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Abstract

Histidine decarboxylase (HDC) plays a critical role in the synthesis of histamine, a central nervous system neurotransmitter used by both vertebrates and invertebrates. Past attempts to create antisera that recognize the HDC protein *in vivo* have not produced satisfactory antisera. While HDC antiserum has been made in other organisms, they appear not to be useful across species, including *Drosophila melanogaster*. As a result, little is known about the localization as well as the biochemistry of HDC in the fly. It has been suggested that HDC undergoes a complex maturation process, including cleavage of the polypeptide at both the N- and C- termini of the predicted protein. We report an approach that should allow the HDC protein to be examined *in vivo* using internal epitope tagging. A plasmid containing a functional *Hdc* gene was modified by insertion of epitope tags, 6xHis into the protein coding region of the *Hdc* gene at specific sites. The location of these tags in the protein structure is predicted to be in the mature HDC protein, and thus, should be present where HDC is active. This project will allow future research investigating the biochemistry and cell biology of HDC, after germline transformation of the tagged *Hdc* constructs is completed. Introduction

KEYWORDS: Histidine decarboxylase, Histamine, Central Nervous System Neurotransmitter

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ABSTRACT

*Histidine decarboxylase (HDC) plays a critical role in the synthesis of histamine, a central nervous system neurotransmitter used by both vertebrates and invertebrates. Past attempts to create antisera that recognize the HDC protein in vivo have not produced satisfactory antisera. While HDC antiserum has been made in other organisms, they appear not to be useful across species, including *Drosophila melanogaster*. As a result, little is known about the localization as well as the biochemistry of HDC in the fly. It has been suggested that HDC undergoes a complex maturation process, including cleavage of the polypeptide at both the N- and C- termini of the predicted protein. We report an approach that should allow the HDC protein to be examined in vivo using internal epitope tagging. A plasmid containing a functional Hdc gene was modified by insertion of epitope tags, 6xHis into the protein coding region of the Hdc gene at specific sites. The location of these tags in the protein structure is predicted to be in the mature HDC protein, and thus, should be present where HDC is active. This project will allow future research investigating the biochemistry and cell biology of HDC, after germline transformation of the tagged Hdc constructs is completed.*

Introduction

The biogenic amine histamine serves many functions in biological systems, including modulating gastric acid release in the stomach (Joseph *et. al.*, 1990) and playing a role in allergic reactions (Lin *et. al.*, 2000; Crockard and Ennis 2001). One of its key roles in both vertebrates (Eriksson *et. al.*, 1998) and invertebrates is that of a neurotransmitter (Melzig *et. al.*, 1996). Cells in the nervous system are known to communicate using a process of regulated exocytosis, known as synaptic transmission (Bajjalieh & Scheller, 1995). Neurotransmitters are responsible for the transfer of chemical messages, which is the basis of thought, learning, memory and motor control (Ludwig & Pittman, 2003). Neurotransmitters are critical to the function of the nervous system, as they are able to pass messages across the synapse, the space between adjacent neurons. In *Drosophila*, 18 pairs of histaminergic cells are found in the central nervous system (CNS) (Melzig *et. al.*, 1998). High concentrations of histamine are also found in the photoreceptors and mechanosensory cells of cuticular hair sensilla (Melzig *et. al.*, 1996). Prior research has demonstrated the importance of histamine as a neurotransmitter for vision and mechanosensory mediated behaviors (Melzig *et. al.*, 1996). Histamine is synthesized from the amino acid histidine through the action of the enzyme histidine decarboxylase (HDC) (Sarthy, 1991).

In order to better understand the regulation and function of HDC in the nervous system, information on the cellular and subcellular localization of this enzyme is needed. Thus far this work has been challenging, as there are limited HDC antibodies available (Dartsch *et. al.*, 2004) and none that are known to be effective in *Drosophila*. Attempts at making antisera specifically for HDC in *Drosophila*

have failed, likely due to its similar structure to other decarboxylases, like dopa decarboxylase (Burg *et. al.*, 1993). Previous work has attempted to overcome this difficulty by creating a gene-fusion for HDC in *Drosophila*. This too has proven unsuccessful (Burg, unpublished data), likely due to the post translational editing that occurs in the initial HDC translational product. Recent work has shown an extensive maturation process for HDC (in vertebrate systems) in which both the carboxy and amino termini are cleaved (Fleming & Wang, 2000). It has been observed in rats that the initial HDC protein is 74-kDa, but it is subsequently cleaved, resulting in a final active enzyme of 48-kDa (Fleming & Wang, 2000). The predicted *Drosophila* HDC protein is larger than rat HDC prior to any possible cleavage, but the amino acid sequence is highly conserved between these species in critical regions necessary for HDC function. The same regions that are cleaved during rat HDC protein maturation are also present in the *Drosophila* predicted protein, suggesting that *Drosophila* HDC also undergoes processing prior to becoming an active protein. This processing of the protein includes the cleavage of both the carboxy and amino termini using “PEST” regions known to be targets of cellular enzymes that cleave proteins containing these sequences (Fleming & Wang, 2000).

Drosophila is an ideal model organism for the study of HDC. The human, rat and *Drosophila Hdc* gene is 87% conserved across species (Burg *et. al.* 1993). Previous research of *Hdc* in *Drosophila* has established a transgenic germline transformation, the wild-type (normal) *Hdc* gene was used to completely restore *Hdc* function back to mutants that normally lacked *Hdc* function totally (Burg & Pak, 1994; Burg & Pak, 1995; Burg & Pak, 1995). This “rescued mutant” demonstrated

that all the regulatory information needed for normal gene expression was confined to 7.4 kb of DNA which has been completely sequenced from a wild-type *Drosophila* strain (Burg & Pak, 1994; Burg & Pak, 1995; Burg & Pak, 1995).

To overcome the problem of the lack of a good antibody for HDC in *Drosophila*, we propose a method of inserting an epitope tag into the open reading frame of the *Hdc* gene. Epitope tags are useful when antibodies are not available for a particular protein (Brault *et al.*, 1999). Many varieties of epitope tags are available (Jarvik & Telmer, 1998). The 6xHis epitope tag consisted of six consecutive histidine residues. Proteins encoding this sequence can be purified by using a divalent metal ion column. Alternately, using the anti-6xHis antibody, it is also possible to analyze the protein via Western blot or by immunofluorescent techniques. Epitope tags are normally inserted at either the carboxy or amino termini, but as was previously stated, in HDC both the termini are likely cleaved during protein maturation. For a tag to be useful in studying HDC, it must be inserted internally (Figure 3). A large tag inserted internally is more likely to interfere with the functionality of HDC than a small tag. There are many epitope tags with established antibodies available, but most are much larger than the 6xHis tag and produce the same results with their respective antibodies (Jarvik & Telmer, 1998). The small size of the 6xHis tag along with the commercially available antibody makes it ideal for an internal insertion into the *Hdc* gene.

The insertion of an epitope tag into the *Hdc* gene will be done at specific restriction enzyme sites, using oligonucleotide primers with extensive overhanging sequences coding for the tag in a PCR-based approach, producing a tagged fragment of the gene. This

tagged *Hdc* fragment will be inserted into the native gene, creating a normal functioning *Hdc* gene containing an epitope tag. Here we outline the location of the inserted tags within the *Hdc* gene and the methods of creating the transformation vector containing a functional *Hdc* gene with an internal epitope tag.

Materials and Methods

Primer design and epitope placement.

The forward oligonucleotide primers were designed to include the sequence of the epitope tags. The composition of each of the primers included: a 7-10bp cap sequence, followed by the *Sall* restriction enzyme recognition sequence, then the sequence encoding for the 6xHis, and 19-24bp complementary to the *Hdc* gene. The sequences of the primers were as indicated in Figure 1. Forward primer 1 and reverse primer 2 were used to insert the 6xHis tag immediately adjacent to the *Sall* restriction endonuclease site which is 1.7kb from the *EcoRI* restriction endonuclease site (Figure 2). The location of the primers used and their sequence was based on the current model of *Hdc* gene structure (Figure 2). By deducing the predicted protein structure of HDC using a known structure of a high related protein (DDC), it was possible to indicate in the structure of DDC where the proposed epitope tag for HDC will likely be located (Figure 3).

Production of 6xHis-tagged Hdc gene fragments.

Using the tagged primers, fragments of the *Hdc* gene were synthesized using a PCR approach (Figure 4). A control reaction that lacked DNA template was also run using the same parameters. The template used was the genomic *Hdc* gene contained in a pCaSpeR-3 that previously was shown to rescue the *Hdc* mutant phenotype completely, indicating that this *Hdc* DNA contained

the normal gene. The *Hdc* gene was linearized with *SalI* and purified using GeneClean. All amplifications were done using an Eppendorf Mastercycler gradient thermocycler. The reactions were set up using Expand High Fidelity Plus PCR system (Roche). Amplification parameters were as follows: 94°C for 20 s, 71°C for 30 s and 68°C for 1 min and 40 s for 18 cycles. The PCR products were initially blunt-ended, but contained restriction sites at both ends, which allowed the PCR DNA fragments to be cut with the enzymes, allowing the fragment to be cloned into a standard cloning vector using the *SalI* (or *SacI*)

and *EcoRI* sites in the pBluescript II SK(+) vector using T4 DNA ligase (NEB). Transformations of XL1blue competent cells with the pBluescript II SK(+) vector containing the tagged *Hdc* fragment were done through a 1 min heat shock. The cells were incubated at 37°C while shaking at 250 rotations/min for 1 hour before applying to plates. The plates were incubated at 37°C for 16 hours and stored at 4°C.

Sequencing of tagged fragments.

Blue/white screening was done to select bacterial cells positive bearing the plasmid with *Hdc* gene fragment

(Fig. 4). Once cells were identified, the plasmids were isolated from these cells through standard alkaline lysis techniques, and the DNA analyzed using restriction enzyme digestions. Once clones with inserts were identified, the DNA was purified (Qiagen) and sent for sequence analysis. For the sequencing of the fragment containing the tag at the *SalI* site, T3 and M13F-20 universal primers were used (Retrogen). T7 and M13R universal primers were used for the sequencing of the fragments with the epitope tag at the *SacI* site. Three clones were sequenced from each of the three tagged groups.

Primer 1
 5'-GGAAAGAGTCGACCACCACCACCACCACCTATATTGCAGACTATTTGGAGAAC-3'

Primer 2
 5'-CCACTCCGGAATTCTCGT GCTGCAGATACAGCGGCTCCACA-3'

Figure 1. Sequence of primers used in PCR reactions to produce epitope-tagged fragments of the *Hdc* gene. Primer 1, 55bp, was used to insert the 6xHis tag immediately adjacent to the *SalI* restriction endonuclease site which is 1.7kb from the *EcoRI* restriction endonuclease site. Primer 2, 41bp, including the *EcoRI* recognition site and was used in the PCR amplification of this region of HDC.

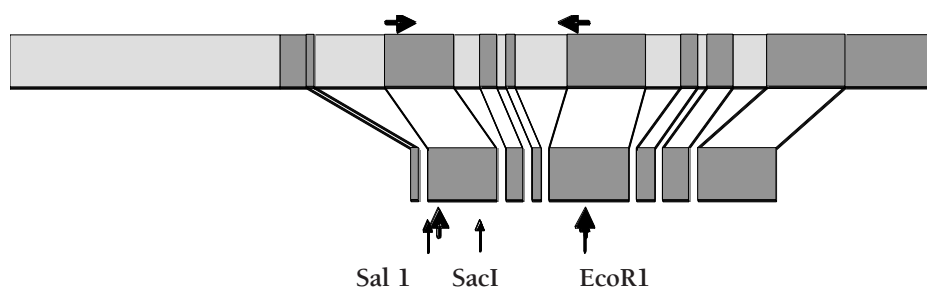


Figure 2. *Hdc* gene intron/exon structure (7.4 kb). The lighter regions represent introns while the darker regions represent exons. The dropped down portion represents the HDC protein coding region. Indicated (by arrowheads above the gene structure) is the location of PCR primers in the genomic fragment used as template and the location of *SalI* and *EcoRI* restriction sites in the coding region.

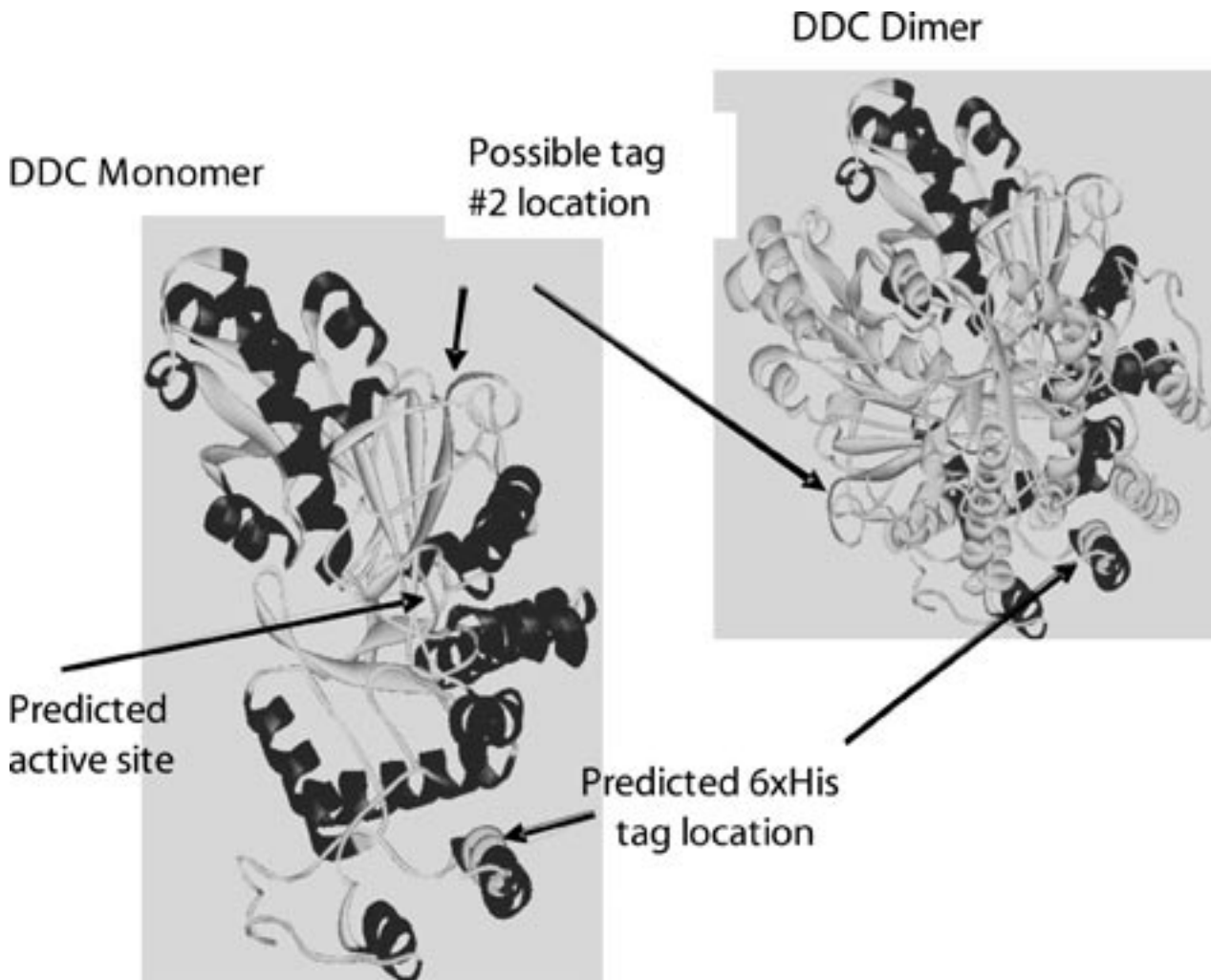


Figure 3. Crystal structure of *Sus scrofa* (pig) dopa decarboxylase (Burkhard et. al., 2001) used to predict structural placement of histidine decarboxylase 6xHis epitope tag (arrows). Within the monomer structure of DDC and dimeric structure DDC, are indicated the predicted position of the 6xHis tag generated currently reported (6xHis) or planned (tag #2). Dimer formation was predicted as solved for the pig DDC enzyme, indicating that the epitope tags are likely outside important structural elements that could disrupt protein structure and function. These structural models were constructed using the Weblab program.

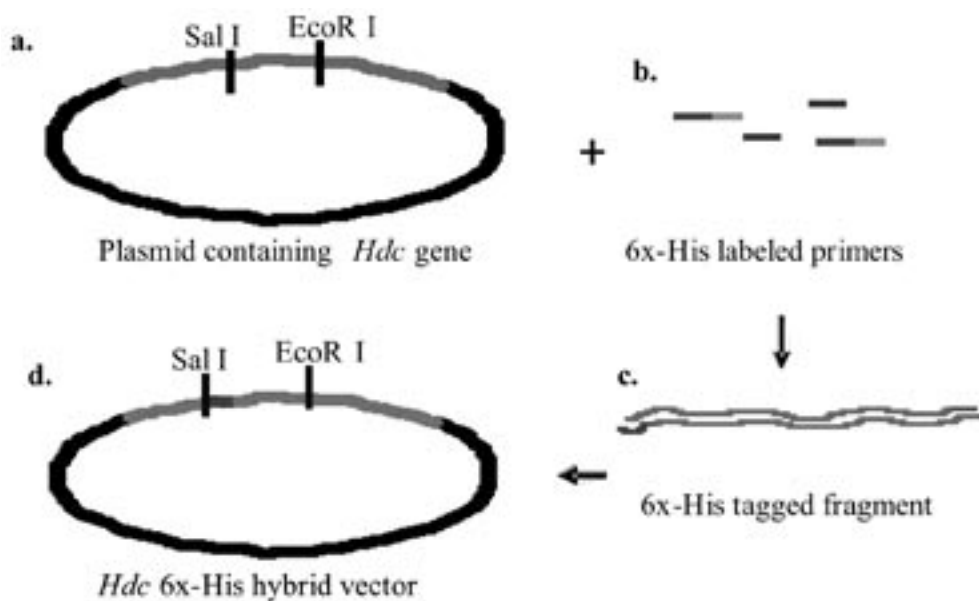


Figure 4. Method used for placing the insertion of a 6xHis tag at the *SalI* restriction endonuclease site in the *Hdc* gene. Restriction endonuclease sites were selected in the *Hdc* gene (a) as a likely location for insertion of the epitope tag. Forward primers coding for the sequence of the tags (b) and reverse primers were designed to amplify a region of *Hdc* containing the tag (c). With the use of the restriction endonuclease digestion, the tagged fragments can then be inserted into the *Hdc* gene, creating an *Hdc*-epitope-tagged hybrid vector (d).

Results

The expected size of the *Hdc* fragments that were amplified using the primer at the *SalI* site in the *Hdc* gene was 1.7kb and the control reaction which lacked DNA template was confirmed to not produce any products (Fig. 5). As apparent by gel electrophoretic analysis (Fig. 6), the digested DNA fragments were of the correct sizes according to the sequence of the *Hdc* gene, confirming the PCR products likely identity. The results of the digest for the *SalI* locus 6xHis indicate the expected two bands of 3kb (pBluescript) and 1.7kb (insert) (Fig. 6).

DNA sequence analysis of the *Hdc* gene fragment containing the suspected

6xHIS indicated that the 6xHis tag at the *SalI* site had been inserted (data not shown). All of the sequencing results confirmed that the expected fragment of the gene had been synthesized properly. In the DNA sequence, it was noted that the *SalI* region 6xHis tag was inserted into the open reading frame with one polymorphism introduced into the primary protein structure. This alteration, located in the second exon, was a single base substitution which caused the change of an alanine to a valine (GCG±GTG). There were seven additional single nucleotide alterations in the amplified DNA. Three of these alterations occurred in intronic regions that likely will not affect the function or

expression of the *Hdc* gene, while the remaining three alterations occurred in positions that did not result in a change in amino acid composition. All alterations were unintentional except for the 6xHis tag insertion. These changes were present in all three of the clones sequenced, and could reflect variability between wild type strains, indicated in previous work (M. Birdsey and M. Burg, Pers. commun.).

Results indicate that the 6xHis tag is positioned in the HDC protein structure as indicated in Figures 3 and 7. Figure 7 indicates precisely where the additional histidine (H) amino acids are inserted into the HDC protein, confirmed by sequence analysis.

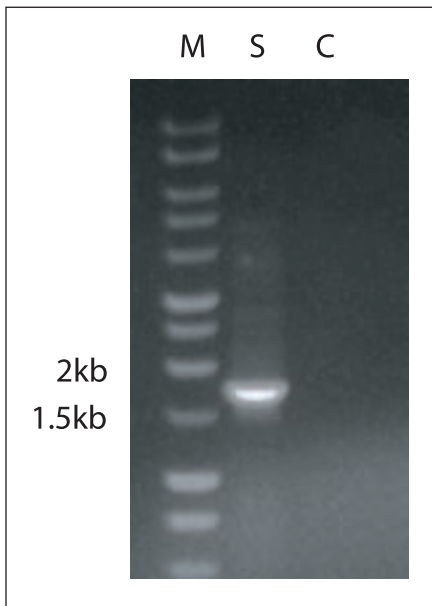


Figure 5. Gel electrophoresis of PCR amplified Hdc fragment (S) using a primer contain the sequence of the 6xHis tag adjacent to the Sall restriction site. A 1 kb DNA size standard (M) was used to confirm size. The sample (S) did appear to contain a fragment of the expected size 1.7 kb. The control (C) lacking template was also run on the gel and did show any product.

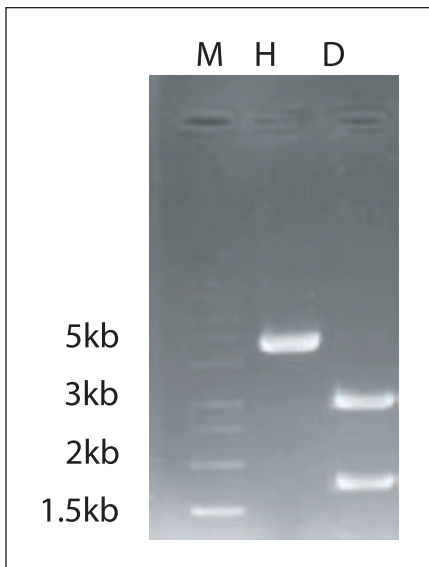


Figure 6. Gel electrophoresis of an Hdc gene fragment containing a 6xHis epitope adjacent to the Sall site and ligated into pBluescript. The undigested (U) sample contains a single fragment of 4.7 kb. The digested sample (D) indicates the inserted fragment digested out of the vector by EcoRI and Sall. The resulting pBluescript fragment is 3 kb and the tagged Hdc fragment is 1.7 kb. A 1 kb DNA size standard (M) was used to determine DNA fragment sizes.

Fly HDC ----MDFKEYRQRGKEMVDHHHHHHYIADYLENIRERRVFPDVSPGYMRQLLPESAPIEG
 Pig DDC ----MNASDFRRRGKEMVD-----YMADYLEGIEGRQVYPDVQPGYLRPLIPATAPQEP
 Rat HDC MMEPSEYHEYQARGKEMVD-----YICQYLSTVRRERQVTPNVKPGYLRAQIPSSAPEEP

 Fly HDC EPWPKIFSDVERIVMPGITHWQSPHMHAYFPALNSMPSLLGDMLADAINCLGFTWASSPA
 Pig DDC DTFEDIQDVEKIIMPGVTHWHSFYFFAYFPTASSYPAMLADMLCGAIGCIGFSSWAASPA
 Rat HDC DSWDSIFGDIEQIIMPGVVHWQSPHMHAYYPALTSWPSLLGDMLADAINCLGFTWASSPA

 Fly HDC CTELEIIVMNWLGMIGLPDAFLHLSSQSGGGVLQTTASEATLVCLLAGRTRAIQRFHE
 Pig DDC CTELETVMDWLGMQLPEAFLAG-EAGEGGGVIQGSASEATLVALLAARTKVVRRLQA
 Rat HDC CTELEMNIMDWLAKMLGLPDDFLHHHPSSQGGGVLQRTVSESTLIALLAARKNKILEMKA

 Fly HDC RHPGYQDAEINARLVAYCSDQAHSSVEKAALIGLVRMRYIEADDDLAMRGKLLREAIEDD
 Pig DDC ASPGLTQGAVLEKLVAYASDQAHSSVERAGLIGGVKKAIPSDGKFAMRASALQEAALERD
 Rat HDC HEPNADESSLNARLVAYASDQAHSSVEKAGLISLVKIKFLPVDDNFSLRGEALQKAIEED

 Fly HDC IKQGLVPFVFCATLGTGSCSFDNLEEIGIVCAEHHLWLHVDAAYAGSAFICPEFRTWLR
 Pig DDC KAAGLIPFFVATLGTSCSFDNLEVGPICHEEDIWLHVDAAYAGSAFICPEFRHLLN
 Rat HDC KQQGLVPFVFCATLGTGVCADFKLSELGPICAREGLWLHVDAAYAGTAFILREPELGRFLK

 Fly HDC GIERADSI AFNPSKWL MVHFDATA LWVRDSTAVHRTFNVEPLYLQHEN--SGVAVDFMHW
 Pig DDC GVEFADSEFNFNPHKWL LVNFDCSAMVVKRRD LTGAFKLDPVYLKHS HQSGLITDYRHW
 Rat HDC GIEYADSEFTFNPSKMMVHFDCTGFVVKDKYK LQQTFSVNP IYLRHAN--SGVATDFMHW

 Fly HDC QIPLSRFRALKVWVFLRSYGIKGLQRHIREGVRLAQKFEALVLADHRFELPAKRHLGLV
 Pig DDC QLPLGRRFRSLKMWFVFRMYGVKGLQAYIRKHVQLSHEFEAFVLQDPRFEVCAEVTGLGLV
 Rat HDC QIPLSRFRS IKLWFVIRSFVGNLQAHVRHGTDMAKYFESLVRSDPVFEIPAERHLGLV

 Fly HDC VFRIRGDNEITEKLLKRLNHRGNLHCIPSSLKQYVIRFTITSTHTLDDIVKDWMEIRQ
 Pig DDC CFRLKGSDGLNEALLERINSARKIHLVPCRLRGQFVLRFAICSRKVESGHVRLAWEHIRG
 Rat HDC VFRLKGNCLTESVLKEIAKTGQVFLIPATI QDKLIIRFTVTSQFTTKDDILRDWNLIRE

 Fly HDC VASTVLEEMNITISNRVYLKETKEKNEAFGSSLLLSNSPLSPKVNGSFAAIFDADEFLA
 Pig DDC LAEELLA AE-----EGKAEIKS-----
 Rat HDC AANLVLSQHCTS-----QPSRAKN-----LIPPPVTR-----

 Fly HDC KTYAGVRIAHQESPSMRRRVRGILMSGKQFSLDSHMDVVVQTTLDAGNGATRTSTTNSYG
 Pig DDC -----
 Rat HDC -----

 Fly HDC HTTSAAQANSERQASIQEDNEESPEETELLSLCRTSNVPSPEHAHSLSTPSRSCSSSSHS
 Pig DDC -----
 Rat HDC --DSKDLTNGLSLESVNEGGDDPVQVRKIFRLPGDS-LETTMDPFDDCFSEEASDTTKHK

 Fly HDC LIHSLTQSSPRSSPVNQFRPITLCAVPSQSQLSMPLAMPLPNRNVTVSVDL LN PVTTCN
 Pig DDC -----
 Rat HDC LSSFLFSYLSVQNKKTMRSLSCNSMPMSAQK-----SPPPDAS

 Fly HDC VYHGKRFLEPLENLAQTSASFSSIFRLPTPIATPTRESPEDPDWPAKTFSQLLERYSS
 Pig DDC -----
 Rat HDC VKHGG-----FFRARIFSG-----FPEEMMMKKGFKKLIKFYSV

 Fly HDC QSQSLGNNSSSTESSLSGGATPTPTPMSLDELVTPLLLSFASPSQPMLSAHGIGEGQRE
 Pig DDC -----
 Rat HDC PSFP-----ECSSQCG---TLQLPCCPLQAMV-----

 Fly HDC QGSDSDATVCSTTSSMESL
 Pig DDC -----
 Rat HDC -----

Consensus key

- single, fully conserved residue
- conservation of strong groups
- conservation of weak groups
- no consensus

Figure 7. Protein sequence alignment of newly constructed *D. melanogaster* Sall-6xHis histidine decarboxylase identified through sequence analysis of PCR-generated fragments (see Figs 5, 6.), *Sus scrofa* (pig) dopa decarboxylase, and *Rattus norvegicus* (rat) histidine decarboxylase. The sequences are highly conserved for the first 465 residues. The tail region of HDC may be cleaved off during protein maturation as it contains PEST-like regions. Underlined region is the “active site” for the enzyme, and amino acids (H) indicates the 6xHis insertion.

Discussion

The epitope tag 6xHis has been successfully placed into the coding region of the *Hdc* gene of *Drosophila* immediately adjacent to the Sall recognition site that is 1.7 kb upstream of the EcoRI site. The tag was inserted into the open reading frame in exon 2 of the *Hdc* gene. The insertion of the 6xHis tag at the Sall site was done without introducing sequence alterations into the gene which would disrupt its function. Although seven possible single nucleotide alterations have been detected, it is unlikely that these alterations will alter gene function. While it is possible that the alterations were caused by the PCR, due to the fact that a high-fidelity polymerase was used, the error rate observed is significantly higher than expected. It is likely that the differences observed are those that have

been confirmed to exist between wild-type strains, and observed recently in the sequence analysis of mutations in the *Hdc* gene (M. Birdsey and M. Burg, pers. commun.). The single base substitution of an alanine to a valine does not occur in or near a region that is likely the active site of the enzyme, thus is likely not to affect HDC enzymatic activity.

The purpose of this project was to produce an epitope tagged *Hdc* gene in order to conduct germline transformation and place this newly constructed gene back into *Drosophila*. For this purpose, *Drosophila* mutants that lack functional HDC will be used as a recipient strain, once the DNA has been introduced successfully into the *Drosophila* germline, using standard techniques. Prior research has established that the HDC mutants

can be transformed with the cloned *Hdc* gene that was used in this project and recover full HDC expression (Burg & Pak, 1994; Burg & Pak, 1995; Burg & Pak, 1995). With the use of these mutants, it will be possible to assess the functionality of the tagged HDC construct both biochemically, physiologically and behaviorally. Once the function of the tagged HDC protein is established, commercially available antibodies will be used to begin answering many questions concerning HDC location in tissue that can only be done with an antibody. Epitope tagged proteins can be detected by using many of the same procedures that are used to detect proteins that have a specific antibody (Jarvik & Telmer, 1998; Harlow & Lane, 1988).

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