THE DOPAMINE β-HYDROXYLASE GENE: UTILIZATION OF TRANSLATION INITIATION CODONS AND REGULATORY ELEMENTS INVOLVED IN TRANSCRIPTION

by

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A DISSERTATION

Presented to the Department of Biochemistry
and Molecular Biology
and the Oregon Health Sciences University
School of Medicine
in partial fulfillment of
the requirements for the degree of

Doctor of Philosophy

June 1993

APPROVED:

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(Professor in Charge of Thesis)

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(Chairman, Graduate Council)

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LIST OF ABBREVIATIONS

BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CAT	Chloramphenicol acetyltransferase
CPT-cAMP	8-(4-chlorophenylthio)-cAMP
CRE	Cyclic AMP response element
CREB	Cyclic AMP response element binding protein
DAG	Diacylglycerol
DBH	Dopamine B-hydroxylase
DMEM	Dulbecco modified Eagle media
FCS	Fetal calf serum
Inr	Initiator
MEM	Minimal Essential Media
PKA	Protein kinase A
PKC	Protein kinase C
PBS	Phosphate buffered saline
PNMT	Phenylethanolamine-N-methyltransferase
RSV	Rous sarcoma virus
TH	Tyrosine hydroxylase
TK	Thymidine kinase
TPA	12-o-tetradecanoyl phorbol-14-acetate
TRE	TPA response element
UTR	Untranslated region

ACKNOWLEDGEMENTS

The completion of this thesis was dependent on the input, influence and support of a large number of people. This aid was both direct and indirect, and of course encompassed much more than the work on this project.

Among others, I wish to thank:

My wife, Sandy, who was always there when the going was tough and my son, Jeremy, who reminded me of what was important, as only a small child can.

My parents, whose support and love never wavered.

My graduate advisor, Dr. Elaine Lewis, for her support and the opportunity to pursue this work.

Previous mentors, including Drs. Paul Haake and Rex Pratt of Wesleyan University, and Drs. Bob Foster and Ralph Yount of Washington State University, who through their examples influenced my attitude toward and appreciation of basic scientific research.

Mavis Patterson, Tom Allen and Maria Schumacher for the camaraderie and their friendship over the last several years.

ABSTRACT

Dopamine β-hydroxylase (DBH) is the enzyme of the catecholamine biosynthetic pathway which converts dopamine to norepinephrine. It is found only in secretory vesicles of certain types of neuroendocrine tissue. There are two forms of the enzyme: soluble and membrane-bound.

Two distinct questions were asked in this research. The first: Is the initial ATG of the DBH mRNA used exclusively to initiate translation, or is it possible that subsequent ATGs are also utilized at least a fraction of the time? Second: What regions of the 5'-flanking sequence of the DBH gene are involved in the regulation of the gene and what do they contribute to this regulation? In order to study this, a genomic clone for rat dopamine β-hydroxylase was isolated. This clone contains 394 bases 5' to the transcription initiation site. The region from -394 to +14 was cloned adjacent to the coding sequence of the reporter gene chloramphenicol acetyl transferase (CAT) resulting in DBH/CAT (-394-(+14)).

In (-394-(+14)) DBH/CAT, there is a translation initiation codon contributed by the DBH gene at +10. Use of this ATG would produce a translation product using codons out-of-frame with those coding for the CAT protein. Therefore, the initial ATG was mutated to AGG, which does not support translation initiation. DBH/CAT (-394-(+14)) was found to

direct approximately 25% of the CAT activity of DBH/CAT (-394-(+14 AGG)). RNase protection assays of cells transfected with those constructs, and the internal control TK-luciferase, revealed that relative levels of DBH/CAT mRNA did not differ for the two constructs and thus did not account for the difference in CAT enzyme activity. The results are consistent with the hypothesis that two translation initiation codons may be used. The use of the 5′ most may give rise to the membranous subunit of DBH anchored by an uncleaved signal sequence. Use of the second translation initiation codon may be responsible for the formation of the soluble subunit and membranous subunits attached by other mechanisms.

Deletion mutants of DBH/CAT (-394-(+14 AGG)) were utilized to define regions of the DBH 5′-flanking sequence which modulate expression of the CAT gene. In the cell line SH-SY5Y, which endogenously expresses the DBH gene, they were used to define regions which contribute to basal expression and mediate inducibility by the protein kinase A (PKA) and protein kinase C (PKC) pathways. In cell lines from diverse origins, these constructs were used to locate regions which contribute to its tissue-restricted expression.

It was shown that the bases between -163 and -189 and between -189 and -232 contain elements which contribute to basal expression in SH-SY5Y cells. The bases between -163 and -189 also convey induciblity by both the PKA and PKC pathways. However, sequences 5′ to -189 reduce the inducibility by both pathways and DBH/CAT (-394-(+14 AGG)) has little inducibility by either. When co-transfected with the internal standard TK-luciferase into SH-SY5Y human neuroblastoma, CV-1 monkey kidney, JEG-

3 human placental and C6 rat glioma cell lines, it was found that the -110/+14 promoter fragment had the highest relative activity in the SH-SY5Y cell line. There were increases in activity upon addition of the bases between -163 and -189 in all cell lines and between -189 and -232 in three of the four cell lines. The region between -232 and -394 was found to harbor a silencing element in the non-expressing cell lines.

INTRODUCTION

DOPAMINE β-HYDROXYLASE

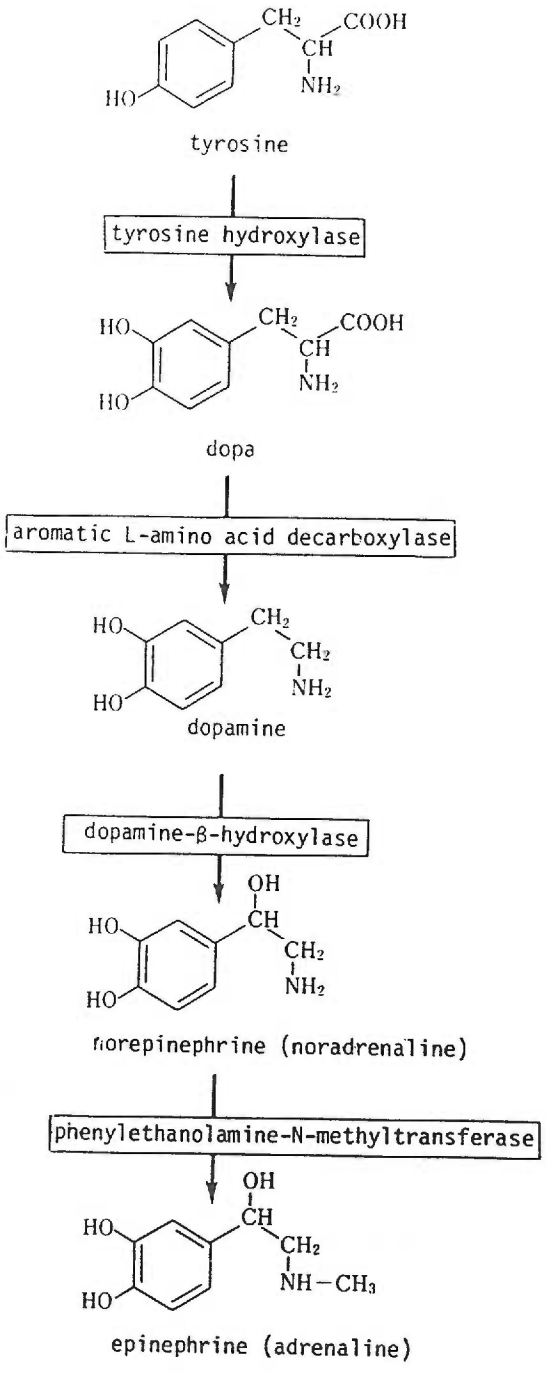
ENZYME LOCATION

Dopamine β-hydroxylase (DBH) catalyses the third step of the catecholamine biosynthetic pathway, the hydroxylation of dopamine to norepinephrine (Figure 1). This is the final step in the catecholamine pathway in noradrenergic tissues which consist of portions of the brain, including the locus coerelus, the peripheral sympathetic nervous system, and the adrenal medulla. It is the penultimate step in adrenergic tissues which are found in portions of the brain and in the adrenal medulla. DBH is not found in dopaminergic tissues. In noradrenergic and adrenergic cells the enzyme is found in intracellular secretory vesicles, including dense-cored synaptic vesicles of the sympathetic nervous system and in chromaffin granules of the adrenal medulla. Here DBH exists in two forms: soluble and membrane-bound. The soluble form is released extracellularly with the other contents of the vesicles upon proper stimulation, whereas the membrane-bound form is recycled. The physiological function of released DBH is unknown.

PHYSICAL PROPERTIES

Both the soluble and membrane-bound forms of the enzyme have a

Figure 1. Biosynthesis of catecholamines.


tyrosine
tyrosine hydroxylase
dopa
aromatic L-amino acid decarboxylase
dopamine
dopamine-β-hydroxylase
norepinephrine (noradrenaline)
phenylethanolamine-N-methyltransferase
epinephrine (adrenaline)

molecular weight of approximately 290,000 (Stewart and Klinman, 1988). Charge-shift immunoelectrophoresis has been used to confirm the existence of the enzyme in amphiphilic and hydrophilic forms (Bjerrum et al., (1979)). Subsequently, Saxena and Fleming (1983) isolated the membrane-bound form. It binds phospholipid tightly and was demonstrated by charge-shift electrophoresis to be amphiphilic. A recent investigation utilizing sedimentation analysis and non-denaturing charge-shift electrophoresis has reported that there are two amphiphilic forms of the enzyme which are capable of binding detergent micelles as well as a hydrophobic form which does not bind micelles (Bon et al., 1991).

Both the soluble and membrane-bound forms of the enzyme are tetramers composed of two disulfide-linked dimers. The dimers are held together by non-covalent interactions. The nature of the monomers is somewhat more controversial. Originally, they were believed to be identical in both forms with molecular weight of 75,000. In 1983, Saxena and Fleming reported the first purification of membranous DBH and demonstrated the existence of two nonidentical subunits of different molecular weight, one of 70,000 and the other of 75,000. Presently, there is disagreement among investigators as to the percentage of each subunit in the holoenzyme and to the molecular weight of each subunit but, there is general agreement as to their presence. The soluble form is generally considered to contain a higher percentage of the smaller subunit and some investigators report no larger subunit. In summary, for both forms of the enzyme heterogeneity in subunit size has been observed and reasons for this are not entirely clear.

DBH is a glycoprotein. Each tetramer contains an average of six N-linked oligosaccharide chains, an average of 1.5 per monomer. Two of the sugars are high-mannose and four are complex biantennary. It has been reported that deglycosylation of the 75,000 kDa subunit results in the formation of a 72,000 kDa subunit (Oyarce and Fleming, 1989). Therefore, it is likely that glycosylation confers some heterogeneity to the enzyme structure though the extent remains unclear.

METHOD OF MEMBRANE ATTACHMENT

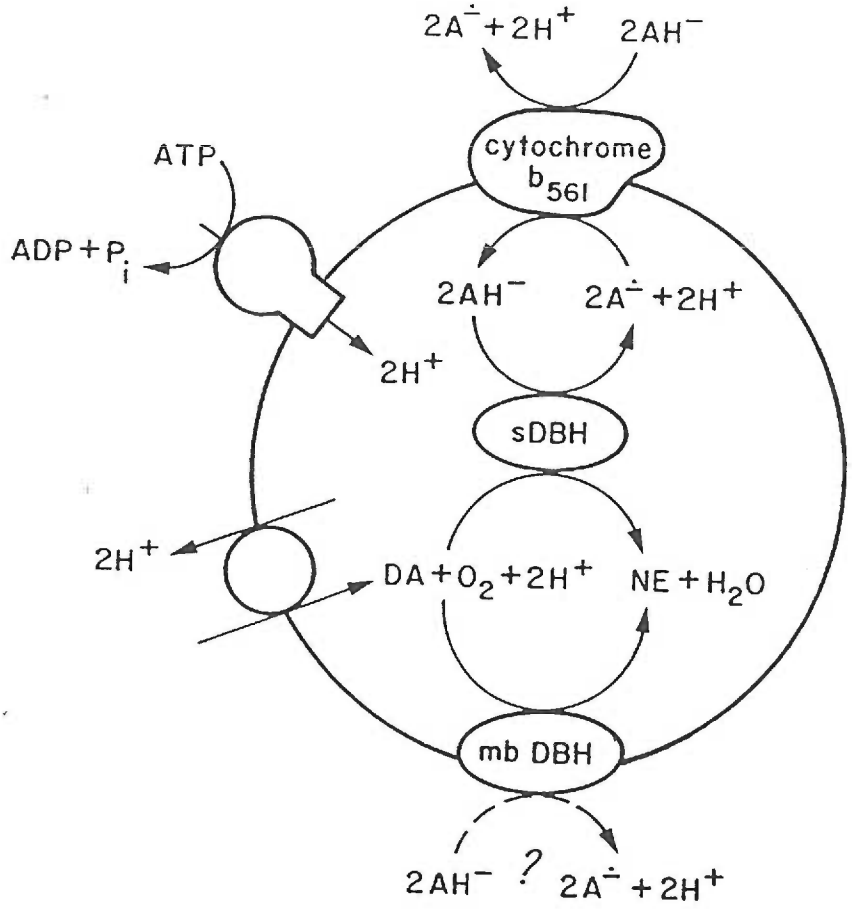
As mentioned previously, DBH is found within intracellular vesicles. The soluble form of the enzyme is completely intravesicular and the membranous form is linked to the membrane with the bulk of the enzyme intravescular. The means by which the enzyme is attached to the membrane has been the subject of numerous studies. Experiments performed to determine if attachment was via phosphatidyl inositol or palmitate resulted in negative conclusions. In order to determine if attachment was via an uncleaved signal sequence, the membranous form of the enzyme has been sequenced. It was originally reported that the membranous subunits had the same N-terminal sequence (Taylor et al., 1989) as that found for the soluble subunits (Skotland et al., 1977, Joh and Hwang, 1986, Robertson et al., 1990). Subsequently, Taljanidisz et al. (1989) reported that sequencing of the 75 kDa subunit resulted in a different sequence than those found in the soluble subunit. About 70% of the amino acids recovered formed a new sequence containing a charged N-terminal followed by a hydrophobic sequence capable of spanning the membrane. This sequence corresponded to the postulated translation

product for the 5' end of the cDNA reported by the same authors. However, it has been reported that the soluble form of the enzyme can be converted to the membrane bound form in a pH- and phosphatidylserine-dependent manner (Taylor and Fleming, 1989). In addition, expression of a cDNA clone which lacks the initial translation start site of the protein, and would therefore be predicted to initiate translation after the N-terminal anchoring sequence near the N-terminus of the predicted membrane spanning region has been expressed in PC12 cells. This expression resulted in the formation of both soluble and membranous forms of DBH (Lewis and Asnani, 1992). Therefore, DBH is anchored, at least in part, by its N-terminal end. However, both the extent of the anchoring due to the extended 5' end and the mechanism of the anchoring by that same end are unresolved.

DBH ACTIVITY IN THE CHROMAFFIN GRANULE

Most studies of the DBH enzyme have focussed on the adrenal enzyme. Several other proteins as well as the cofactor ascorbic acid are necessary for DBH to be active inside the chromaffin granule (Figure 2). This vesicle has a pH of approximately 5.5. This pH is maintained by active transport of protons into the granule by an H^+-ATPase. The catecholamine transporter uses energy from the resultant electrochemical gradient to transport dopamine in from the cytosol against a concentration gradient which has been estimated to approach 135,000:1. Ascorbate is a necessary cofactor of DBH; it donates an electron to maintain a copper ion in the reduced form necessary to maintain the enzyme in the active form. Ascorbate is found in the granule at a

Figure 2. "Proposed model for dopamine B-hydroxylation within adrenal chromaffin granules, where sDBH and mbDBH reflect the soluble and membrane-bound forms of enzyme. AH^-, AH^-, and DA are ascorbate, semihydroascorbate, and dopamine, respectively. As shown, cytosolic ascorbate is used to recycle intragranular semidehydroascorbate via cytochrome b_{561}. Additionally, direct reduction of DBH from the external face has been proposed. The mechanism and mediators for the latter process are not yet known." (This figure and legend are from Stewart and Klinman (1988).


2A· + 2H+
2AH−
cytochrome b561
ATP
ADP + Pi
2AH−
2A· + 2H+
2H+
sDBH
2H+
DA + O2 + 2H+
NE + H2O
mb DBH
2AH− ?
2A· + 2H+

concentration of approximately 10 mM (Menniti et al., 1987). It is not transported in or out of the granule, and it is believed that cytochrome b_{561} is capable of reducing internal dehydroascorbate to ascorbic acid using external ascorbate as the electron donor (Huyghe and Klinman, 1991). It is not known how norepinephrine is exported from the granule to be further modified by phenylethanolamine-N-methyltransferase (PNMT) in the cytosol of adrenergic cells.

MOLECULAR BIOLOGY OF DBH

The original cloning of DBH was reported in 1987 (Lamouroux et al.). Since then isolation of both cDNA and genomic clones of human, rat and bovine DBH have been reported in the literature. Their findings are summarized below. The gene has been localized to human chromosome 9q34 (Craig et al., 1988).

Lamouroux et al. isolated a nearly full length human clone of DBH and predicted that there were less than 16 bases to the start of transcription from the 5' end of their clone. Later studies using genomic and longer cDNA clones have shown that there is a translation initiation codon in the missing region. Analysis of the incomplete clone led to the assumption that translation initiated further downstream than is now believed. A consequence of this was that, in the earlier analyses, a portion of the signal sequence was not originally detected.

The cloning of both human cDNA and genomic clones was reported by Kobayashi et al. (1989). They found that two different forms of the mRNA are present (2.7 and 2.4 kb) and differ only in the use of

different polyadenylation sites. They also found minor differences in nucleotide sequence between cDNAs and also between cDNA and genomic clones which result in single mutations in the DBH enzyme. Examination of the genomic clone showed that it covers approximately 23 kb and is composed of 12 exons. Expression of two of the cDNA clones in COS cells (Ishii et al, 1991) demonstrated different specific enzyme activities.

5.8 kb of human DBH 5′ to the transcription initiation site was cloned and fused to the E. coli lacZ gene by Mercer et al. (1991). This construct was expressed in transgenic mice and lacZ activity was found in all expected noradrenergic and adrenergic tissues. Activity was also found in some tissues where DBH activity has not been found including the enteric nervous system, retina, and cranial parasympathetic ganglia. These observations suggest that regulatory elements for the DBH gene are found elsewhere in the gene. This expression of this construct was also studied during embryogenesis (Kapur et al., 1991). It was found that a number of cell types expressed the gene during development, and that not all the cells derived from the expressing embryonic cells would later synthesize catecholamines.

The creation of transgenic mice with four kb of the DBH promoter from the human gene fused to the cDNA for PNMT was reported by Kobayashi et al. (1992). Noradrenergic tissues were converted to adrenergic. The authors found no significant differences in metabolism, including levels of blood sugar and blood pressure, between transgenic animals and nontransgenic controls.

Several groups have reported cloning the bovine cDNA, although no genomic clones have been reported. Taljanidisz et al. (1989) reported

the sequence of a full-length bovine cDNA. They used this sequence to verify the protein sequence of the membranous enzyme subunits they isolated. Lewis et al. (1990) reported the sequence of a nearly full length cDNA which originated four bases after the initial ATG. They were still able to express the protein in PC12 cells, however no enzyme activity was obtained. The lack of enzyme activity was later explained to be due to the presence of an inhibitor in the crude protein extract. Lewis and Asnani (1992) later expressed this cDNA as well as the one corresponding to that of Taljanidisz et al.. Enzyme activity was obtainable for both proteins. Interestingly, both cDNAs produced membrane bound and soluble forms of DBH. Wang et al. (1990) reported the isolation of a cDNA clone which was used to verify their protein sequence. Two differences between the protein and the coding cDNA sequences were found; this is consistent with that found by Kobayashi et al. (1989) for the human clone. The presence of different amino acids found in protein sequences, and predicted by cDNA and genomic DNA coding sequences, may explain the variability in DBH enzyme activity found clinically. Wu et al. (1990) also reported the isolation of a nearly full length clone. This clone also started after the initial ATG. The authors expressed the gene in bacteria and obtained a single 65 kDa peptide which reacted with DBH antisera. Northern analysis of bovine adrenal mRNA resulted in the hybridization to a single band of 2.6 kb. This contrasts with hybridization to two bands for both the human (Kobayashi et al., 1989) and the rat (McMahon et al., 1990) mRNAs.

The cloning of a full-length cDNA of rat DBH was reported by McMahon et al. (1990). Northern analysis of rat adrenal and PC12 RNA

detected the presence two RNA species in both. This is likely due to the use of different polyadenylation sites as two potential consensus sequences were identified. McMahon and Sabban (1992) have isolated a genomic clone for rat DBH. No functional studies were performed.

PHYSIOLOGICAL AND MEDICAL ASPECTS OF DBH ACTIVITY

CATECHOLAMINES AND STRESS

Catecholamines, in particular norepinephrine and epinephrine, are known to be involved as mediators in the "fight or flight" response to stress. In this response, they are released as neurotransmitters by the nervous system and brain, and as a hormone by the adrenal gland. How the biosynthetic enzymes of the pathways are involved in, and react in, response to stress has been a question of interest since their discovery.

Animal studies have allowed the examination of how externally applied stress can affect the levels of the biosynthetic enzymes and mRNAs as well as the rate and conditions necessary for catecholamine release. Use of cells in culture allows factors which are thought to act directly at the cellular level to be tested and their mechanisms of action elucidated. Both of these types of studies are essential if we are to reach a true understanding of the effects of stress on living organisms.

Previous investigations have focussed on determining the activity and amounts of biosynthetic enzymes and examining the levels of mRNAs in side-by-side comparisons of stressed and unstressed subjects or samples. Many of these studies have focussed on tyrosine hydroxylase (TH) as it

is the biochemical point of commitment and rate limiting step in the biosynthesis of the various catecholamines under many conditions. It was thus expected that increases in catecholamine biosynthesis would be evident at this step. However, where it has been examined, enzyme activity and accompanying protein amounts as well as mRNA levels of DBH have been found to be increased by external factors. Analysis of these studies suggests that at least some regulation of DBH activity may be due to increased amounts of enzyme. This may be due to an increased level of transcription.

ANIMAL STUDIES

The effect of immobilization stress on rats has been reported to affect levels of TH and DBH mRNA in rat adrenals (McMahon et al., 1992). A single two hour immobilization gave rise to a transient increase in TH mRNA levels but did not effect the level of DBH mRNA. However, a second immobilization 24 hours later resulted in significant increases in both TH (six fold increase) and DBH (four fold increase) which did not decrease between immobilizations. Subsequent immobilizations did not change the levels of mRNA for either enzyme.

It has been demonstrated that reserpine causes an increase in DBH enzyme activity when it is fed to animals (Viveros et al., 1969). (Reserpine is a naturally occurring alkaloid used medically to lower blood pressure and act as a tranquilizer. It has been reported to interfere with the binding of norepinephrine in the synaptic vesicle (Sjarne, 1964). At higher doses reserpine also causes catecholamine depletion (Viveros et al., 1969). Lima and Sourkes (1986) report that

feeding rats a 2.5 mg/kg dose of reserpine for four days resulted in a 50% increase of DBH activity in isolated adrenal glands. If the rats were given a single dose of ten mg/kg and fed one mg/kg of cycloheximide daily instead, there was no increase in DBH activity compared with rats not fed the cycloheximide. This implies that the observed changes in DBH activity are due to changes in the amount of enzyme.

Treatment of rats with a single dose of reserpine led to a 3 to 4 fold increase in DBH mRNA over 2 to 3 days (McMahon et al., 1990). It was not determined if this was due to increased transcription or an increase in the stability of DBH mRNA.

STUDIES WITH CULTURED CELLS

Cyclic AMP functions as a second messenger for a number of hormones and other factors which effect cell metabolism through receptors coupled to adenylate cyclase. In many cases activity due to increased amounts of cAMP can result in gene transcription (Montminy et al., 1990). Cyclic AMP itself is incapable of crossing the cell membrane due to its highly polar nature. However, there are a number of analogs which have been used that are capable of activating cAMP's target molecule, protein kinase A. The addition of a hydrophobic moiety to cAMP allows these compounds to diffuse into the cell.

Waymire et al. (1978, 1979) have studied the effects of cAMP on a cultured mouse neuroblastoma cell line (NBD-2) that produces DBH. It was found that treatment of the cells for 48 hours with 1.0 mM 8Br-cAMP resulted in 15 fold increases in both TH and DBH activities and that time courses for these inductions were similar. If the cells were

treated with phosphodiesterase inhibitors instead of cAMP (Waymire et al., 1979), the phosphodiesterases which result in increased cAMP concentration (papaverine, Ro20-1724) increased TH and DBH activity in parallel. Theophylline, which has no effect on cAMP levels, had no effect on TH or DBH activity. The authors also found (1979) that overall protein synthesis rates did not correspond with the changes in TH or DBH activity. Experiments performed in which actinomycin D and cycloheximide were included in the media either alone or together (1978) led these authors to the conclusion that "an increased production of messenger RNA" may be one of the explanations for the increased enzyme activities.

In a study utilizing primary bovine adrenal chromaffin cells (Acheson et al., 1984), treatment of the cells with 1 mM 8Br-cAMP for 12 hours resulted in three fold increases in both TH and DBH enzyme activity. This induction was blocked by including α-aminitin in the media. As α-aminitin is an irreversible inhibitor of RNA polymerase II (Chambon, 1975), this increase is probably due to increased transcription of the genes.

Other studies with cells in culture have shown that DBH mRNA levels can be increased by treatment with glucocorticoids, dibutyryl cAMP and NGF (Badoyannis et al., 1991, McMahon and Sabban, 1992), as well as with reserpine (McMahon et al., 1990).

In summary, these studies show that DBH activity and mRNA levels are modulated by a variety of external factors. They also provide a basis for the supposition that DBH gene transcription may be responsible for a portion of the increases in DBH activity and enzyme levels

associated with external stimuli.

MEDICAL ASPECTS OF DOPAMINE β-HYDROXYLASE

A large percentage of DBH enzyme is soluble and is released from secretory granules along with norepinephrine. DBH activity can be measured in plasma and in cerebrospinal fluid.

DBH activity has been found to vary widely between individuals. One recent study with 40 healthy volunteers serving as their control group reported an activity of 46.3 (4.3) mean (SEM) with a range of 13 to 133 umol/min/l serum (Barakat et al., 1988). However, it is only in the last decade that the lack of DBH activity has been found to be associated with physiological abnormalities. Several cases of orthostatic hypotension, in which blood pressure decreases when the patient goes from a supine to a more upright position, have been found to be caused by a near-complete lack of DBH activity in serum (Robertson et al. 1986, man in't Veld et al. 1987, Rea et al. 1990, Biaggioni 1990). Along with negligible or no DBH activity, these patients have exceptionally low levels of NE and epinephrine in their plasma and spinal fluid but, otherwise normal sympathetic nerve activity (Rea et al. 1990). Dopamine levels in the plasma are highly elevated implying that unmodified dopamine is being released instead of NE.

TRANSLATION INITIATION

Translation of mRNA initiates at an ATG codon in nearly all cases known. The present model for translation initiation in eukaryotes requires that the ribosomal 40S subunit and its associated factors bind

near the 5′ capped end of the mRNA and scan downstream to a AUG codon. Approximately 95% of known translation occurs at the initial ATG (Kozak, 1984) but, the use of subsequent ATGs in addition to or instead of the most 5′ one has resulted in modification of this model to one has been described as "leaky scanning" (Kozak, 1989b)

A number of factors have been found to influence the efficiency of usage of particular ATG to initiate translation. These include the sequence surrounding the codon, the length of the 5′ untranslated sequence and the occurrence of secondary structure in the region surrounding the initiation codon.

Comparison of sequences surrounding the ATG initiation codon resulted in the consensus sequence CCA/G ATG G (Kozak, 1984, 1987a). The 1984 study, which had a sample base of 166 cellular mRNAs, concluded that while only 10 of the mRNAs conformed perfectly to this sequence, >50% have 3 or 4 nucleotides in common with the CCACC preceding the ATG. The 1987 study reported that 95% of the published sequences had a purine at −3 and G predominated at +4. The Cs in the other positions appeared to be less important. Mutagenesis studies have shown that the optimal context for initiation was (GCC)GCCA/GCC ATG G (Kozak, 1987b).

Vertebrate mRNAs have a 5′ noncoding sequence that averages approximately 90 nucleotides. Most of them are between 20 and 100 nucleotides long (Kozak, 1987b). The effect of an especially short untranslated sequence has been found to affect the utilization of the initial ATG for translation initiation. Sedman et al., (1990) reported that decreasing the length of an untranslated region (UTR) from 44 to 6 bases resulted in a 30% decrease in translation initiation at the first

and a 3 fold increase in initiation at a second ATG. A second construct had the same context for the initial ATG but had a different context for the second ATG. Utilization of the initial ATG decreased 70% and use of the second increased 30% when the untranslated sequence was decreased. Kozak (1991) has demonstrated that in the absence of secondary structure, untranslated regions of 3, 6, 9 or 12 nucleotides show decreased utilization of the first initiation codon and increased use of the second codon compared to the identical construct with a 32 base untranslated region. Use of the first:second codons ranged from 2:3 for the 3 base UTR, 3:2 for the 9 base UTR and approximately 2:1 for the 32 base UTR.

Secondary structure has been found to influence the effects of sequence context and short UTRs. Recognition of an ATG in a suboptimal context was found to be higher than expected if the adjacent downstream structure was capable of forming a hairpin (Kozak, 1989a, 1989b). A hairpin 5′ to the ATG was found capable of inhibiting translation of the mRNA. Also, the effect of a short UTR was reduced if hairpin formation 3′ to the ATG was possible.

As there are only 10 nucleotides in the untranslated region of the DBH mRNA, we expect that translation might be affected by the length of its untranslated region. Translation initiating at a second or subsequent ATG might result in different processing of the protein than translation initiation at the initial one. Use of the initial ATG may result in membrane-bound subunit held in by the uncleaved signal sequence while use of a subsequent ATG might result in preferential formation of soluble subunits or subunits attached to the membrane by

different means.

GENE EXPRESSION

Recent study of gene expression has resulted in the realization that gene promoters are composed of modules (Dynan, 1989). Each 7–20 bp module is capable of binding one or more different proteins. It is the interactions of the different protein/DNA modules that determine if a gene will be transcribed, and if it is transcribed, to what extent. Promoter structure is often described as being composed of several regions. These include the proximal promoter, which contains the TATA box and the transcription start site and upstream elements, which include the GC box which binds the nuclear factor Sp1, and the CAAT box which can bind one or more different nuclear factors. Enhancers bind one or more proteins and can influence gene expression either positively or negatively in a position and orientation independent fashion (Mitchell and Tjian, 1989).

Regulation of transcription can be controlled on the level of whether a protein binds the appropriate DNA segment, or whether the protein is properly modified. An example of the first is that of activation by steroids. It is generally believed that the steroid must bind the receptor which in turn binds the appropriate DNA response element (Beato, 1989). Protein modification occurs in the case of cAMP activation via the protein CREB. CREB binds the same DNA element whether or not it is phosphorylated (Goodman et al., 1991). However, phosphorylation is essential for CREB to increase the level of gene expression (Gonzalez and Montminy, 1989).

PROMOTER ELEMENTS AND BINDING FACTORS

INITIATORS

The sequences surrounding the transcription initiation site were shown to be important both for the level of transcription and for dictating the specific nucleotide of transcription initiation (Corden et al., 1980). However, possibly because of difficulties in determining a consensus sequence motif at that site, and variations in the extent of the effectiveness of that site in the mentioned roles, its importance in gene regulation was not widely studied until recently.

Smale and Baltimore (1989) reported a characterization of the initiator (Inr), which they described as the "simplest functional promoter" yet discovered. The Inr contains the bases surrounding the transcription start site of the murine terminal deoxynucleotidyltransferase (TdT) gene, and through mutagenesis studies a functional Inr was shown to have the consensus sequence CTCANTCT. Transcription initiates at the A (Smale et al., 1990). This sequence was found to support transcription initiation at a single nucleotide with a requirement for transcription factor TFIID in the absence of a TATA box. Transcription is enhanced in the presence of an Sp1 binding sites to a similar extent as for a TATA box without the Inr. The Inr was also found capable of directing the assembly of a TFIID–dependent transcription complex (Carcamo et al., 1991). It has been shown that Inr elements with similar sequences are involved in transcription repression of the interleukin–6 promoter by glucocorticoids (Ray et al., 1990). In addition, the transcription factor YY1 has been shown to bind to the initiator sequence and activates transcription (Seto et al.,

1991). Recently it has become clearer that this region is important in regulating transcription.

PROXIMAL PROMOTER AND UPSTREAM ELEMENTS

A number of DNA sequence elements are found in many gene promoters and could be considered general sequence motifs. These include the TATA box, the GC box and the CCAAT box (Mitchell and Tjian, 1989). The TATA box is bound by the nuclear factor TFIID, which along with other general initiation factors is involved in forming a transcription initiation complex with RNA polymerase II (Salwadogo and Sentenac, 1990; Conaway and Conaway, 1991). The GC box has the consensus sequence GGGCGG (Mitchell and Tjian, 1989) and is bound by the ubiquitous transcription factor Sp1. This site is usually found within 100 bases of the start site of transcription and is active in both orientations. In studies of model minimal promoters consisting only of a TATA box and/or an initiator element, addition of Sp1 binding sites to the promoter was found to greatly increase the rate of transcription (Smale et al., 1990; Pugh and Tjian, 1990). In addition, multiple Sp1 sites have been found to be essential for promoter activity in genes which are transcribed by RNA pol II but lack TATA boxes (Hoshiko et al., 1990; Yamaguchi et al., 1987). Gene structure is commonly found in "housekeeping genes" (Dynan, 1986). Unlike TATA and GC boxes, the other common sequence motif, the CCAAT box, does not bind a single, unique protein. There have been a large number of proteins identified which can bind the pentanucleotide CCAAT (Johnson and McKnight, 1989; Dorn et al., 1987). The strength of the protein-DNA interaction is greatly dependent on the nucleotides

flanking the core motif and the specific binding protein. CCAAT binding proteins have been implicated in performing a number of regulatory roles in transcription including mediating the response to activation by calcium calmodulin-dependent protein kinase (Kapiloff et al., 1991).

ENHANCERS

In addition to the common, proximal promoter elements described above, enhancers have been found to be imperative for regulating gene transcription in many genes. Enhancers are protein-binding DNA elements which can function in a position and orientation independent manner. Frequently, these elements are located in the 5' flanking region of the gene.

The expression of a number of genes active in the nervous system is dependent on enhancer elements whose activity is in turn affected by external stimuli. Often the external stimuli results in an increase in kinase activity in the cell. Two kinases whose effects on gene expression have been well documented are protein kinase A (PKA) and protein kinase C (PKC) (Comb et al., 1987).

PKA, also known as cyclic AMP-dependent protein kinase, is the only known effector of cAMP in eukaryotes. The kinase consists of two types of subunits, a regulatory (R) and a catalytic (C). The subunits are associated in a R_2C_2 complex in the absence of cAMP. Activation of the kinase occurs when cAMP binds the R subunits, which then dissociate from the complex leaving two active C monomers.

PKC is activated in vivo by diacylglycerol (DAG), a product of the cleavage of phosphatidyl inositol 4,5-bisphosphate by phospholipase C.

Association of DAG with PKC causes an increased affinity of the kinase for calcium, which is essential for its activation. Also essential for activation is phosphatidyl serine (Nishizuka, 1986).

The DNA sequence elements shown to confer activity on a number of promoters in response to the activation by PKA and PKC are very similar. The DNA consensus sequence TGACGTCA has been named the cAMP response element (CRE). This palindromic octamer, or slight variants thereof, are found in a large number of cAMP responsive genes. The consensus heptad TGAC/GTCA is also called the TRE. This motif, or very similar ones have been found in many genes responsive to the phorbol ester 12-o-tetradecanoyl phorbol-14-acetate (TPA). This phorbol ester is an analog of DAG and is known to activate PKC. A third element, AP-2, has the consensus sequence CCCCAGGC. This element was originally described as being capable of mediating induction by both PKA and PKC pathways (Imagawa et al., 1987). It has also been shown to act synergistically with the cAMP/TPA-inducible enhancer of proenkephalin (Hyman et al., 1989).

CRE BINDING PROTEINS

The CRE octamer from the somatostatin gene was used to purify the nuclear protein CREB (CRE binding protein) from rat brain tissue and PC12 cells by affinity chromatography (Montminy and Bilezikjian, 1987). This protein has a molecular mass of 43 kDa and binds to the octamer as a dimer. This protein has been shown to be phosphorylatable by PKA in vitro (Yamamoto et al., 1988) and is phosphorylated in vivo in cells treated by the PKA activator forskolin. This phosphorylation has been

shown to be essential for the activation of gene transcription by CREB (Gonzalez and Montminy, 1989). A second 37 kDa protein was isolated from JEG-3 cells (Hoeffler et al., 1988), which are derived from a human placental choriocarcinoma. This protein was highly homologous to the rat protein with the most striking difference being the deletion of 14 amino acids. It was found capable of binding to CREs from several genes which have different flanking sequences. If the CREs were mutated to the corresponding TREs, so that the identical flanking sequences were maintained, protein/DNA binding was abolished. Subsequently, screening of a human cDNA library resulted in the isolation of two cDNA clones corresponding to each of the above CREBs. Testing of a number of cell lines derived from different mammalian species, and the testing of a number of tissues has implied that both forms of CREB are ubiquitous (Berkowitz and Gilman, 1990). A large number of other proteins have been found capable of binding the CRE octamer. ATF-1 is also responsive to cAMP and has also been found in all cell lines thus far examined (Rehfuss et al., 1991). It is one member of a much larger family of related proteins (Hai et al., 1989). This family of proteins is capable of cross-dimerizing with CREB (Hai and Curran, 1991, Rehfuss et al., 1991) and at least some heterodimers are capable of conferring cAMP activation to genes. Other proteins have been found to bind the CRE octamer but their functions have not been determined (Andrisani and Dixon, 1990; Andrisani et al., 1988; Merino et al., 1989).

TRE BINDING PROTEINS

Phorbol ester-inducible genes were found to share the 7 base

consensus heptad TGAC/GTCA and were found to bind the transcription factor AP-1 (Angel et al., 1987, Lee et al., 1987). AP-1 was subsequently shown to be composed of two separate proteins, fos and jun (Rauscher III et al., 1988). Both of these proteins are members of larger families. The jun family consists of at least three members: c-jun, junB and junD; fos of at least four: c-fos, fra-1, fra-2 and fosB (Ryder et al., 1988, 1989, Hai and Curran, 1991). The jun family is capable of forming DNA-binding homodimers and heterodimers with each of the other jun proteins (Nakabeppu et al., 1988; Ryseck and Bravo, 1991) and bind to TRE sequences. All three jun proteins are capable of forming DNA-binding heterodimers with each of the fos proteins (Ryseck and Bravo, 1991). With each of the jun proteins, it was found that association with c-fos increased DNA-binding affinity (Nagabeppu et al., 1988). The fos proteins fra-1 and fosB formed even stronger complexes with the jun proteins than c-fos (Ryseck and Bravo, 1991). It was also found that both jun homodimers and jun/fos heterodimers could also bind to CRE sequences (Ryseck and Bravo, 1991, Nagabeppu et al., 1988) under conditions of electrophoretic mobility shift assays, though the stregth of the binding is not clear. Fos and jun associate through regions of each proteins containing regularly spaced leucine residues (Schuermann et al., 1989; Turner and Tjian, 1989); this motif has acquired the name "leucine zipper". The protein-protein association domains are distinct from the protein-DNA association domains. Though fos will not bind the TRE either as a monomer or homodimer, evidence suggests that fos protein does interact with the DNA and that the interaction of the heterodimer with the TRE is asymmetrical (Risse et al., 1989).

SIMILARITIES AND DIFFERENCES OF CRES AND TRES

The consensus sequences of CREs (TGACGTCA) and TREs (TGAC/GTCA) differ by only a single base. As the previous sections have noted, the protein binding properties of these response elements are different though the specifics are not absolute. These DNA sequence motifs are found to be important in the regulation of a large number of genes in many different cell types (Comb et al., 1987, Goodman et al., 1991). The overall regulation of genes is tightly controlled and it is reasonable to expect that not all CREs or TREs will be equally active under any particular circumstance. It has also been noted that the two kinase signal transductions pathways can converge in their activation of genes, such as through activation of the transcription factor AP2 (Imagawa et al., 1987).

The effects of flanking sequences of the CREs and TREs on the inducibility by their respective activators has been examined (Deutsch et al., 1988a, Deutsch et al., 1988b). In addition, the effects of deleting a single base to change a CRE to a TRE while not changing the flanking sequences was approached in the same studies. The response elements were placed adjacent to the proximal promoter for the α-chorionic gonadotropin gene which was in turn fused to the coding region for the bacterial reporter gene chloramphenicol acetyl transferase (CAT). It was found that the CREs conferred response to cAMP but not TPA and that flanking sequences had a major influence on the degree of the induction. When the CREs were mutated to TREs, CAT activity was induced by both cAMP and TPA. Again, the degree of induction was greatly dependent on the sequences flanking the response elements. An

unexpected phenomena appeared when constructs containing TREs were tested. Not only did they confer responsiveness to both inducers, but when both inducers were added at the same time, a superadditive induction was observed; i.e. the induction was equal to more than the inductions of each added together (Hoeffler et al., 1989). Though the above studies show that CREs mediate responsiveness only to cAMP and not TPA and TREs mediate responsiveness to both, this is not an unequivocal rule. A separate study has reported a contrary result. The somatostatin CRE, which contains the consensus octamer, is necessary for the activation of a somatostatin-CAT fusion gene by TPA (Goodman et al., 1991).

A number of genes which are responsive to cAMP or TPA do not contain consensus CREs or TREs. Functional assays of the promoters inducibility has led to the location of response elements in many genes (Bowlus et al., 1991, Fisch et al., 1989). In efforts to study the importance of specific bases in CREs and TREs, mutagenesis has been done on several of them. Chorionic gonadotropin (Deutsch et al., 1988b) has a consensus CRE. Mutation of the first T to A, the first C to G or final A to T resulted in decreases in activity in excess of 95%. Mutagenesis has also been done for vasoactive intestinal peptide (VIP) (Fink et al., 1988, Fink et al., 1991). This gene contains a 17 bp element which confers both cAMP and TPA inducibility when the promoter is linked to the CAT coding sequence. This element contains a CGTCA motif separated from a near-consensus CRE by five bases (sequence: CGTCA TACTG TGACGTCC). The first five bases of the CRE read CGTCA on the non-coding strand. Mutations of either the 5' CGTCA or the CRE

reduce forskolin induced activation and mutation of the CRE to TGCTATC reduced activation by both forskolin and TPA. Comb et al. (1988) performed extensive mutagenesis to the promoter region conferring cAMP and TPA activation to the proenkephalin gene. This gene includes a seven base sequence, TGCGTCA, labelled ENKCRE-2 by the authors, which is important for activation by both reagents. The proenkephalin gene also has an second seven base element 12 bases upstream, ENKCRE-1, which augments the response. Mutagenesis of ENCRE-2 to a consensus TRE, TGAGTCA resulted in reductions in both basal and induced expression of a CAT fusion construct by a combination of PKA and PKC pathway activators (Comb et al., 1988). All but one of the other mutations of this heptad also led to reduced activity. The sole mutation which led to increased activity gave the heptad TACGTCA, on the other strand this reads TGACGTA which contains the first six bases of a consensus CRE. It has since been demonstrated that a portion of the proenkephalin 5'-flanking sequence containing ENKCRE-2 can be positively regulated by JunD and negatively regulated by JunB (Kobierski et al., 1991). ENKCRE-2 is also capable of binding AP-1 (Comb et al., 1988, Sonnenberg et al., 1989).

In summary, consensus sequences for elements responsive to cAMP and to TPA have been determined. The activity mediated by these elements to gene transcription depends on a number of factors. These include the exact sequence of the element, the flanking sequence and the gene being regulated.

CRES AND TRES AS BASAL AND TISSUE-SELECTIVE ELEMENTS

CREs and TREs have also been shown to be involved in both basal

activity and in tissue selective activity. Deletion of the core sequences of the phosphoenolpyruvate carboxykinase gene (Quinn et al., 1988) resulted in an 85% decrease in basal activity and the loss of cAMP responsiveness. The CRE of the glycoprotein hormone α-gene raises basal activity of heterologous promoters in a manner dependent on the identity of the individual promoter. In addition, the degree of cAMP inducibility was found to be dependent of the cell line (Jameson et al., 1989) suggesting its involvement in tissue-selective regulation. The ENKCRE-2 element of proenkephalin has been demonstrated to be necessary for basal as well as induction by cAMP and TPA (Comb et al., 1988). When linked to a minimal α-chorionic gonadotropin promoter, the glucagon CRE is not capable of mediating induction to cAMP analogs in JEG-3 cells which do not express the endogenous glucagon gene. However, it can mediate responsiveness in a glucagon-expressing pancreatic cell line (Knepel et al., 1990). The CRE of the somatostatin gene is necessary for the expression of a somatostatin/CAT gene fusion in pancreatic islet cells (Powers et al., 1989). Timmers et al. (1990) report that a TRE-like element (TGACTCC) is necessary for basal activity of the rat JE immediate early gene but is not responsive to TPA. They also demonstrated, by gel shift assays in the presence of competitive DNAs, that this site was capable of binding many of the same proteins as the consensus TRE from the collagenase gene. The binding specificities were not identical. In summary, it is clear that CREs and TREs are responsible for more than induction via the PKA and PKC pathways, they contribute to the fine-tuning of gene expression.

RESTRICTED GENE EXPRESSION

Gene expression is often restricted in a cell-type selective pattern by proteins which bind to enhancers. This restriction can be either positive, where expression is increased by the binding of a particular nuclear factor, or negative, where transcription is inhibited by the protein binding. Most often the promoter contains a number of both positive and negative factors. These cis-elements and the corresponding proteins can be located by either physical or functional studies.

The somatostatin gene contains a negative element between bases -65 and -250 which allowed expression of the gene in cell lines derived from pancreatic islet cells but not in other cell lines tested (Powers et al., 1989). The SCG10 gene was found to be negatively regulated by an element which suppresses activity of the gene in non-neuronal cells (Mori et al., 1990). Also the type II sodium channel gene normally expressed in rat brain was found to be controlled by a negative element in skeletal muscle cells (Maue et al., 1990).

Analysis of the genes coding for digestive enzymes including α-amylase 2, elastase 2 and trypsin[a] were found to specifically bind the same protein in cells derived from the acinar pancreas. It was shown that the DNA binding motif was necessary for enhanced expression of the α-amylase 2 promoter in a cell-type selective manner (Cockell et al., 1989). The synapsin I gene was found to contain positive regulatory elements that contributed to its expression in some cell types (Thiel et al., 1991). The rat ceruloplasmin gene was found to contain an element which allowed hepatocyte-specific expression by the binding of a

specific protein factor (Fleming and Gitlin, 1992).

Often both positive and negative regulatory elements are found to be active in the same gene. An example of this is the gene coding for human factor X, which is involved in blood coagulation (Miao, 1992). It was also reported for the mouse gene coding for proteoglycan peptide core (Avraham et al., 1992). The presence of a combination of positive and negative regulatory elements would give a high degree of sensitivity to the control of transcription for genes. This is advantageous to the organism where appropriate gene expression is necessary for proper function.

SPECIFIC AIMS

The specific aims of the research reported in this dissertation were twofold. The first aim was to determine if the 5'-proximal ATG of the DBH gene is the only one used to initiate translation. This was accomplished through the use of hybrid DBH/CAT constructs. Dopamine β-hydroxylase is found in two forms in secretory granules, a soluble and a membrane-bound. At least a portion of the membrane-bound form appears to be attached by subunits which contain an uncleaved signal sequence with a charged N-terminal present in the cytosol. The soluble form has not been found to contain subunits with a signal sequence. Searches for different mRNAs to explain the formation of the two types of subunits have been unsuccessful. For the rat gene, use of the initial ATG would allow the translation of a peptide with a hydrophobic segment capable of spanning the membrane and a charged N-terminal end, capable of anchoring the protein and residing in the cytosol. Use of the subsequent ATG would result in a peptide where translation initiated in the hydrophobic region and therefore would not have the anchoring amino acids. This might result in formation of the soluble subunits of DBH or subunits attached to the membrane by other means. Therefore, utilization of different translation initiation codons, and differential processing of the resultant peptides, might explain the formation of the two different

types of subunits and the existence of the two forms of DBH.

The second aim was to identify regions in the 5′ flanking sequence of the DBH gene which are responsible for regulating its level of transcription. DBH is present only in certain tissues. In addition, there is evidence that the level of transcription may be a factor in regulating the level of DBH enzyme activity in these tissues. DBH 5′ flanking sequence was fused to the coding sequence of the reporter gene for chloramphenicol acetyl transferase and deletion mutants were constructed. They were utilized to locate regions of the 5′ flanking sequence involved in regulating expression of the gene in cells which transcribe the endogenous DBH gene. Their use also revealed regions which are involved in induction via the protein kinase A and protein kinase C pathways, and the location of elements which contribute to cell-type restricted expression of DBH.

MATERIALS AND METHODS

ISOLATION OF GENOMIC CLONES

A rat genomic library in the lambda phage vector EMBL3 (provided by O. Civelli) was screened by plaque hybridization (Maniatis et al., 1982). Plaques were originally screened with 1.0 or 1.4 kb probes corresponding to the 3' end of the bovine DBH cDNA (Lewis et al., 1990). Probes were labelled with α-^{32}P-dCTP (3000 Ci/mMol, Amersham) using the Nick Translation Reagent Kit (BRL). Subsequent screenings were performed with ^{32}P-labelled oligonucleotides kinased with γ-^{32}P-ATP (3000 Ci/mmol, Amersham); oligonucleotide sequences were chosen on the basis of sequence homology between the human (Lamouroux et al., 1987) and bovine cDNA clones (Lewis et al., 1990). The second screenings of phage plaques were performed with the degenerate oligonucleotide TCTGG/ATCG/ACTCCAG/A/TGCG/ATC which corresponds to nucleotides 338-355 of the originally reported human sequence. Final screenings were performed by Southern blotting with the oligonucleotide ATGACCAGGAAGATGGCCAG which corresponds to nucleotides 73-92 of the same sequence. Positive clones were amplified by the plate lysate method (Maniatis et al., 1982) and the DNA was extracted and purified using LambdaSorb (Promega). After digestion with restriction enzyme(s), the resultant fragments were separated on 1% agarose gels and then

transferred to nitrocellulose.

To determine the 5' end of the cloned DNA, we took advantage of the knowledge that Sal I can remove the non-phage insert from EMBL3 without cleaving the phage arms (Frischauf et al., 1987). Isolated phage DNA was cleaved with a series of restriction enzymes which do not cut the arms of EMBL3, both as single digests and as double digests with Sal I. Southern blotting, using the kinased 73-92 oligonucleotide as the probe, allowed determination of the 5' end of the clone.

DNA SEQUENCE ANALYSIS

Sequence analysis of the 5' most 600 bases of the DBH clone was performed by a modification of the method of Sanger et al. (1977) using the TacTrack Sequencing System (Promega) and the directions provided by the manufacturer. Initial sequencing was performed on double-stranded DNA subcloned into the plasmid pGEM-3Z (Promega) on the full-sized subclone. Subsequent use of an internal Eco RI site allowed smaller subclones to be used. Both strands of the clone were sequenced. Ambiguities due to "compressions" which appear in the autoradiograph of the sequencing gel due to the high guanosine content of certain regions of the DBH gene, were resolved by the use of deaza dGTP in the sequencing reaction.

PLASMID CONSTRUCTION

The 5' Sal I/Sst I fragment which hybridized to the 73-92 probe was subcloned into pGEM-3Z (Promega). Subsequently, a Sst I/Eco RI fragment was removed from the original subclone and recloned into pGEM-

3Z. The Sst I and Eco RI ends of the original plasmid were blunted and ligated. All of these plasmids were used for sequencing.

In order to assess promoter activity the 5' flanking region of the DBH gene was fused to the coding region of the bacterial gene chloramphenicol acetyl transferase (CAT). Two different constructs which contain the promoter fragments extending 5' to -394 were originally constructed: DBH (-394-(-10)) and DBH (-394-(+14)). The -10 construct contains the endogenous DBH TATA box but deletes the transcription start site. The +14 construct contains the start site but also contains a translation start codon at +10-12. This ATG was subsequently mutated (see below) resulting in DBH (-394-(+14 AGG)). DBH (-394-(-10)) was constructed by complete digestion of the original pGEM clone with FOK I, separation of the fragments on an 1% agarose gel and isolation of DNA of the 1.5 kb band (GENECLEAN (Bio 101)). Overhanging ends were blunted with the Klenow fragment of DNA Polymerase and mononucleotides, and the fragment was redigested with Hind III. The 400 bp fragment was isolated and subcloned into the promoterless plasmid PUCCAT (Lewis et al., 1987). DBH (-394-(+14)) was constructed by the same method with the restriction enzyme Fnu 4HI substituted for Fok I. RSV-luciferase and -109 TK-luciferase, in which the coding region of the luciferase gene (de Wet et al., 1987) is under transcriptional control of promoter from the Rous sarcoma virus (RSV) long terminal repeat or the herpes simplex thymidine kinase (TK) promoter (Nordeen, 1988) were used as controls to monitor transfection efficiency in CAT assays where necessary.

For ribonuclease protection assays, probes were generated from

portions of the DBH/CAT and TK/luciferase genes inserted into pGEM-3Z. For the CAT template, the Eco RI fragments extending from -79 of the DBH promoter 250 bases into the CAT gene were excised from DBH (-394-(+14)) and DBH (-394-(+14 AGG)) and individually subcloned into the Eco RI site of pGEM-3Z. Orientation of the fragments was determined by restriction mapping and plasmids in which the antisense strand could be generated by RNA polymerase T7 were chosen for probe generation. For the luciferase template, the fragment extending from the Eco RI site of the TK promoter to the Xba I site of the luciferase gene was inserted into Xba I/Eco RI digested pGEM-3Z. Orientation of the fragment required that SP6 RNA polymerase be used to generate this probe.

MUTAGENESIS OF DBH/CAT (-394-(+14))

Due to the presence of a translation start site at nucleotides +10-12 in the DBH/CAT fusion (-394-(+14)), oligonucleotide-directed mutagenesis was used to change this ATG to AGG, which will not initiate translation (Peabody, 1989). This resulted in the creation of the plasmid DBH (-394-(+14 AGG)). Mutagenesis was performed using the Altered Sites in vitro Mutagenesis System (Promega). The method employed relies on the concomitant creation of a second mutation in the supplied mutagenesis plasmid which repairs the ampicillin resistance gene, and thereby confers resistance to transformed bacteria. Because of the conditions employed, over 50% of the phagemids screened may harbor the mutation desired and screening is performed by sequence analysis. Directions provided by the manufacturer were followed. The primer used for mutagenesis was complimentary to nucleotides 6-20 of the

DBH coding sequence (primer sequence gctccctg cCt gggctggga, the mutated base is capitalized). Briefly, the 2 kb Bam HI fragment containing the DBH promoter and CAT gene was removed from DBH (-394-(+14)) and subcloned into the phagemid pSELECT-1. Proper orientation was confirmed by restriction mapping and single strand DNA was generated using helper phage R408. After annealing of the mutagenesis and ampicillin repair primers, second strand synthesis was performed with T4 DNA polymerase and ligation was accomplished concomitantly with T4 DNA ligase. After transformation of the repair defective bacterial strain BMH 71-18 mut S, ampicillin and tetracycline resistant mutants were amplified. Final selection was by sequence analysis utilizing a primer complimentary to a portion of the 5' untranslated region of the CAT gene (CAACGGTGGTATATCCAGTG) (Gorman et al., 1982).

PROMOTER-DELETION MUTANTS

Deletion mutants of DBH (-394-(+14 AGG)) were created with the reagents from the Erase-a-Base System (Promega). The procedure recommended by the manufacturers was followed with sight modifications. Briefly, DBH (-394-(+14 AGG)) plasmid DNA was enriched for the supercoiled form by extraction with acid phenol (Zasloff et al., 1978), and subsequently cut to completion with Sph I and Sal I. Exo III digestion of 5 μg of plasmid was done at 34° in a total volume of 60 μl. Two μl aliquots were removed at 15 second intervals and the reaction was terminated by diluting the reaction mixture into 7.5 μl of S1 nuclease buffer on ice. After treatment with S1 nuclease and blunting of uneven ends with the Klenow fragment of DNA polymerase and dNTPs, plasmids were

ligated with T4 DNA ligase in the presence of polyethylene glycol. JM109 bacteria were transformed, plated on agar and selected for ampicillin resistance. Individual colonies were amplified in mini-cultures and screened by analysis of DNA digested with Hind III and Pvu II on 2.5% agarose. Recombinants containing the appropriate sized fragments were further screened by sequence analysis.

CELL CULTURE

All cell lines were maintained in a humidified atmosphere consisting of 95% air, 5% CO_2 at 37°C. All media was supplemented with 100 units per ml penicillin and 100 μg per ml streptomycin. SH-SY5Y human neuroblastoma-derived cells which express endogenous DBH enzyme activity (Ross et al., 1983) were obtained from Dr. June Biedler. They were grown in minimal essential media (MEM):Hams Nutrient Mixture F12 (F12) 1:1 supplemented with 10% fetal calf serum. JEG-3 human placental choriocarcinoma cells, CV-1 monkey kidney cells and C6 rat glioma cells were maintained in Dulbecco modified Eagle medium (DMEM) supplemented with 10% fetal calf serum (FCS). All media was from JRH Biosciences and sera was from Hyclone.

DNA TRANSFECTIONS

SH-SY5Y cells were plated at $1x10^6$ cells per 60 mm dish or $3x10^6$ cells per 100 mm dish 72 hrs. before transfection. Alternatively, they were plated at 50% higher density 48 hrs. before transfection. CV-1, JEG-3 and C6 cells were plated 24 hrs. prior to transfection. CV-1 cells were plated at 5×10^5 cells per 100 mm plate, JEG-3 and C6 cells

were plated at a cell density of approximately 10% of confluence. Media was changed three hours prior to transfection.

Plasmid DNA was transfected into cultured cells by the calcium phosphate coprecipitation method (Graham and van der Eb, 1973) using the reagents from the Transfinity Calcium Phosphate Transfection System (BRL). DBH/CAT fusion plasmids were transfected with 15 μg of plasmid per 100 mm plate or 5 μg of plasmid per 60 mm plate. When used as an internal control for transfection efficiency RSV-luciferase was cotransfected at 2 μg per 60 mm plate, alternatively TK-luciferase was co-transfected at 5 μg per 100 mm plate. The calcium phosphate/DNA precipitates were prepared according to directions provided by the manufacturer and were applied to plates of cells at the amount of 1 ml to 8 mls of media for 100 mm plates or 0.5 ml to 3 mls of media for 60 mm plates. The precipitate was allowed to remain on the cells for four hours; at the end of that period the media was removed. Except as noted, the media was replaced by 15% glycerol in PBS for two minutes. In all cases cells were washed twice with PBS and refed with fresh media containing serum. For experiments where the inducibility of CAT activity by the PKA and PKC pathways was determined, media was replaced with fresh media containing 1% FCS 20 hours after initial transfection. Inducers, a final concentration of 200 μm 8-(4-chlorophenylthio)-cAMP (CPT-cAMP), 100 nm TPA or both together were added six hrs. later. Harvest was 48 hrs. after initial transfection.

CAT ASSAYS

Cells were harvested by scraping into 1 ml of PBS and centrifuging

in a microfuge for 5 minutes (4°, 9,500 rpm, 7,500 x g). Pellets were resuspended in 1 ml of PBS and respun under the same conditions. Pellets were then resuspended in 110 μl of 0.1 M potassium phosphate (pH 7.9) 1 mM DTT if CAT and luciferase assays were to be performed, or 250 mM Tris (pH 7.6), if only CAT assays were to be done. Cells were lysed by three cycles of freeze/thaw and cellular debris was removed by centrifugation at full speed in a microfuge. Protein concentration was assayed by the method of Bradford (Bradford, 1976), using the dye reagent from BRL, and BSA (Sigma) as the protein standard. Luciferase assays were done in triplicate for each CAT assay and the results were averaged. The final assay solution for luciferase contained 25 mM glycylglycine (pH 7.8), 15 mM $MgSO_4$, 5 mM ATP and 1-5 μl of cell extract. The reaction was initiated by the addition of 100 μl of 0.1 mM luciferin and monitored for ten seconds on a Packard Picolite luminometer. Immediately prior to assay for CAT activity, extracts were heated at 65° for ten minutes to remove interfering deacetylase activity (Crabb and Dixon, 1987). Assay solutions contained cell extract (30-200 μg protein), 0.5 mM acetyl CoA (Pharmacia) and ^{14}C-chloramphenicol (0.1 mCi per assay minimum, NEN 60 mCi/mMol). Volume was adjusted to 180 μl with 250 mM Tris (pH 7.6). Reactions were allowed to proceed for one to two hrs. at 37° and terminated by extraction with one ml of ethyl acetate. 0.9 ml of ethyl acetate was dried with heat on a vacuum desiccator, products were resuspended in 30 μl of ethylacetate and separated by thin layer chromatography. Acetylated derivatives were separated from unreacted chloramphenicol by thin-layer chromatography and quantitated by liquid scintillation counting after visualization of

the products by autoradiography. For experiments where basal promoter activity was determined, both in SH-SY5Y and other cell lines, relative CAT activity was determined by correcting for transfection efficiency estimated with luciferase activity. For experiments where induction via PKA or PKC pathways was to be determined CAT activity was corrected for protein content. For each determination of CAT activity, two or three separate plates were transfected with the same precipitate solution, assayed independently and the results were averaged.

RIBONUCLEASE PROTECTION ASSAYS

RNA was extracted from SH-SY5Y cells by the LiCl/urea precipitation method (Auffrey and Rougeon, 1980) with slight modifications. Cell pellets were homogenized in 5-10 volumes of 6 M urea, 3 M LiCl and 10 mM vanadyl ribonucleoside, sonicated for 3 x 15 seconds on ice, and incubated at 4° for 18 hours. The pellet was collected by centrifugation in a microfuge (15 min, 4°, full speed) and resuspended in buffer (20 mM Tris (pH 7.5), 5 mM EDTA (pH 7.5), 0.1% SDS. This solution was extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (50:49:1) and precipitated twice with ethanol. RNA quantitation was determined by absorbance at 260 nm and purity was assessed by the ratio of absorbance at 260 and 280 nm.

RNA probes for RNase protection assays (RPAs) were generated from the T7 (DBH) or SP6 (luciferase) promoters of pGEM-3Z using reagents from the Riboprobe kit (Promega) and the directions provided by the manufacturer. They were internally labelled with α-32P-UTP (800 Ci/mmol, Amersham). Full-length DBH-derived probes were 414 nucleotides

long and full-length luciferase-derived probes were 210 nucleotides long. After digestion of the DNA with RNase-free DNase, probes were purified with RNaid (Bio 101). Full length transcripts were size-selected by electrophoresis on a 6% denaturing gel followed by elution of the proper band and a second purification with RNaid. Probe was used within five days of preparation.

RPAs were performed essentially as described by Sambrooke et al. (1989). 1 x 10^4 dpm of probe was hybridized with 50 μg of RNA in a total volume of 20 μl of hybridization buffer (40 mM Pipes (pH 6.4), 0.4 M NaOAc (pH 6.4), 1 mM EDTA, 80% formamide) at 44° for 16 hours. Digestion was carried out with 2500 units of ribonuclease T1 (BRL) for one hour at 30° in a total volume of 200 μl. At the end of this time, 20 μl of 10% SDS and 10 μl of freshly reconstituted Proteinase K (10 mg/ml, Boehringer) was added and the incubation was continued at 37° for 15 minutes. The solution was extracted one time with phenol/chloroform, 5 μg of carrier tRNA was added and the RNA was precipitated with ethanol. Size fractionation of the RNA was carried out on a 6% acrylamide denaturing gel. An end-labelled Sau 3A digest of pGEM-3Z was used as a size marker.

RESULTS

ISOLATION OF A CLONE OF DOPAMINE β-HYDROXYLASE FROM A RAT GENOMIC LIBRARY

To isolate a genomic clone of the rat gene for dopamine B-hydroxylase an amplified library of rat DNA, cloned into the bacteriophage EMBL3 was screened. Initially 2.5 x 10^5 plaques were screened with nick-translated cDNAs corresponding to the 3' end of the bovine dopamine β-hydroxylase gene (Lewis et al., 1990). 29 plaques were isolated and phage from 15 of these were subjected to a secondary screen. Plaques from 11 of the 15 tested positive. Subsequently, eight of these clones were screened with a ^{32}P-labelled oligonucleotide corresponding to nucleotides 333-355 of the previously published sequence for a human cDNA clone (Lamouroux et al., 1987). Of these two tested positive, two negative and the results for the other four were ambiguous. The six non-negative clones were analyzed by Southern blotting of Eco RI, Sst I and Bam HI digested DNAs using a ^{32}P-labelled oligonucleotide corresponding to nucleotides 73-92 of the same human cDNA clone for the probe. Four of the six hybridized to the oligonucleotide; three had identical restriction patterns and one differed in that a single band was approximately 200 bp shorter. One of the larger clones was chosen for further analysis.

DETERMINATION OF THE 5' END

The primary reason for our isolating a genomic clone was to identify regions of the DBH gene's 5'-flanking sequence which are important in regulating expression of the gene. Since most transcription control elements have been found to exist 5' to the transcribed portion of the gene, it was important for us to determine how much of this region our clone contained. We took advantage of the ability of the restriction enzyme Sal I to remove the rat-derived DNA insert from the phage without cleaving the phage arms. Purified DNA was cleaved with a series of restriction enzymes which are known to not cleave the arms (Eco RI, Bam HI, Sst I, Xba I and Xho I) both as single digests and as double digests with Sal I. When analyzed on agarose gels, several of these enzymes gave restriction patterns where the single digests had two fewer bands than the double digests. When the 73-92 oligonucleotide was used as a probe in Southern blotting, it hybridized to a ten kb fragment from the Sst I digest and to a 600 bp fragment in the Sst I/ Sal I digest. The 600 bp fragment was subcloned into the plasmid pGEM-3Z for sequence analysis.

SEQUENCING STRATEGY

DNA inserted into the multiple cloning site of pGEM-3Z is amenable to sequencing by the dideoxy method (Sanger et al., 1977) using a protocol which has been adapted for sequencing double-stranded templates. Primers used were complimentary to the promoter sequences for SP6 and T7 RNA polymerases. Initial sequencing revealed an Eco RI site approximately in the center of the fragment. The Sst I/Eco RI

fragment was subcloned into pGEM-3Z and the plasmid remnant which resulted from removal of the fragment was recircularized, All of the plasmids were sequenced in both directions. The region between the TATA box and the transcription start site is guanosine-rich and autoradiographs of the sequencing gel were difficult to read due to a number of compressions. Ambiguities were resolved by the use of deaza dGTP in the reaction mixture. The DNA sequence is shown in Figure 3.

COMPARISON WITH PUBLISHED SEQUENCES

After this work was initiated, the sequence of a cDNA clone corresponding to rat DBH was reported (McMahon et al., 1990). The 200 bases on the 3' end of our genomic fragment are identical to the 5'-most bases of the published sequence. Sequences for human cDNA (Lamouroux et al, 1987) and genomic clones (Kobayashi et al., 1989) as well as bovine cDNA clones (Lewis et al., 1990; Taljanidisz et al., 1989) have also been reported in the literature. The transcription initiation sites have been estimated by primer extension for human and bovine mRNA and are consistent with those noted in Figure 3. RNase protection assays have corroborated these results for the rat transcript. Comparison of the sequences for the three species (Figure 4A) show high homology in the 12 bases immediately downstream from the transcription start site. A translation start codon (ATG) is present +10 in all transcripts. Immediately 3' to the start codon is a region where there are deletions in the human and bovine sequences as compared to the rat sequence. The human sequence is nine bases shorter than the rat and the bovine sequence is 30 bases shorter; both differences are in multiples of

Figure 3. Nucleotide sequence of DBH genomic DNA. This sequence corresponds to the 5′ most 596 bases of a clone isolated from a library in EMBL3. Numbering is relative to the predicted start site of transcription (+1). Possible binding sites for known transcription factors are noted; shaded sequences have high homology to their known consensus binding sequences. Also noted, and shaded, are the TATA box and the transcription and translation start sites.

```
                                        AP1                    -344
AACGGATCCA GGAACCCACC ACTCACTGTC ACTCAGGAAG GAGCCCCTTG

                                                               -294
AACCTCAGTT GGAGCAGGTA GAGAGGCCCT GACTGCCTAT GAGGCATTCA

                                                               -244
GCAGGGCCTG GCTGGAGGTG TCCTTGGGAC CTATGTCTGC AGAGAGTAGC

                    CRE             CAAT                       -194
TGTTTCCAAC AGGCGTCAGA GATCCATTGG AGGACATGGC CATTCTGCTT

                              CRE/AP1          CAAT              -144
CGATTCTCTT GATGATGTCC ATGCGTCATT AGTGTCAATT AGGGGAGGAT

                         AP2                                   -94
CGGAGCAAAG TGTTTGCCCC AGGGCATGGG CTGGTGGGAG AGCCACCAGG

 CAAT                                                          -44
ACAATTGAAT TCCCCACCAG ACAAATGTGA TTAGGTACAG CCTGGCCCAA

                      TATA                      AP2      TRANSCRIPTION
CCCCACCGAA CAGACATAAA TGGCCCAGTG GGGCTGGGGT GCTCATCCCA

 TRANSLATION                                                    56
GCCATGCAGC CTCACCTCAG CCACCAGCCT TGCTGGAGCC TCCCCAGCCC

                                                              106
CAGCGTCCGT GAGGCGGCTT CCATGTATGG CACTGCTGTG GCCATCTTCC

                                                              156
TGGTCATCCT GGTGGCTGCA CTGCAGGGCT CGGAGCCTCC GGAGAGCCCC

                                                          202
TTCCCTTACC ACATCCCCCT GGACCCTGAA GGGACTTTAG AGCTCG
```

Figure 4. Comparison of DBH sequences between species. A. Nucleotide sequences. Comparison of sequences beginning at the TATA box for rat and human genes, and from a full-length cDNA for the bovine gene, aligned for highest homology. B. Comparison of the amino acid sequences derived from the nucleotide sequence in A. aligned for highest homology. Translation is believed to initiate at the first ATG codon in A.

A.

```
Rat     CAT AAA TGG CCC AGT GGG GCT GGG GTG CTC ATC CCA GCC
Human   CAT AAA TGG CCA GGT GGG ACC AGA GAG CTC ACC CCA GCC
Bovine                                          C CCA GCC

Ra      ATG CAG CCT CAC CTC AGC CAC CAG CCT TGC TGG     AGC
Hu      ATG     CCC GCC CTC AGT             CGC TGG GCC AGC
Bo      ATG CAG

Ra      CTC CCC AGC CCC AGC GTC CGT GAG GCG GCT TCC ATG TAT
Hu      CTG CCC GGC CCC AGC ATG CGG GAG GCA GCC TTC ATG TAC
Bo      GTC CCC AGC CCC AGC GTG CGC GAG GCG GCC TCC ATG TAC

Ra      GGC ACT GCT GTG GCC ATC TTC CTG GTC ATC CTG GTG GCT
Hu      AGC ACA GCA GTG GCC ATC TTC CTG GTC ATC CTG GTG GCC
Bo      GGC ACC GCG GTG GCC GTC TTC CTG GTC ATC CTC GTG GCT
```

B.

```
Rat     Met Gln Pro His Leu Ser His Gln Pro Cys Trp     Ser
Human   Met     Pro Ala Leu Ser             Arg Trp Ala Ser
Bovine  Met Gln

Ra      Leu Pro Ser Pro Ser Val Arg Glu Ala Ala Ser Met Tyr
Hu      Leu Pro Gly Pro Ser Met Arg Glu Ala Ala Phe Met Tyr
Bo      Val Pro Ser Pro Ser Val Arg Glu Ala Ala Ser Met Tyr

Ra      Gly Thr Ala Val Ala Ile Phe Leu Val Ile Leu Val Ala
Hu      Ser Thr Ala Val Ala Ile Phe Leu Val Ile Leu Val Ala
Bo      Gly Thr Ala Val Ala Val Phe Leu Val Ile Leu Val Ala

Ra      Ala Leu Gln Gly Ser Glu Pro Pro Glu Ser Pro Phe Pro
Hu      Ala Leu Gln Gly Ser Ala Pro Arg Glu Ser Pro Leu Pro
Bo      Ala Leu His Gly Ser Ala Pro Ala Glu Ser Pro Phe Pro
```

three. When corresponding amino acid sequences are compared, it is found that there is a high degree of sequence homology in the amino acids present. Many of the base-substitutions in the nucleotide sequence result in no change in the amino acid sequence or in conservative substitutions (Figure 4B). The largest differences in protein structure found between species occur in the N-terminal end.

STUDY OF TRANSLATION INITIATION OF THE DBH/CAT FUSION

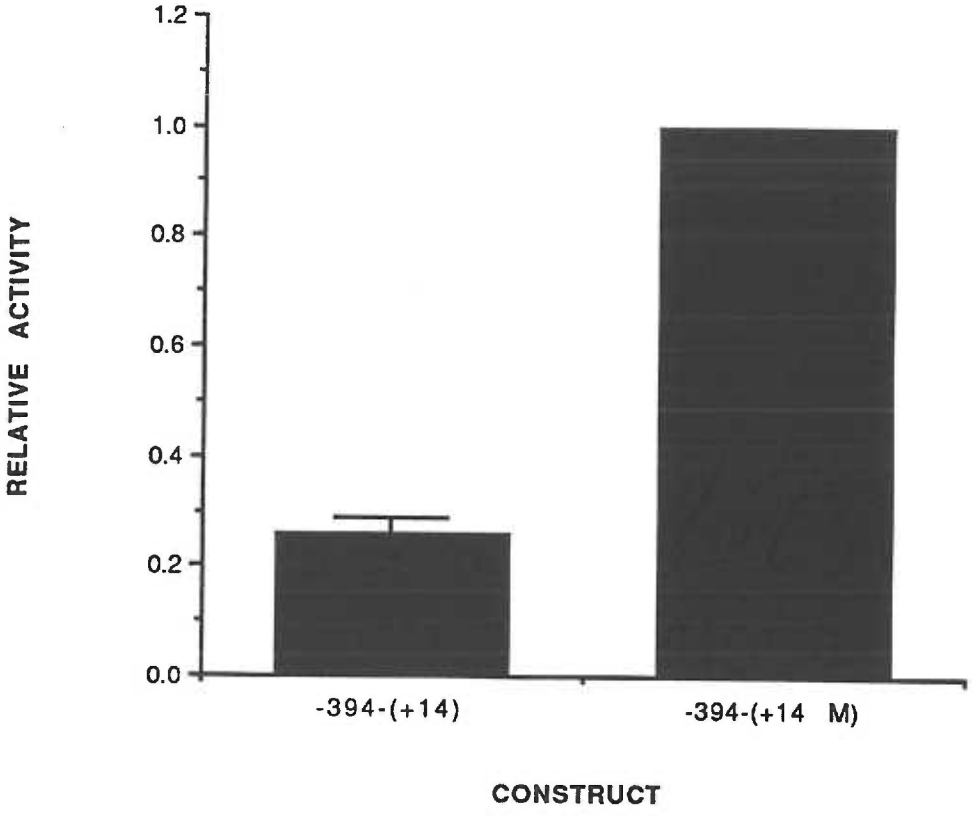
The translation initiation codon (ATG) 10 bases after the predicted transcription initiation site is in a good sequence environment to be used as a start site for translation (Kozak, 1984). However, it has been shown that ATGs within 15 bases of the 5′ end of the mRNA are inefficiently used to initiate translation (Kozak, 1991). If the second ATG were to be used for translation, the physical characteristics of the N-terminal end would be drastically changed. Translation would start in the hydrophobic sequence for the rat and bovine genes and three amino acids towards the N-terminal end from the hydrophobic sequence in the human gene. We wished to explore the possibility that the DBH gene may in fact have two different translation initiation start sites. If in fact two different ATGs can be used to initiate translation, the existence of the two different forms of DBH may be related to the physical properties of the translation products or in differences in protein processing.

If our DBH/CAT fusion gene -394-(+14) is translated using the initial ATG, the CAT gene would be translated as a frame-shift mutant (see Figure 10 p.75). The protein translated would not express CAT

activity. The second ATG is the endogenous translation start site for the CAT gene and translation utilizing this as the initial codon would result in active CAT enzyme. The -394-(+14 AGG) construct is expected to utilize only the endogenous CAT translation start site. Therefore a comparison of the corresponding CAT enzyme and mRNA levels for each of the constructs should indicate the relative use of each translation initiation codon. We carried out CAT assays and RNase protection assays in order to answer this question.

When transfected in parallel and corrected for transfection efficiency, by the use of the internal control TK-luciferase, the DBH/CAT fusion -394-(+14 ATG) construct gave approximately 25% of the activity of -394-(+14 AGG) (Figure 5). This implies that the first ATG is used a large percentage of the time and produces translation products which do not have CAT activity. It also implies that the residual CAT activity is the result of enzyme produced by translation initiating at subsequent ATGs. Other possible explanations for residual CAT activity include the presence of cryptic promoters upstream from the endogenous DBH promoter supporting DNA transcription which extends into the CAT gene. The RNA thus synthesized would then be translated in frame with the CAT protein. The fusion proteins created would then have CAT enzyme activity. Alternatively, translation may in fact initiate at the second or subsequent ATGs in all cases but, RNA levels resulting from transcription of the unmutated plasmid are proportionately lower than those for the mutated one. A third possibility is that the RNA resulting from transcription of the unmutated plasmid is not translated as efficiently as the mRNA of the mutated one.

Figure 5. Comparison of relative CAT activity for unmutated vs mutated DBH/CAT gene fusions. SH-SY5Y cells were tranfected with DBH/CAT and TK luciferase as in Materials and Methods. The graph represents the average of 3 separate transfection experiments. The CAT activity is standardized by the luciferase activity for each sample. The standardized CAT activity for (-394-(+14 AGG)) was set to 1.00 for each experiment and the relative activity for the unmutated construct was subsequently calculated. The error bar represents the standard error.


RELATIVE ACTIVITY
1.2
1.0
0.8
0.6
0.4
0.2
0.0
-394-(+14)
-394-(+14 M)
CONSTRUCT

Analysis of the 5′-flanking sequence shows that the -394 DBH/CAT construct contains translation initiation codons (ATGs) in all reading frames 5′ to base -170. The promoter also contains translation stop codons in all three frames, at -28, -63 and -91. Therefore, if mRNA initiating upstream to or in the DBH promoter was translated, translation would terminate prior to the coding sequence of the CAT gene. The CAT gene would not be translated and thereby contribute to background CAT activity.

To approach the second possibility, that mutagenesis of the 5′ ATG to AGG results in increased amounts of CAT mRNA, we transiently transfected cells with each of the CAT constructs. We performed both enzyme and RNase protection assays on the same cellular extracts. Separate RNA probe templates were made for each of the CAT constructs and TK-luciferase was used as an internal control for both the enzyme and RNA assays. All procedures were done in parallel from the same stock solutions.

As shown in Figure 7A, the unmutated DBH/CAT fusion showed 39% of the relative CAT enzyme activity of the mutated one.

The RNase protection assays resulted in several bands corresponding to protected DBH/CAT RNA in lanes for both of the constructs (Figure 6). Both lanes contain bands corresponding to 360 and 272 bases. The 360 base band results from "read-through" RNA products (Gizang-Ginsberg and Ziff, 1990) which originate at sites in the vector 5′ to the DBH gene fragment. This RNA is shorter than the undigested probe because the latter contains linker sequences from the pGEM plasmid used to generate the probe. The 272 base band corresponds

Figure 6. RNase protection assays of DBH/CAT constructs and controls. After cotransfection of each DBH/CAT construct with TK-luciferase, total cellular mRNA was isolated from a portion of the sample. RNase protection assays were performed with 50 ug of RNA as described in materials and methods. Samples in lanes 1 and 2 contained both DBH/CAT and luciferase probes. Lane 1, DBH/CAT (-394-(+14 AGG); lane 2, DBH/CAT (-394-(+14)); lane 3, blank; lane 4, 50 ug tRNA with probe from DBH/CAT (-394-(+14)); lane 5, TK luciferase alone (5 ug from transiently transfected CV-1 cells); lane 6, TH-CAT mRNA (15 ug from E. Lewis); lane 7, DBH/CAT (-394-(+14 AGG) undigested probe; lane 8, DBH/CAT (-394-(+14) undigested probe; lane 9, TK-luciferase undigested probe.

The symbol > corresponds to "read-through" RNA, + to the artifactual band, * to the expected DBH/CAT RNA band and # to the 95 base luciferase mRNA band used to standardize the intensities of the DBH/CAT RNA bands.

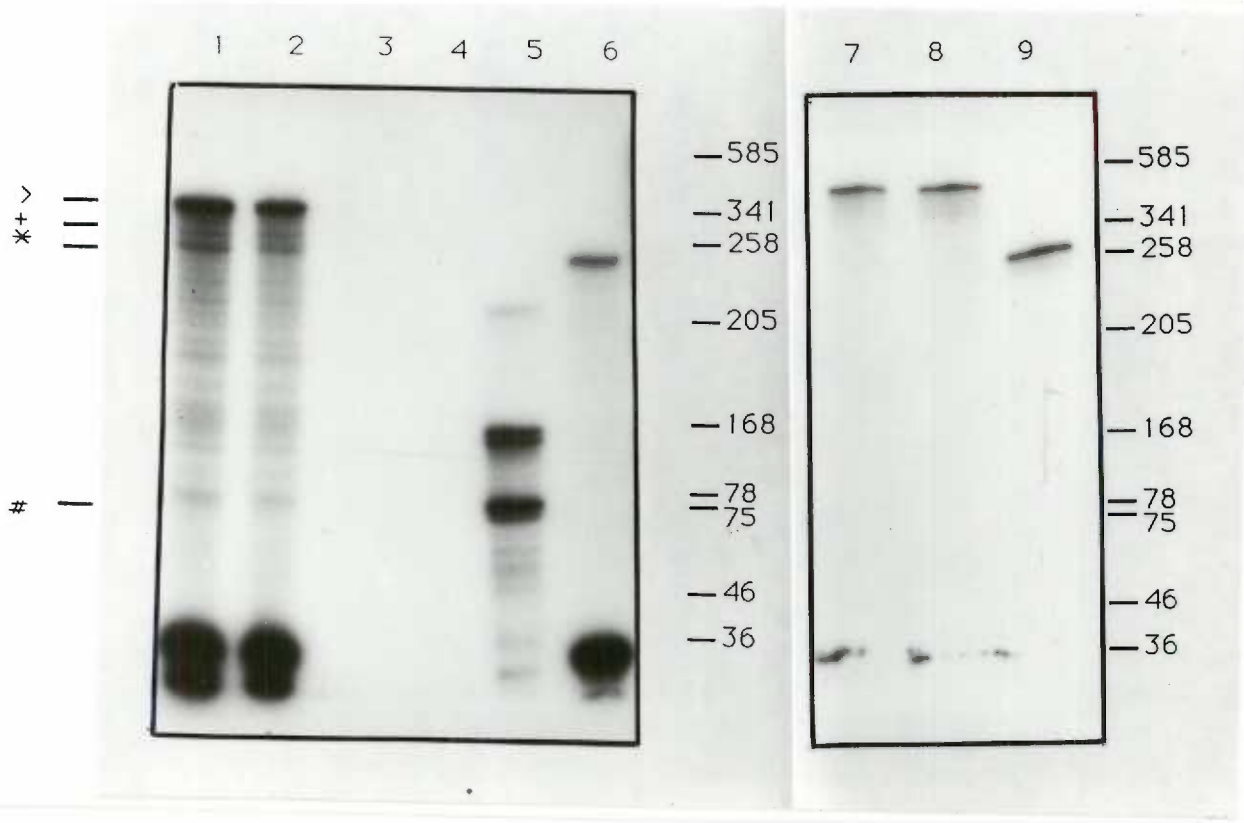

1
2
3
4
5
6
7
8
9
585
341
258
205
168
78
75
46
36
585
341
258
205
168
78
75
46
36
>
+
*
#

Figure 7. CAT enzyme assays of samples used for RNase protection assays and possible RNAse T1 cutting sites of RNAs used in RNAse protection assays. A. Corrected CAT enzyme activity to determine relative amounts of luciferase and CAT activity in transiently transfected cultures of SH-SY5Y cells used for RNAse protection assays. B. RNAse protection assays were performed with ribonuclease T1. This enzyme cleaves on the 3′ side of G. Because of this specificity, it is believed that an artifact has occurred in the results for this procedure, i.e. the appearance of an band approximately 30 bases longer than the expected full-length protected band. A possible loop structure has been found in the region between the TATA box and the transcription initiation site that may explain this artifact. In B. / represents possible cutting sites for ribonuclease T1, +1 designates the start site of transcription, and the nucleotides capable of forming a stem-loop structure are underlined.

A.

CONSTRUCT	LUCIFERASE (COUNTS)	CAT (CPM)	CAT/LUCIF.	RELATIVE CAT/LUCIF.
(-394-(+14))	33562	16719	0.498	0.39
(-394-(+14 AGG))	18465	18465	1.28	1.00

B.

-34 -30 +1

/GTCT /GTATTTACCGGGTCACCCCGACCCCAC /GA /GTAGGGT antisense sequence

to protection of transcribed RNA initiates at +1 of the DBH gene. The intermediate-sized band may be an artifact caused by RNA secondary structure. We used only RNase T1 in our protection experiments to digest unhybridized RNA. This ribonuclease cleaves only on the 5′ side of guanosine. Analysis of the antisense sequence shows several guanosines immediately around the +1 nucleotide (Figure 7B), which should be the 5′ most unprotected nucleotide. There are guanosines at -30 and -34 where if cleavage occurred would give rise to the observed intermediate-sized band. There are additional guanosines at -11, -19, -20 and -21 which are protected from cleavage. Examination of the RNA sequence in this region reveals a potential stem-loop structure with 5 bases paired in the stem. The paired sequence would include GGGTC (-17 to -21). Four of the five bases would form G-C base pairs in which three hydrogen bonds are formed between the bases. In addition, RNA-RNA base pairings are very stable (compared to DNA-DNA or RNA-DNA base pairings). Therefore, this region of the probe may be resistant to cleavage by RNase T1 under the condition we have employed.

TK-luciferase was used as an internal control. The full-length probe is approximately 210 bases long and the protected RNA was expected to be approximately 130 bases. In all experiments in which we performed RPAs of TK-luciferase, there was an additional protected fragment of 95 bases. We attribute this to melting of the RNA-RNA duplex at an AT-rich region and subsequent cleavage by RNase. The band corresponding to the 95 base fragment was used as the internal control so that we could obtain relative intensities of the bands corresponding to CAT mRNAs.

Densitometry analysis of the autoradiographs for the RNase

protection assays are shown in Figure 8. Because of difficulties in determining accurate peak areas corresponding to luciferase bands in the densitometric scans, the area of the peak corresponding to the CAT band was compared to the height of the appropriate luciferase peak. It was found that if the relative CAT/luciferase value for the mutated CAT (AGG) construct was set to 1.00, (relative CAT enzyme activity 1.00) the value for the unmutated (ATG) construct was 1.32 or 1.42 for experiments 1 or 2 respectively (relative CAT enzyme activity 0.39). In other words, transfection of the construct containing the endogenous DBH translation start site resulted in lower enzyme activity but higher amounts of mRNA in transfected cells than did the corresponding construct where this site was mutated. Therefore, we conclude that the lower level of CAT enzyme activity associated with the construct containing the endogenous translation start is not due to a lower amount of mRNA coding for the CAT enzyme. It must be due to a post-transcriptional property of the mRNA.

SEQUENCE ANALYSIS OF THE PROMOTER REGION

A comparison of sequences both 3′ and 5′ to the proposed transcription start site, as well as the results found through primer extension and RNase protection studies done by us and others, have led us to believe that our clone contains 394 bases 5′ to the start of RNA transcription. Examination of this sequence has led to the discovery of a number of sequence motifs which have been found important for the activity of other promoters.

Surrounding the start site of transcription is the sequence

Figure 8. RNase protection assays of cells transfected with (−394−(+14)) and (−394−(+14 AGG)) were performed as described in Materials and Methods. A. Densitometry data. Autoradiograms were scanned, peak heights and areas corresponding to protected fragments of luciferase (95 bases) and CAT (272 bases) RNA are noted. B. Results. Ratio of the area under the peak corresponding to the CAT probe fragment to the height of the peak corresponding to the luciferase probe fragment for each experiment. C. Graphical representation of the relative values in B..

A.

DENSITOMETRY DATA

EXPERIMENT 1

	CONSTRUCT	HEIGHT	AREA
LUCIFERASE	(-394-(+14))	0.64	2.18
	(-394-(+14 AGG))	0.60	1.96
CAT	(-394-(+14))	1.89	5.63
	(-394-(+14 AGG))	1.69	4.01

EXPERIMENT 2

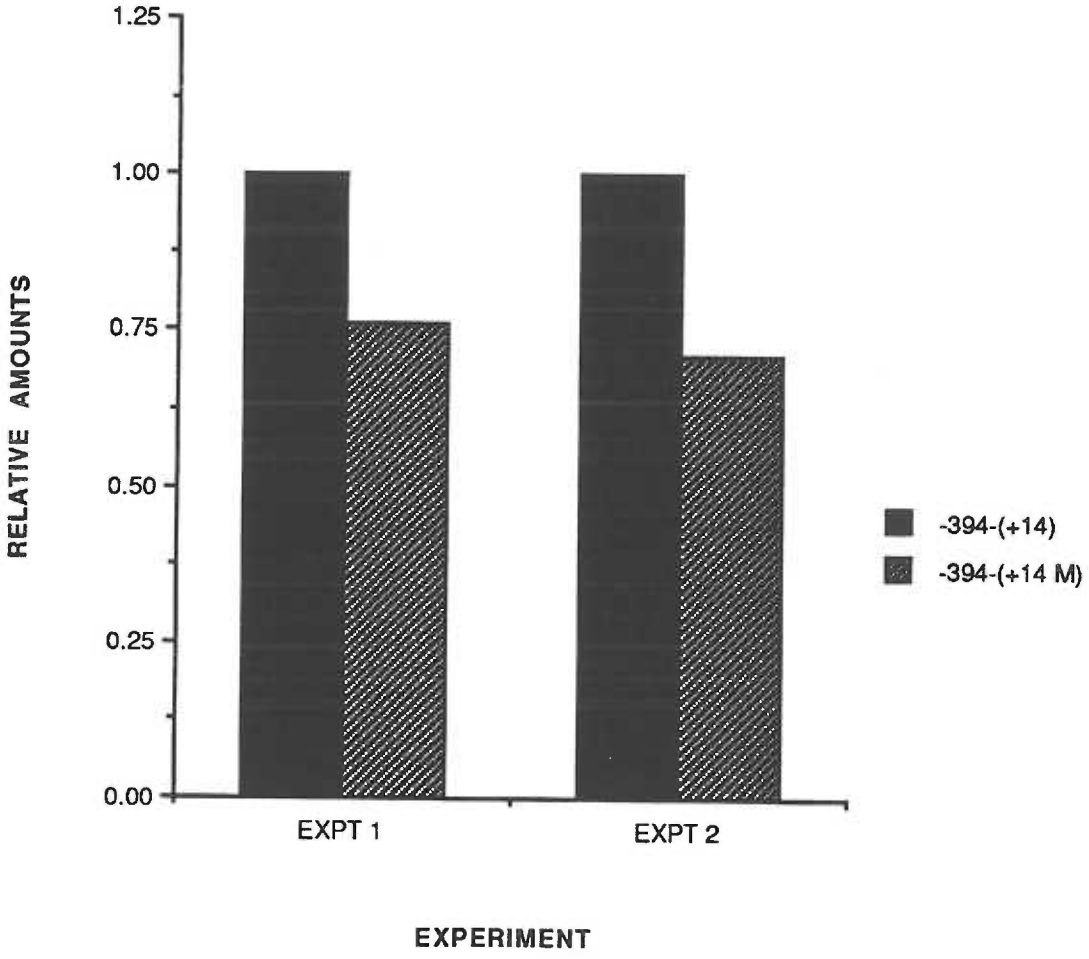
LUCIFERASE	(-394-(+14))	0.45	1.59
	(-394-(+14 AGG))	0.33	<1.31
CAT	(-394-(+14))	1.34	3.59
	(-394-(+14 AGG))	0.77	1.86

B.

RESULTS

CAT/LUCIF (AREA/HEIGHT)

	EXPERIMENT 1		EXPERIMENT 2	
	RAW VALUE	RELATIVE VALUE	RAW VALUE	RELATIVE VALUE
(-394-(+14))	8.80	1.00	7.97	1.00
(-394-(+14 AGG))	6.68	0.76	5.63	0.71


1.25
1.00
0.75
0.50
0.25
0.00
RELATIVE AMOUNTS
EXPT 1
EXPT 2
EXPERIMENT
-394-(+14)
-394-(+14 M)

CTCATCCC. This sequence fits the weak consensus sequence PyPyCAPyPyPyPyPy originally noted by Corden et al. (1980) where transcription initiates at the adenine. The DBH sequence is very similar to the "initiator" (Inr) sequence originally studied in the murine terminal deoxynucleotidyltransferase gene (Smale and Baltimore, 1989) and has been well characterized as being able to support transcription by RNA polymerase II (pol II) in the absence of the characteristic TATA box (Carcamo et al., 1991, Smale et al., 1990). Inr sequences are also found in many eukaryotic genes which do have TATA boxes.

Centered between 24 an 29 bases 5' to the predicted start site of transcription is the motif ATAAAT. This is likely to serve as the binding site for the "TATA box" binding factor TFIID (consensus TATAAAT) which is found in most genes transcribed by pol II.

Other common sequence elements found in genes transcribed by pol II are SpI binding sites, also called GC-boxes (consensus GGGCGG), and CAAT boxes (consensus GGC/TCAAT/ACT)(Darnell et al., 1986). These binding sites for regulatory proteins have been found to be effective in both orientations. There are no consensus binding sites for the factor SpI in the DBH gene, however there are a number of GC-rich regions in the 110 bases proximal to the transcription start site where the Sp1 sites are often found. There are three CAAT motifs with weak homology to the CAAT box, two in the forward orientation at -92 and -158 and one in the reverse orientation at -219. The latter two are further from the transcription start than is usually observed.

In addition, there are several sequence elements similar to those

found to mediate increased activity to genes responsive to induction via the protein kinase A and/or protein kinase C pathways. AP2 is a transcription factor which has been shown to be present in cells derived from the neural crest during mouse embryogenesis. These include those of the peripheral nervous system and the adrenal medulla (Mitchell et al., 1991). AP2 has also been found either to mediate induction by both PKA and PKC pathways (Imagawa et al., 1987) or to act synergistically with other elements and increase induction without being able to act alone (Comb et al., 1986, Hyman et al., 1988). Putative AP2 sites are found in the DBH sequence at -127 in the forward orientation and at -13 in the reverse orientation. There are two copies of the motif CGTCA which is found in elements known to confer induction by cAMP (Goodman, 1990). The proximal one occurs at -172 and has the sequence TGCGTCA. This heptad has close homology to both the cyclic AMP response element (CRE) (TGACGTCA) and the TPA response element (TRE) (consensus TGAC/GTCA). It is the same heptad found to confer both phorbol ester and cAMP induction to the proenkephalin gene (Comb et al., 1986) and is also found adjacent to the serum response element in the c-fos gene (Fisch et al., 1989). The more distal element occurs at -231 and has the sequence GCGTCA. As well as the GCGTCA motif, these putative response elements also have in common that they occur 6 or 7 bases 5′ to CAAT sequences. There is also an element at -365 which has high homology to the TRE.

COMPARISON OF HUMAN AND RAT PROMOTER SEQUENCES

When the human and rat promoter sequences are aligned for highest

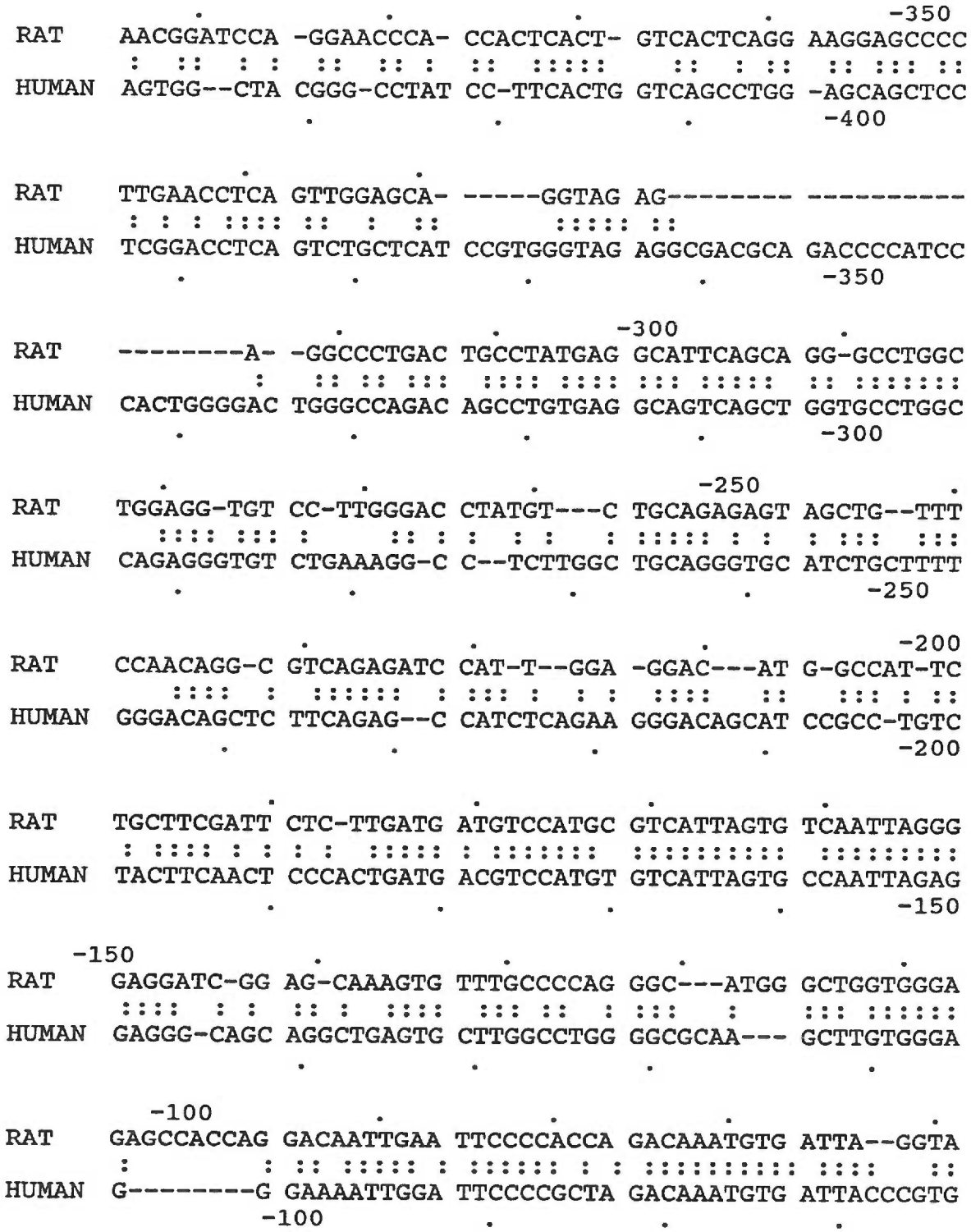
homology, the 394 bases of the rat gene fragment have 75% homology with the corresponding 438 bases of the human gene, with fewer deletions and insertions closer to the TATA box and Inr (Figure 9). A search for putative regulatory elements in the human promoter revealed that the Inr sequence was identical to that of the rat gene except for a T to C substitution at +2. The TFIID binding site was also identical. The CAAT element at -93 of the rat gene had a counterpart at -96 of the human gene in the opposite orientation and the one at -159 occurs in a region of exceptionally high homology (31 of 32 bases). The single difference is a substitution resulting in a change of TCAAT to CCAAT. Of the AP2 sites, the one at -128 in the rat gene occurs in the human gene in the same position, but the opposite orientation. The AP2 site at -13 does not appear in the human gene at this position but there is one at +2. The TGCGTCA motif, which occurs at -173 in the rat gene becomes TGTGTCA in the analogous region of the human gene. Though this motif has little resemblance to either the TRE or CRE, at -181 of the human promoter there is a near-consensus CRE: TGACGTCC. Therefore, it is possible that the functionality of cAMP response element may be conserved in location for the two genes. The more distal putative cAMP and TPA response elements of the rat gene are not conserved between species.

FUNCTIONAL STUDIES OF THE DBH PROMOTER

In order to study functional aspects of our DBH gene fragment we created chimeric constructs in which the promoter region was fused to the coding sequence for the bacterial reporter gene for chloramphenicol

Figure 9. Comparison of the 5′ flanking sequences of dopamine B-hydroxylase genes from rat and human clones. A. Comparison of sequences from −394 to −1 aligned for highest homology. The top sequence is from the rat gene and the bottom from the human (Kobayashi et al.). B. Percentage of nucleotide identity for the two genes. Comparisons start at −1 and proceed from 3′ to 5′. Percentages were calculated at 50 nucleotide intervals.

A.


RAT AACGGATCCA -GGAACCCA- CCACTCACT- GTCACTCAGG AAGGAGCCCC
HUMAN AGTGG--CTA CGGG-CCTAT CC-TTCACTG GTCAGCCTGG -AGCAGCTCC
-350
-400
RAT TTGAACCTCA GTTGGAGCA- -----GGTAG AG-------- ----------
HUMAN TCGGACCTCA GTCTGCTCAT CCGTGGGTAG AGGCGACGCA GACCCCATCC
-350
RAT --------A- -GGCCCTGAC TGCCTATGAG GCATTCAGCA GG-GCCTGGC
HUMAN CACTGGGGAC TGGGCCAGAC AGCCTGTGAG GCAGTCAGCT GGTGCCTGGC
-300
-300
RAT TGGAGG-TGT CC-TTGGGAC CTATGT---C TGCAGAGAGT AGCTG--TTT
HUMAN CAGAGGGTGT CTGAAAGG-C C--TCTTGGC TGCAGGGTGC ATCTGCTTTT
-250
-250
RAT CCAACAGG-C GTCAGAGATC CAT-T--GGA -GGAC---AT G-GCCAT-TC
HUMAN GGGACAGCTC TTCAGAG--C CATCTCAGAA GGGACAGCAT CCGCC-TGTC
-200
-200
RAT TGCTTCGATT CTC-TTGATG ATGTCCATGC GTCATTAGTG TCAATTAGGG
HUMAN TACTTCAACT CCCACTGATG ACGTCCATGT GTCATTAGTG CCAATTAGAG
-150
-150
RAT GAGGATC-GG AG-CAAAGTG TTTGCCCCAG GGC---ATGG GCTGGTGGGA
HUMAN GAGGG-CAGC AGGCTGAGTG CTTGGCCTGG GGCGCAA--- GCTTGTGGGA
-100
-100
RAT GAGCCACCAG GACAATTGAA TTCCCCACCA GACAAATGTG ATTA--GGTA
HUMAN G---------G GAAAATTGGA TTCCCCGCTA GACAAATGTG ATTACCCGTG
-100

```
          -50                .                .                .
RAT    CAGCCTGGCC CAACCCCA-C C-GAAC-AG- ACATAAATGG CCCA-GTGGG
       : :::::: : : ::::::   : : :: ::  :::::::::: :  : :::::
HUMAN  CTGCCTGGAC CCACCCCATT CAGGACCAGG GCATAAATGG CC-AGGTGGG
         .          50           .           .           .

         .         -1
RAT    GCTGGGGTGC TC
       :  : : :: ::
HUMAN  ACCAGAGAGC TC
         .         -1
```

B.

39/50 = 78%	190/250 = 76%
79/100 = 79%	227/300 = 76%
112/150 = 75%	265/350 = 76%
153/200 = 77%	294/394 = 75%

acetyl transferase (CAT). The original promoterless CAT plasmid, PUCCAT, was a fusion of 2.5 kb from pSV2CAT containing the CAT transcription unit with the plasmid pUC 13. The original DBH/CAT gene fusion contained DBH sequences from -394 to +14: DBH/CAT (-394-(+14)). Due to the arrangement of restriction enzyme sites, and the proximity of a putative translation initiation codon to the transcription initiation site, this construct contains both the endogenous DBH transcription start site and translation initiation codon (ATG) at +10. Because this codon might be used to a significant extent and is out-of-frame with the coding sequence of the CAT gene, translation would result in a high percentage of nonsense peptides with no CAT enzymatic activity. We therefore mutated the codon to AGG which does not initiate translation and allows use of the CAT initiation codon: DBH/CAT (-394-(+14-AGG)).

To locate sequence elements of the 5'-flanking region which are responsible for regulation expression of the DBH gene. A series of deletion mutants was made of plasmid DBH -394-(+14-AGG) (Figure 10). These deletion mutants were used to define elements in the 5'-flanking region which are responsible for basal activity in cells that express the endogenous DBH gene, effecting response to activators of PKA and PKC pathways or contribute to the cell-type selective expression of DBH.

ACTIVITY OF THE DBH/CAT FUSION GENE IN A CELL LINE WHICH EXPRESSES THE ENDOGENOUS DBH GENE

The cell line SH-SY5Y is derived from a human neuroblastoma and expresses the DBH enzyme (Ross et al., 1983). We co-transfected cultures of these cells with our deletion mutants and either RSV-

Figure 10. Deletion mutants of the CAT/DBH gene fusion. Deletion mutants were prepared using Exonuclease III as described in Materials and Methods. The 5' most base of each deletion mutant is directly beneath the "ones" digit of the corresponding number. Also noted are the TATA box, a putative AP-2 binding site, and the transcription and translation start sites. A construct with 24 bases removed (-394-(+10)) was used in experiments seeking DNA sequences involved in the cell-type selective expression of DBH. The bases removed in the formation of this construct are underlined and contain the endogenous DBH start sites for transcription as well as translation of the resultant mRNA.

-355
AACGGATCCA GGAACCCACC ACTCACTGTC ACTCAGGAAG GAGCCCCTTG

-330
AACCTCAGTT GGAGCAGGTA GAGAGGCCCT GACTGCCTAT GAGGCATTCA

-282
GCAGGGCCTG GCTGGAGGTG TCCTTGGGAC CTATGTCTGC AGAGAGTAGC

-232
TGTTTCCAAC AGGCGTCAGA GATCCATTGG AGGACATGGC CATTCTGCTT

-189 -163
CGATTCTCTT GATGATGTCC ATGCGTCATT AGTGTCAATT AGGGGAGGAT

-131 -110
CGGAGCAAAG TGTTTGCCCC AGGGCATGGG CTGGTGGGAG AGCCACCAGG

ACAATTGAAT TCCCCACCAG ACAAATGTGA TTAGGTACAG CCTGGCCCAA

DBH SEQUENCE

TATA AP2 TRANSCRIPTION
CCCCACCGAA CAGACATAAA TGGCCCAGTG GGGCTGGGGT GCTCATCCCA

TRANSLATION
GCCATGCA TRANSLATION
CC CTCGTTGGCG AGGTTTTCAG GAGCTAAGG AAGCTAAAATGG

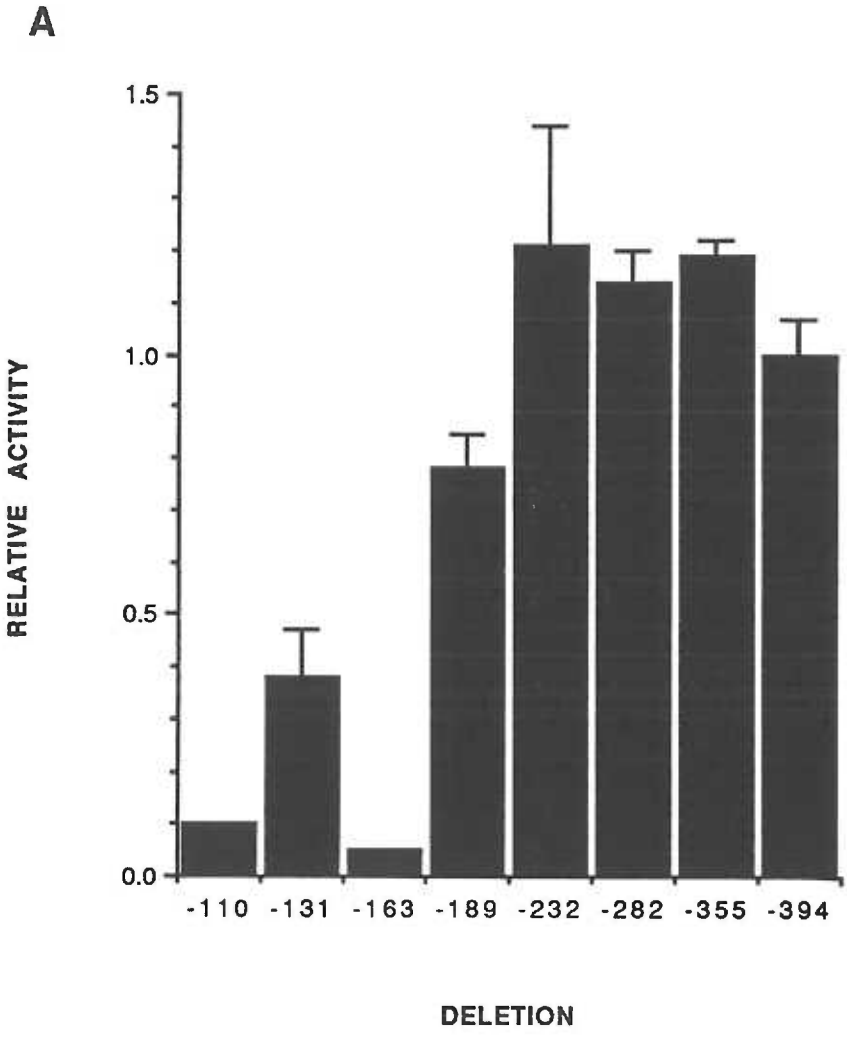
CAT SEQUENCE

luciferase or TK-luciferase plasmid as an internal control for transfection efficiency. Representative results are shown in Figure 11A. Deletion of bases between -232 and -394 did not effect basal activity of the DBH promoter. However, as emphasized in Figure 11B, bases between -163 and -232 contribute greatly to expression. There are a minimum of two elements in this region, with one between -163 and -189 and the second between -189 and -232. The total increase in activity ranged from five to 20 fold. This region contains both of the putative CREs and one of the CAAT boxes. The -163 deletion occurs between the proximal CAAT box and the first CRE while the -189 deletion includes the proximal combination. The -232 deletion is immediately 5' to the second CRE. This change in activity implies that proteins binding to the elements in these regions are involved in maintaining basal activity of the DBH gene in a cell line where it is normally transcribed. There is a negative element between -131 and -163, A positive element is found between -110 and -131. This region contains a putative AP2 binding site. It is clear that there are a combination of regulatory elements in this 394 bases of DBH 5'-flanking sequence that effect the activity of the DBH promoter in an expressing cell line.

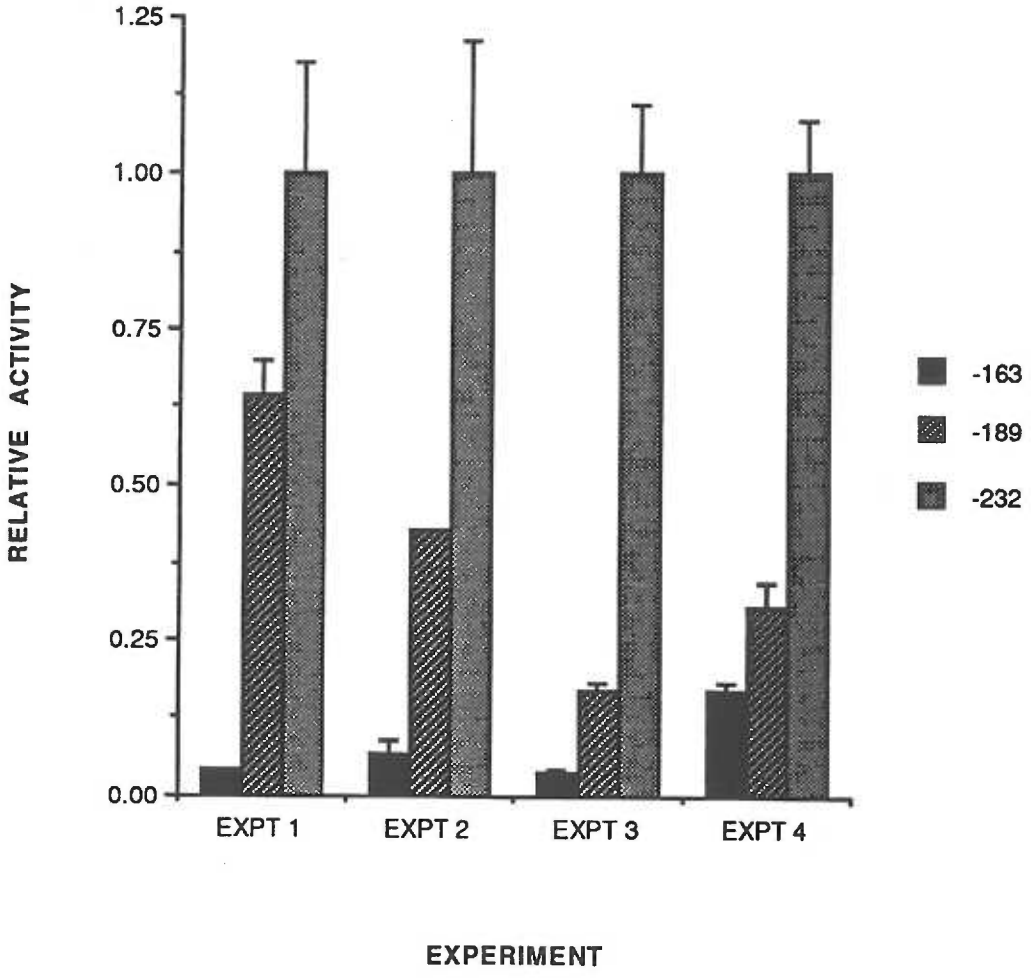
INDUCTION BY PKA AND PKC ACTIVATORS

Catecholamines are known to mediate many responses to stress. Long term stresses have been shown to correlate with changes in mRNA and enzyme levels. These changes may be the result of increased transcription of genes of enzymes in the pathway. Expression of a number of genes has been shown to be effected through second messenger

Figure 11. Elements in the proximal 394 bases of the DBH promoter region contribute to basal activity in the SH-SY5Y cell line. A. 5ug of each DBH deletion mutant was cotransfected with 2ug of RSV luciferase as described in Materials and Methods. Relative CAT/luciferase activity of the -394 construct was set to 1.00; error bars represent the range of duplicate samples. This experiment was repeated four times with qualitatively similar results. B. There are at least 2 elements between bases -163 and -232 which contribute to basal activity. Constructs were transfected into SH-SY5Y cells with either RSV or TK luciferase as an internal control. Each value represents the average of either duplicate or triplicate samples; error bars represent the range or standard error of mean. This experiment was repeated four times with the results shown above.


A
RELATIVE ACTIVITY
1.5
1.0
0.5
0.0
-110
-131
-163
-189
-232
-282
-355
-394
DELETION

B

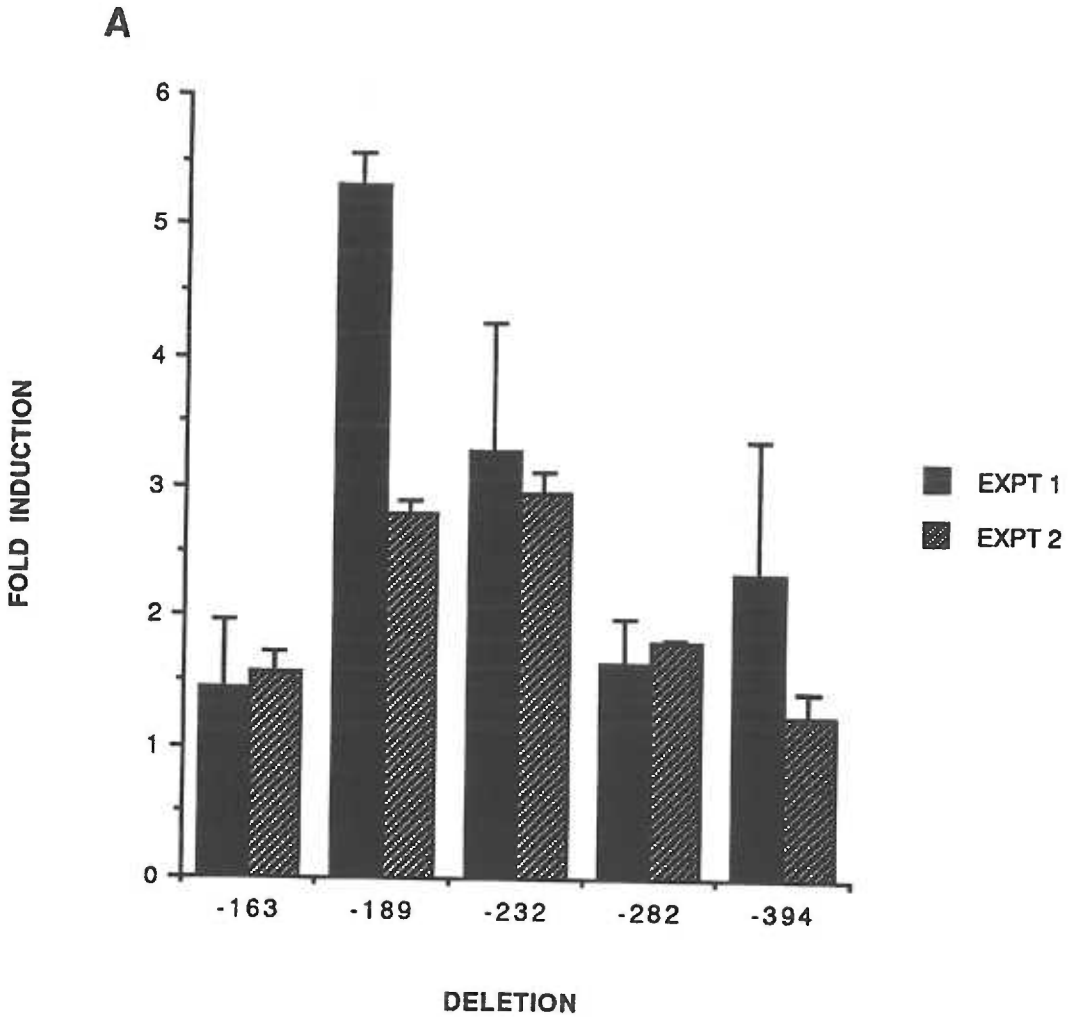

1.25
1.00
0.75
0.50
0.25
0.00
RELATIVE ACTIVITY
EXPT 1
EXPT 2
EXPT 3
EXPT 4
EXPERIMENT
-163
-189
-232

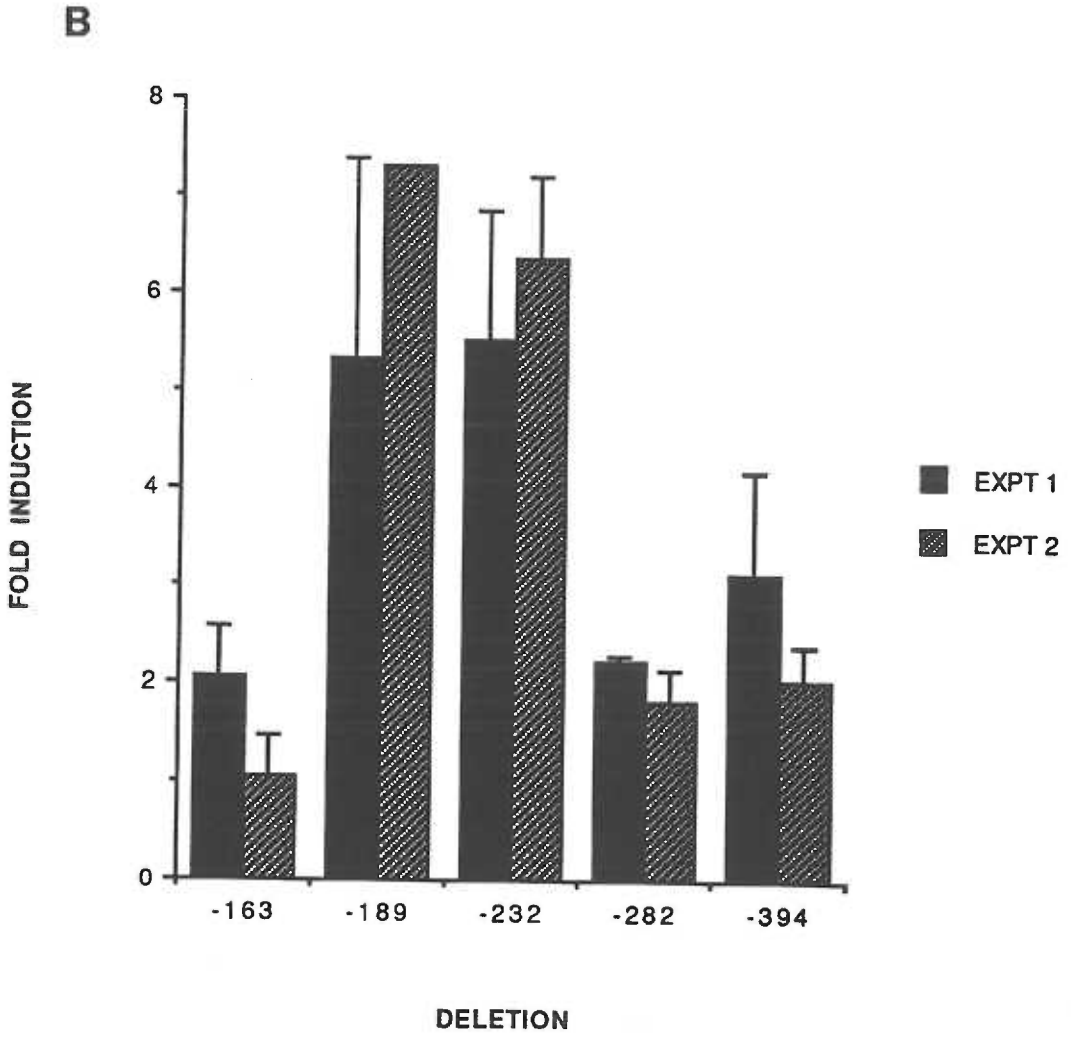
pathways including protein kinases (Comb et al., 1987, Goodman et al., 1991). The DBH/CAT deletion mutants were utilized to determine which regions of the DBH promoter might be involved in activating expression by two of these pathways. SH-SY5Y cells were transfected with DBH/CAT fusion constructs and treated with of the cAMP analog 8-(4-chlorophenylthio)-cAMP (CPT-cAMP) which activates protein kinase A, the diacylglycerol analog 12-O-tetradecanoyl-phorbol-13-ester (TPA) which activates protein kinase C, or a combination of the two (Figure 12A-C). For each experiment shown, all deletion mutants tested were transfected in parallel with aliquots of the same precipitate. In Experiment 1 cells were not subjected to glycerol shock whereas in Experiment 2 they were. The results did not differ appreciably. As serum can desensitize cells to activation via the PKC pathway, cells were refed with fresh media containing reduced serum 24 hours after initial transfection,. Six hours subsequent to feeding, inducer was added from a single bottle where it had been diluted to the proper concentration in a large volume of fresh media. Cells were harvested 16 hours after induction.

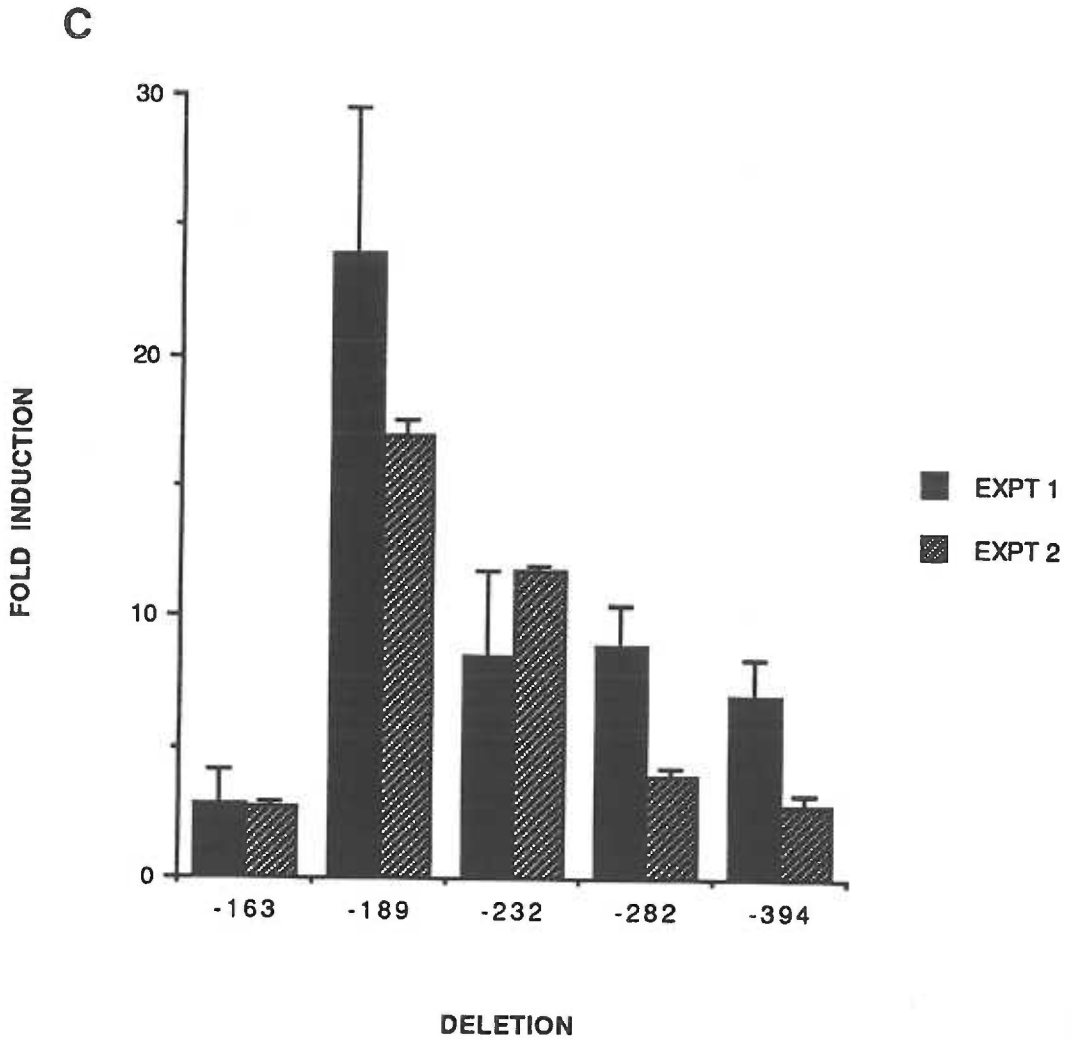
As shown in Figure 12A, maximum induction by CPT-cAMP occurs when sequences between -163 and -189 are included in the DBH/CAT fusion construct. Induction is maximum at approximately three to five fold in our system. DBH sequences 5' -189 do not increase the induction, and inclusion of those after -232 actually results in a decrease in induction. Maximum induction by TPA (Figure 12B) also occurs when the sequences between -163 and -189 are included in the promoter, reaching a maximum of approximately six fold. In this case, induction is the same when DBH sequences between -189 and -232 are included but, like

Figure 12. Regions of the DBH promoter are responsive to activators of the Protein Kinase A and Protein Kinase C pathways. SH-SY5Y cells were transfected with 5ug of DBH plasmid as described in Materials and Methods, and either subjected (Expt. 2) or not subjected (Expt. 1) to glycerol shock after four hours. 20 hours after transfection media was replaced with fresh media containing 1% Fetal Calf Serum. Six hours later cells were induced with a final concentration of A. 100 nM TPA, B. 200 uM cAMP analog or C. the two combined. Cells were harvested 18 hours after induction. Note change of scale in C.

All cells for each experiment were transfected on the same day; sample results are standardized for protein amounts. This experiment was repeated twice with the results shown above. Error bars represent standard error for triplicate transfections. One fold induction = basal activity.


A
FOLD INDUCTION
6
5
4
3
2
1
0
-163
-189
-232
-282
-394
DELETION
EXPT 1
EXPT 2


B
FOLD INDUCTION
8
6
4
2
0
-163
-189
-232
-282
-394
DELETION
EXPT 1
EXPT 2


C
30
20
10
0
FOLD INDUCTION
-163
-189
-232
-282
-394
DELETION
EXPT 1
EXPT 2

with CPT-cAMP, decreases when further 5'-flanking sequences are present.

When both inducers are added together the effect on CAT activity is greater than the additive effect of each one individually (Figure 12C). Minimal induction is observed for the -163 construct which contains the AP2 site at -127 as well as the TATA box and Inr. An exceptionally large induction is observed when the 23 bases between -163 and -189 are included: 17 to 24 fold. If the effect were additive, we would have expected to observe approximately an 11 fold effect. Including bases past -189 results in a decrease in induction. For the -232 deletion induction is half of what it is for the -189 and is approximately equivalent to the sum of the individual inductions. Addition of bases to -282 and again to -394 result in increasingly smaller inductions. By the addition of 205 bases of the DBH promoter to the maximally inducible construct, activation is decreased from 20 to four fold.

Analysis of results obtained with our deletion mutants clearly demonstrates that bases between -163 and -189 are necessary for the regulation of the DBH promoter by activators of both the PKA and PKC pathways. The lack of inducibility of the -163 deletion construct implies that the AP2 elements at +13 and -127 confer little or no activation. However, their presence may be necessary for full induction to take place. The bases between -163 and -189 contain the motif TGCGTCA which has homology to both the CRE and TRE. This heptad is identical to ENKCRE-2 of the proenkephalin gene, which has been shown to be essential for its inducibility by both CPT-cAMP and TPA (Comb et al., 1988). The addition of bases to -232, which contains the second CGTCA

motif, does not result in an increase in PKA- or PKC-directed activation ability by themselves. Increased CAT activity due to each inducer present individually is approximately the same for this construct as for the -189. However, the superadditive response of both together is eliminated. The inclusion of subsequent bases further reduces response to the two activators.

CELL-TYPE SELECTIVE EXPRESSION OF DBH[1]

DBH is expressed in a cell-type selective pattern in mammalian organisms. Cis-elements which contribute to cell-type selective gene expression have been discovered in the first several hundred bases of the 5' flanking region in a number of other genes. We used our deletion mutants to determine if cis-elements in our DNA fragment contribute to the restricted expression of DBH.

As a first step towards identifying tissue-selective elements four distinct cell lines including SH-SY5Y, C6 rat glioma, JEG-3 human

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1

When a reporter gene under control of a spectfic promoter and accompanying control elements is transfected into different cell lines it is often found that activity of the transcription product is present in higher relative amounts in some cell-lines, than in others. The terms tissue selective, tissue specific and tissue restricted expression of a gene are often used in the literature to describe this circumstance.

The SH-SY5Y cell line has a higher relative CAT activity when transfected with the various DBH/CAT constructs as compared to the other cell lines tested. When describing DNA elements which contribute to the higher relative activity in SH-SY5Y cells, I have chosen to refer to them as contributing to "tissue selective expression".

Though cells in cell lines differ from those found in organisms in a number of ways, and the cell lines chosen are from different mammalian species, (human, monkey and rat), I believe that regions found to be important for regulation the expression of the DBH gene in the cell lines will have similar functions in regulating the expression of the DBH gene in vivo.

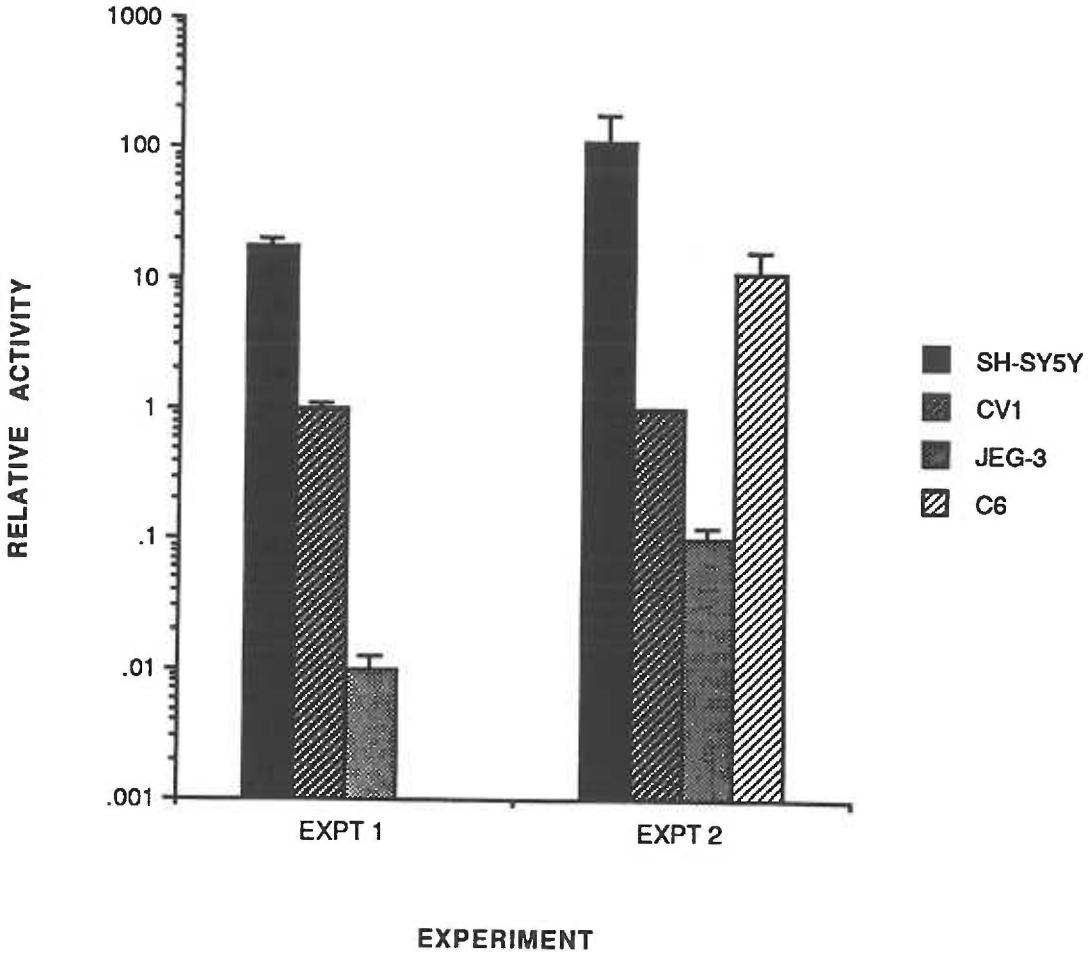
placental choriocarcinoma and CV-1 monkey kidney cells were selected for study. These cell lines were selected because of their diverse origins and their ability to be transfected by the calcium phosphate co-precipitation method. Plates of cells of each cell type were transfected in parallel with the same calcium phosphate precipitate. In addition, two or more DBH/CAT deletion constructs were transfected during the same experiment so that differences in the conditions between experiments would not create artifacts which could color our interpretation of the results.

TK-luciferase plasmid was cotransfected as an internal control to correct for transfection efficiency. It was chosen because the promoter region has been well characterized and contains cis-elements only for the ubiquitous Sp1 transcription factor and a CAAT box. We expect that the TK 5'-flanking sequence will show smaller differences in cell-type selective expression than other internal standards containing viral enhancer/promoters such as the RSV long terminal repeat.

ELEMENTS BETWEEN -110 AND +14

Transfection of the -110 construct, which contains the smallest 5'-flanking sequence of our constructs, resulted in a major difference in relative CAT activity for the four cell lines (Figure 13). The relative CAT activity in SH-SY5Y cells was one order of magnitude greater than for C6 glioma cells. It was between one and two orders of magnitude greater than in CV-1 cells and approximately three orders of magnitude greater than in JEG-3. If the assumption holds, that activity of the TK-109 promoter is relatively constant across cell lines, we can

Figure 13. The first 110 bases of the DBH promoter confer higher relative CAT activity in SH-SY5Y cells than in cells which do not endogenously produce DBH. Cells from each of the four cell lines (SH-SY5Y, CV-1 monkey kidney, JEG-3 human choriocarcinoma, C6 glioma) were transfected with 15ug of -110 DBH/CAT and 5ug of TK luciferase from the same $CaPO_4$ precipitate, as described in Materials and Methods. Values shown are the average of three individual samples. This experiment was repeated two times with the results shown. Experiment 1 had no C6 samples. Error bars represent standard error for triplicate transfections. Note log scale.


1000
100
10
1
.1
.01
.001
RELATIVE ACTIVITY
EXPT 1
EXPT 2
EXPERIMENT
SH-SY5Y
CV1
JEG-3
C6

conclude that this region contributes greatly to the restricting expression of DBH in certain cell types.

Because the first 110 bases of the promoter seem to confer cell-type selective expression of the DBH/CAT fusion gene, the region surrounding the transcription start site was examined for an ability to contribute to that property. The plasmid DBH/CAT (-394-(-10)) differs from DBH/CAT (-394-(+14 AGG)) in that the 24 bases from -10 to +14 are deleted. These 24 bases contain the endogenous start site of transcription which has a high homology to a sequence which has been named the Initiator (Inr). This sequence has been found capable of binding the ubiquitous transcription factor YY1. Similar sequences, in the analogous positions have also been found to be important in the regulation of genes in a cell-type selective manner in other systems (Lewis and Manley, 1985, Seto et al., 1991). We have found that in the SH-SY5Y cell line, deletion of this region had no effect on relative CAT activity (data not shown). However, when these constructs were transfected in parallel into the other cell lines, we found that the (-394-(-10)) construct had two to five fold higher relative activity than (-394-(+14 AGG)) (Figure 14). Therefore, the 24 bases which are deleted in the -10 construct are responsible for repressing CAT activity in non-DBH expressing cell lines relative to the expressing SH-SY5Y cell line.

ELEMENTS BETWEEN -232 AND -110

The bases between -110 and -232 conferred an increase in activity in all four cell types (Figure 15). This activity increase was the highest in JEG-3 cells where it was approximately 100 fold. C6 cells

Figure 14. Bases between -10 and +14 confer cell-type selective expression of CAT activity. 15ug of either -394-(-10) or -394-(+14 AGG) were cotransfected with 5ug of TK luciferase into each of the four cell lines and relative CAT/luciferase activities were determined. The relative activity for the -394-(+14 AGG) construct was assigned a relative value of 1.00 in all cell lines. The (-394-(-10) construct was assigned a relative value of 1.00 in the SH-SY5Y cell line. Relative values for the activity of this construct in the other cell lines were calculated. This experiment was performed twice with the results shown. C6 cells are not represented in Experiment 2.

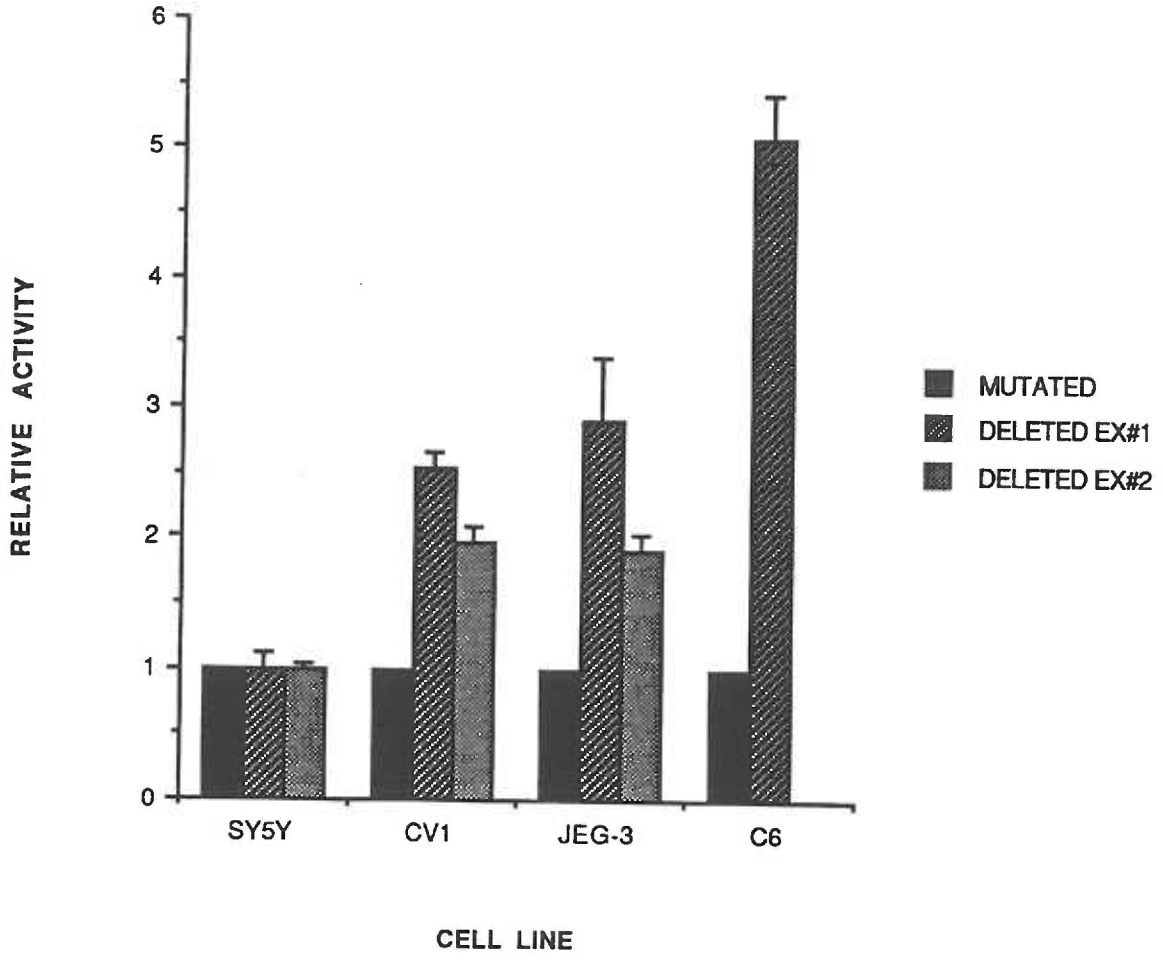
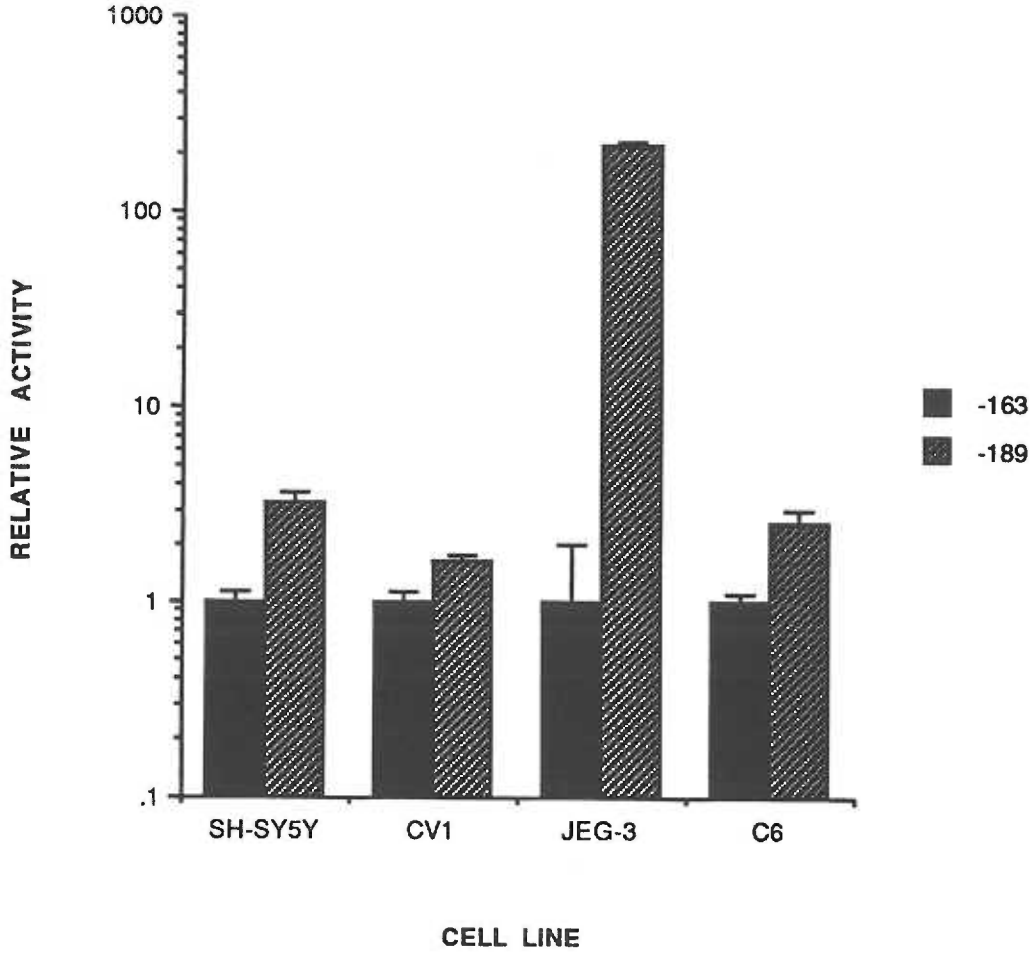
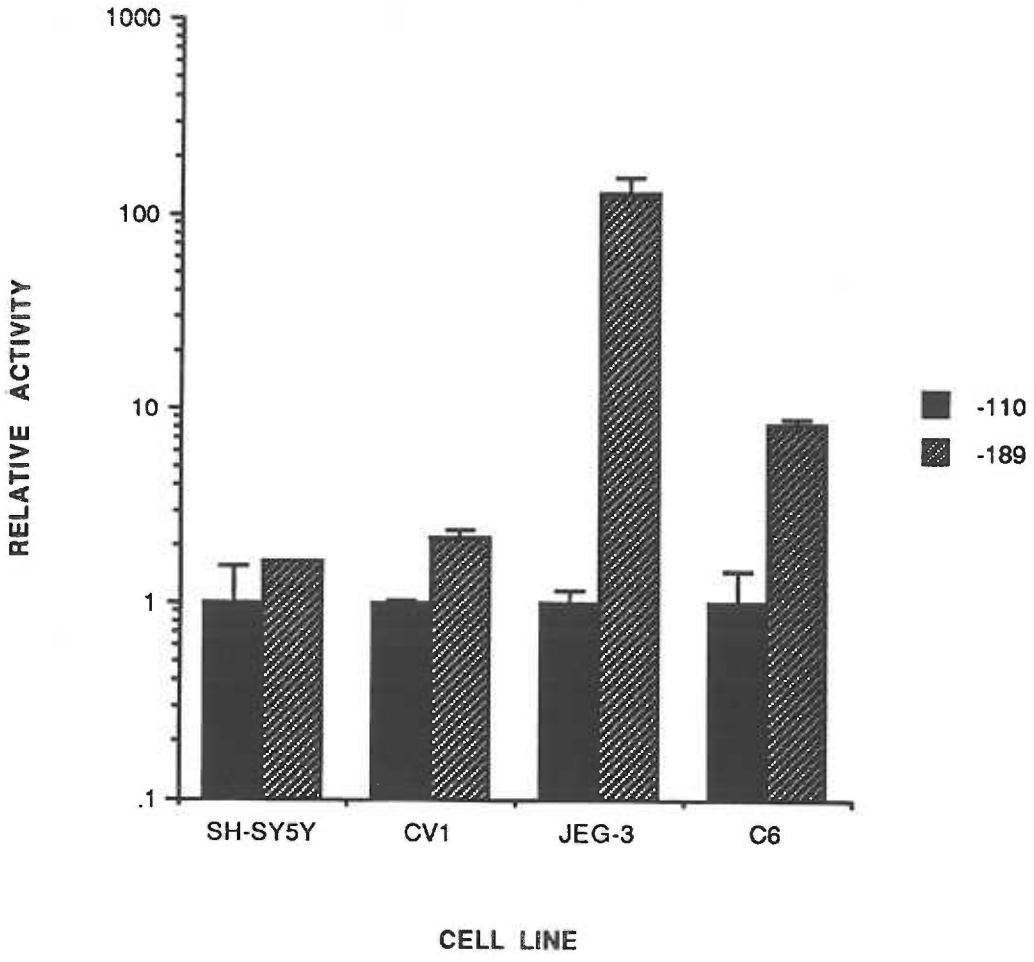

RELATIVE ACTIVITY
6
5
4
3
2
1
0
SY5Y
CV1
JEG-3
C6
CELL LINE
MUTATED
DELETED EX#1
DELETED EX#2

Figure 15. There are a minimum of two cis-elements between bases -163 and -232 involved in determining CAT activity in the studied cell lines. A. Cells of all four cell lines were transfected with 15 ug of either -163 or -189 DBH and 5 ug of TK luciferase as an internal standard. Relative CAT/luciferase activities were determined for each cell line assigning the -163 DBH activity a value of 1.00. Values shown are the average of three individual samples. This experiment was performed one time with the results shown. Note log scale. B. Same as for A. except the -110 DBH construct was used in place of the -163. This experiment was performed one time with the results shown. C. Same as for A. however, the constructs used were -189 and -232. Activity corresponding the -189 construct was assigned a relative value of 1.00. This experiment was performed twice with the results shown. Note linear scale.

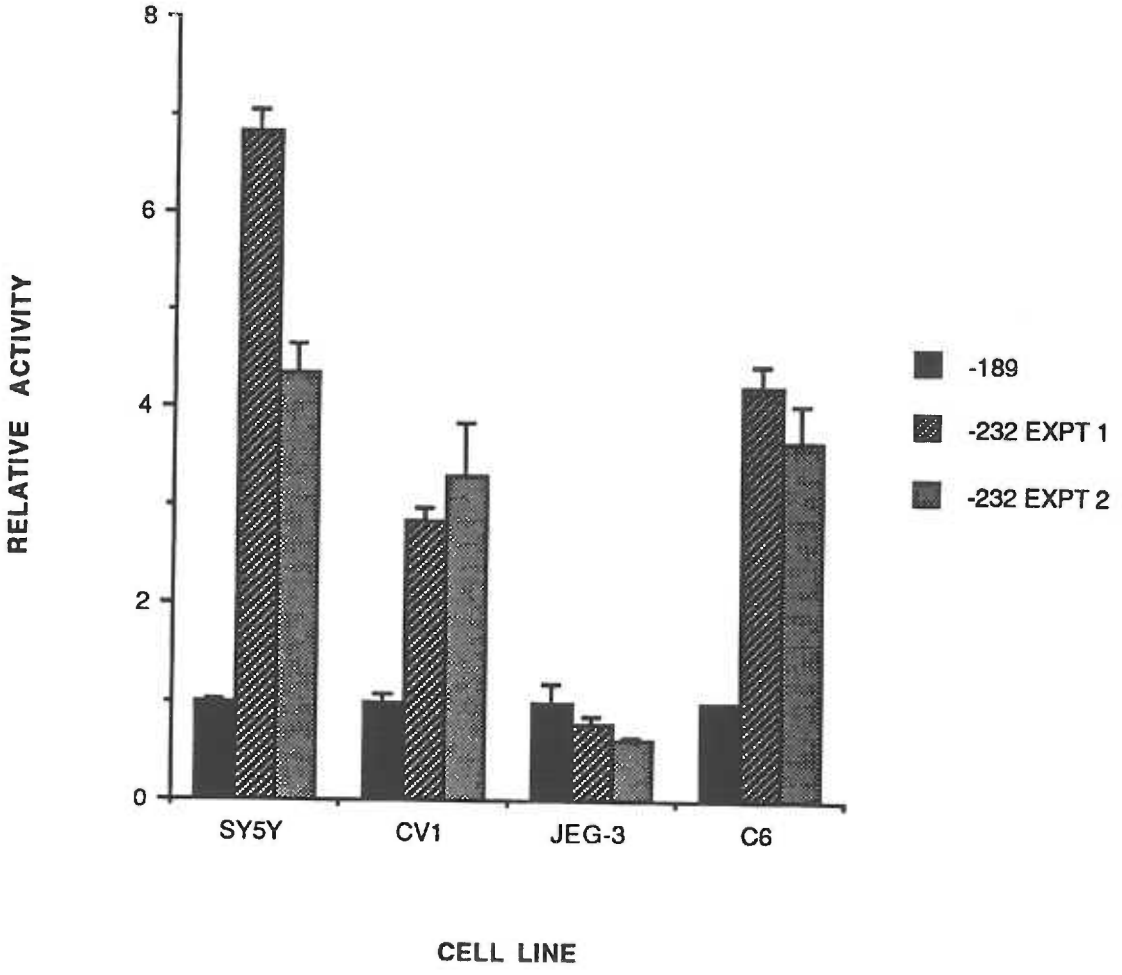
A


1000
100
10
1
.1
RELATIVE ACTIVITY
SH-SY5Y
CV1
JEG-3
C6
CELL LINE
-163
-189

B


1000
100
10
1
.1
RELATIVE ACTIVITY
SH-SY5Y
CV1
JEG-3
C6
CELL LINE
-110
-189

C


8
6
4
2
0
RELATIVE ACTIVITY
SY5Y
CV1
JEG-3
C6
CELL LINE
-189
-232 EXPT 1
-232 EXPT 2

showed the next highest increase at 35 fold and the other non-DBH-expressing cell line CV-1 showed an increase of six fold. SH-SY5Y cells showed an increase of 11 fold which is consistent with the results found in the experiments described in the section of basal activity. These results indicate that the bases between -110 and -232 mediate and increase in the amount of CAT activity in all cell lines tested.

Our studies on the basal expression of the DBH promoter in SH-SY5Y cells presented earlier demonstrated that this region carries at least three separate cis-elements. Between -163 and -232 there are a minimum of two elements which contribute to activity in SH-SY5Y cells. When activity of the -163 and -189 constructs was tested it was found that there was a 3.25 fold increase in activity in SH-SY5Y cells (Figure 15A). There were parallel 1.7 and 3.6 fold increases in CV-1 and C6 cells respectively. However, in JEG-3 cells there was an anomalous increase of over 200 fold. This unexpected result was confirmed in a separate experiment where activity corresponding to the -110 and -189 constructs was compared (Figure 15B). The addition of the 79 bases resulted in an increase of 130 fold. Therefore, the region between -163 and -189 contains sequences which can increase basal activity in all cell types examined.

However, the magnitude of this increase differed according to cell type. The bases between -189 and -232 confer an increase in basal activity of approximately five fold in SH-SY5Y cells (Figure 15C). They also confer parallel increases of three fold in CV-1 cells and four fold in C6 cells. In JEG-3 cells, however, these bases confer a decrease in relative activity of 20 to 40%. Therefore, though these bases contain

an element which acts in a positive manner in a number of cell lines, this is not a universal phenomenon. It also contains an element which acts as a negative regulator in at least one cell line.

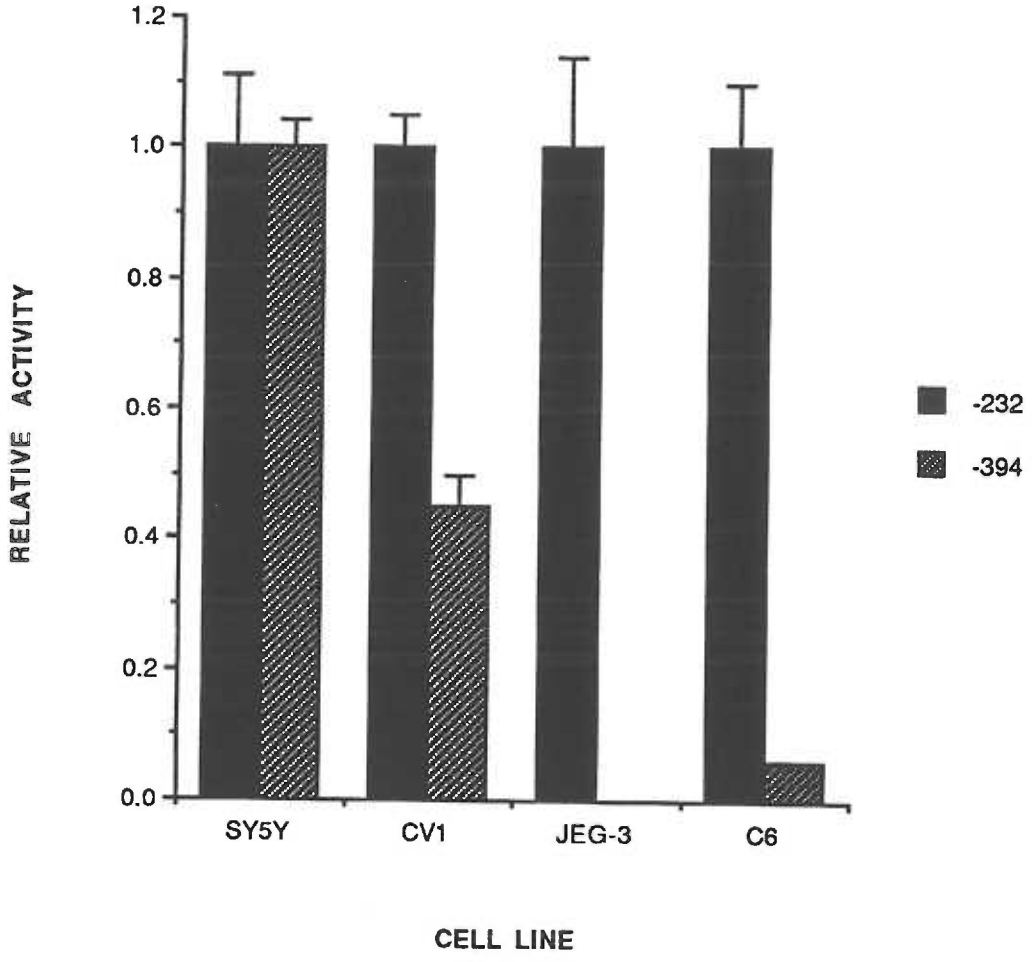
The -110 to -163 region, which includes a putative AP2 element, was not studied directly. However, comparisons of the experiment in which the -110, -189 and -232 constructs were used with the one in which the -163, -189 and -232 were used, implies that the region is not critically important for cell-type selective expression of DBH.

ELEMENTS BETWEEN -394 AND -232

The -394 and -232 DBH/CAT constructs were transfected in parallel (Figure 16). We found that, compared to the SH-SY5Y cell line, all other cell lines showed a decrease in CAT activity when the additional 5'-flanking sequences were present. In the CV-1 cell line relative activity fell by 50%. In the other two non-DBH expressing cell lines activity fell by a much higher greater amount; in C6 cells activity fell by at least 35 fold and in JEG-3 cells by over 1000 fold. In these experiments activity of the of the -394 DBH/CAT construct was less than two times background in the JEG-3 and C6 cell lines. Luciferase activity, however, was comparable to that found in samples where TK-luciferase had been co-transfected with the -232 construct. Therefore, these decreases in relative activity for the two cell lines may actually be greater. We conclude that there is a negative regulatory element in the 5'-flanking region between -232 and -394 which is involved in the cell-type selective expression of the DBH promoter.

The overall region between -163 and -394 was analyzed as a single

Figure 16. Bases between −232 and −394 contain an element which reduces CAT activity in cell lines which do not endogenously produce DBH. Cells of all four types were transfected with 15ug of either −232 DBH or −394−(+14 AGG) DBH and 5ug of TK luciferase as an internal standard. Relative CAT/luciferase values were determined assigning the −232 construct a relative value of 1.00 for all cell lines, and −394−(+14 AGG) a relative value of 1.00 in the SH−SY5Y cell line. This experiment was performed once with the results shown.


1.2
1.0
0.8
0.6
0.4
0.2
0.0
RELATIVE ACTIVITY
SY5Y
CV1
JEG-3
C6
CELL LINE
-232
-394

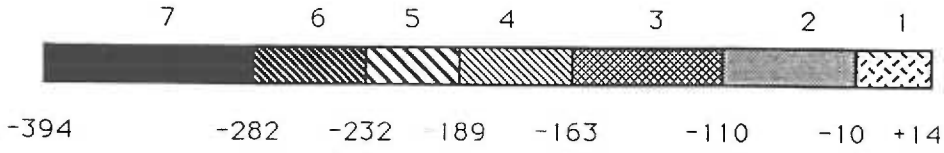
entity in order to determine the net change in promoter activity due to the bases in this segment of the 5'-flanking sequence (see appendix). It was found that if the SH-SY5Y relative activity increase is set to 7 fold, that relative activity of the JEG-3 cells increases less than 3 fold and that relative activity of the CV-1 and C6 cell lines actually decreases by 30 and 70% respectively. These results are confirmed in a separate experiment in which the -110 and -394 constructs are compared.

SUMMARY OF RESULTS FOR CELL-TYPE SELECTIVE EXPRESSION EXPERIMENTS

Taken together, the data reveals that bases in the 5'-flanking region of the DBH gene between -394 and +14 contribute to the restriction of its expression in certain cell types. We conclude that bases between -110 and +14 contribute to cell-type selective expression. Bases between -10 and +14 are at least partially responsible for this. Elements further 5' can increase promoter activity in all cell lines. In addition, there is a very strong negative regulatory element which obviates the effect of these positive elements in the cell lines tested.

SUMMARY OF EXPERIMENTS WITH DELETION MUTANTS

A summary of the function of the transcription control elements located in the bases of the DBH 5' flanking sequence between -394 and +10 is found in Figure 17.



Region of 5' Flanking Sequence		Functional Importance
1	-10 to +14	-ve element cell-type selective CV1, JEG-3, C6
1&2	-110 to +14	-ve element cell-type selective CV1, JEG-3, C6
3	-163 to -110	no definitive function
4	-189 to -163	+ve element basal expression induction by CPT-cAMP and TPA
5	-232 to -189	+ve element basal expression SH-SY5Y, CV1 and C6 cell lines
6	-282 to -232	-ve element CPT-cAMP and TPA
7	-394 to -282	-ve element CPT-cAMP and TPA
6&7	-232 to -394	-ve element cell-type selective CV1, JEG-3, C6

DISCUSSION

A clone of the rat gene for dopamine β-hydroxylase was isolated. The putative 5'-flanking region was fused to the bacterial gene coding for chloramphenicol acetyl transferase. We have demonstrated the functionality of this DNA fragment. It has the properties of other well studied promoters. These properties include the ability to direct transcription of the CAT gene in a cell line which expresses the endogenous DBH gene. In addition, the level of CAT enzyme is increased when inducers that might be expected to increase transcription of the DBH gene are added to the media of transfected cells. CAT activity is also expressed in a tissue-selective manner with highest relative activity in the DBH-expressing cell line. That this transcriptional activity is not due to the effect of cryptic promoter elements in the plasmid and the translation of fusion proteins is assured by the occurrence of translation start and stop codons in all reading frames of the DBH gene fragment.

In addition, the combination of CAT assays and RNAase protection assays shows that, with our gene fusion construct, that the endogenous DBH translation start site is used extensively but not exclusively. These results imply that the DBH gene may also utilize two translation

initiation sites. If this is the case, we expect that the different translation products may be processed in different ways. If this is true, use of the 5′ most ATG may result solely, or preferentially in a subunit with a membrane-spanning domain and an N-terminal anchor in the cytosol. Use of the subsequent ATG may result in solely or preferentially in the formation of soluble subunits.

DNA SEQUENCE MOTIFS

The DBH 5′-flanking sequence has a number of sequence motifs which are common to a large number of promoters including a TATA box, CAAT boxes and putative CRE/AP-1 sites. The sequences of these, however, are generally not 100% homologous to the recognized consensus sequences (Mitchell and Tjian, 1989).

Comparison of the sequences of the human and rat DBH genes shows high interspecies sequence conservation at both the Inr and TATA box sites. In the region of the Inr, between -4 and +12 there is a single base difference occurring at +2. Around the TATA box, between -21 and -30, ten of ten bases are identical. The bases between -4 and -21 as well as those immediately 5′ of the TATA box show a much lower degree of homology. The bases at the TATA and Inr sites also show no similarity to those of the genes coding for another catecholamine biosynthetic enzyme, tyrosine hydroxylase, of the corresponding species (Lewis, et al., 1987). This interspecies conservation implies that these regions may be important in control of the expression of DBH.

The importance of the transcription initiation site for expression of a number of genes was recognized at an early date (Corden

et al., 1980). However, there have been difficulties in finding consensus sequences at this site (Corden et al., 1980; Bucher and Trifonov, 1986; Nussinov, 1986). The Inr was initially characterized as a minimal promoter capable of supporting RNA polymerase II directed transcription in the absence of a TATA box (Smale and Baltimore, 1989). Elements with high homology with this element have been found in a large number of genes transcribed by RNA pol II including many which contain TATA boxes. It has also been implicated in regulating expression of them. The functional importance and wide variation in sequences at the transcription initiation site suggests that this region may be an important control point for gene regulation.

The TATA box is an especially prevalent element and the one most associated with genes transcribed by RNA pol II. It binds the transcription factor TFIID and would seem to have a well-defined role in transcription regulation. However, there is a wide variation in TATA box sequences between genes and the importance of this has not been fully explored. It has been reported that the TATA box of the human myoglobin gene is involved in the tissue specific regulation (Wefald et al., 1990).

Several other putative elements found in the rat gene are conserved in position if not in identical sequence in the human gene. These include the CAAT boxes at -94 and -155 and the AP2 elements at -120 and -6. Conservation of these elements implies that they may be important. Physical and functional studies must be done to be certain of this point.

The putative CRE/TRE-like element at -173 is not conserved at the

same position. However, nine bases upstream, or approximately one turn of the double helix, in the human gene is a near-consensus CRE. The function of the CRE/TRE-like element therefore may be conserved in approximately the same position of the flanking sequence even though the DNA sequence is different between the two species.

The putative CAAT box at -218, the CRE at -231 and the AP1 site at -365 do not have corresponding elements in the human gene. Functional studies will be necessary to determine if these elements contribute to the expression of the rat gene and if there are elements in the human gene which duplicate their function. It is also possible that differences in human and rat metabolism require expression of this gene in different manners.

Both the CRE/AP-1 site at -170 and -232 are separated from CAAT boxes by about 14 bases. The CAAT box at -232 is in the reverse orientation. Fourteen bases is approximately 1.5 turns of the DNA α-helix. This raises the question, that if these are in fact binding sites for regulatory proteins, what is the function relating to the protein binding? Do proteins have to bind to each of these sites at the same time for transcriptional activation? Or is there competitive binding and the binding of only one of these sites affect transcription positively and binding to the other negatively? That the spacing of the two binding sites is conserved lends credence to the question of their functional importance.

TRANSLATION INITIATION

Based on the position of the TATA box and the functionality of the

initiator in positioning the start site of transcription, we predict that the DBH mRNA has a relatively short untranslated region, containing only 10 bases. Comparison of the relative CAT enzyme activity and CAT RNA levels for our construct leads to the conclusion that the initial ATG is used to initiate translation but is not used exclusively. In our construct, the second ATG is the endogenous translation initiation codon of the CAT gene. The initial DBH ATG is used approximately 75% of the time and the CAT ATG is used about 25% of the time. For the DBH gene, the results will undoubtedly vary from those of the fusion gene. There is an analogous case, with the gene coding for the SV40 proteins LP1 and VP1 (Sedman et al., 1990). Here the initial ATG is 10 bases from the translation initiation site, and use of different ATGs is responsible for the translation of the LP1 and VP1 genes. In investigating this system, untranslated regions (UTR) of 44 and 6 bases were used. Decreasing the UTR from 44 to 6 bases resulted in a decrease of 30% in LP1 initiation and an increase of 300% in VP1 initiation. When the CAT gene was substituted for VP1, decrease from a 44 to 6 base UTR resulted in a 70% decrease in LP1 translation and a 30% increase in CAT synthesis.

The sequence surrounding the first ATG of DBH (CCAGCC ATG C) is fairly close to the consensus sequence (GCCA/GCC ATG G) (Kozak, 1987). It contains the G at −3 and Cs at −1, −2 and −5. It does not contain the G at −4 found in many other flanking sequences. The sequence of the rat gene immediately 5′ to the ATG does not have any potential hairpins that would otherwise affect the use of the initiation codon. The next ATG in the rat sequence occurs in frame and 66 nucleotides downstream.

Its flanking sequence is GCGGCT TCC ATG T, has neither the purine at -3 or the G at +4 as found by Kozak. However, it does contain homology with the optimal sequence GCCGCCA/GCC ATG G (Kozak, 1987).

Proteins which contain signal sequences when they are translated either have them cleaved and are secreted, or the sequences remain uncleaved and the proteins are anchored in the membrane by them. The anchored protein can be oriented with either the N- or C-terminal remaining cytoplasmic. Those with the C-terminal remaining cytoplasmic are referred to as type I and those with the N-terminal cytoplasmic, are type II.

For the rat DBH gene, use of the initial ATG would result in the rat homolog to the bovine protein sequenced by Taljanidisz et al. (1989) (Figure 4). The authors propose that DBH is anchored to the membrane by an uncleaved signal sequence found in their membranous subunit, but not found in the sequence of the soluble form or in the membranous form by Taylor et al. (1989). (The sequence of the soluble form starts at ser ala pro and ala glu ser in the bovine protein. These starting sequences are found in a 1 to 1 ratio.) For this protein to be bound in the membrane, the charged N-terminus would be found in the cytoplasm, a hydrophobic core sequence would span the membrane and the C-terminal body of the protein would be found in the secretory granule. The DBH held in the membrane by its uncleaved signal sequence is a type II protein.

The signal sequence is composed of three adjacent domains which dictate the fate of the protein: 1) an amino terminal (n) which is hydrophilic and has a net charge, 2) a hydrophobic sequence (h) and 3) a

second charged (c) region (von Heijne, 1985). The difference in charges of the n and c regions dictate whether the protein is type I or II. (For this analysis the n and c regions are defined as the 10 or 15 amino acids immediately flanking the hydrophobic region. If [Δ(C-N)>or =0] the N-terminal is cytoplasmic and the C-terminal is exoplasmic. If [Δ(C-N)<0] then the N-terminal is usually exoplasmic and the C-terminal cytoplasmic (Hartmann et al., 1989). The sequences flanking the hydrophobic regions in all three sequenced DBH genes fit the pattern described.

The difference between type II membrane proteins and excreted proteins has been ascribed to the lack of a suitable cleavage site for signal peptidase (Lipp and Doberstein, 1986; Shaw et al., 1988). This cleavage site generally has a small neutral amino acid at +1 and a non-aromatic, non-polar residue at -3 (von Heijne, 1985). It has been also been shown that for a model type I protein, which contained artificial leader sequences adapted from the cytochrome p-450 protein fused to the interleukin 2 protein, that a signal anchor sequence could be converted into a signal sequence by changing the length of a hydrophobic sequence. A hydrophobic region of 7 to 10 leucines functioned as a signal sequence whereas 12 to 15 leucines functioned as a signal sequence or signal-anchor sequence depending on the N-terminal charge (Sakaguchi et al., 1992). Therefore, it is the difference between the c and n charges which determines the orientation of membrane-attached proteins. The difference between the secreted proteins and both the type I and II proteins the length of the hydrophobic sequence as well as the charge of the n region.

Alteration of the N-terminal sequences of other type II proteins has resulted in changes in the behavior of the protein. It has been demonstrated with the asialoglycoprotein receptor H1, that if charged residues flanking the hydrophobic region are mutated so that the n and c regions change their charge relationship, that insertion of the protein into the membrane occurs in the opposite orientation (Beltzer et al., 1991). Removal of the charged N-terminal sequences of the invariant chain of class I histocompatibility antigen resulted in cleavage of the signal peptide within the transmembrane segment (Lipp and Doberstein, 1986). Removal of the analogous segment of the asialoglycoprotein receptor resulted in cleavage at the carboxyterminal end of the transmembrane segment (Schmid and Spies, 1988).

Conversion of a secretory to a membrane bound protein was achieved by adding a 30 amino acid charged N-terminal end to the coding sequence for multilineage colony-stimulant factor (Haeuptle et al., 1989). Also,changing the charge at the N-terminal end or the length of the hydrophobic segment was found sufficient to convert a secreted protein to a membrane bound in the model system described above (Sakaguchi et al., 1992).

Rat DBH (Figure 4) contains a possible n region of 20 amino acids with a charge of +2. It contains a possible h region of 20 amino acids extending to the gln at amino acid #41. All but one of the amino acids in the h region are predicted to have a positive free energy for the transfer of the residue in an α helix from the membrane interior to water (Engelman et al., 1986). The tyrosine has a small negative free transfer energy. The c region, if postulated to contain 15 amino acids

has a charge of -2.5. Use of the second translation initiation codon results in a peptide starting four amino acids into the h region. The predicted cleavage site for the signal sequence is after glycine, two bases into the c region.

It is therefore possible that the use of different translation initiation sites results in the formation of different DBH subunits if the translation products are processed differently. Use of the 5' most translation initiation site might result in the formation of a membrane-bound subunit attached via an uncleaved signal sequence. Use of the subsequent ATG might result in the formation of the subunit found in the soluble form and in membrane-bound forms which are attached by other means. Lewis and Asnani (1992) have shown that translation of a bovine cDNA lacking the 5'-most ATG gives rise to a membrane binding form of the enzyme. The translation of this construct is expected to initiate in the transmembrane section of the protein and not contain an N-terminal anchoring region. Without the anchor, a different mechanism of attachment may occur such as attachment by association with phosphatidyl serine (Taylor and Fleming, 1989).

ACTIVITY OF THE DBH PROMOTER

BASAL STUDIES

Examination of the basal activity of the DBH promoter in the cell line SH-SY5Y shows that there are several elements which are responsible for the basal activity. There is a minimum of one between -189 and -232 and another between -163 and -189. The -232 deletion occurs immediately 5' of the second putative CRE and the -163 deletion is found between the

first putative CRE and its adjacent CAAT box. The -189 deletion occurs about half way between the two compound motifs. Overall there is an 80 to 95% loss in activity between -232 and -163. The relative change between each of the two segments varies but there is always a two step change. CREs and TREs have been shown to function as basal regulatory elements in a number of genes. This may be true in this case, but a definitive answer must await the results of further studies.

The fluctuation of activity seen in the deletions smaller than -163 suggests that there may be other elements important for basal expression in this region of the promoter. However, overall activity levels are low and the effects are too small for any conclusions to be drawn from the data at this time.

INDUCTION OF DBH GENE TRANSCRIPTION VIA PKA AND PKC PATHWAYS

Induction of the DBH promoter by the cAMP analog CPT-cAMP and the diacylglycerol analog TPA were tested both separately and together. Activity corresponding to the full-length constructs was only modestly increased in response to the inducers. Larger inductions were seen when constructs containing the smaller 5'-flanking sequences were tested.

The -163 deletions showed negligible induction by CPT-cAMP and TPA demonstrating that the AP2 elements, by themselves were not capable of conveying the response of either inducer. Addition of the 16 bases between -163 and -189 resulted in induction by both CPT-cAMP and TPA and a super-induction when both were used together. An oligonucleotide containing bases -151 to -180 was capable of causing a large (12 fold)

induction to a CAT construct linked to the TK promoter when the cells were treated with a combination of CPS-cAMP and TPA (Shaskus et al., in press). The motif TGCGTCA occurs in position -166 to -172, found is in both the -150 to -180 oligonucleotide and in the -189, but not the -163 deletion mutant. This region has homology to both the CRE and TRE. It is also identical to the ENKCRE-2 heptad found to be necessary for basal activity as well as conferring responsiveness to both PKC and PKA activators in the proenkephalin gene (Comb et al., 1986, 1988). The TGCGTCA heptad also occurs in the c-fos gene between -289 and -295. Study of the importance of this heptad has been more limited than that of the one in the proenkephalin gene. However, it has been shown to be important in directing c-fos gene expression (Fisch et al., 1989).

The sequence homology of the DBH heptad with the proenkephalin ENKCRE-2 element makes the comparison of function necessary. ENKCRE-2 is necessary for basal activity of the proenkephalin gene as well as cAMP and phorbol ester mediated inductions (Comb et al., 1986, 1988). cAMP response was increased more than three fold by the addition of the phosphodiesterase inhibitor IMX and this inhibitor was necessary for any induction by phorbol esters in CV-1 cells (Comb, 1986). In contrast, the addition of IMX had no effect on the induction of either agent for the DBH gene (data not shown). Maximal response of the proenkephalin gene requires the presence of a second element, called ENKCRE-1 immediately 5′ to ENKCRE-2 for both basal and induced expression. Interestingly the spacing between the ENKCRE-1 and -2 elements in the proenkephalin gene is the same as that of the putative CAAT box and heptad elements in the DBH gene. Because the CAAT box occurs 3′ to the

heptad element, its necessity for the maximum response by the heptad was not testable by the use of 5′ deletion mutants and awaits further study.

CAT activity of a proenkephalin/CAT gene fusion has been shown to increase dramatically when the construct was cotransfected with the gene for the CRE binding protein CREB in an expression vector (Huggenvik et al., 1991). However, there have been no studies in the literature reporting the binding of CREB to the proenkephalin 5′-flanking region and therefore its direct action on the proenkephalin promoter has not been demonstrated. The transcription factor AP-1 has, however, been shown to bind the promoter (Comb et al., 1988, Sonnenberg et al., 1989). In addition, cotransfection with the AP-1 protein junD has been shown to activate proenkephalin transcription in a PKA dependent manner and junD was shown to bind ENKCRE-2 as a homodimer. Another AP-1 protein, junB was shown to inhibit the activation mediated by junD and was shown to bind the motif as a heterodimer with c-fos (Kobierski et al., 1991).

A promoter region containing the TGCGTCA motif confers response to both cAMP and TPA and for the proenkephalin genes In the proenkephalin gene this DNA motif has not been shown to bind CREB but has been shown to bind AP-1 proteins. These are characteristics more representative of the classical TRE than a CRE (Deutsch et al., 1988, Hoeffler et al., 1989). A 17 base region of the DBH gene containing this motif also confers induction by TPA and cAMP and is thus also more characteristic of a TRE than a CRE.

That both the proenkephalin and DBH genes are active in the same cells leads to questions of whether common elements are responsible for the tissue-selective expression of both genes. Physical studies such as

electrophoretic mobility shift assays and footprints using oligonucleotides containing the heptads and flanking sequences from each gene would be very informative.

In the c-fos gene, this same heptad element has been found to be a constitutive activator in HeLa cells, and to be responsive to cAMP via PKA in PC12 cells when it was linked to the β-globin promoter and the CAT gene (Velcich and Ziff, 1990). It has also been shown to be confer response to cAMP when linked to a minimal c-fos promoter (Fisch et al., 1989).

The -232 deletion mutant of the DBH/CAT gene fusion contains an additional CGTCA motif with an adjacent CAAT box compared to the -189 construct. However, there is no further increase in promoter activation by phorbol esters and a slight decrease in activity in cAMP induction. There is also a decrease in the synergistic induction when the two inducers are added together. This may be due to less effective binding of factors necessary to increase the rate of transcription. Alternatively, it may be due to secondary structure of the DNA or to binding of a negative regulatory factor. Addition of further 5' DBH sequences result in even greater decreases in induced activity. There may be other DNA sequence elements outside of this 5'-flanking region which contribute to regulating induction via the PKA and PKC pathways. They might function by modulating the binding of factors in this region and partially overcome the negative effects of the -189 to -394 region shown here.

CELL-TYPE SELECTIVE STUDIES

On the basis of the observed CAT and luciferase enzyme activities, we propose that the first 110 bases of the DBH promoter contain cell-type selective elements. The -110 minimal DBH promoter is over an order of magnitude more active in SH-SY5Y cells than in CV-1 cells, approximately an order of magnitude more active in C6 cells, and three orders of magnitude stronger than in the JEG-3 cell line. Analysis of this region is based on the assumption that TK-luciferase promoter will have smaller variations in activity between cell types than will the DBH promoter. The location of elements which determine cell-type selective activity in this region of the promoter is not uncommon. The TATA box sequence of the human myoglobin gene was necessary for interaction with the muscle-specific enhancer (Wefald et al., 1990). Transcription directed by the -229 to -160 fragment of the tyrosine hydroxylase activates the -44 to +27 TH minimal promoter better than the RSV promoter (Fung et al., 1992). This implies that there are specific elements in the -229 to -160 region that contribute to TH promoter activity. Since the -229 to -160 fragment is an enhancer involved in tissue specific expression, the core TH promoter must also contain tissue selective activity. The glycoprotein hormone α-gene promoter region between +44 and -100 was found to contribute approximately five fold to expression of the gene in JEG-3 cells versus the other cell lines tested (Jameson et al., 1989). A liver specific promoter element was found at -42 to -63 in the gene for the human factor X gene, which is a blood coagulation factor (Miao et al., 1992). It therefore seems likely that there may be several factors in the -110 to +14 region of the DBH gene which contribute to tissue selective expression.

The region between -110 and -232 was shown to contain at least two elements which confer basal activity to the DBH gene in the SH-SY5Y cell line. It was also shown that this region functions to increase DBH promoter activity when it is tested in other cell lines. However, the extent and pattern of these increases are not the same for all four cell lines tested. There is an increase in all cell lines upon addition of the -163 to -189 fragment. This increase is relatively small, under 5 fold for the SH-SY5Y, CV-1 and JEG-3 cell lines. However, it was over 100 fold in the JEG-3 cell line. Therefore, this region, which contains the TRE-like heptad TGCGTCA is a constitutive activator in all cell lines. However, the increase in activity in JEG-3 cell lines implies that a factor which binds DNA of a similar sequence might be important in placental cells. Also, unlike some other CREs and TREs that have been studied, this heptad, in its present sequence context, is not important for cell-type selective gene expression.

The region between -189 and -232 is also important for the expression of the DBH gene. It contains the second CGTCA element and adjacent CAAT box. For the SH-SY5Y, CV-1 and C6 cell lines, this region again contributes to increased basal activity of the DBH promoter. In these cell lines, it contributes three to seven fold increases in induction. However, in JEG-3 cells this region functions as a negative regulatory element resulting in a 30 to 40 % decrease in basal activity. Therefore, this region is important for the cell type selective expression of the DBH gene in some cell lines. This result also implies that the regulatory factors which bind the cis-elements between -150 and -189 are not the same as those which bind between -189 and -232.

Another possibility is that there is a negative regulatory element present which is active only in some cell lines.

The region between -232 and -394 contain a negative regulatory element which is active in all cell lines except SH-SY5Y. The strength of this negative regulatory element varies across the cell lines resulting in only a two fold decrease in CV-1 cells and a decrease in over 30 fold in C6 cells and over 1000 fold in JEG-3 cells. The effectiveness of this negative regulatory element was proportional to the positive increases in activity found for the other elements. Where the increase in activity of the CV-1 cells was approximately six fold for the -163 to -232 region, the negative effect was about two fold. Where the increase in activity was over 100 fold in JEG-3 cells for the same region, the decrease was approximately 1000 fold. Overall, the effect of the negative element was to decrease the DBH promoter activity back to approximately the level of the -110 deletion in the CV-1, JEG-3 and C6 cell lines. The SH-SY5Y cells had a corresponding increase in promoter activity of approximately five fold for the same region.

Though elements in the region between -163 and -232 increased promoter activity greatly in the cell lines which do not endogenously express DBH, the specific activity i.e., the CAT/luciferase activity was always highest in the SH-SY5Y cell line. This too would seem to imply that elements in the first 110 bases of the promoter contribute greatly to the cell-type specific expression of the DBH gene. Negative regulation of gene expression is very common (Foulkes and Sassone-Corsi, 1992). This type of regulation fits well with the modular construction of promoters. If the same activating factors, or activating factors

with overlapping binding specificities, are found in many different cell types, the presence of strong negative regulatory factors would act as a switch that can turn off a gene which would otherwise be inappropriately expressed. As an example, in this case, the region containing the -163 to -189 DBH sequences is so powerful a positive regulatory element in JEG-3 cells that the DBH gene might otherwise be inappropriately expressed if not for the presence of the negative regulatory element.

Use of the DBH/CAT constructs -394 -(-10) and -394-(+14 AGG) allowed us to determine if the 24 bases surrounding the transcription start site are important for the regulation of transcription. We found that these constructs gave equal activity in SH-SY5Y cells (data not shown). However, this region conferred some cell-type selective expression to the DBH gene. This varied from about two fold in CV-1 and JEG-3 cells to five fold in C6 cells. Two to five fold increases are not spectacular when compared to the large effects contributed by the silencer found between -232 and -394. But, this contribution is consistent with the modular hypothesis of gene transcription. It is also consistent with what has been found for other elements contributing to the transcription of the DBH gene as well as elements contributing to the transcription of a number of genes including the TH gene (Gandelman et al., 1990) and the glycoprotein hormone α-gene (Jameson et al., 1989).

Initiation site sequences have recently been found capable of binding transcription factors which influence expression of a number of genes. These include a protein which bound the adenovirus major late promoter (AdMLP) Inr as well as HIV-1 and TdT Inr elements (Roy et al.,

1991). The transcription factor YY1 (Shi et al., 1991), also known as common factor 1 (Riggs et al., 1991) has been shown to bind Inr sequences of the adeno-associated virus type 2 P5 (Seto et al., 1991). It was found capable of restoring basal transcription from this element in a HeLa cell extract depleted of YY1. The 3' end of deletion mutants of the AdMLP was found to be important for the regulation of expression of transcription in a cell-type selective manner (Lewis and Manley, 1985). Their construct containing DNA sequences form -405 to -2, +5 or +7 relative to the transcription initiation site showed reduced expression, of up to 25 fold less than constructs in which the 3' end contained nucleotides up to +33 in the 293 cell line as opposed to HeLa cells.

It has been found that the partially purified TATA box binding factor TFIID has a size that differs greatly from the factor cloned from a number of sources (Pugh and Tjian, 1992). The cloned factors are found to be approximately 38 kDa while the partially purified mammalian factors are considerably larger (Sawadogo and Sentenac, 1990, Conaway and Conaway, 1991). The DNAse 1 footprints of this region also differ in their coverage and patterns on gels depending on the factor used. This implies that the purified factor consists of multiple subunits. It has been proposed that there may in fact be multiple TFIID complexes (Pugh and Tjian, 1992; Sharp, 1992). A factor isolated from uninfected K562 cells, CAP-site binding factor (CBF) has been shown to bind the AdMLP Inr site and be necessary for optimal transcription activity. Its relationship to TFIID however has not been shown (Garfinkel et al., 1990).

The nature of the contributions to transcription regulation by the Inr region are as yet unclear. It seems evident that this region will be found to be a general contributor to gene regulation. Whether this is through the binding of specific factors or though the presence of multiple relatively specific factors constituting what is now call TFIID is not certain.

There are a number of DNA elements and corresponding factors which contribute to the activity of the DBH promoter in the promoter fragment studied. These elements and factors contribute to all aspects of DBH expression in a permissive cell line as well as induction via the PKA and PKC pathways. Several elements which contribute to the restriction of DBH gene expression to certain cell types have also been located.

SUMMARY AND CONCLUSIONS

Our interest in studying the gene for dopamine β-hydroxylase was twofold. We wanted to determine if the first translation initiation codon was used exclusively, or if a subsequent one might be used all or part of the time. We also wanted to locate sequence elements in the DBH 5'-flanking sequence which contribute to the regulating the expression of the gene.

In order to perform these studies a genomic clone of the rat gene coding for DBH was isolated. It was determined that this clone contained 394 bases 5' to the transcription initiation site. The region between -394 and +14 of the DBH gene was fused to the coding region for the reporter gene chloramphenicol acetyl transferase (CAT) resulting in the DBH/CAT fusion (-394-(+14)). Because the first potential translation initiation codon for DBH is at +10, this codon is contained in the original construct. This initiation codon is out of frame with the coding sequence for CAT. The second ATG is the endogenous CAT translation initiation codon. To determine if the DBH codon is used, that ATG was mutated to AGG which does not allow translation initation. This construct is DBH/CAT (-394-(+14 AGG)). Side-by-side transfections

of both constructs with an internal control were performed. The (-394-(+14)) construct had about one fourth the activity of (-394-(+14 AGG)). RNAase protection assays demonstrated that the difference in CAT activity was not due to differences in transcription efficiency. The results imply that the endogenous DBH codon was being used about 80% of the time with the CAT ATG used for the remainder. This use of two different ATGs in the fusion construct implies that a similar thing may happen in the DBH gene. Use of two different initiation codons, and differential processing of the translation products, may be an explanation for the occurance of the two different types of DBH subunits known. These are the one corresponding to the full length translation product and capable of being anchored in the secretory granule membrane by an uncleaved signal sequence, and a second which has the charged N-terminal and hydrophobic portions of that sequence cleaved.

Analysis of the sequence located a number of possible sequence motifs which have homology to the consensus binding sites for factors already shown to be important in gene regulation. Deletion mutants of DBH/CAT (-394-(+14 AGG)) were constructed. They were used to locate sequence elements important for regulating transcription of the gene. Studies in the cell line SH-SY5Y, which expresses the DBH gene, demonstrated that the bases between -163 and -189 and between -189 and -232 contained elements which contributed to basal expression of the gene. It was also shown that bases between -163 and -189 mediate induction by the protein kinase A and protein kinase C pathways. When the -189 construct was tested, it was found that if inducers for both pathways were present at the same time, induction was higher than the

sum of the inductions for both inducers present individually. Bases 5' to -189 reduced the level of induction.

When the contribution of bases in the 5'-flanking region of the gene were tested for their contribution to the cell-type dependent expression of CAT activity, it was found that the deletion containing 110 bases 5' to the transcription start site was expressed best in the SH-SY5Y cell line. Relative expression was 10 to 1,000 times higher than in the CV-1, JEG-3 or C6 cell lines. This implies that there are elements in this region contributing to the restricted expression of the DBH gene. Parallel transfections of the (-394-(-10)), which deletes the DBH transcription and translation initiation sites, and (-394-(+14 AGG)) with an internal control TK-luciferase were performed. The results demonstrated that the region between -10 and +14 of the DBH gene contributes to its cell type selective expression. The differences in relative activity of the two constructs was about two fold in CV-1 and JEG-3 cell lines and about 5 fold in the C6 cell line. It was found that the region between -163 and -189 contained a positive element in all four tested cell lines. This element was most active in the JEG-3 cell line where it increased relative activity by over two orders of magnitude compared to about 3 fold in fold in SH-SY5Y cells. The region between -189 and -232 also increased activity in three of the four cell lines, again implying that the effects of the elements were not restricted to certain cell types. The region between -232 and -394 contained an element which repressed expression in all cell lines except SH-SY5Y cells. It reduced expression to approximately the level found in the -110 construct. It therefore functions to aid in the cell-type

specific expression of DBH.

The region between -394 and +14 of the DBH gene contains a wealth of information. It contains a DBH-contributed translation initiation codon which is not utilized 100% of the time. In the DBH gene. use of this and the subsequent ATG may provide the explanation for the two different forms of the enzyme. The gene fragment studied also contains elements which effect the expression of the gene in a variety of ways. These elements contribute to control of basal expression, induced expression and the restriction of DBH expression to certain cell types.

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APPENDIX

SUMMARY OF EXPERIMENTS PERFORMED FOR THE DETERMINATION OF CELL TYPE-SELECTIVE CIS-ELEMENTS

CONSTRUCTS UTILIZED	RATIO OF ACTIVITY OF LONGER PROMOTER TO THAT OF SHORTER PROMOTER			
	CELL LINE			
	SY5Y	CV-1	JEG-3	C6
Experiment 1				
(-395 AGG)------(-110)	4.76	0.37	1.16	--
Experiment 2				
(-395 AGG)----(-163)	7.00	0.71	2.75	0.31
Experiment 3				
(-232)-----(-110)	11.1	6.22	103	35.5
(-189)--(-110)	1.62	2.20	130	8.42
(-232)(-189)	6.84	2.83	0.79	4.21
Experiment 4				
(-232)(-189)	4.34	3.31	0.62	3.64
(-189)(-163)	3.25	1.66	>200	2.58
(-232)--(-163)	14.1	5.52	>100	9.41
Experiment 5				
(-394 AGG)-(-232)	0.51	0.23	<0.001	<0.03
(-232)-----(-110)	4.74	----	------	>451

(-394 AGG)------(-110)	2.40	----	-----	not determinable