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Epitope Labeling of Histidine Decarboxylase in Drosophila melanogaster

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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 (Braut 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: 94ºC for 20 s, 71ºC for 30 s and 68ºC 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 37ºC while shaking at 250 rotations/min for 1 hour before applying to plates. The plates were incubated at 37ºC for 16 hours and stored at 4ºC.

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’-GGAAAGAGTCGACCACCACCACCACCACCACTATTGCAGACTATTTGGAGAAC-3’

Primer 2
5’-CCACTCCGGAATTCTCGT GCTGCAGATAACACGGCGCTCCACA-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.
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 \(6x\)His 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 \(6x\)His indicated that the \(6x\)His 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 \(6x\)His 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 (\(GCG\rightarrow GTG\)). 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 \(6x\)His 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 \(6x\)His 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 containing the sequence of the 6xHis tag adjacent to the SalI 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 SalI 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 SalI. 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.
**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).

**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 thorough 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.
References


Crockard, A.D. and M. Ennis. 2001. Basophil histamine release tests in the diagnosis of allergy and asthma. Clinical & Experimental Allergy. 31(3): 345


