	Spliced leader
TPH	Tryptophan hydroxylase
TH	Tyrosine hydroxylase
5-HT	Serotonin (5-Hydroxytryptamine)

GENERAL INTRODUCTION

Tryptophan hydroxylase (TPH) plays a key role in mammalian physiology as the rate-limiting enzyme in the biosynthesis of the essential neurotransmitter, serotonin, 5-hydroxytryptamine (5-HT). 5-HT has widespread action in maintaining proper mental functions and stability. In addition, studies have revealed the presence of 5-HT in invertebrates, where it functions as a vital neuroactive agent (Isaac et al, 1996; Pax et al, 1992). In helminths, including the free-living nematode, *Caenorhabditis elegans*, 5-HT has been associated with carbohydrate metabolism, glucose utilization, motility and reproductive behaviour (Mansour, 1984; Rahmen et al, 1995).

Mammalian TPH has not been extensively studied, mainly due to its paucity in neuronal tissue; consequently little is known about its properties. However, recent investigations have suggested that mammalian TPH activity is regulated directly by phosphorylation of the enzyme and, indirectly, by mechanisms that alter expression of TPH protein. The mechanisms that affect TPH activity and protein levels are believed to be mediated by multiple signal transduction pathways (Boularand et al, 1995).

Considerably less is known about TPH in lower invertebrates, particularly helminths. Though there is evidence for the presence of 5-HT in all helminths, it has been suggested in *Schistosoma mansoni*, for example, that the predominant source of 5-HT is from the host and not from endogenous synthesis. In this type of scenario the need for a TPH mediated pathway is abolished and in fact the majority of parasitologists in the field believe that TPH is absent at least in some parasites. However, in *Hymenolepis diminuta* and *Ascaris suum*, two parasitic helminths closely related to *C. elegans*, TPH enzyme activity has been observed (Chaudhuri et al, 1988; Ribeiro and

Webb, 1983). In addition, recent evidence suggests the presence of a TPH-like gene in *S.mansoni* and, more recently, in *C. elegans* as well. Nucleotide sequence of *C. elegans*, generated from the *C. elegans* genome sequencing project, reveals a stretch of DNA that is highly homologous to mammalian TPH and contains several recognizable TPH signature peptides. Based on sequence analysis, the *C. elegans* TPH gene has 12 predicted exons that spans approximately 3 Kb of DNA.

C. elegans is an ideal model system to use to study TPH in helminths. *C. elegans* is a good model system for a number of reasons: it can be used as a model system for closely related parasitic helminths (nematodes and platyhelminths) which are more difficult to manipulate *in vitro*; its genome is nearly fully sequenced, allowing for manipulation of TPH at the genetic level, and its nervous system is well characterized and relatively simple, making it more amenable to manipulation.

In light of the present controversy on the origin of helminth serotonin, as described above, the purpose of this thesis is to determine whether a TPH-like gene is present in *C. elegans* and encodes a functional TPH enzyme. Toward that end, I have cloned a full-length TPH-like cDNA from *C. elegans* (CeTPH) and partially characterized the gene product using a bacterial heterologous expression system. Localization studies have been performed and the CeTPH distribution pattern in adult and larvae *C. elegans* has been established. Knowledge gained from this study may provide a better understanding of the role of 5-HT in evolutionary related parasitic helminths, and may be useful in the development of nematocidal and antihelminthic drugs. In addition, any observations made of the *C. elegans* system, will provide insight into the molecular mechanisms of serotonergic neurons in general and might be useful in the synthesis of more

effective drugs for the treatment of a number of mental dysfunctions and neuro-degenerative diseases.

CHAPTER I

LITERATURE REVIEW

1.0 Introduction: 5-HT as a neuroactive agent

Tryptophan hydroxylase (TPH) is the initial and rate-limiting enzyme in the biosynthesis of the neurotransmitter 5-HT (5-hydroxytryptamine: 5-HT) (Jequier et al, 1967). In mammals, TPH is present in the brain stem raphe nuclei, the pineal gland in the central nervous system, and in several peripheral tissues, including the pancreas, intestine, mast cells and in carcinoid and mastocytoma tumors (Boularand et al, 1995). The serotonergic neurons of the raphe nuclei form a neuronal system that controls the basic activity of many regions throughout the forebrain, the cerebellum, and the spinal cord, which in turn modulates a variety of processes, including thirst, appetite, sleep, memory, pain perception and reproduction (Jacobs et al, 1992; Tipper et al, 1994). A disruption of these neural transmissions has been implicated in some very serious pathologies, including obsessive compulsive disorder, sexual dysfunction, aggression, eating disorders, schizophrenia, depression and dementia in Parkinson's syndrome, dementia associated with Alzheimer's disease and the neurodegenerative symptoms of Huntington's disease (Tipper et al, 1994). In the pineal gland, 5-HT serves as an intermediate in the synthesis of melatonin, a hormone associated with a variety of functions including, skin pigmentation, sexual behaviour, body temperature and sleep (Boularand et al, 1995). In each of these tissues, TPH catalyses the first step in the synthesis of 5-HT, and as such sets the pace for the formation of 5-HT.

In the case of platyhelminths, 5-HT is present, based on immunocytochemical studies, predominately in the central nervous system including, the anterior ganglia, longitudinal and transverse nerve cords, and in several peripheral tissues, including, subtegumental muscles, the

musculature of the suckers and rostellum, and in the case of several helminths, the muscular lining of various reproductive structures (Horvitz et al, 1982). Low levels of 5-HT have been shown to induce muscle fibre contraction in *S. mansoni*, causing an increase in motility of the parasite. Thus 5-HT is believed to serve as an excitatory neuromusclular transmitter in some helminths (Day et al, 1994). In addition, 5-HT is thought to be involved in a variety of metabolic functions that include stimulation of glucose uptake, glycolysis and glycogenolysis (Mansour et al, 1984).

Strong evidence exists for the presence of 5-HT in nematodes, for example, *A. suum* and *C. elegans* (Schafer and Kenyon, 1995) . The cellular distribution of 5-HT in both *A. suum* and *C. elegans* has been localized to neurosecretory neurons in the pharynx and to motorneurons in the vulva muscles that are involved in egg-laying in *C. elegans*, as was demonstrated by staining with an anti-5-HT antibody (Desai et al, 1988). In *C. elegans*, 5-HT appears to have a stimulatory effect on egg-laying by acting directly on the vulva musculature structures that surround this area (Trent et al, 1983) and an inhibitory effect on pharyngeal pumping (Horvitz et al, 1982). In addition, six neurons in the head of *C. elegans* have been shown to contain 5-HT immunoreactivity and, in the males of both *A. suum* and *C. elegans*, there are five additional 5-HT immunoreactive cells in the tail (Desai et al, 1988).

TPH and 5-HT in helminths have received very little research attention as compared to their mammalian counterparts. Therefore, for the purpose of this review, information concerning TPH enzyme and 5-HT have been obtained largely from studies of the mammalian system.

2.0 Mammalian aromatic amino acid hydroxylases

TPH is a member of the aromatic amino acid hydroxylase family, which also includes tyrosine hydroxylase (TH) and phenylalanine hydroxylase (PAH) of liver. Both TH and TPH were first documented in 1964, in brain and other nerve tissue preparations (Kaufman and Ribeiro, 1996). These three enzymes are very similar at structural and biochemical levels, but perhaps their most distinct property is their absolute requirement for a reduced pterin cofactor (tetrahydrobiopterin, BH_4) to hydroxylate their respective amino acid substrates (Boularand et al, 1995).

The parallel between TH and TPH is of particular interest because they both play similar roles in the physiology of the nervous system. TH mediates the rate limiting step in the synthesis of catecholamine neurotransmitters, specifically dopamine and noradrenaline (Nankova et al, 1996). These neurotransmitters play as key a role in the physiology of the nervous system as does 5-HT, and thus must be under tight regulatory control. It is generally believed that both TH and TPH are subject to similar types of regulation and, in recent years, a great deal of research efforts have been made to characterize the properties of TH regulation. Biochemical characterization of TPH, on the other hand, has greatly lagged behind that of TH, due to its limited availability and extreme instability (Yang and Kaufman, 1994) although recently a bacterial expression system for rabbit TPH has been developed that has served as a valuable tool for the characterization of this enzyme (Tipper et al, 1994; Vrana et al, 1994). The majority of information available on the properties of TPH is a result of *in situ* studies, for example of brain slices, or from studies of only partially purified enzyme (Yang and Kaufman, 1994). Therefore, results obtained from studies of TH have led to a framework for understanding TPH, and have served to define parameters that are likely to be important for TPH regulation and future study.

2.1 Catecholamine and 5-HT biosynthesis

An outline of the reactions catalysed by TH and TPH is shown in Fig 1. Tyrosine hydroxylase catalyses the hydroxylation of substrate tyrosine to 3,4 dihydroxyphenylalanine (DOPA). Subsequent steps in the biosynthetic pathway that lead to the formation of dopamine, noradrenaline and adrenaline are catalysed by a nonspecific aromatic amino acid decarboxylases (AAADC), dopamine-β-hydroxylase (DBH) and phenylethanolamine-N-methyl transferase (PNMT), respectively (Kumer and Vrana, 1996).

Similarly, TPH regulates the formation of 5-HT by catalysing the hydroxylation of tryptophan to 5-hydroxytryptophan (5-HTP). This compound is then rapidly metabolized to form 5-HT by an AAADC, thought to be the same enzyme found in catecholaminergic neurons (Kaufman and Ribeiro, 1996). 5-HT and the catecholamines, are classified as biogenic amines.

In each biosynthetic pathway, the aromatic amino acid hydroxylase functions as the rate-limiting enzyme, and as such sets the pace of biogenic amine production. The reaction of TH and TPH requires oxygen, iron, and the cofactor, BH_4. The latter is oxidized to dihydrobiopterin, BH_2, concomitant with the oxidation of the aromatic amino acid substrate. Oxygen serves as an electron acceptor and is reduced to water. BH_2 is regenerated to BH_4 through the activity of a second enzyme in the system, DHPR, in the presence of NADH, thereby allowing the cofactor to function catalytically (Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996).

2.2 The TH and TPH gene and alternative RNA processing

In mammals, TH is encoded by a single gene that spans approximately 8.5 kb and is composed of 14 exons. In humans, the primary mRNA transcript undergoes alternative RNA splicing to generate four different mRNA/protein products (Fig. 2) (Kumer and Vrana, 1996). Alternative processing occurs through the use of two different splice donor sequences within the first and second exons. The differences between the four mRNA species lie within a small area at the 5' termini where a short stretch of 12 bp or 81 bp or both are included (Kumer and Vrana, 1996; Kaufman and Ribeiro, 1996). Type 1 human mRNA shares the highest homology to the rat gene and encodes a 497 amino acid (aa) protein. Types 2 and 3 contain an insertion of 12 bp and 81 bp, respectively and type 4 contains both the 12 bp and 81 bp addition. The resulting protein sizes for these isoforms are, 501 aa, 524 aa and 528 aa, respectively. Types 1 and 2 are the most abundant among the forms of TH, being widely distributed in both the CNS and periphery (Kaufman and Ribeiro, 1996).

In contrast very little is known about alternative splicing in the TPH gene. The mammalian TPH gene is 2.9 Kb in size and contains at least 11 known exons (Boularand et al, 1995). The coding region of TPH reveals very similar positions of exon/intron boundaries to those found in TH and in PAH (Boularand et al, 1995). Implying that TPH likely undergoes alternative RNA processing that may be similar to that of TH. However, further investigation is necessary before any conclusions can be drawn.

2.3 TH and TPH structure and function

Native forms of TH and TPH are thought to exist as tetramers with molecular weights of approximately 240 kDa and approximately 250 kDa respectively, as was estimated by gel filtration, and subunit sizes of approximately 60 kDa and approximately 62 respectively, as was observed by polyacrylamide gel electrophoresis. Based on these findings it was concluded that these enzymes are composed of four identical subunits (Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996). More recently, TH and TPH cDNAs have been isolated, cloned, expressed and sequenced (Tipper et al, 1994). Analysis of the nucleotide sequence has revealed that the deduced molecular weights of the enzymes are 56 kDa and 51 kDa respectively, values which compare to the ones obtained from studies of native enzymes (Kaufman and Ribeiro, 1996).

Each subunit is thought to be composed of an N-terminal regulatory domain and a C-terminal catalytic region as is depicted in Fig. 3. Several studies support this model. Grenett and co-workers reported that both TH and TPH share similar reaction characteristics, and have highly conserved C-terminal amino acid sequences, hypothesising that similar catalytic domains reside in the C-terminus (Grenett et al, 1987). Campbell and Haycock showed that phosphorylation of TH occurs within the first 40 amino acid sequence in the N-terminal region, establishing the N-terminal region as the regulatory portion of the molecule (Campbell et al, 1986; Haycock et al, 1990). This was confirmed by mild proteolysis of the enzyme by trypsin or chymotrypsin and later by deletion mutagenesis of the N-terminal regions (Daubner et al, 1993; Ribeiro et al, 1993). The results of these studies confirmed each other, in that removal of the outer region of the N-terminal region in TH, either by deletion mutagenesis or by proteolysis, caused an increase in catalytic activity as compared to wild type TH. These mutant forms of TH are no longer

phosphorylated as mutagenesis removes the phosphorylation sites. Thus it would appear that the outermost portion of the N-terminus exerts a regulatory constraint on the molecule which may be reversed upon phosphorylation. Phosphorylation and other molecular mechanisms that govern the activity of these enzymes will be discussed throughout the remainder of this paper.

2.4 Mechanisms of regulating TH and TPH activity and gene expression

The physiological importance of TH has been perhaps most dramatically demonstrated by two independent studies performed in the same year by Kobayashi and colleagues (1995) and Zhou and colleagues (1994). They found that disruption of the TH gene in transgenic mice (a homozygous null mutation) results in failure of >97% of the embryos to develop to term (midgestational lethality). Of the surviving embryos, 100% of them had stunted growth and died just over 1 month of life. These studies clearly illustrate that catecholamines are required for mouse fetal development. It would be of great interest to perform a TPH gene knock out experiment in mice to observe the physiological importance of 5-HT. Likely this type of investigation would reveal the same sort of drastic results as was observed in the TH study.

Given the central role that TH and TPH play in the physiology of the nervous system it is not surprising that these enzymes are subject to regulation by a wide variety of mechanisms. Regulation of these enzymes can be categorized under two main headings: acute or short-term regulation, and chronic or long-term regulation. Acute regulation leads to a rapid and transient change in the catalytic activity of the enzyme, it includes such mechanisms as, feedback inhibition, allosteric modulation and phosphorylation (Ribeiro et al, 1992; Daubner et al, 1992; Wu et al; 1992 and Gahn et al, 1993). On the other hand, long-term regulation has long lasting effects that

encompass alterations at the transcriptional level, which in turn affects the expression of the enzyme. To date, little is known about the molecular mechanisms of TPH regulation. In contrast, the nature of TH regulation has been more thoroughly investigated and is better understood.

2.5 Feedback Inhibition

It is well known that both TH and TPH are subject to feedback inhibition by their end products. All catecholamine products have been shown to cause a decrease in the activity of TH (Zigmond et al, 1989). In the same way, 5-HT appears to inhibit TPH by direct product inhibition (Boadle-Biber, 1982). In addition, inhibition of TPH by dopamine and the other catecholamines has been reported for *in vitro* and *in vivo* studies (Goldstein et al, 1971; Jequier et al, 1969; Makoto et al, 1994; McGeer et al, 1969). Catecholamines demonstrate a competitive inhibition with the pterin substrate, tetrahydrobiopterin (BH_4) (Goldstein, 1995). This means that the inhibitor binds to the same free enzyme form as the pterin substrate, preventing pterin from binding. Therefore, an increase in pterin concentrations causes a decrease in catecholamine inhibition. Thus, TH follows the classic kinetic-mediated type of inhibition that is readily reversed depending on the concentrations of end product. As will be discussed later, phosphorylation activates TH activity, in part, by alleviating this type of inhibition.

2.6 Allosteric Inhibition

TH is also subject to allosteric inhibition. By definition, allosteric effectors regulate enzyme activity at a site other than the active site of the enzyme. The first type of TH regulation to be

documented was the activation of the enzyme by heparin (Kuczenski et al, 1972). Later it was discovered that certain polyanions like polyglutamic acid (Katz et al, 1976) and certain phospholipids, such as, phosphatidyl-L-serine are able to activate TH (Lloyd and Kaufman, 1974). These modulators of TH activity are examples of allosteric effectors of this enzyme. All have been shown to interact with TH in a reversible manner and result in TH activation that is primarily expressed as a decrease in the Km for the pterin substrate (BH_4) (Kuczenski et al, 1972; Lloyd and Kaufman, 1974 and Katz et al, 1976)

2.7 TH and TPH phosphorylation

It is well accepted that TH acts as a substrate for a variety of different protein kinases and that phosphorylation is a very important means of regulating TH activity *in vivo*. Research efforts have been made to identify the various protein kinase systems and it is now generally believed that TH is phosphorylated by at least six such systems *in vitro* and many of these phosphorylating events have been shown to be associated with an increase in TH activity (Kaufman and Ribeiro, 1996). By way of a brief summary; Tischler et al (1985) and Waymire et al (1991) demonstrated that the neuroactive peptide, vasoactive intestinal peptide (VIP), is capable of stimulating TH activity of adrenal chromaffin cells by increasing cyclic AMP (cAMP)-dependent phosphorylation (commonly known as protein kinase A, PKA). Roskoski and Ritchie (1991) demonstrated that PKA phosphorylates Serine (Ser) 40 of TH (Figure 3).

Ca^{+2} calmodulin-dependent protein kinase (CaM-PKII) has been shown to be involved in the activation of TH as was demonstrated through the use of CaM-PKII specific inhibitors, W-7, KN-62 and trifluoperazine. These caused inhibition of phosphorylation and subsequent inhibition of

TH activity (Lee et al, 1985; Hirato and Nagatsu, 1985 and Ishii et al, 1991). Campbell and co-workers (1986), demonstrated that CaM-PKII phosphorylates Ser 19 and Ser 40 of TH (Figure 3). TH activation by CaM-PK II has been shown to have an absolute requirement for a protein known as the 14-3-3 activator protein. The activator protein is not required for CaM-PK II to phosphorylate its enzyme substrate, its presence does however result in an increase in expression of enhanced activity of phosphorylated TH (Yamauchi and Fujisawa, 1981; Ichimura et al, 1987). Further studies are needed to better elucidate the mechanism(s) and significance of this interaction.

Similarly, Ca^{+2}/ phospholipid-dependent protein kinase (commonly known as, protein kinase C, PKC), is capable of regulating the activity of TH, as was demonstrated by the use of phorbol esters and nerve growth factor in rat pheochromocytoma PC12 cells (Haycock 1990; Mitchel et al, 1990). It was found that these agents, which are known activators of PKC (Pocotte et al, 1986; Tachikawa et al, 1987), elicit phosphorylation of Ser 31 (Haycock, 1990; Mitchell et al, 1990). This is an example of "cross talk" between second messenger/kinase systems, whereby stimulation of one system influences another system. In this case stimulation of PKC by phorbol esters leads to the activation of another pathway that results in the phosphorylation of Ser 31 (Campbell et al, 1995). Therefore PKC is involved indirectly in the activation of TH. A group of basic protein and microtubule-associated (MAP) kinases including, ERK1 and ERK2, have been identified as the kinases responsible for phosphorylation of Ser 31 *in vivo*. (Halloran and Vulliet, 1994; Haycock et al, 1992). PKC is also known to phosphorylate Ser 40 directly (Figure 3) (Cited in Kumer and Vrana, 1996).

Less is known about the remaining kinase systems but for the sake of completion, these include,

cyclic GMP-dependent protein kinase, PKG (Roskoski et al, 1987); cell cycle-dependent, proline-directed protein kinase (cdc2/cyclin A) (Hall et al, 1992) which has been shown to phosphorylate Ser 8; and Ser 19 is a substrate for the MAP kinase-activated protein kinases (MAPKAP kinases) 1 and 2 (Sutherland et al, 1993). It remains to be seen whether phosphorylation by these last three kinase systems modulates TH activity.

Interestingly, the notion that phosphorylation alleviates catecholamine inhibition was further elucidated upon discovering that phosphorylation by PKA (at Ser 40) serves to increase the rate of dissociation of TH-bound catecholamines while also facilitating the reduction of Fe^{+3} to Fe^{+2}, the form required for TH activity (Goldstein, 1995). In addition, phosphorylation by PKA leads to an increase in Vmax and a decrease in the Km for the pterin (BH_4). Therefore, release of inhibiting catecholamines as mediated by phosphorylation appears to be the first step toward TH activation, a likely procedure for inducing TPH activity as well.

2.8 Long term regulation of TH and TPH; Transcriptional regulation of TH and TPH gene expression *in vivo*

It is generally presumed that the long term increases in TH and TPH protein and activity levels are a result of an increase in the respective mRNA concentrations and an increase in expression of TH and TPH protein, respectively. Park and co-workers (1994), reinforced this belief by demonstrating that rats treated with high levels of TPH inhibitor exhibit an increase in brain levels of TPH mRNA. To date, there are only a few studies of TPH gene expression and the mechanisms involved await further investigation. On the other hand, numerous *in vivo* studies have been performed on TH expression. Since it is beyond the scope of this paper to address all such reports a few general examples will be given.

Extensive deletional and mutational analysis of the 5' region of the TH gene (500 bp) have identified at least six putative transcriptional regulatory elements (Fig. 4) including, a glucocorticoid regulatory element (GRE) (about 450 bp upstream from the start site) (Coker et al, 1988), an AP1 sequence (the Fos/Jun immediate early protein binding site) (approximately 200 bp upstream from the start site) (Icard-Liepkalns et al, 1992), and a cAMP responsive element (CRE) (approximately 40 bp upstream from the start site) (Kim et al, 1993b, 1994).

The sequence of the AP1 site (5'-TGATTCA-3') differs from the consensus sequence (5'-TGACTCA-3') by a single base; nevertheless it allows Fos/Jun binding. The significance of the site has been tested using a phorbol ester, 12-O-tetradecanoylphorbol 13-acetate, a common activator of PKC, and it was shown by deletion analysis (Caroll et al, 1991; Goc and Stachowiak, 1994) and point mutagenesis (Icard-Liepkalns et al, 1992) that the phorbol ester stimulated increases in TH transcription. This was achieved through the binding of Fos/Jun complexes to the AP1 site, thereby suggesting that this sequence plays a key role in TH gene expression via stimulation of the PKC pathway (Caroll et al, 1991; Goc and Stachowiak, 1994 and Icard-Liepkalns et al, 1992).

Transcriptional activation of TH is also thought to be induced by cAMP, mediated through a PKA signalling pathway that leads ultimately to the phosphorylation and activation of a cAMP responsive element binding protein (CREB). Phosphorylated CREB is then believed to induce transcription of the TH gene by binding to the cAMP responsive element (CRE). This flow of information in the stimulation of a CRE in the TH promoter region is strongly supported by a study that demonstrated that cells lacking in PKA activity (PKA-deficient cell lines derived from PC12 cells), were unable to activate TH transcription when treated with cAMP (Kaufman and

Ribeiro, 1996). On the other hand, transcription activity was fully restored when these cells were cotransfected with an expression vector containing the catalytically active subunit of PKA. This is a strong indication that the effect of cAMP is mediated by PKA.

2.9 TPH regulation

TH is known to be regulated by numerous protein kinases, as was discussed above. It is therefore a reasonable possibility that TPH may be activated by the same kinase systems.

Grennet and co-workers (1987) and Darmon and co-workers (1988) demonstrated the presence of a PKA phosphorylation site (Ser 58) in TPH (Fig. 5). Since this early study, a recombinant form of TPH was expressed in bacteria and shown to be phosphorylated by PKA (Vrana et al, 1994). In addition, Johansen and colleagues (1995) have established that TPH is phosphorylated by PKA but that this phosphorylation is not accompanied by an increase in the activity levels of TPH. This does not diminish the biologically significant role potentially played by TPH. For instance, some kinases phosphorylate a variety of substrates without inducing the activity of these substrates, for example, the casein kinase (Johansen et al, 1995). However, phosphorylation by such kinases can alter phosphorylation by subsequent kinase pathways (Picton et al, 1982). The possibility exists that phosphorylation of TPH by PKA may have this same type of effect, whereby phosphorylation by other protein kinases may lead to enzyme activation (Johansen et al, 1995). Studies have demonstrated that TPH is phosphorylated by CaM-PK II (Ehret et al, 1989) (Fig. 5). Perhaps TPH is activated by CaM-PK II following phosphorylation by PKA. Further study is needed to better identify these kinase systems and to define the "cross talk" relationship between

them.

Although PKA does not have any direct effect on TPH activity it is however thought to be involved in long term regulation of the enzyme. Foguet et al, (1993) demonstrated that TPH mRNA levels are increased by cAMP in rat serotonergic neuronal cultures from raphe nuclei. Therefore, it would appear that cAMP is able to regulate TPH activity by inducing transcription of the TPH gene (Boularand et al, 1995). It is interesting to note that the promoter elements normally implicated in a cAMP-dependent induction of mRNA in cAMP responsive genes are not the ones responsible for inducing transcription of the TPH gene. For instance, the elements identified in a large number of cAMP-responsive genes include, the consensus CRE sequence, the AP-1 and AP-2 sites (cited in Boularand et al, 1995), however in the case of TPH, an inverted CCAAT box, an AP-4 site, was identified. This motif has been designated by Boularand et al, (1995) as the element implicated in transcriptional regulation of TPH by cAMP. Evidence for this decision was based on deletion mutagenesis of the promoter region and site-directed mutagenesis of the inverted CCAAT box (Boularand et al, 1995). This finding is in contrast to what is known about the TH promoter region. As was mentioned previously TH transcription is induced via a putative CRE site located about 40 bp upstream of the start site (Kim et al, 1993b; 1994).

There are a couple of noteworthy distinctions between TH and TPH that may offer some explanation for the observed differences in their responsiveness to the same protein kinase systems and different regulation of their genes. The N-terminal region (regulatory domain) of the two proteins differ in length and in sequence (Darmon et al, 1988). In addition, the five phosphorylation sites that are responsible for the activation of TH reside in the N-terminal region (Haycock, 1990; Haycock and Haycock, 1991), whereas only one serine residue is located within

the regulatory domain of TPH (Ser 58), all other predicted sites are in the catalytic domain (C-terminal end) (Darmon et al, 1988). Further investigation is necessary to better understand the significance of these differences.

There is as yet very little known concerning the activation and regulation of TPH as compared to TH which is itself only in infancy stages of being understood. A direct link between phosphorylation of TPH by any protein kinase and a change in activity has not yet been established. Nor has any direct evidence been presented to show that cAMP or other compounds modulate transcription of TPH gene.

3.0 Serotonin in Helminths

5-HT has been reported in a the tissues of a wide variety of parasitc and free-living platyhelminths, where it has been demonstrated to have a variety of biological effects.

Addition of exogenous 5-HT has been shown to stimulate rhythmical movements in numerous platyhelminths, including the trematodes, *Fasciola hepatica* (Mansour, 1957) and *S. mansoni* (Tomosky et al, 1974) and cestode, *H. diminuta* (Mettrick et al, 1981). Based on the above observations, 5-HT was suggested to function as an excitatory neuromuscular transmitter or modulator in platyhelminths.

In immunocytochemical studies of trematodes and cestodes, serotonergic staining is localized to the Central Nervous System, CNS and the Peripheral Nervous System, PNS. 5-HT-like immunoreactivity is most pronounced in CNS structures (anterior ganglia, longitudinal and

transverse nerve cords) but it is also seen in peripheral nerve fibres associated with subtegumental muscles, musculature of the sucker and rostellum, in addition to muscles associated with various reproductive structures and ducts (Lee at al, 1978, Fairweather et al, 1987, Halton et al, 1987). Interestingly, the localization of putative 5-HT staining in these organisms roughly follows the distribution pattern of the cholinergic element, acetylcholinesterase (Halton et al, 1987, Wilson and Schiller, 1969), suggesting that 5-HT and acetylcholine may have antagonistic effects in flatworms and other platyhelminths. This is supported by the finding that acetylcholine will inhibit muscle activity in *S. mansoni* (Barker et al, 1966) and *H. diminuta* (Wilson and Schiller, 1969), whereas, 5-HT will stimulate motor activity in the flukes *S. mansoni* (Tomosky et al, 1974), *F. hepatica* (Mansour, 1979) and in *H. diminuta* (Mettrick et al, 1981).

3.1 5-HT in nematodes : *Ascaris suum and C. elegans*

Similarly, 5-HT has been reported in numerous parasitic and free-living nematode species, including, *Gooeyus ulmi*, (Leach et al, 1987), *C. elegans* (Desai et al, 1988) and *Ascaris suum* (Sretton and Johnson, 1985). The cellular localization of 5-HT has been studied more extensively in *C. elegans* (Desai et al, 1988) and *A. suum*. In both species, antisera raised against 5-HT labelled a pair of neurosecretory neurons in the pharynx (the NSM neurons) of both sexes and five cells in the ventral cord of the male tail (Desai et al, 1988; Loer and Kenyon, 1993, Brownlee et al, 1994). In *C. elegans*, but not in *A. suum*, the HSN neurons (hermaphrodite-specific neurons), a pair of motorneurons that control egg-laying at the vulval muscles, show prominent staining with an anti-5-HT antibody as do six additional neurons in the head of both sexes (Cited in Horvitz et al, 1982).

In *A. suum*, 5-HT stimulates glycogenolysis in muscles (Donahue et al, 1982) and increases cAMP levels, activates phosphorylase enzyme while inactivating glycogen synthase (Donahue et al, 1981). Injection of 5-HT into *A. suum* gives rise to a reversible paralysis, thereby suggesting a probable role in motility (Buchanan and Stretton, 1991).

In *C. elegans*, 5-HT stimulates egg-laying and inhibits pharyngeal pumping (Horvitz et al, 1982). Trent and colleagues demonstrated that when a 5-HT uptake inhibitor, imipramine, is administered to *C. elegans* mutants defective in the functioning of the HSNs, these mutants which are egg-laying defective do not lay eggs but do lay eggs in response to exogenous 5-HT (Desai and Horvitz, 1989; Trent et al, 1983). This suggests that 5-HT acts directly on the egg-laying muscles, and that the source of 5-HT is the HSN neurons (Trent et al, 1983).

3.2 Serotonin deficient *C. elegans* mutants

Analysis of 5-HT-deficient mutants is useful to define how a neuron controls the expression of a serotonergic phenotype. *C. elegans* mutants have been employed to identify and characterize neurons required for various behaviours, for example, Loer and Kenyon have identified 5-HT-immunoreactive neurons required for tail curling in *C. elegans* males, a behaviour typical of *C. elegans* males during mating. They found that males mutant in three different genes that are known to reduce 5-HT expression, cat-1, cat-2, and bas-1, exhibit defects in tail curling behaviour similar to the wild-type males in which these neurons were ablated. The defective phenotype is reversed in response to exogenous 5-HT. An observation that is consistent with the idea that this behavioural defect is due to a lack of 5-HT (Loer and Kenyon, 1993).

3.3 The presence or absence of TPH in helminths: the controversy

5-HT has been found in the tissues of a wide variety of free-living and parasitic platyhelminths including, *C. elegans*, *S. mansoni* and *H. diminuta* (Chou et al, 1972, Desai et al, 1988, Ribeiro and Webb, 1984). However, the long standing belief is that TPH is absent at least in some parasites. For example, *S. mansoni* and *H. diminuta* are thought to obtain their source of 5-HT from their respective host (Bennett and Bueding, 1973; Mansour, 1979; Cho and Mettrick, 1982; Hillman, 1983). However, two reports by Ribeiro and Webb and one by Chadhuri, challenged that dogma by providing evidence of TPH activity in *H. diminuta*, and in *A. suum*, respectively. These studies suggested the presence of an endogenous biosynthetic pathway for the production of 5-HT (Chadhuri et al, 1988; Ribeiro and Webb, 1983, 1984), a finding which has since been reinforced by the recent discovery of a TPH-like gene in *S. mansoni* (preliminary results, unpublished finding). In light of the conflicting evidence, there is a need to re-examine the notion that TPH is absent in parasitic helminths.

It is well established that TH and AAACD are present in *C. elegans* and thus a complete biochemical pathway for the synthesis of dopamine exists (Davis and Stretton, 1995). While TH and AAACD are known to be present in *C. elegans*, TPH has not been identified. Strong evidence exists for the presence and biological activity of 5-HT in *C. elegans*, suggesting that TPH must be present in the free-living nematode. Therefore, we propose that *C. elegans* TPH may be a useful model system for studies of helminth TPH, for reasons outlined below.

3.4 Features of the *C. elegans* model system

3.4a Development and cell lineage

In 1965, Brenner began to take advantage of *C. elegans* as a model organism to study for the following reasons; rapid (3 day) life cycle, small size (1.5-mm-in length, adult), ease of laboratory cultivation, high production of progeny (a single adult worm is capable of producing 300-350 progeny) and because *C. elegans* is a simple biological system to study, with a small genome (only 20 times that of *E. coli*) and anatomical simplicity (<1000 cells) and a nervous system of only 302 neurons (Riddle et al, 1997; White et al 1986).

There are two sexes in *C. elegans*, the hermaphrodite and the male. Fertilization is internal in the hermaphrodite and can also be effected by sperm supplied by the male (Brenner, 1974). Development is completed in 72 h at 25'c and includes an embryonic phase of about 14 h and 4 distinct larval or juvenile stages interrupted by molts. In response to environmental cues associated with starvation or crowding an alternative pathway is initiated (Golden and Riddle, 1984). The third larval phase is replaced by the dauer larva which can survive for several months without food, upon returning to food the dauer larva molts and lives out a normal adult life (Klass and Hirsh, 1976).

3.4b *C. elegans* as a model system for studying neurotransmitters; Molecular and genetic analysis.

C. elegans is an ideal model organism for investigating the role of neurotransmitter function. The nervous system has been characterized and a complete circuitry diagram has been worked out (White et al, 1986). Also, the *C. elegans* genome project has provided a wealth of information

that can be used for neurobiological study of this organism, as indicated above. A physical map of ordered cosmids have been generated (Coulson et al, 1986) and this map is forming the basis for sequencing the entire genome of the organism. The nucleotide sequence of the cosmids is assembled into a series of continuous sequence which then may be searched by the computer program Genefinder. Genefinder searches the sequence for motifs found in genes like, open reading frames, splice sites and others, and highlights them (Isaac et al, 1996). Also available is a mapping of thousands of mutations that have been created for this organism and techniques for insertional mutagenesis (Plasterk, 1995). Such information is essential for the pursuit of meaningful investigation into nervous system function.

3.5 Transcription in *C. elegans*: mechanism of trans-splicing

Mature mRNA transcripts of *C. elegans* are composed of two different elements, the SL (spliced leader), which constitutes the 5' end of the molecule, and the pre-mRNA, which contributes the body of the RNA that represents the region that codes for protein (Singer and Berg, 1991). The joining of these two molecules occur by means of trans-splicing. This type of RNA processing is very similar to that of the snRNP-mediated removal of introns from pre-mRNA. Interestingly, helminths and trypanosomes represent the only eukaryotes which transcribe their genes in this way (Hannon et al, 1990; Marr and Muller, 1996). Thus, it is possible to take advantage of the presence of the spliced-leader sequence in *C. elegans* in order to determine where the 5' end of the gene begins.

The studies in this thesis were designed to determine whether TPH is present in the free-living nematode, *C. elegans*, and if so to express and partially characterize *C. elegans* TPH (Chapter

II) as well as localize this novel protein *in situ* (Chapter III).

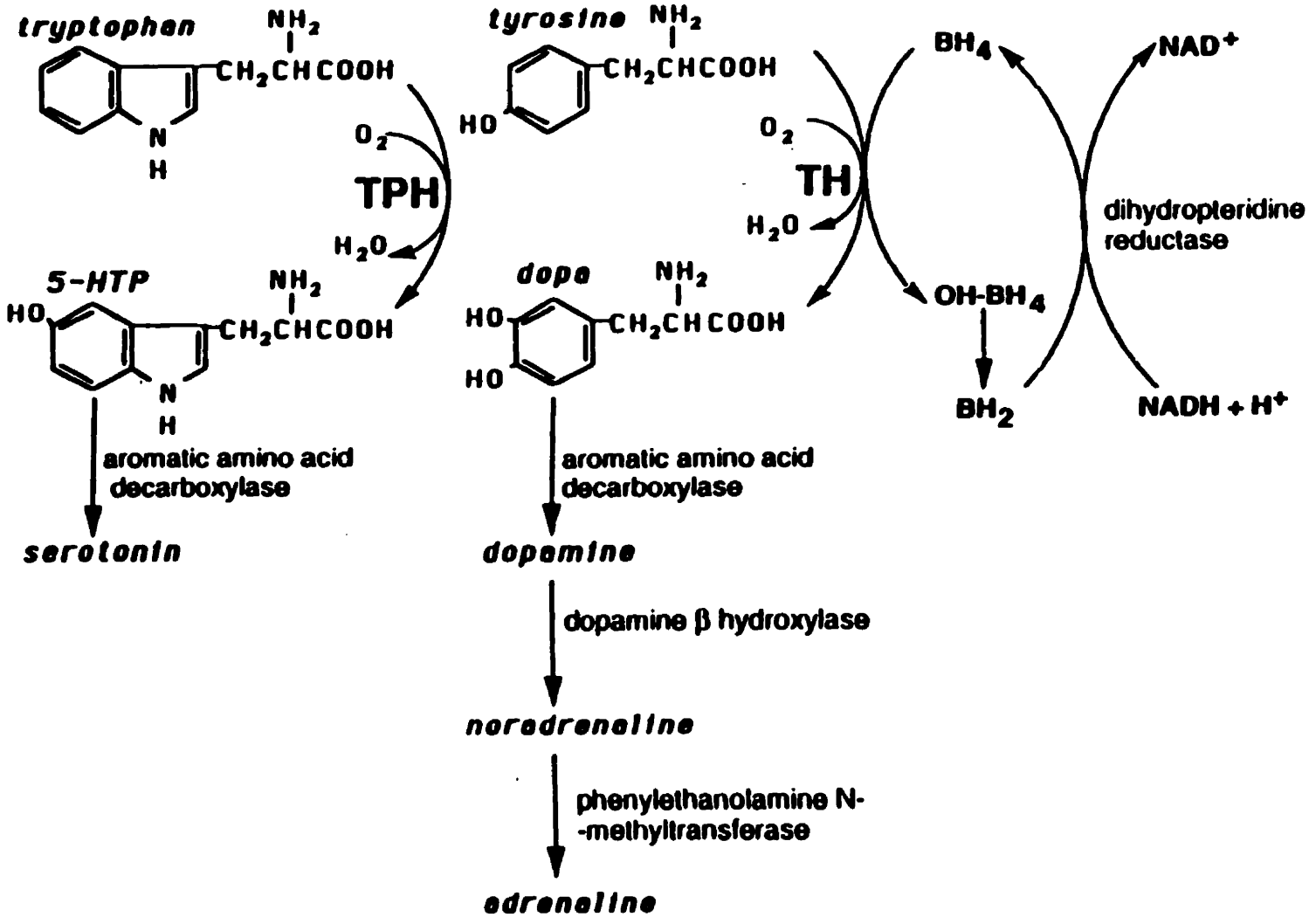

Figure 1. Schematic representation of the reaction of tyrosine hydroxylase and tryptophan hydroxylase and the pathways leading to the biosynthesis of catecholamines and serotonin, respectively.

(Taken from Kaufman and Ribeiro, 1996)

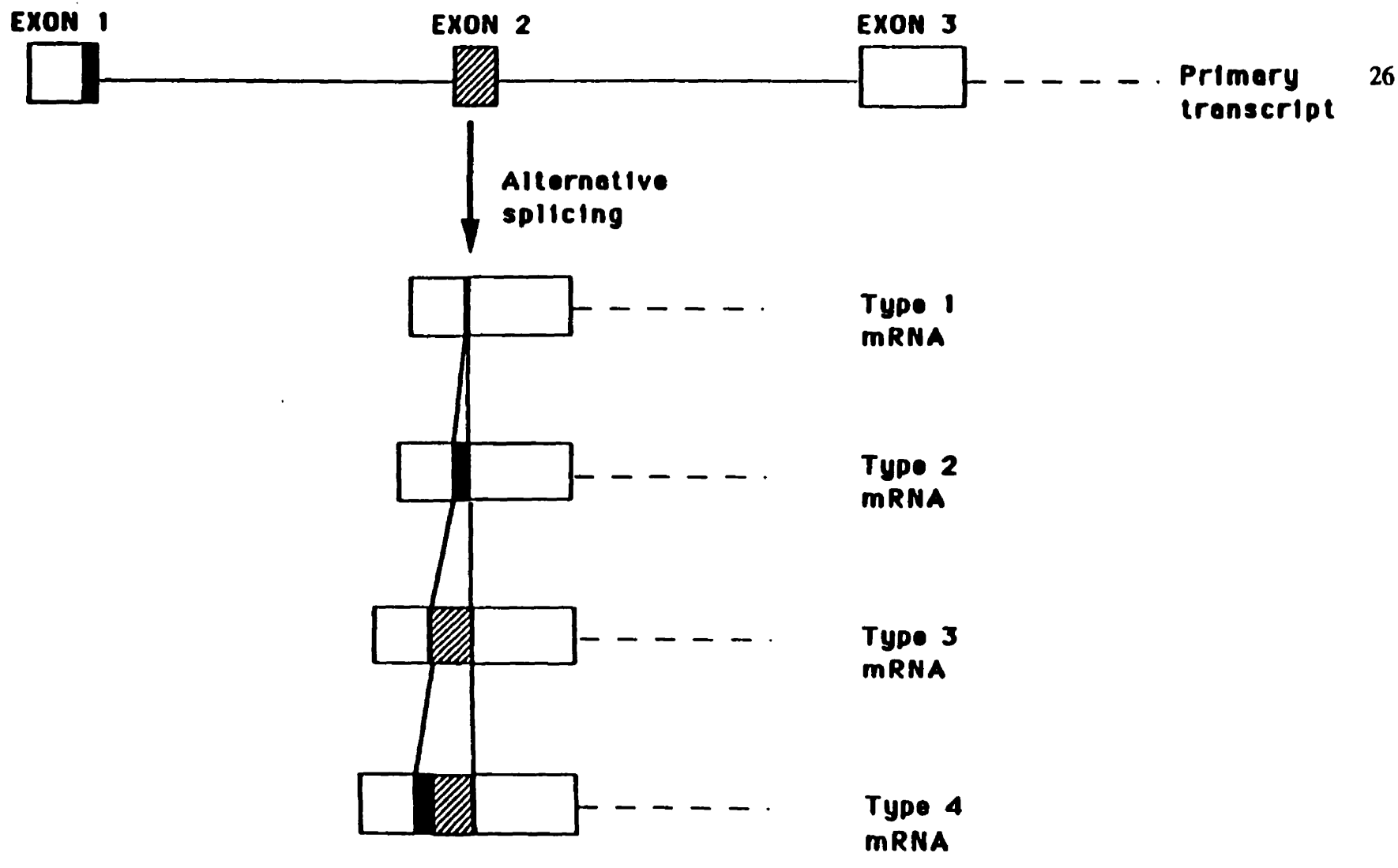

Figure 2. Structure of the human tyrosine hydroxylase gene illustrating the alternative splicing pathways that generate four enzyme species from a single mRNA transcript.

(Taken from Kaufman and Ribeiro, 1996)

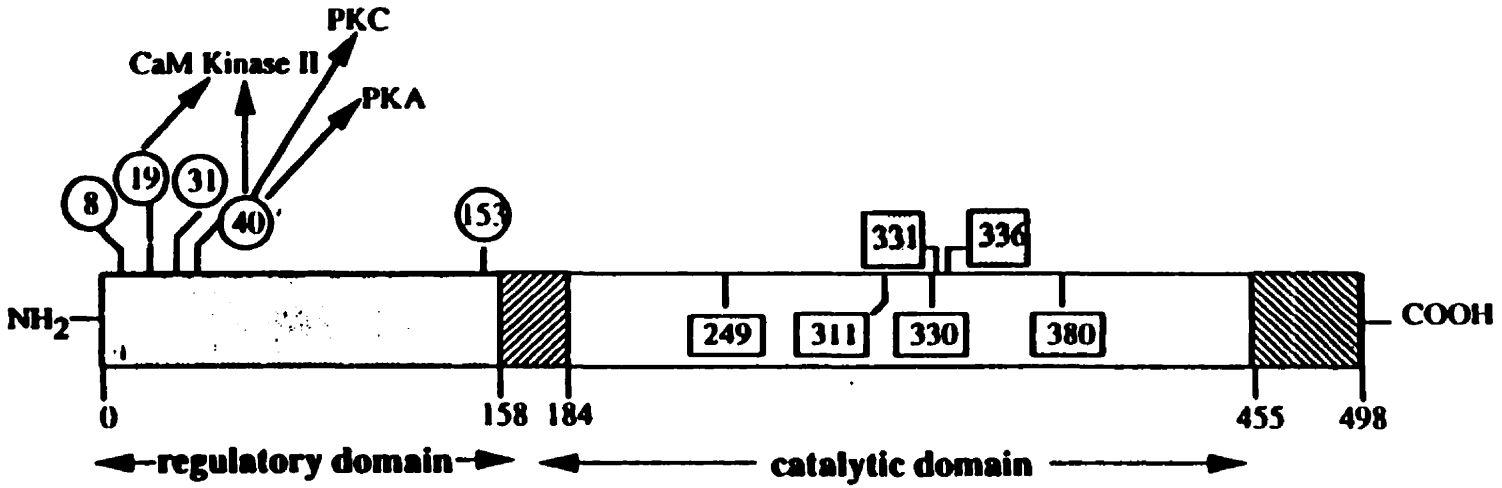

Figure 3. Diagrammatic representation of the regulatory and catalytic domains of rat pheochromocytoma tyrosine hydroxylase: circles represent phosphorylation sites (Ser 8, Ser 19, Ser 31, Ser 40, and Ser 153) with their corresponding protein kinases.

(Taken from Kaufman and Ribeiro, 1996)

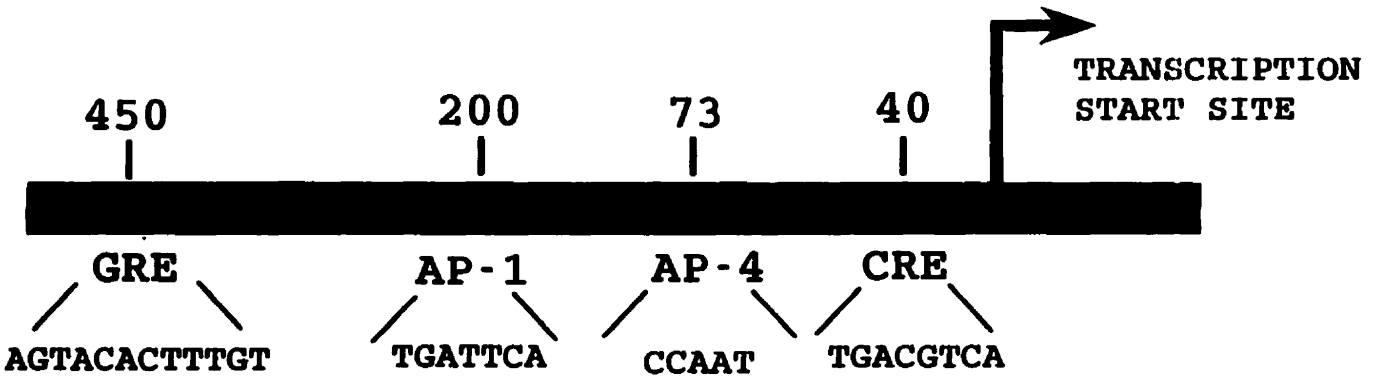

Figure 4. Promoter structure of the tyrosine hydroxylase gene. Several putative transcriptional regulatory elements are represented within the 5' region of the gene. The sequences for these elements are displayed at the top and bottom and the putative regulatory elements are given in the comments below. Modified from Kumer and Vrana, 1996

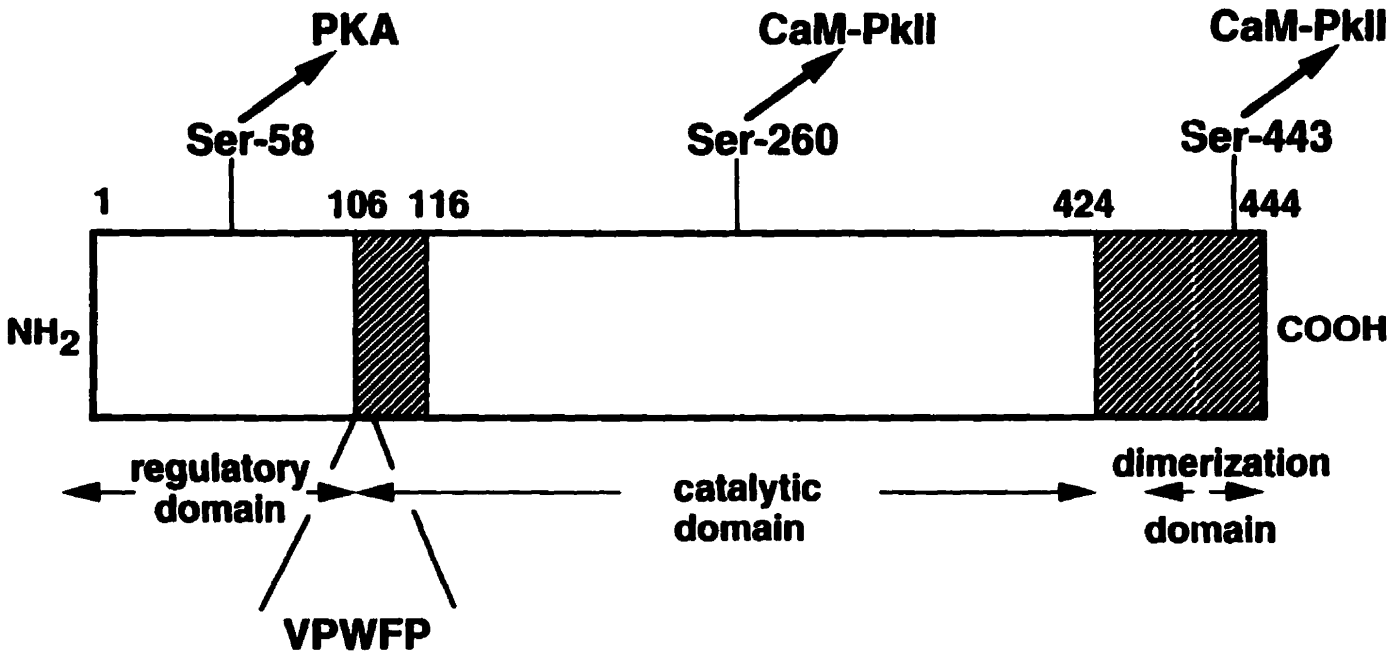

Figure 5. Rabbit TPH monomeric subunit.

CHAPTER II

Cloning, expression and partial characterization of tryptophan hydroxylase in *Caenorhabditis elegans* (Manuscript I)

Suzanne D. Hill and Paula Ribeiro

(In preparation)

ABSTRACT

Serotonin (5-hydroxytryptamine: 5-HT) is a monoamine neurotransmitter that has widespread function in both vertebrates and invertebrates. In helminths, including *C. elegans*, 5-HT acts as an important neuromuscular transmitter and modulator. We have cloned a novel *C. elegans* tryptophan hydroxylase (CeTPH) cDNA that shows high homology to mammalian tryptophan hydroxylase (TPH), the enzyme that catalyzes the first and rate-limiting step in the biosynthesis of serotonin. The CeTPH transcript is trans-spliced at its 5' end to a *C. elegans* spliced-leader (SL) sequence and encodes a protein of 541 amino acids with a predicted size of 62 kDa. Analysis of CeTPH protein sequence reveals two CaM-PKII phosphorylation sites at serine positions 43 and 69 and two PKC phosphorylation sites at serine position 88 and threonine position 63. Further amino acid analysis indicates that the deduced CeTPH protein has 45% identity with *Schistosoma mansoni* TPH, ~40% identity with human, rabbit, rat and mouse TPH homologues and up to ~70% identity in the predicted catalytic domain relative to mammalian hydroxylases. Expression of CeTPH in *Escherichia coli* produced a functional enzyme with a final specific activity of 0.80 nmol/min/mg. CeTPH enzyme was found to have the same absolute requirement for the tetrahydrobiopterin cofactor, BH_4, as the mammalian enzyme. Thus, we propose *C. elegans* as an ideal model system for studies of TPH and 5-HT not only in evolutionary related parasitic helminths but in higher organisms as well.

INTRODUCTION

Serotonin (5-hydroxytryptamine: 5-HT) is an important neuroactive agent in both vertebrates and invertebrates. The first step in the biosynthesis of 5-HT is catalysed by tryptophan hydroxylase (TPH; tryptophan-3-monooxygenase; EC 1.14.16.4), the enzyme that converts tryptophan to the short-lived intermediate 5-hydroxytryptophan (5-HTP). The latter is rapidly metabolised by an aromatic amino acid decarboxylase to form 5-HT. TPH catalyses the first and rate-limiting step in the biosynthesis of 5-HT and consequently regulates the pace of 5-HT production (Kaufman, 1987; Kaufman and Ribeiro, 1996). A disruption of serotonergic transmissions has been implicated in pathologies, including obsessive compulsive disorder, sexual dysfunction, aggression, eating disorders, schizophrenia, depression and dementia in Parkinson's syndrome, dementia associated with Alzheimer's disease and the neurodegenerative symptoms of Huntington's disease (Tipper et al, 1994). In the pineal gland, 5-HT serves as an intermediate in the synthesis of melatonin, a hormone associated with a variety of functions including, skin pigmentation, sexual behaviour, body temperature and sleep (Boularand et al, 1995).

Genes that encode TPH have been isolated from mammals and other vertebrate species (Boularand et al, 1995; Tong and Kaufman, 1975; Stoll et al, 1990). Analysis of the gene products revealed that TPH belongs to a family of aromatic amino acid hydroxylases that also includes phenylalanine hydroxylase (PAH) of the liver and neuronal tyrosine hydroxylase (TH). PAH is the rate-limiting step in the only metabolic pathway by which phenylalanine is completely catabolized to carbon dioxide and water. TH catalyses the rate-limiting reaction in the biosynthesis of catecholamine neurotransmitters, dopamine and noradrenaline.

The three enzymes share common catalytic properties, including an absolute requirement for a reduced tetrahydrobiopterin, BH4, as the obligatory cofactor. In addition, these enzymes share important structural properties as well as functional organization that includes a variable N-terminal third containing the regulatory domain and a highly conserved catalytic region

comprising two-thirds of the carboxyl end of the protein (Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996). Interestingly, both TPH and TH undergo similar forms of regulation, both at the level of transcription and by phosphorylation at multiple serine residues (Campbell et al, 1986; Zigmond et al, 1989; Haycock and Haycock, 1991; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996).

5-HT and catecholamines have been reported in the tissues of a wide variety of parasitic and free-living platyhelminths, where they play important roles as neuromuscular transmitters and modulators (Pax and Bennett, 1992; Davis and Stretton, 1995; Mettrick et al, 1981). In the trematodes, *Fasciola hepatica* (Mansour, 1957) and *Schistosoma mansoni* (Tomosky et al, 1974) and cestode, *Hymenolepis diminuta* (Mettrick et al, 1981) 5-HT is an excitatory neuromuscular transmitter and modulator of motor activity. In parasitic helminths, both 5-HT and catecholamines have been localized to the CNS and PNS including, muscle groups associated with the sucker, rostellum and reproductive structures (Lee at al, 1978; Fairweather et al, 1987; Halton et al, 1987). Similarly, 5-HT has been reported in numerous parasitic and free-living nematode species, including, *Gooeyus ulmi*, (Leach et al, 1987), *C. elegans* (Desai et al, 1988) and *Ascaris suum* (Stretton and Johnson, 1985). In the latter two species, antisera raised against 5-HT labelled a pair of neurosecretory neurons in the pharynx (the NSM neurons) of both sexes and five cells in the ventral cord of the male tail (Desai et al, 1988; Loer and Kenyon, 1993, Brownlee et al, 1994). In *C. elegans*, but not in *A. suum*, the HSN neurons (hermaphrodite-specific neurons), a pair of motorneurons that control egg-laying at the vulval muscles, show prominent staining with an anti-5-HT antibody as do six additional neurons in the head of both sexes (Cited in Horvitz et al, 1982). In *C. elegans*, 5-HT stimulates egg-laying and inhibits pharyngeal pumping (Horvitz et al, 1982). Trent and colleagues demonstrated that when a 5-HT uptake inhibitor, imipramine, is administered to *C. elegans* mutants defective in the functioning of the HSNs, these mutants which are egg-laying defective do not lay eggs but do lay eggs in response to exogenous 5-HT (Desai and Horvitz, 1989; Trent et al, 1983). This suggests that 5-HT acts directly on the egg-laying muscles, and that the source of 5-HT is the HSN neurons (Trent et al, 1983). In *A. suum*, 5-HT is believed to play a role in motility (Buchanan and

Stretton, 1991).

Though there is evidence for the presence of 5-HT in all helminths, it has been suggested in *S. mansoni*, for example, that the predominant source of 5-HT is from the host and not from endogenous synthesis. In this type of scenario the need for a TPH mediated pathway is abolished and in fact the majority of parasitologists in the field believe that TPH is absent at least in some parasites (Bennett and Bueding, 1973; Mansour,1979; Cho and Mettrick, 1982; Hillman, 1983) . However, in *H. diminuta,* and *A. suum*, two parasitic helminths related to *C. elegans*, TPH enzyme activity has been observed (Chaudhuri et al, 1988; Ribeiro and Webb, 1983;). Recently, Hamdan and Ribeiro cloned of a TPH-like gene from *S. mansoni*. Although these studies have challenged this long-standing dogma, the question of whether parasitic helminths have mammalian-like TPH has not been undisputably resolved.

The present study reports the cloning and partial characterization of a TPH-like enzyme in *C. elegans* (CeTPH). The results show CeTPH shares some structural and catalytic properties with the mammalian enzyme, although the N-terminal regulatory region of the mammalian counterpart appears to be absent in CeTPH. The high degree of conservation in the catalytic domain as well as the obligatory requirement for the cofactor, BH4, suggests that CeTPH plays as important a role in the biosynthesis of 5-HT as does mammalian TPH.

Materials and Methods

Cloning of a full-length CeTPH cDNA

Total RNA was purified with the use of the TRIzol total RNA isolation reagent, according to the manufacturer's instructions (GibcoBRL), from *C. elegans* wild-type adult worm tissue. Worm strains were obtained from the Caenorhabditis Genetics Center (CGC) of the University of Minnesota. Reverse transcription of *C. elegans* total RNA was performed with the use of cDNA cycle kit (Invitrogen). Five μg of total RNA was used either with oligo dT or specific antisense oligonucleotide primers (PR57 5'-AAACACGGAAACTCAAACTACAGGAAA-3' and PR50 5'-CTACAGGATGTAGTGGAGAGCTCCG-3'), that correspond to a predicted *C. elegans* TPH sequence generated from the *C. elegans* genome project (Cosmid number ZK 1290). The resulting cDNA was used directly for PCR according to standard protocols (see below).

In order to determine the 5' end of predicted TPH transcripts nested PCR reactions were performed using the spliced-leader (SL) primer (PR93) (5'-GGTTTAATTACCCAAGTTTGA-3') and an antisense primer PR50 (5'-CTACAGGATGTAGTGGAGAGCTCCG-3'). Typically each PCR reaction mixture contained 3 μl of cDNA, 200 mM deoxynucleotide triphosphates (dNTPs), 1 μM of each primer and 1.5 mM of $MgCl_2$ in a total volume of 50 μl. The cycling protocol was as follows: Denaturation for 3 min at 94'C was followed by addition of 2.5 units of Taq polymerase. This variation on the standard PCR protocol (known as Hot-Start) is known to minimize artefacts in the subsequent PCR reaction. A Touch-down PCR step included denaturation for 1 min at 94'C, followed by annealing at 60'C for 2 min, with a decrease in temperature by 0.8'C per cycle and extension at 72'C for 3 min. This pattern was repeated for a total of 10 cycles resulting in a final annealing temperature of 52'C. Denaturation step was for 1 min at 94'C, followed by annealing at 52'C for 2 min and extension at 72'C for 3 min. This pattern

was repeated for a total of 30 cycles with a final extension time of 7 min at 72'C in the last cycle. PCR products were cloned into the PCRII vector (Invitogen) and sequenced. The full-length TPH cDNA sequence was subcloned into the prokaryotic expression vector pET-15b (Novagen) for high level expression of TPH in *Escherichia coli*. Reverse-transcribed *C. elegans* cDNA was subject to 30 cycles of PCR using primers that targeted the beginning and the end of the predicted coding sequence of CeTPH. Enzyme restriction sites NdeI and BamHI were incorporated at the 5' end of the sense and antisense primers, respectively, to facilitate further subcloning into the expression vector. The final PCR product (1,582 bp) was gel-purified, digested with NdeI and BamHI and ligated to the prokaryotic expression vector pET-15b (Novagen) that had been linearized by the same two restriction enzymes. The construct in pET- 15b was confirmed by DNA sequencing of the entire insert and used to transform *E. coli* host strain BL_{21} (DE_3) pLys (Novagen), according to standard protocols (Sambrook et al, 1989).

Expression of CeTPH in *E. coli*

Transformed cultures of *E. coli* {BL_{21} (DE_3) pLys} cells were grown in Lb-ampicillin / chlorempheniçol medium to an OD of ~0.6-1.0 and induced with 1mM isopropyl β-D-thiogalactopyranoside (IPTG) for 2 h at 37°C with constant shaking. Ferrous sulfate (0.1mM) was added to expressing cultures to ensure that iron concentrations were high enough to be incorporated into the recombinant enzyme for subsequent measurements of enzyme activity (Wang et al, 1991). Following induction, the cells were pelleted by centrifugation for 10 min at 5,000g and 4°C and washed once in a buffer saline (pH 8.0) before freezing at -80°C.

For extraction of CeTPH protein, pellets of 25-ml induced bacterial cultures were thawed and resuspended in 2.5 ml of sonication buffer (pH 7.5) containing 50 mM Tris, 0.2% Tween 20, 10 % glycerol, and a cocktail of protease inhibitors (leupeptin, 5μg/ml; aprotinin, 5μg/ml; and phenylmethylsulfonyl fluoride, 1 mM). Cells were subjected to two cycles of rapid freezing and

thawing to promote cell lysis by the resident T7 lysozyme and then sonicated through five cycles of 10-s pulses separated by cooling periods of 30-s on ice. Cell lysates were centrifuged at 12,000g for 15 min and 4°C. The resulting pellet was resuspended in 2.5 ml of the same buffer, sonicated and centrifuged as described above. The two supernatant fractions were designated S1 and S2.

TPH assay

Tryptophan hydroxylase activity was measured by the tritiated water release assay (Nagatsu et al, 1964; Reinhard et al, 1986) with the modifications described by Ribeiro et al (1991). The assay was carried out in a total volume of 100 µl of 50 mM HEPES buffer (pH 7.0), 0.4 mM NADH (Sigma), 10 mU dihydropteridine reductase, 0.2 mg/ml catalase (Boehringer Mannheim), 50 µM ferrous sulfate, 400,000 cpm of ^{3}H-tryptophan and crude lysate fractions, S1 or S2 (~20µg of protein). The reaction was initiated by addition of 250 µM (6R)-5,6,7,8, tetrahydrobiopterin (BH4; Research Biochemicals International), unless otherwise stated. Samples were incubated for 10 min at 37°C. The reaction was terminated by addition of 1 ml of activated charcoal [7.5 % (wt/vol)] to each sample and following centrifugation at 1,000g for 10 min, aliquots of the supernatant containing [^{3}H]OH were radioassayed in 10 ml scintillation cocktail.

Computer analysis

Nucleic acid and protein sequences were analyzed and aligned using the MacVector Sequence Analysis version 6.0 software package (Oxford Molecular). Oligonucleotide primers for DNA sequencing and PCR were designed using Oligo 4.0 software (NBI, Plymouth, MN, U.S.A.). Protein sequences were compared to sequence data bases at the National Center for Biotechnology using BLAST software for protein and nucleotide similarities.

Other methods

Protein concentrations were measured by the Bradford method (1976), using the Bio-Rad protein assay kit and bovine serum albumin as a standard. Reducing SDS-polyacrylamide gel electrophoresis was performed according to the specifications of Laemmli (1970) using precast 10% acrylamide gels from Novex. For Western blot analysis of CeTPH, aliquots of crude lysate fractions were electrophoresed , transferred onto nitrocellulose membranes (Sambrooke et al, 1989), and reacted with a sheep polyclonal antibody (1:500 dilution) raised against recombinant rabbit tryptophan hydroxylase (Chemicon). A peroxidase-conjugated rabbit anti-sheep IgG (Pierce) was used as the secondary antibody (1:1,000 dilution).

Results

Isolation of CeTPH cDNA clone

We employed the Polymerase Chain Reaction (PCR) method to amplify a TPH cDNA from *C. elegans* tissue. The full-length tryptophan hydroxylase cDNA, CeTPH, was cloned from *C. elegans* by RT-PCR, using sequence information derived form the *C. elegans* genome database (Cosmid number ZK 1290). Earlier efforts to amplify a full-length *C. elegans* TPH clone by the use of RT-PCR (not described in Materials and Methods) gave a product size within the expected range: however, when this product was cloned and sequenced, 5 of the predicted 11 introns were found to be present which introduced several stop codons in the reading frame. Three separate clones of *C. elegans* TPH were sequenced and all were found to contain the same 5 introns (data not shown). Therefore an alternative RT-PCR strategy was designed. Since the majority of nematode pre-mRNA is known to acquire a SL sequence, we decided to design an oligonucleotide primer corresponding to published sequence of the most prevalent spliced-leader sequence in nematodes. The SL primer (PR93) and reverse primer PR50 were used to amplify the full-length coding sequence of TPH giving a ~1.6 Kb product (Fig.3).

A comparison of the CeTPH cDNA with the predicted genomic sequence (Cosmid number ZK 1290) revealed that the CeTPH gene is approximately 2.7 kb and consists of 12 exons separated by 11 introns (Fig. 2). The mature CeTPH transcript carries a spliced-leader, SL1, sequence (GGTTTAATTACCCAAGTTTGAG) which is trans-spliced onto exon 2. A continuous open reading frame begins at the start codon residing at the beginning of exon 2 and runs through to the end of available sequence, resulting in a 1582 bp stretch. A consensus polyadenylation site (AATAAA) is present 188 bp downstream of the stop codon (Fig. 2).

Analysis of the predicted CeTPH protein sequence

CeTPH encodes a protein of 526 amino acids with a predicted molecular mass of ~ 62 KDa. Protein subsequence analyses revealed that CeTPH contains two consensus sites for phosphorylation by Ca^{+2}/calmodulin-dependent protein kinase of type II (CaM-PKII) (amino acid positions Ser^{43} and Ser^{69}) and two Protein Kinase C (PKC) phosphorylation sites at serine position 88 and threonine position 63 (Fig. 4). BLAST analysis of the predicted protein sequence shows that CeTPH is most closely related to tryptophan hydroxylase from other species. Based on pairwise protein alignments, CeTPH shares identity with tryptophan hydroxylase from *Schistosoma mansoni* (45%), *Drosophila* (36%), rat (41%), mouse (41%), rabbit (39%) and human (40%). The degree of conservation is greatest in the midregion sequence, the predicted catalytic domain, (amino acid positions 181-377), where homology values relative to hydroxylases from the above mentioned species increases to 68-69%. A predicted protein alignment of CeTPH with other known tryptophan hydroxylase sequences is shown in Fig. 5. The alignment identified four cysteine residues (CeTPH amino acid positions 192, 264, 278 and 326), which are conserved in other species and are believed to play an important role in catalysis (Nagatsu and Ichinose, 1991; Ramsey et al, 1995; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996). The iron-binding site of the enzyme, consisting of two histidines and a glutamate residue (Ramsey et al, 1995; Goodwill et al, 1997) is present in CeTPH (His 346, His 351 and Glu391). The highly conserved PEPD-CHELLGHVP motif of all aromatic amino acid hydroxylases is present in CeTPH except that the highly conserved cysteine residue is replaced with a valine and one of the conserved leucine residues and the conserved valine are replaced with methionines.

Expression of CeTPH in *E. coli*

To establish if CeTPH encodes a functional tryptophan hydroxylase, the predicted coding sequence of the cDNA was amplified from reverse-transcribed *C. elegans* cDNA and subcloned into the bacterial expression system pET 15b. The resulting construct was used to transform *E. coli* strain BL_{21} (DE_3) pLys for subsequent induction of CeTPH protein. Previous studies have

shown that *E. coli* is a suitable system for expression of recombinant mammalian tryptophan hydroxylase, provided that the bacterial cultures are supplemented with iron, which is limiting in the bacterium (Tipper et al, 1994). Iron is coordinated to two histidines and a glutamate residue in the enzyme's iron-binding site (Ramsey et al, 1995; Goodwill et al, 1997) and is required for functional expression of the enzyme. In the present study, iron was added to expressing cultures of *E. coli*, and the conditions for expressing CeTPH protein were the same as those established for the mammalian enzyme (Tipper et al, 1994).

Transformed *E. coli* cells were induced with isopropyl β-$_D$-thiogalactopyranoside (IPTG) and produced considerable amounts of protein. The soluble (S1) and the insoluble (pellet) protein fractions were analysed by SDS-PAGE followed by Western immunoblotting with an anti-rabbit TPH polyclonal antibody (Fig. 6). The results identified a Western positive protein band of the correct size (~ 62KDa) which is present in the soluble S1 fraction but is mainly enriched in the pellet. This is similar to mammalian TPH which also forms insoluble aggregates (inclusion bodies) when expressed in E. coli (Tipper et al, 1994). No Western positive band was detected in bacterial lysates transfected with pET vector only (data not shown).

TPH assay

Aliquots (0.1 mg protein) of soluble (S1) extracts containing CeTPH were assayed for TPH activity (Tipper et al, 1994) in the presence and absence of the cofactor BH_4 (250 μM). A similarly prepared S1 extract of bacterially-expressed rabbit TPH was also tested for comparison. Expression of CeTPH in *E. coli* in the presence of BH_4 had a final specific activity of 0.80 nmol/min/mg which is comparable to that of rabbit TPH. Both enzymes have the same absolute requirement for the tetrahydrobiopterin cofactor, BH_4, as the mammalian enzyme (Table 1).

DISCUSSION

The hydroxylase described in this study is one of the most primitive forms of the enzyme ever cloned or characterized at the molecular level. With the exception of TPH sequence from the parasitic helminth *S. mansoni* and *Drosophila* (Neckameyer and White, 1992), all other tryptophan hydroxylase sequences available have been from vertebrates, for the most part mammalian species (Grenett et al, 1987; Tipper et al, 1994). Comparisons between the *C. elegans* hydroxylase and TPH in *S. mansoni*, *Drosophila* and vertebrate counterparts reveal numerous similarities and some interesting differences. Based on sequence analyses, CeTPH appears to be most closely related to *S. mansoni* TPH (45% identity) and more closely related to the mammalian enzymes, particularly human and mouse (40% and 41% respectively), than the lower vertebrate (xenopus) and invertebrate (*Drosophila*) enzyme species. Among all different species of tryptophan hydroxylase, including CeTPH sequence, the degree of conservation is greatest in the C-terminal half to two-thirds of the protein, the predicted catalytic domain (Yang and Kaufman, 1994). Several of the signature peptides of tryptophan hydroxylase are present in this region of CeTPH, including the previously described iron-binding site (Kuhn et al, 1980) and the conserved cysteines of the catalytic domain. It is interesting to note that one of the most highly conserved cysteines in vertebrates, a residue that is part of the motif characteristic in all vertebrate aromatic amino acid hydroxylases (PEPD-CHELLGHVP) (Nagatsu and Ichinose, 1991; Kaufman and Ribeiro, 1996), is replaced in CeTPH by another nonpolar amino acid residue, valine. Although the significance of this difference is undetermined, the finding nonetheless suggests that this residue is not critical for enzyme activity. Interestingly, this same cysteine residue in the TH aromatic amino acid hydroxylase is similarly replaced with an isoleucine in the two invertebrate sequences, *S. mansoni* and *Drosophila*.

The boundary between the regulatory and catalytic domains in TPH and TH species is defined by a stretch of highly conserved 5 amino acids (Val-Pro-Try-Phe-Pro or VPWFP) (Grennett et al,

1987). In CeTPH, this boundary is in the vicinity of amino acid 181. Curiously, whereas in all the different species of TPH observed in Fig. 5 this pentad motif is entirely conserved. In CeTPH only the last 3 amino acids are conserved (WFP). Similarly, the TH sequence in *Drosophila*, quail and bovine show sequence variation in the first 2 amino acids of the pentad and conservation of the following WFP amino stretch. Tyrosine hydroxylase in *S. mansoni* has the lowest degree of conservation in this region, with only the W*P residues remaining. Taken together, these observations suggest that the VP residues in the pentad sequence are not necessary in defining the boundary of the highly homologous C-domain. On the other hand, the last W*P residues may be important in defining the catalytic/regulatory border in all TPH and TH species.

Based on sequence analysis, CeTPH diverges from other enzyme forms at both its N-terminal and C-terminal ends. CeTPH shows divergence at the C-terminus, within the last 50 amino acid residues. Compared with mammalian and mouse/rat/rabbit hydroxylases, CeTPH contains in total ~50 additional N-terminal amino acid residues and its following amino acid sequence up to position 106 shows very little homology with other forms of the enzyme. On the other hand, comparison between the *C. elegans* hydroxylase and TPH from other species more closely related in evolutionary terms (Xenopus, *Drosophila* and *S. mansoni)*, reveals a more similar protein sequence pattern in that all hydroxylases have less protein sequence divergence at both the amino and carboxyl terminal ends. The evolutionary divergence observed across TPH sequences in different species appears to be targeted towards the N-terminal regulatory domain. Similarly, this notion is observed among members of the hydroxylase family (TPH, TH and PH) where evolutionary divergence is directed towards the amino terminal region. The N-terminus of all AAAH's correspond to the regulatory domain of the enzyme (Kaufman and Ribeiro, 1996; Yang and Kaufman, 1994; Kumer et al, 1997), thus the additional sequence in this area in CeTPH suggests that this enzyme has a different functional organization and may be subjected to different forms of regulation.

Protein subsequence analysis reveals that CeTPH contains 2 consensus sites for phosphorylation by CaM-PKII (Ser^{43}, Ser^{69}) and 2 PKC phosphorylation sites at Ser^{88} and Thr^{63}, thereby suggesting that *C. elegans* hydroxylase may be regulated by multisite phosphorylation *in vivo*. In mammals, TPH is phosphorylated by PKA and CaM-PKII (Ehret et al, 1989; Vrana et al, 1994a; Johansen et al, 1995, 1996). Serine-58 serves as the phosphoryl acceptor for PKA (Kuhn et al, 1997; Kumer et al, 1997). To date, the phosphorylation site for CaM-PKII has not been identified; however two potential candidates exist; Ser^{260} and Ser^{443} (Darmon et al, 1988). Interestingly, these two sites are within the catalytic domain. If these sites do in fact act as kinase substrate sites, TPH would be the only member in the AAAH superfamily to be phosphorylated within its C-domain. Mammalian TH is regulated through multisite phosphorylation by CaM-PKII, PKA and PKC at four highly conserved serine or threonine residues all located in the N-terminus of the enzyme. Each phosphorylation event leads to specific changes in the kinetic behaviour of the enzyme resulting in an overall increase in activity (Zigmond et al, 1989; Kaufman and Ribeiro, 1996). Whereas the mammalian phosphorylation sites in TPH may be found not only within the N-terminus but within the C-terminus as well, the four potential phosphorylation sites of CeTPH are clustered at the amino end of the enzyme. Interestingly, as detailed above, this is similar to the functional organization of the mammalian TH N-terminus. Just as CeTPH has a potential variation to the mammalian phosphorylation scheme, so TH in *S. mansoni* has a variation to its mammalian counterpart's phosphorylation clustering and has potential phosphorylation sites in both regulatory and catalytic domains (Hamdan and Ribeiro, 1998). In addition, the PKA phosphorylation site is not conserved in CeTPH. If CeTPH activity is mediated through phosphorylation then the mechanisms of phosphorylation and the corresponding changes in enzyme kinetics are likely to be unique to this organism.

Expression of CeTPH in *E. coli* produced an enzyme that has TPH activity and an absolute requirement for the reduced pterin cofactor, BH_4. BH_4 dependency is a characteristic of all known AAAH's. Thus, CeTPH represents a novel member of this important enzyme family.

Immunoblot analysis of expressed *C. elegans* TPH with an anti-rabbit TPH polyclonal antibody identified a band of the predicted size (62 KDa). Although the majority of CeTPH protein resides in the pellet fraction in the form of insoluble aggregates, soluble CeTPH has a level of enzyme activity in the presence of BH_4 that is slightly lower but nonetheless comparable to that of rabbit TPH when expressed in *E. coli* cells (0.80 nmol/min/mg of *C. elegans* protein, 1.51 nmol/min/mg of rabbit protein) (Table 1). Bacteria have a tendency to sequester overexpressed proteins in the form of insoluble inclusion bodies. In the case of TPH expression systems, it has been estimated that 70-80 % of the expressed protein is aggregated in this manner (Vrana et al, 1994a). Although, it is possible to resolubilize inclusion bodies, the treatments are harsh and denaturing and proper re-folding of the protein to give a functional enzyme is highly unlikely.

Sequence analysis revealed that CeTPH is trans-spliced at its 5' end to a conserved nematode spliced leader sequence (SL1) (Blumenthal and Steward, 1997). Trans-splicing of RNAs to an SL sequence is a well known phenomenon in lower invertebrates, including nematodes, where the SL is thought to play a role in the stability of the transcript and the binding to the ribosomal complex, as well as the processing of polycistronic transcripts (Blumenthal and Steward, 1997). In the case of CeTPH, the SL sequence is trans-spliced onto an internal acceptor site, specifically the 5' end of exon 2, which generates a truncated mature CeTPH transcript. CeTPH transcripts that included exon 1 were not trans-spliced to an SL and also contained several intron sequences, an indication of improper RNA processing (data not shown). Thus, the trans-splicing event appears to select for a shorter, properly processed TPH species. Similar trans-splicing of SL sequences onto internal acceptor sites of immature transcripts has been reported for nematodes and other invertebrates (Davis and Stretton, 1995; Blumenthal and Steward, 1997).

In summary, the present study has cloned a novel cDNA from the free-living *C. elegans*. Sequence analysis of the predicted coding sequence identified high homology with tryptophan hydroxylase, (TPH), the rate-limiting enzyme in the biosynthesis of the neurotransmitter,

serotonin. In addition, CeTPH has tryptophan hydroxylase activity and also shows an absolute dependency for the cofactor, BH4, just as shown previously for the mammalian enzyme. With *C. elegans* we have a model system in which it should be possible to study the role of serotonin in evolutionary related parasitic helminths, and any knowledge gained may be useful in the development of nematocidal and antihelminthic drugs. In addition, any observations made of the *C. elegans* system, will provide insight into the molecular mechanisms of serotonergic neurons in general and might be useful in the synthesis of more effective drugs for the treatment of a number of mental dysfunctions and neuro-degenerative diseases. Additional research is needed to further characterize the kinetic and regulatory properties of this novel *C. elegans* enzyme.

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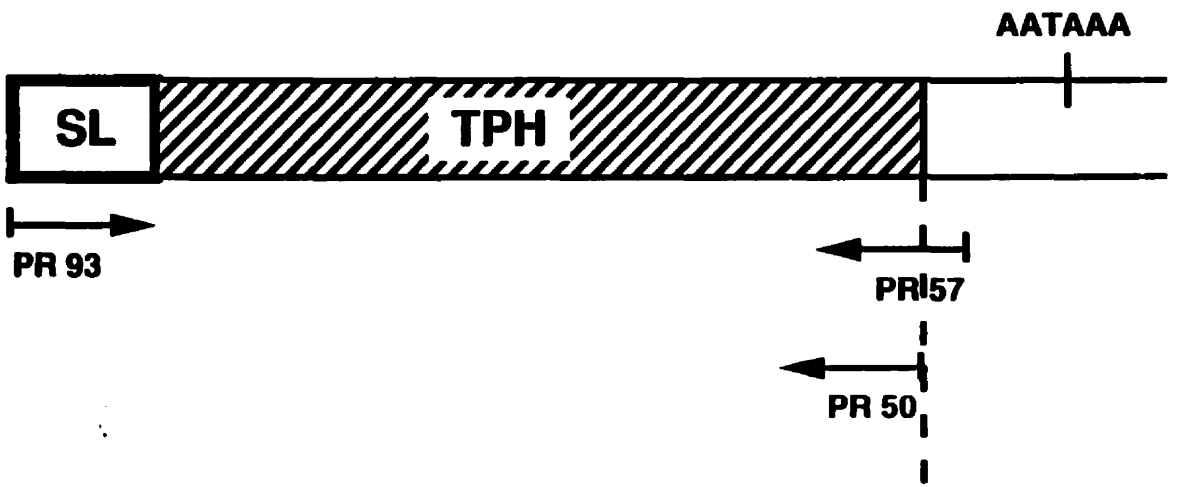

Figure 1. Schematic representation of the predicted genomic *C. elegans* TPH sequence. PCR primers used in the reverse transcription and amplification of *C. elegans* TPH cDNA are depicted below.

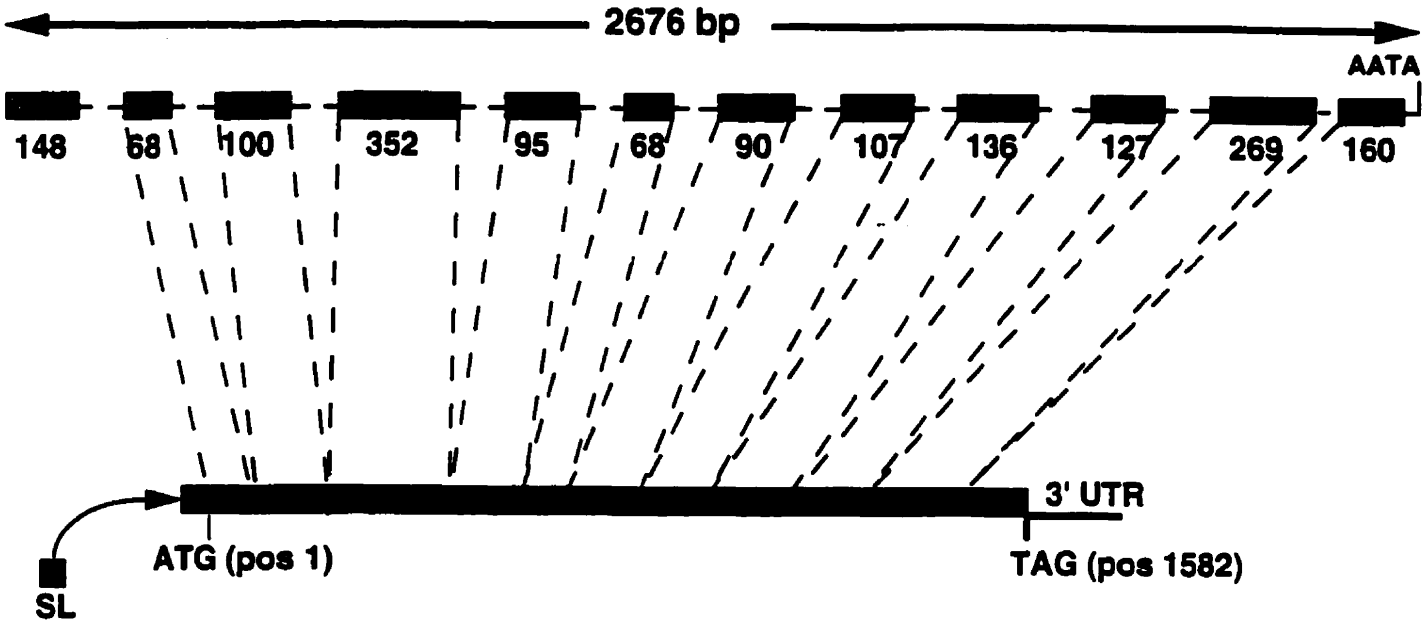

Figure 2. The full-length tryptophan hydroxylase cDNA, TPHCe, was cloned from *C. elegans* by RT-PCR, using sequence information derived from the *C. elegans* genome database (Cosmid ZK 1290). A comparison of the TPHCe cDNA and predicted genomic sequences revealed that the TPHCe gene is approximately 2.7 Kb and consists of 12 exons separated by 11 introns; their respective lengths are shown. The mature TPHCe transcript carries a spliced-leader, SL1, sequence (GGTTTAATTACCCAAGTTTGAG) which is trans-spliced onto exon 2. A continuous open reading frame begins at the start codon residing at the beginning of exon 2 and runs through to the end of available sequence, resulting in a 1582 bp stretch that encodes a protein of 526 amino acids. A consensus polyadenylation site (AATAAA) is present 188 bp downstream of the stop codon.

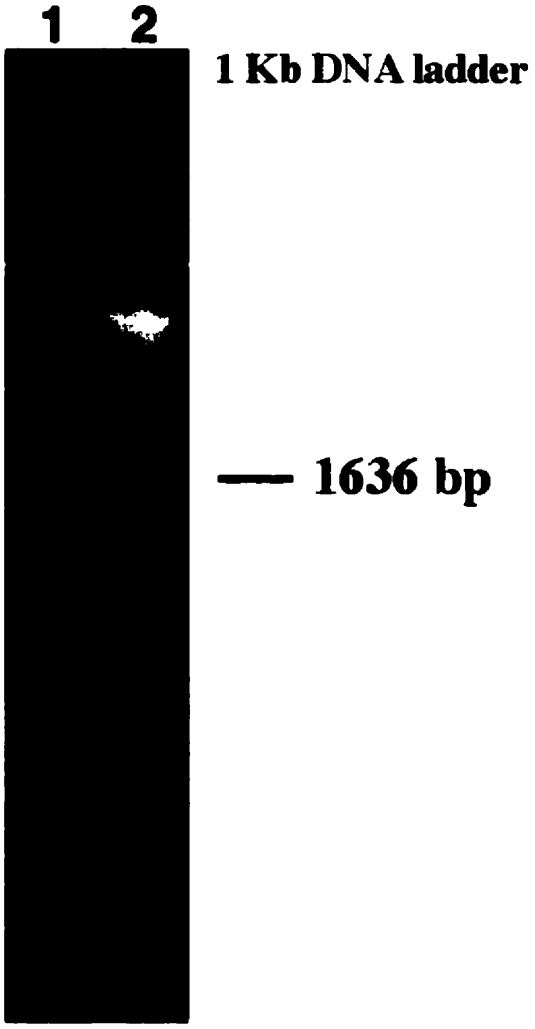

Figure 3. Amplification of the full-length coding sequence of TPH (~1590bp) from *C. elegans* DNA by PR93(SL) and by PR50 (lane 1) as shown on a 1% TBE agarose gel. The molecular weight standard is measured in kilodaltons (lane 2).

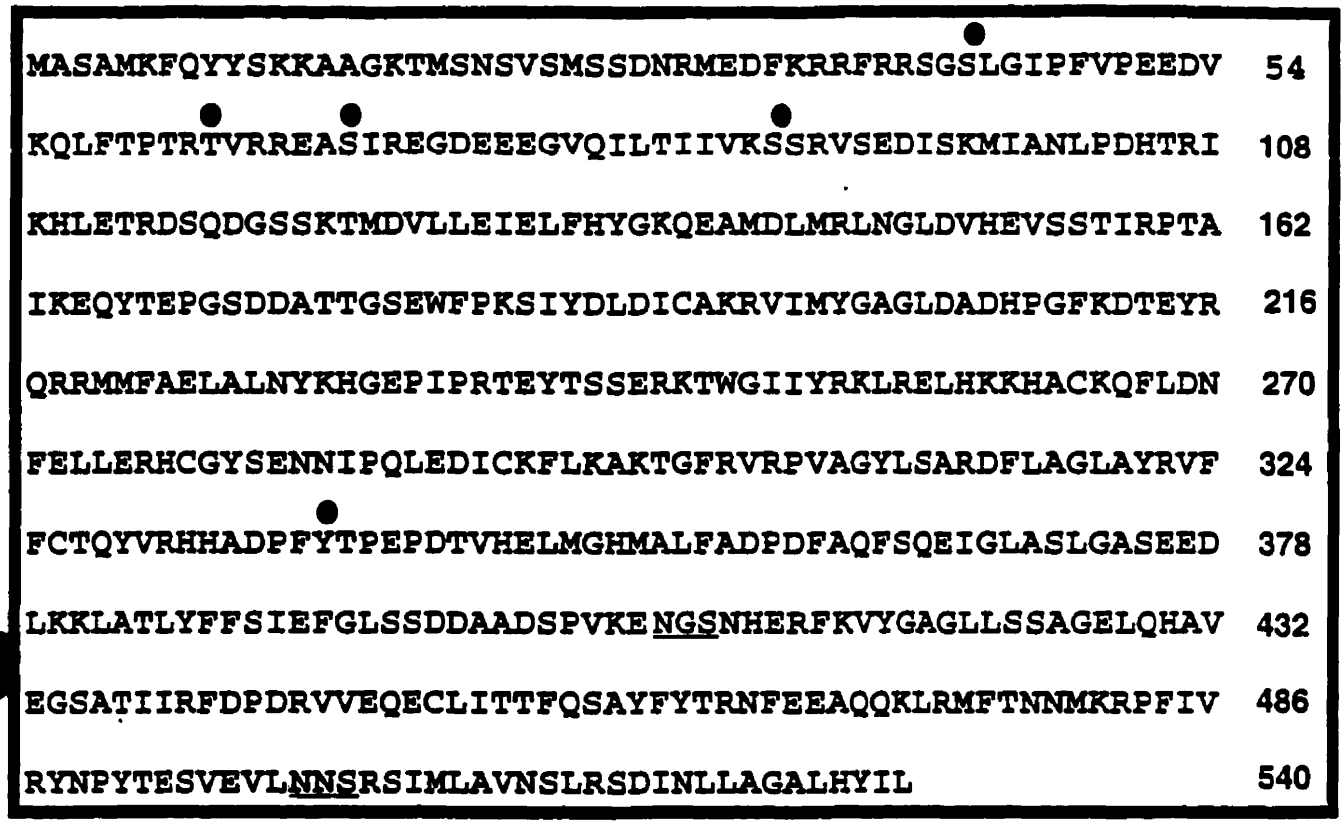

Figure 4. Deduced amino acid sequence of CeTPH. CeTPH encodes a predicted protein of 526 amino acids with a calculated molecular weight of ≈62 kDa. Protein subsequence analysis reveals that CeTPH contains consensus sites for phosphorylation by Cam-PKII (amino acid positions Ser^{43} and Ser^{69}), PKC (Ser^{88} and Thr^{63}) and tyrosine kinase (Tyr^{331}). Potential phosphorylation sites are marked with solid circles. Two N-glycosylation sites are underlined.

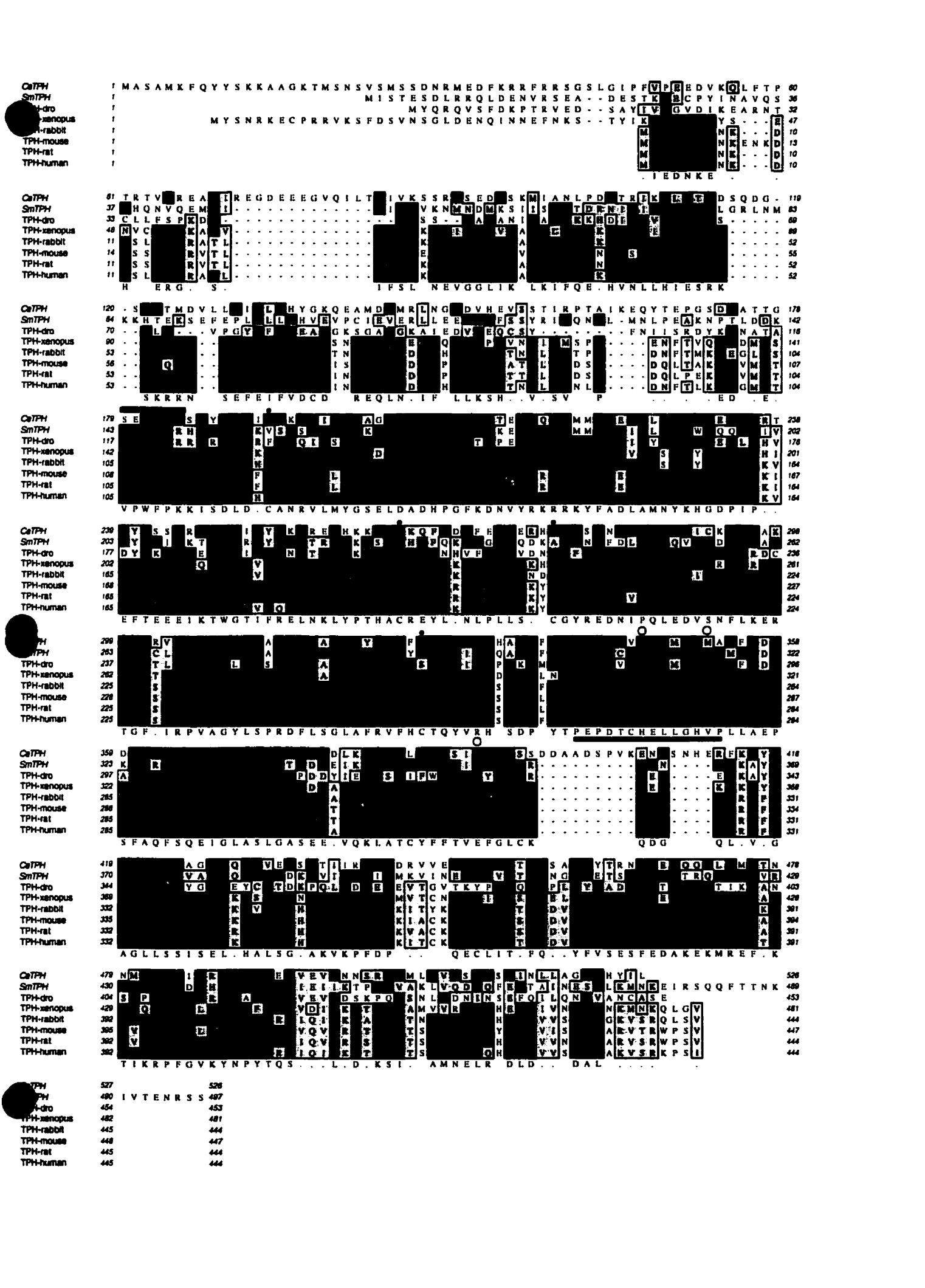
CaTPH
SmTPH
TPH-dro
TPH-xenopus
TPH-rabbit
TPH-mouse
TPH-rat
TPH-human

Figure 5. The predicted protein alignment of CeTPH with other known tryptophan hydroxylase is shown. The filled circles in the sequence mark four conserved cysteine residues (CeTPH amino acid positions 192, 264, 278 and 326), which are thought to play an important role in catalysis (Nagatsu and Ichinose, 1991; Ramsey et al, 1995; Kaufman and Ribeiro, 1996; Kumer and Vrana, 1996). The iron-binding site of the enzyme, consisting of two histidines and a glutamate residue (Ramsey et al, 1995; Goodwill et al, 1997), is present in CeTPH (CeTPH His^{346}, His^{351} and Glu^{391}) and is denoted by open circles. The highly conserved PEPD-CHELLGHVP and VPWFP motif of all aromatic amino acid hydroxylases are underlined and overlined in bold. respectively.

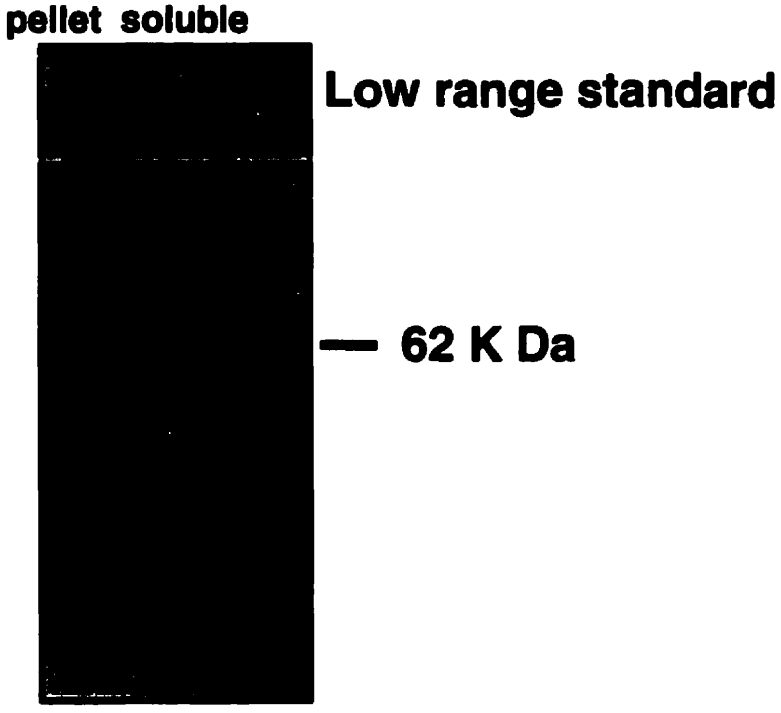

Figure 6. Immunoblot analysis of expressed *C. elegans* TPH. Induced bacterial lysates were centrifuged at 12,000g and the resulting soluble (S1) and insoluble (pellet) protein fractions were analyzed by SDS-PAGE followed by Western immunoblotting with an anti-rabbit TPH Polyclonal antibody. The results identified a Western positive protein band of the correct size (appr. 62 KDa) which is present in the soluble S1 fraction but is mainly enriched in the pellet. No Western positive band could be detected in bacterial lysates transfected with pet vector only (data not shown).

Table 1: Measurement of CeTPH activity in the presence and absence of cofactor, BH_4

	+ BH_4 (nmol/min/mg of protein)	- BH_4 (pmol/min/mg of protein)
TPHCe	0.80	0
RABBIT TPH	1.51	0

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Statement of Continuity

In the previous study (Chapter II) we have cloned by RT-PCR, a full-length *C. elegans* cDNA (CeTPH) homologous to TPH from other species. CeTPH has enzymatic activity comparable to that of its mammalian counterpart and shows an absolute requirement for the pterin cofactor, tetrahydrobiopterin, BH_4. BH_4 dependency is a characteristic of all known aromatic amino acid hydroxylases. Thus, CeTPH represents a novel member of this important enzyme family.

By way of characterizing further CeTPH it was of interest to examine the *in situ* distribution of TPH within intact adult worms and to compare CeTPH staining patterns to the known localization of serotonin in *C. elegans*. In the following study (Chapter III) we examine the localization of TPH in whole mounts of *C. elegans* by immunofluorescence using a polyclonal antibody against TPH.

CHAPTER III

Localization of tryptophan hydroxylase in whole mounts of *Caenorhabditis elegans*

Suzanne D. Hill and Paula Ribeiro

(In preparation)

Abstract

Tryptophan hydroxylase (TPH) is the initial and rate-limiting enzyme in the biosynthesis of the biogenic amine serotonin (5-HT). It has been well established that 5-HT is present and is biologically active in the tissues of *C. elegans* and, more recently, it has been demonstrated that TPH is present in *C. elegans* as well. TPH immunoreactivity in whole mounts of *C. elegans* was studied by fluorescence microscopy, using an antibody raised against TPH. TPH-like immunofluorescence is seen in the head region, including the nerve ring and the area of the pharyngeal nervous system, consistent with the location of previously identified 5-HT neurons. Fluorescence was also observed in the uterine and vulva region, and in several discrete cells of the hermaphrodite tail which may be associated with the posterior dorsorectal and preanal ganglion of the posterior nervous system.

Introduction

Caenorhabditis elegans is a free-living soil nematode of approximately1.5 mm in length. It feeds primarily on bacteria and reproduces with a life-cycle of about 3 days under optimal conditions. There are two sexes, males and hermaphrodites. Hermaphrodites produce both oocytes and sperm and are self-fertilizing. Males, which spontaneously arise at a low frequency, can fertilize hermaphrodites (Figure 1). The *C. elegans* nervous system is extremely simple and has been well characterized by electron micrographs of serial sections. It contains only 302 neurons, each with a precisely determined and invariant position (White at el, 1986). Most of the cells in the nervous system are found surrounding the pharynx, along the ventral midline and in the tail. Processes from these neurons form a ring around the pharynx (nerve cord), emanate from the nerve ring on the ventral side and run the length of the body (ventral cord). The anterior end of the ventral cord runs into the retrovesicular ganglion, a small ganglion of mainly motor neurons, and the posterior end of the cord terminates in the preanal ganglion where the outputs of some of the posteriorly located sensory receptors are integrated (Figure 2). Interestingly, the *C. elegans* nervous system can be subdivided into two almost independent nervous systems: the extrapharyngeal nervous system, which in the hermaphrodite consists of 282 neurons of 104 anatomical types (White et al, 1986) and the pharyngeal nervous system, which consists of 20 neurons of 14 types and is thought to modulate feeding (Albertson and Thomson, 1976). The pharynx is located at the anterior end of the animal and opens to the outside by the buccal cavity. The procorpus and the metacorpus together make up the structure called the corpus. The terminal bulb connects to the intestine by the pharyngeal-intestinal valve. The T-shaped grinder is located in the terminal bulb and the isthmus connects the two bulbs of the pharynx (Fig. 3).

Serotonergic neurons in *C. elegans* have been identified by formaldehyde-induced fluorescence (Horvitz et al, 1982) and by anti-serotonin immunostaining (Desai et al, 1988). A pair of

neurosecretory motor neurons (NSM) have been identified in the pharynx (Albertson and Thomson, 1976; Horvitz et al, 1982) that are believed to be involved in modulating pharyngeal pumping. Worms eat by pumping in bacteria, processing these food particles through the grinder in the terminal bulb, then passing the debris back to the intestine. The NSM cells lie at the outside edge of the pharynx and have their neuronal endings in the metacorpus. It is thought that the NSM cells secrete a humoral factor into the pseudocoelom when their sensory endings detect food in the lumen causing contraction of the isthmus muscles. By changing the tone of these muscles, food is pulled back to the grinder and pulled into the corpus (Fig. 3) (Albertson and Thomson, 1976). In addition, antisera raised against 5-HT labeled six neurons in the head of both sexes (cited in Horvitz et al, 1982) and five cells in the ventral cord of the male tail that are necessary for normal turning behavior during mating (CP cells) (Loer and Kenyon, 1993). Males turn at approximately 10% or 90% hermaphrodite body length if they have not yet located the vulva. They must recognize the approaching end of the hermaphrodite and turn in a coordinated and well-timed fashion, followed by relaxation of the turn. Exogenous supplies of 5-HT stimulates tail curling by causing contraction of sex-muscles (Loer and Kenyon, 1993). It is hypothesized that the CP neurons release 5-HT that serves to stimulate these sex- muscles. Interestingly, males without sex-muscles have been shown to still respond slightly to serotonin, indicating that another role for CP neurons exists (or other serotonergic neurons). In addition, CP-ablated males are not completely defective in turning, suggesting that there is another input to the sex-muscles (Loer and Kenyon, 1993). Similarly, staining is seen in the two HSN motor neurons of the hermaphrodite that control egg-laying at the vulva muscles. The vulva and uterine muscles are predominately innervated by the HSN motor neurons. Laser ablation experiments by Trent and colleagues have shown that the HSN neurons are essential for egg-laying (Trent et al, 1983) as well, Horvitz and colleagues have used various pharmacological agents , including, acetylcholine agonists, 5-HT analogues and an octopamine blocking agent to stimulate egg laying (Horvitz et al, 1982).

In vertebrates, serotonin (5-HT, 5-hydroxytryptamine), is synthesized from tryptophan in two steps: hydroxylation of the enzyme by tryptophan hydroxylase (TPH) and decarboxylation by AAAD. 5-HT has also been reported in the tissues of a variety of free-living and parasitic platyhelminths, where 5-HT is known to have numerous hormone-like effects on the stimulation of glycogenolysis, glycolysis and activation of adenylate cyclase (Webb and Mizukawa, 1985). Although it is well established that 5-HT is present in the tissues of helminths, the long standing belief is that TPH is absent at least in some parasites. For example, *S. mansoni* and *H. diminuta* are thought to obtain their source of 5-HT from their respective host (Bennett and Bueding, 1973; Mansour, 1979; Cho and Mettrick, 1982; Hillman, 1983). However, two reports by Ribeiro and Webb and one by Chadhuri, challenged that dogma by providing evidence of TPH activity in *H. diminuta*, and in *A. suum*, respectively. These studies suggested the presence of an endogenous biosynthetic pathway for the production of 5-HT (Chadhuri et al, 1988; Ribeiro and Webb, 1983, 1984), a finding which has since been reinforced by the recent discovery of a TPH-like gene in *S. mansoni* (preliminary results, unpublished finding). In *C. elegans*, exogenous 5-HT stimulates egg-laying and pharyngeal pumping and inhibits locomotion and defecation (Horvitz et al, 1982). Serotonin has also been shown to be involved in male-mating behavior (Loer and Kenyon, 1993), as detailed above. Since it is known that both 5-HT and, more recently, TPH are present and biologically active in *C. elegans*, we propose *C. elegans* as a useful model system for studies of helminth TPH. In the present study, the sensitive technique of immunofluorescence, with an antibody directed to TPH, was used to study the localization of TPH immunoreactive cells in whole mounts of *C. elegans*.

Materials and Methods

Preparation of *C. elegans* for Immunofluorescence

Wild-type *Caenorhabditis elegans* N_2 strain was cultured as described (Sulston and Hodgkin, 1988) on petri dishes containing nematode growth medium (NGM) agar preseeded with *Escherichia coli* strain OP50. Worms were gently washed off NGM plates with M9 buffer and pelleted by centrifugation (1, 000 rpm for 3.5 min). The worm pellets were rinsed in M9 buffer, pipetted onto a poly-L-lysine-coated slide (APES method, Sigma) and affixed to the slide by overlaying the drop with a coverslip. Slides were plunged into liquid nitrogen, the coverslip was immediately flipped off with a razor blade and the specimens were fixed with methanol and acetone. Specimens were fixed first in methanol for 2.5 min and then in acetone for 5 min (Sulston and Hodgkin, 1988). Fixatives were maintained at -20°C and fixation was performed on ice. After fixation the slides were either stored at -20°C in a desiccator or used immediately.

Immunofluorescence

For immunofluorescence studies, the slides were washed twice with Ab buffer (1 x PBS, 1% BSA, 0.5% Triton X-100, 1 mM EDTA, 0.05% sodium azide) dried and incubated with sheep anti-rabbit TPH polyclonal Ab (Chemicon) (10µg/ml) in Ab buffer (300 µl/slide) in a humidity chamber for 3 hours at room temperature. After three washes with Ab buffer (0.1% BSA), the first bound antibody was visualized by incubation with a 1:500 dilution of fluorescein-conjugated rabbit anti-goat IgG antibody (Chemicon) for 30 min at room temperature (300µl/slide). The slides were washed twice with Ab buffer (0.1% BSA), dried and mounted in DABCO solution (80% glycerol, 0.2 M Tris-HCl, pH 8.0, 2.5% 1,4-diazobicyclo-[2.2.2]-octane, Sigma) to prevent

photobleaching. Staining was examined on an epifluorescence microscopy (Nikon, Optiphot-2) equipped with a fluorescein filter. Photographs were taken on Kodak extrachrome ASA-400 color reversal film.

Results and Discussion

Localization of TPH protein in the *C. elegans* worm

We employed the immunofluorescence technique to study the distribution of tryptophan hydroxylase (TPH) in the tissues of wild-type N_2 strains of *Caenorhabditis elegans*. To visualize TPH in these worms a polyclonal sheep anti-rabbit TPH was used with an FITC-conjugated rabbit anti-goat IgG. We previously established that this antibody recognizes *C. elegans* TPH as seen from immunoblot analysis (Fig. 6, chapter I). Figure 4 shows fluorescence and corresponding Nomarski photomicrographs of a wild-type hermaphrodite stained with anti-TPH polyclonal antibody. The results show extensive fluorescence throughout the length of the worm in whole mounts incubated with anti-TPH antibody (Fig. 4b). A negative control incubated with secondary antibody alone did not show any appreciable background staining (Fig. 4c), suggesting that the fluorescence is antigen specific. Distinctive areas of immunofluorescence were seen in the anterior end of the wild-type hermaphrodite (Fig. 5), particularly in the area of the nerve ring and the pharyngeal nervous system. Though no discrete neuronal cell bodies could be identified, the presence of fluorescence in this anterior region is consistent with the location of previously described serotonin-containing neurons, including the serotonergic NSM neurons of the pharyngeal system (Albertson and Thomson, 1976), and several neurons (RIG, RIH) located in the area of the pharyngeal terminal bulb and isthmus. In addition, some animals exhibited immunofluorescence in the region of the amphids (Fig. 5), the sensory organs that project anteriorly towards the tip of the head region. TPH immunoreactivity in the amphids may be associated with amphid-related ADF sensory neurons, which are thought to contain serotonin (5-HT), (Rand and Nonet,1988).

Wild-type hermaphrodites showed several apparent immunoreactive cells in the posterior end and tail region (Fig. 6). Although the precise identity of these cells is unknown, their relative positions in the whole mount are consistent with those of preanal and dorsorectal ganglion neurons (PAG and DRG, respectfully), which constitute the posterior nervous system of the worm and are thought to modulate enteric muscle activity and defecation. 5-HT has been implicated in the regulation of defecation in the hermaphrodite (Thomas, 1990) but the source of this serotonin has not yet been identified. The finding of TPH-like immunoreactivity in the tail region raises the possibility that 5-HT may be produced and thus released from neuronal cells of the posterior nervous system. It is noteworthy that the tail-associated immunofluorescence shown in Figure 6 is unrelated to the well defined serotonergic CP neurons, which occur only in male worms. No adult males, typically a small proportion of the population, could be identified throughout these experiments.

The best characterized serotonergic neurons in *C. elegans* are the HSN neurons, whose cell bodies are located about midventrally in the region of the vulva. The activity of these neurons in the serotonergic regulation of vulva activity and egg-laying has been well established (Desai et al,1988). In addition, there is ample evidence that the HSN stain very strongly with antiserum raised against 5-HT, an indication that 5-HT is present, and therefore likely to be produced within these neurons. In the present study, we identified TPH-like immunoreactivity in the area of the vulva (Fig. 4). However, there is extensive and diffuse fluorescence in surrounding areas as well, particularly in the gonad and throughout the entire uterine region. This widespread fluorescence makes the identification of discrete immunoreactive cells in the vulva and other regions difficult.

The finding of TPH-like immunorectivity throughout the animal, both in neuronal structures and outside of the nervous system, is difficult to reconcile with earlier localization of 5-HT to only a few discrete neurons. One possible explanation is that serotonergic cells (ie. TPH-containing) may have a wider spread distribution than was previously believed. A second, perhaps more likely

explanation, however, is that the anti-TPH antibody used in the present study is cross-reacting with another structurally related protein, probably another member of the aromatic amino acid hydroxylase (AAAH) superfamily. In higher organisms, it has been well established that antibodies raised against TPH do not cross-react with cognate neuronal enzyme, tyrosine hydroxylase (TH). However, there may be some cross-reactivity with the third member of the AAAH family, phenylalanine hydroxylase (PAH) (Tipper et al, 1994). It is unknown, at present, if anti-TPH antibody cross reacts with *C. elegans* PAH, since this latter enzyme has not yet been identified in the worm. The immunoreactivity observed in the nerve ring and other apparent neuronal structures, particularly in the anterior end, is unlikely to be caused by cross-reactivity with PAH since this enzyme is not known to occur in nervous tissue. On the other hand, the extensive immunoreactivity associated with clearly non-neuronal regions, in particular the reproductive tract, may be due, at least in part, to a *C. elegans* form of PAH which is widely distributed in the worm. Additional, research is needed to clarify these results and to provide a more conclusive localization of TPH in *C. elegans*

Acknowledgments

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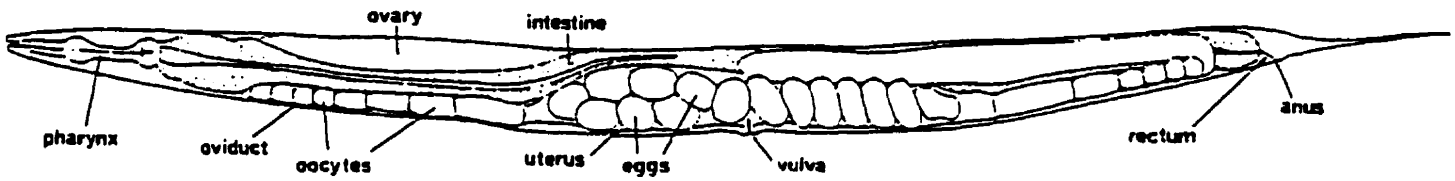

Figure 1. Major anatomical features of the adult hermaphrodite *C. elegans*. Taken from "The nematode *Caenorhabditis elegans*", 1988.

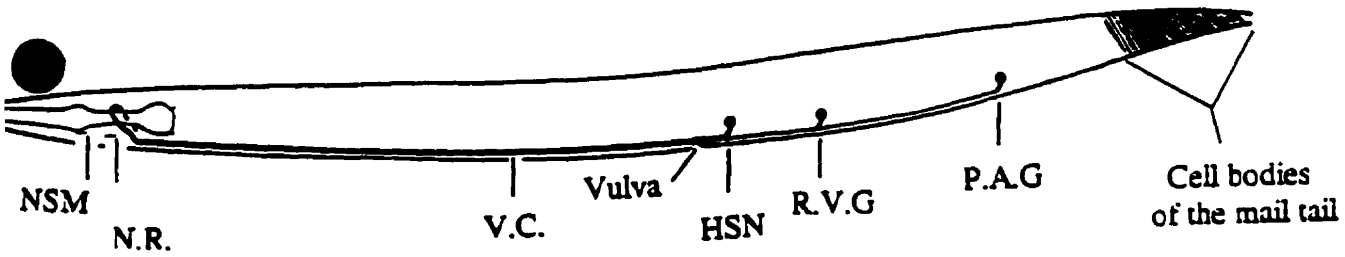

Figure 2. Major features of the nervous system of the adult *C. elegans*. N.R (nerve ring), NSM (neurosecretory motor neurons), V.C. (ventral cord), HSN (hermaphrodite specific neurons), R.V.G (retrovesicular ganglion), P.A.G (preanal ganglion).

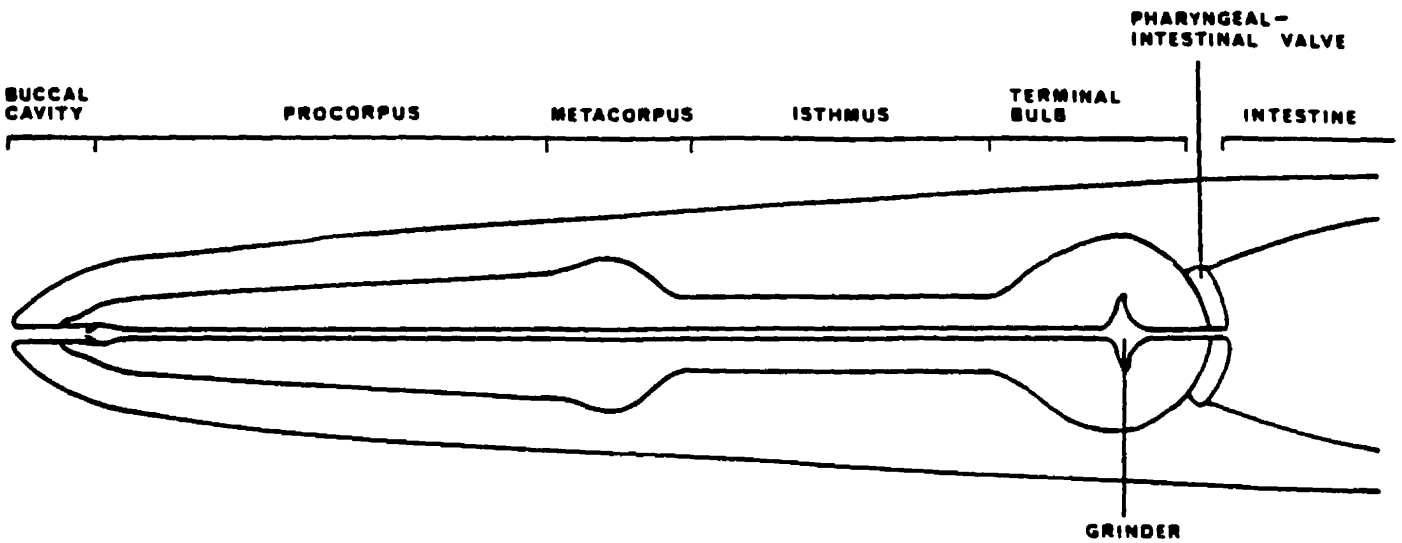

Figure 3. Principal regions of the pharynx. Taken from "The nematode *Caenorhabditis elegans*",1988.

Figure 4. Wild-type N_2 larval hermaphrodites at magnification 20X. **a**, **b**, Nomarski and fluorescence photomicrographs stained with antisera to TPH. **c**, Negative control incubated with secondary antibody only.

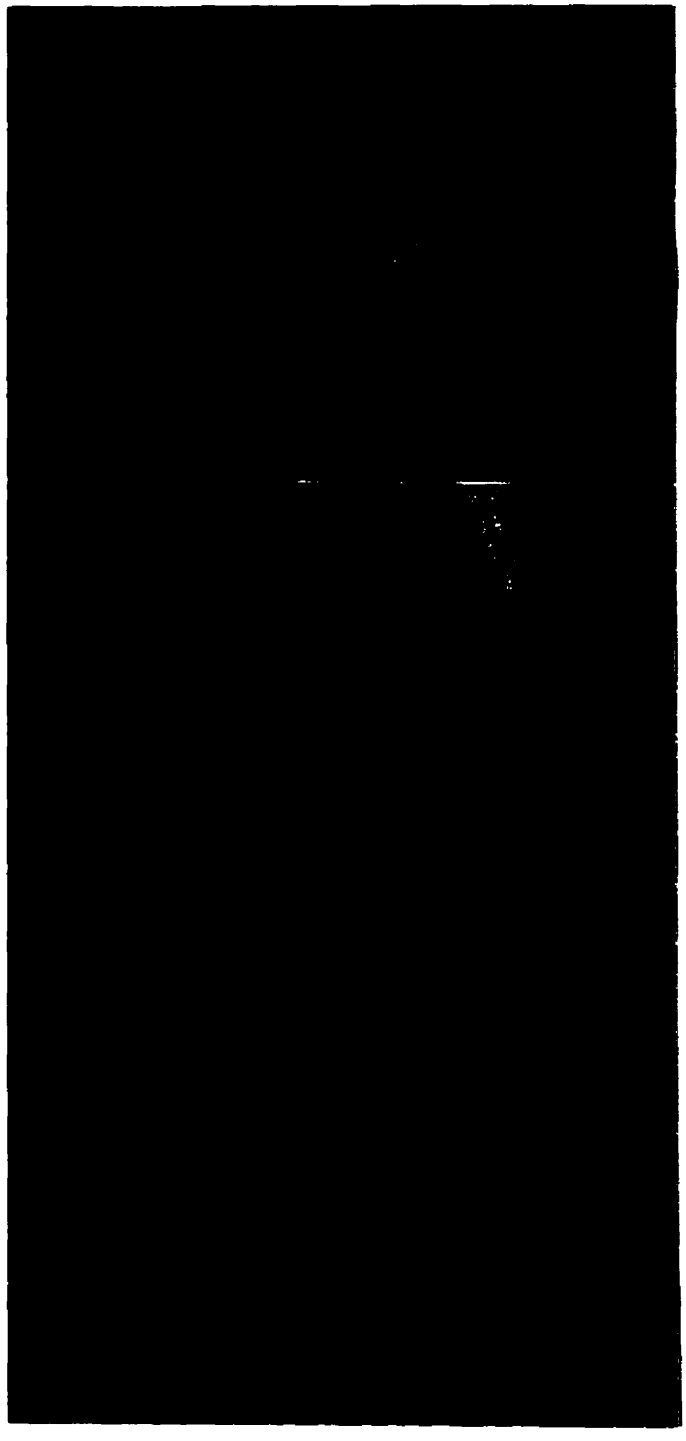

Figure 5. Anterior end of a wild-type N_2 adult hermaphrodite at magnification 20X. The arrows mark, from top to bottom, the nerve ring, pharyngeal nervous system and amphid neurons.

Figure 6. Posterior end of a wild-type N_2 adult hermaphrodite at magnification 20X. Arrows mark the preanal and dorsorectal ganglia of the posterior nervous system.

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General Discussion

Results presented herein, report the cloning and partial characterization of a TPH enzyme in *C. elegans* (CeTPH) which resembles mammalian TPH in peptide sequence and in enzymatic activity. In addition, CeTPH appears to be expressed in the nervous system of *C. elegans* in areas that are consistent with the location of the serotonergic neurons. Taken together, this research supports the notion that a complete biological pathway for the synthesis of serotonin (5-HT) exists in *C. elegans* and that CeTPH plays as important a role in the biosynthesis of 5-HT as does mammalian TPH.

It is thought that the majority of nematode pre-mRNA acquire a spliced-leader (SL) sequence. This phenomenon, observed in CeTPH sequenced reported here, further supports the notion that the acquisition of a trans-spliced leader is crucial for the maturation of gene transcripts in *C. elegans*.

There have been numerous comments on the utility of *C. elegans* as a model for parasitic nematodes (Ward et al, 1988, Fleming et al, 1993). Not only does it have a short life cycle and is easy to maintain in the laboratory but because of the genome project and high level of research activity benefits have overflown to parasitology. Many researchers hold that *C. elegans* is as useful as a model for parasites as mice are for humans (Grant, 1994; Sangster, 1996). Undeniably analogies exist between the free-living worm and parasites. For example, comparison of the sequences of an acetylcholine (Ach) receptor gene from *C. elegans* and *Trichostrongylus colubriformis* show a 92% amino acid similarity (Wiley et al, 1996). *C. elegans* has provided molecular information which was crucial for the successful cloning of the vast majority of drug receptors in parasitic nematodes, in addition to the TH cDNA in *S. mansoni* (Hamdan and Ribeiro, 1998). Similarly, undeniable differences exist between *C. elegans* and its parasitic counterparts as well. The biology of free-living and parasitic organisms will differ,

understandably, as each faces varying challenges with respect to feeding, reproduction and life cycle. While recognizing its limitations, we support the study of parasite biology based on the *C. elegans* model organism. Knowledge gained from the study of this free-living helminth will provide a better understanding of the role of 5-HT in closely related parasitic helminths, and might be useful in the development of nematocidal and antihelminthic drugs. Just as the biology and lifestyle of mice differs greatly from that of humans, the study of human biology has advanced through the utility of the mouse model. In addition, any observations made of the *C. elegans* TPH system will provide insight into the molecular mechanisms of serotonergic neurons in general and might be useful in the synthesis of more effective drugs for the treatment of a number of mental dysfunctions and neurodegenerative diseases.

With the advent of more sensitive biochemical and biological assays, we are now able to study the properties of an enzyme that has been very difficult to characterize in the past due to its low abundance in tissue. Future studies should focus on attempting to characterize the kinetic and regulatory properties of this novel *C. elegans* enzyme.

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