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University of Nevada, Reno

The Isolation of Juvenile Hormone Epoxide Hydrolase Genes in the Malpighian Tubules of the Tobacco Hornworm, *Manduca Sexta*

A thesis submitted in partial fulfillment
of the requirements for the degree of

Bachelor of Science in Biochemistry and Molecular Biology and the Honors Program

by

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UNIVERSITY OF NEVADA RENO

THE HONORS PROGRAM

We recommend that the thesis
prepared under our supervision by

ALEXANDER J. FIANNACA

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requirements for the degree of

BACHELOR OF ARTS, BIOCHEMISTRY AND MOLECULAR BIOLOGY

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David A Schooley, Ph.D., Thesis Advisor

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Tamara Valentine, Ph. D., Director, **Honors Program**

May 2011

Abstract

Understanding the regulation of Juvenile Hormone (JH) titer in insects is critical for understanding the biochemical processes related to metamorphosis. The cDNAs from several potential isoforms of the enzyme Juvenile Hormone Epoxide Hydrolase have been isolated from the Malpighian tubules of *Manduca sexta*. Modified PCR methods are being used to search for any other potential isoforms of this enzyme that may be contained in a Malpighian tubule expressed sequence tag library. To date, this protocol has yielded three potential genes, in addition to the two originally isolated. Future research will allow the creation of baculovirus vectors containing the isolated potential JHEH genes and the identified isoforms will be expressed in an insect cell culture. The isoforms will be assayed for activity in metabolizing the three forms of JH present in *M. sexta* through the use of radiolabeled JH's.

Acknowledgements

Special thanks go to Chong Tang, Sharon Young, Alaine Garrett, and Dr. David Schooley for their guidance in developing my experimental methods and troubleshooting thereon. Also, thank you to Dr. Kristina Kruse of the Nevada Genomics Center for her endless help and knowledge in sequencing the EST clones. Finally, thank you to Nevada INBRE for providing the funds for this research.

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Introduction

In the insect *Manduca sexta,* one particular isoprenoid hormone, known as Juvenile Hormone (JH), plays a key role in the regulation of development and reproduction. It is known that the primary hormone controlling the ecdysis of *M. sexta* is the steroid hydroxyecdysone; however the function of hydroxyecdysone is highly modulated by JH (Riddiford et al., 1986). When the titer of JH decreases sharply at the end of the fifth larval stadium it signals that pupation will occur, rather than molting (deKort et al., 1981). Therefore, understanding the metabolic pathways responsible for the breakdown of JH is integral to understanding the role JH plays in the development of *M. sexta* from a larva to an adult.

JH is a sesquiterpene methyl ester with an epoxide ring and several different possible R groups projecting off of the main sesquiterpene backbone. Due to the different possible R groups on the JH molecule, there are three types of JH's in *Manduca sexta* (JH I-III). Metabolism of JH is carried out by three enzymes: Juvenile Hormone Esterase (JHE), Juvenile Hormone Epoxide Hydrolase (JHEH), and Juvenile Hormone Diol Kinase (JHDK) (Hammock et al., 1985.) JHE and JHEH function as the primary modes of JH degradation, while JHDK phosphorylates the product of JHEH only (Maxwell et al., 2002b and 2002c). JHE is a soluble enzyme which has been isolated from *Manduca sexta* and well-studied. On the other hand, Juvenile Hormone Epoxide Hydrolase is less well studied primarily because it is much harder to isolate an integral membrane protein than a soluble protein. Research in the early-1990s gave improved methods for analyzing the products of JHEH using *M. sexta* Malpighian tubules and partially characterizing the enzyme using radiolabeled JH's. (Halarnkar et al., 1993 and Grieneisen et al., 1995). It is known that JHEH functions by hydrolyzing the epoxide ring at the end of the molecule distal from the methyl ester; thereby, allowing JHDK to attach a phosphate group to the

newly formed alcohol residue on carbon 10. It is also known that JHEH catalyzes an irreversible reaction, whereas JHE is reversible (Seino et al., 2010). This may indicate that JHEH is the more important of the two enzymes in clearing the insect of JH prior to metamorphosis. In fact, a good deal of recent research has pointed to this exact hypothesis (Gilbert et al., 2000).

Wojtasek *et al.* (1996) first determined the amino acid sequence of JHEH and Debernard *et al.* (1998) used this to express and characterize JHEH. Debernard et al. (1998) managed to express JHEH in an insect cell culture and determined several things. First JHEH is a microsomal protein, meaning that it has an integral membrane domain in addition to its catalytic and regulatory domains. The structure of JHEH has not yet been determined by x-ray crystallography or 2-D NMR. Second, Debernard determined the activity of JHEH on JH-I, JH-II, and JH-III and showed that it has the greatest activity on JH-III. Finally, Debernard proposed a mechanism for the reaction catalyzed by JHEH. In addition, a recent paper by Seino *et al*. (2010) fully characterized the JHEH from *Bombyx mori*, a species closely related to *M. sexta*, and confirmed that the Wojtasek JHEH is most likely the most active JHEH in *B. mori*. However, Harris *et al.* (1999) discussed the probability that JHEH's are not as specific as once believed, so it stands to reason that there are other JHEH isoforms present in *M. sexta*.

We recently discovered, by way of multiple sequence alignment analysis of the Wojtasek sequence vs. 2000 partially sequenced cDNAs from an expressed sequence tag (EST) library constructed from the Malpighian tubules of *M. sexta*, that there are several other isoforms of the JHEH. DNA sequencing confirmed that these are epoxide hydrolases, although more research will be needed to determine if these are specific for JH. It was found that one of these sequences is predicted to be cytosolic using the computer algorithm TMHMM. This is significant due to the fact that no soluble epoxide hydrolases have been discovered in insects to date. Furthermore,

three additional genes from publicly available *M. sexta* EST libraries were discovered via the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information. Here we report the partial isolation of these genes for further study.

Methods

Discovery of Potential Genes in the M. sexta EST Library:

The sequence of the Wojtasek JHEH (GenBank ID: U46682.1) was compared to 2000 partially sequenced EST's from the *M. sexta* EST library by way of a multiple sequence alignment using the DeCypher database. Sequences with high levels of homology were picked and the *E. coli* containing the respective EST inserts were grown in 1 x Terrific Broth. The QIAGEN Spin Miniprep plasmid isolation kit was used to extract the plasmids from these overnight cultures following the manufacturer's protocol. DNA concentrations were obtained using the Picogreen method at the Nevada Genomics Center. Extracted plasmids were sequenced using the universal primers M13-20, M13-reverse, T7, and T_{mix}. When the initial attempt at sequencing did not obtain the complete gene sequence, elongation primers were designed using the Primer3 computer program, and an additional attempt at sequencing was performed. Sequences were analyzed using the computer program VectorNTI, and the structure prediction algorithm TMHMM available through the Center for Biological Sequence Analysis at the Technical University of Denmark.

Isolation of Potential Genes from EST Library by Degenerate PCR:

Degenerate primers were designed based on regions of high amino acid identity in an alignment of JHEH genes from both *M. sexta* and *B. mori*. These primers along with the universal primers seen above were used to perform a touch-down nested PCR protocol. The master mix for these reactions contained 1x PCR Buffer (Promega GoTaq Flexi), 2.5 mM $MgCl_2$, 0.2 mM dNTP's, 0.4 µM of one universal primer, 0.4 µM of one degenerate primer, and 1.25 units of GoTaq Flexi DNA Polymerase (Promega). The thermocycler protocol consisted of 5 minutes at 95°C, then 10 cycles of 45 seconds at 95°, 1 minute at 50° (minus one degree per

cycle), and 2 minutes at 72°. This was followed by 30 cycles of 45 seconds at 95°, 1 minute at 40°, and 2 minutes at 72°. Finally the thermocycler ended with a period of 10 minutes at 72°. Gradient PCR was used to optimize the annealing temperature for this protocol. Primers were grouped into an external and internal pair. An initial run of the PCR protocol used the external primers and a master mix of plasmids from the EST library as template. A second run at the PCR protocol used the internal primers and the resulting products of the initial run as template. The products from the second/internal run were separated on a 1.0% agarose gel made in 1x TAE. The gel was stained with ethidium bromide and imaged using ultraviolet light.

Isolation of Potential Genes from the EST Library Based on NCBI BLAST Searches:

Exact primers were designed from sequences found by submitting the *M. sexta* JHEH sequence to the BLAST algorithm search of *M. sexta* EST sequences at the National Center for Biotechnology Information. An initial run of PCR using the reaction mixture conditions, as seen above, was performed. A second batch of primers was then designed. The primers consisted of pairs of 3' primers located at the 5' end of the gene and 5' primers located at the 3' end of the gene (i.e. the primers were oriented towards the ends of the gene rather than towards each other; so-called inverse PCR). Nested PCR, as described above, was then used to extract these genes from our EST library. After the nested PCR protocol, the resulting linear DNA (consisting of the ends of the gene of interest and the pBluescript vector) was digested using Dpn I to break down template DNA. This was then purified by gel electrophoresis using a 1.0% agarose gel and the MoBio Ultra Clean 15 DNA Purification Kit following the manufacturer's guidelines. The ends of this purified DNA were blunted using the NEB Quick Blunting Kit following the manufacturer's guidelines. Blunted DNA was then ligated back into a functional plasmid using the NEB Quick Ligation Kit following manufacturer's guidelines. These plasmids were then

transformed into DH5α competent cells. Colony PCR was performed using the universal M13 primers. The colony with the largest insert was grown up in TB and its plasmids were extracted using the QIAprep Spin Miniprep Kit. This DNA was sequenced at the Nevada Genomics Center.

Results

Discovery of Potential Genes in the M. sexta EST Library:

The multiple sequence alignment of the Wojtasek sequence against the 2000 partially sequenced ESTs from the Malpighian Tubules EST library yielded four EST hits with high levels of homology to the Wojtasek sequence. Of these hits, two were found to be unique: MT010A2D07 and MT010B2B02. These two EST's were amplified, purified, and sequenced. This allowed for the discovery of the full sequences of the cDNAs of these two genes. The sequencing results of the two full genes yielded several key facts. First, MT010A2D07 was found by the TMHMM algorithm to have region of hydrophobic amino acids which is most likely a transmembrane domain. Also, MT010A2D07 was found to align strongly with the microsomal Epoxide Hydrolase from *Aedes aegypti* and the Abhydrolase Domain-Containing Protein from *Culex quinquefaciatus* (Figure 1). Secondly, MT010B2B02 was found by the TMHMM algorithm to have no regions of hydrophobic amino acids long enough to span the plasma membrane and therefore has no transmembrane domains, ie, it is a soluble epoxide hydrolase. In addition MT010B2B02 aligned strongly with the *Bombyx mori* Juvenile Hormone Epoxide Hydrolase (Figure 2).

Isolation of Potential Genes from EST Library by Degenerate PCR:

The Nested PCR protocol required a large amount of optimization (Figure 3) in order to reduce the amount of background due to the nature of PCR with degenerate primers. Once the protocol was optimized, it yielded several results. First, no genes were directly isolated due to the fact that a large number of different segments of DNA were replicated in the degenerate PCR. However, the final optimized gels (Figure 4) show that many bands were obtained in the library screening lane of both the internal and external PCR gels. These bands ranged from 500 to 1000

base pairs, meaning they are similar in size to the MT010A2D07 and MT010B2B02 EST's. Due to the vast number of bands present and how similar they were in size, it was not possible to isolate them from this gel.

Isolation of Potential Genes from the EST Library Based on NCBI BLAST Searches:

Three potential JHEH-like genes with high similarity to the Wojtasek gene were found using a BLAST analysis. These genes were labeled GR921488.1, GR921028.1, and FK816866.1 in the NCBI GenBank database. These sequences were found to be from unpublished EST sequences deposited in GenBank from the *Manduca sexta* Malpighian Tubules and midgut. The initial check for the presence of these genes in our EST library yielded positive results for two of the genes, indicating that GR921488.1 and GR921028.1 are both expressed in the Malpighian Tubules while FK816866.1 is not (Figure 5). The next step was to find the complete sequence of the genes by confirming their 5' and 3' regions. Preliminary results show that the 5' and 3' regions of the genes have been successfully isolated (Figure 6). Colony PCR indicated that the GR921488.1 and GR921028.1 5' and 3' regions had been successfully cloned out (Figure 7). From this, exact primers were designed and the full sequence of two genes will be cloned.

Figures

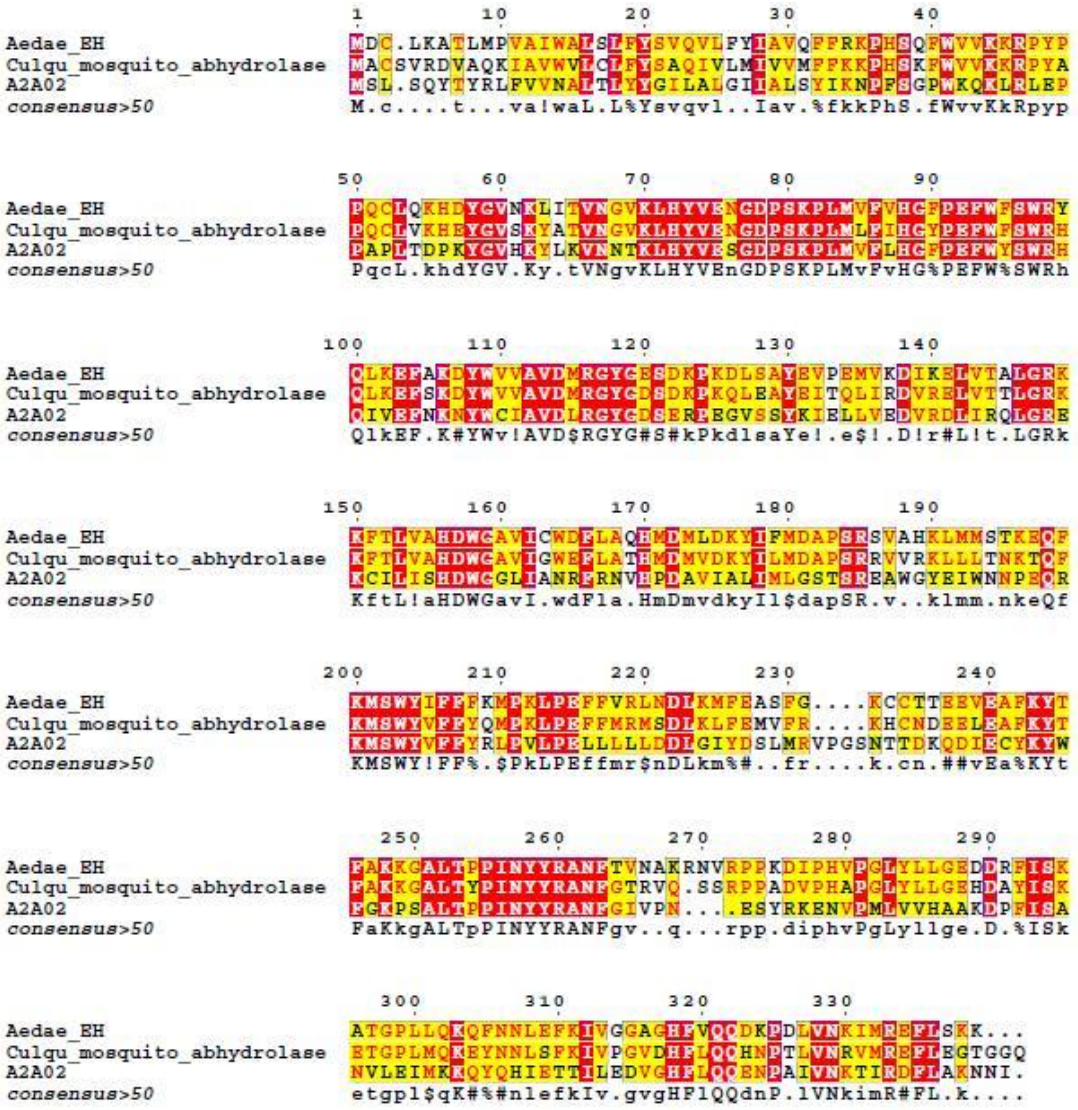

Figure 1. Multiple Sequence Alignment of the EST MT010A2D07 against the Top Two BLAST Search Results.

This alignment shows the translated amino acid sequence of MT010A2D07 on the bottom and the amino acid sequences of *Aedes aegypti* Epoxide Hydrolase and *Culex quinquefasciatus* Abhydrolase Domain-Containing Protein on the top. Red amino acids align exactly, while yellow amino acids have similar properties, and white amino acids are unrelated.

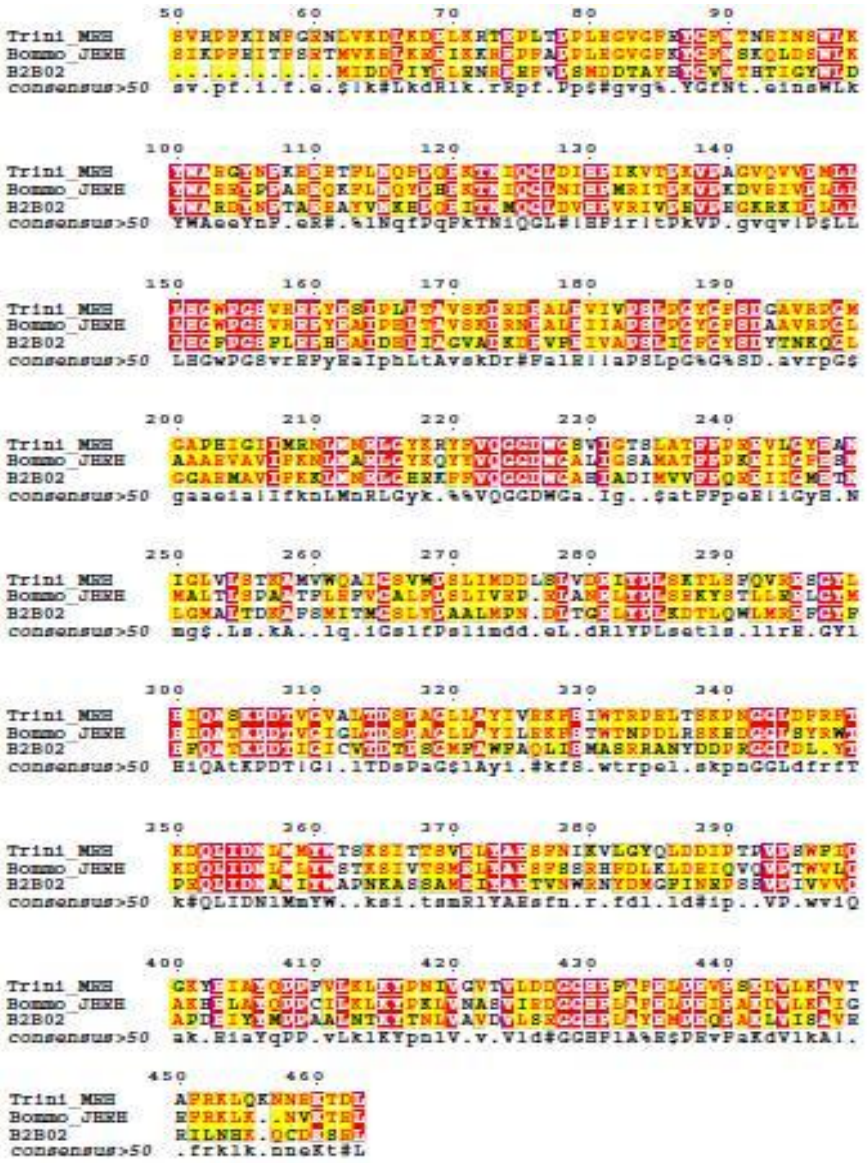

Figure 2. Multiple Sequence Alignment of the EST MT010B2B02 against the Top Two BLAST Search Results.

This alignment shows the translated amino acid sequence of MT010B2B02 on the bottom and the amino acid sequences of *Trichoplusia ni* Microsomal Epoxide Hydrolase and *Bombyx mori* Juvenile Hormone Epoxide Hydrolase on the top. Red amino acids are identical, while yellow amino acids have similar properties, and white amino acids are unrelated.

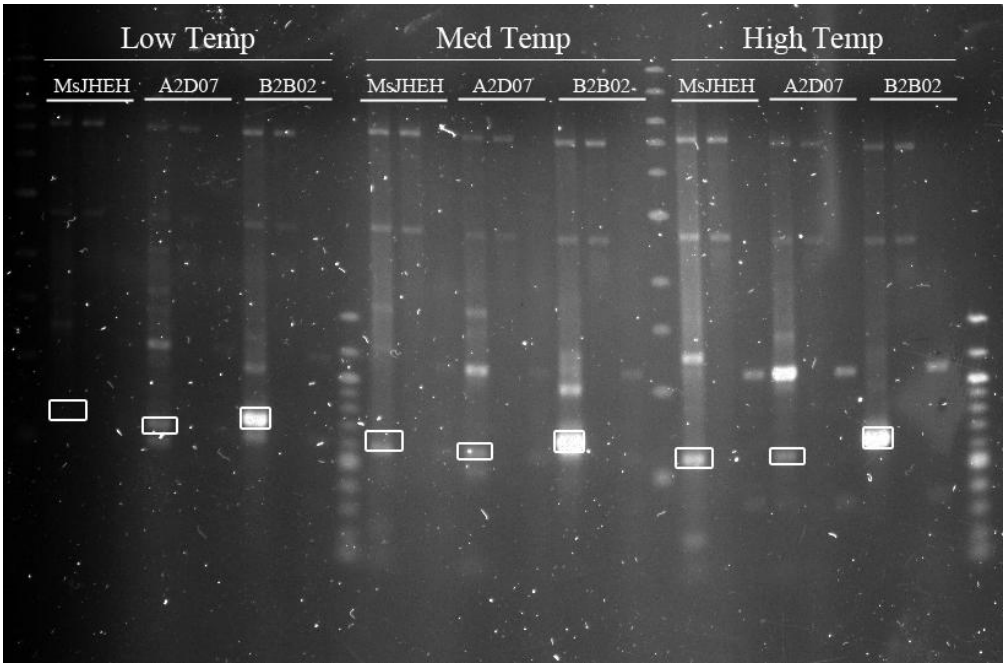

Figure 3. Determining the Optimal PCR Conditions by Temperature Gradient PCR

Three identical sets of samples were run at three temperatures (37.0 °C, 43.4 °C, and 52.0 °C). At each temperature, a reaction was setup containing 0.4 μM Manse_1F degenerate primer, 0.4 μM M13 reverse primer, 1 x PCR Buffer, 2.5 mM $MgCl_2$, 0.2 mM dNTP's, and 1.25 u Taq Polymerase, along with either MsJHEH, MT01013A2D07, or MT01011B2B02 as a template. The second and third lanes for each of the templates are single primer controls (SPC) and no template controls (NTC), respectively. This gel shows that the least background is achieved at 37 °C. The "positive" results bands are highlighted on the image.

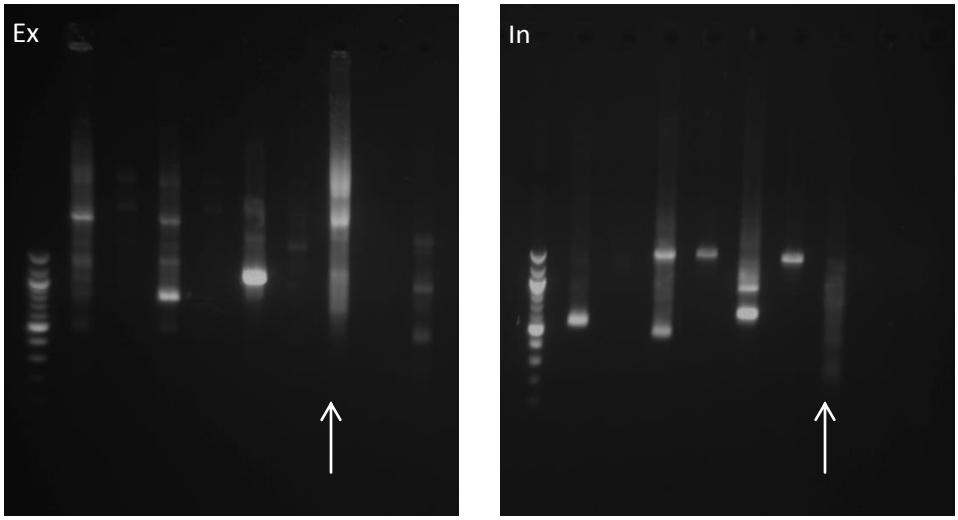

Figure 4. Nested PCR Library Screening Results

(Ex) External PCR attempt 3, (In) Internal PCR attempt 3. Both gels were setup in the same way with lanes 1 through 10 containing the following (respectively): NEB 100 Bp Ladder, MsJHEH positive control (PC), single primer control (SPC), MT01013A2D07 PC, SPC, MT01011B2B02 PC, SPC, library template (indicated by an arrow), SPC, and a no template control (NTC).

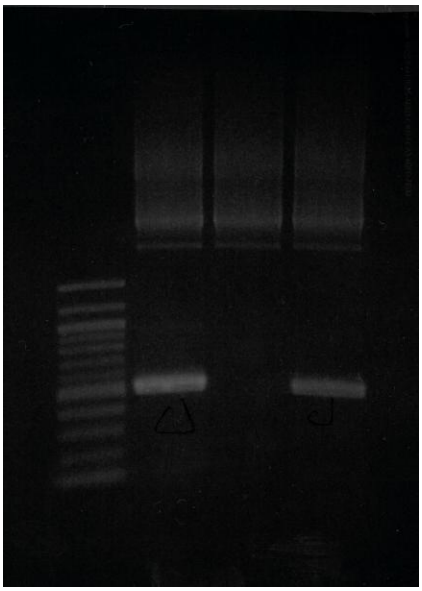

Figure 5. Preliminary Test for the Expression of GR921488.1, GR921028.1 and FK816866.1 in the Malpighian Tubules of *M. sexta*

Lane 1 contains a NEB 100 bp DNA ladder and lanes 2-4 contain GR921488.1, FK816866.1, and GR921028.1 respectively. Both of the bands in lanes 2 and 4 align with the 500 bp band in the ladder.

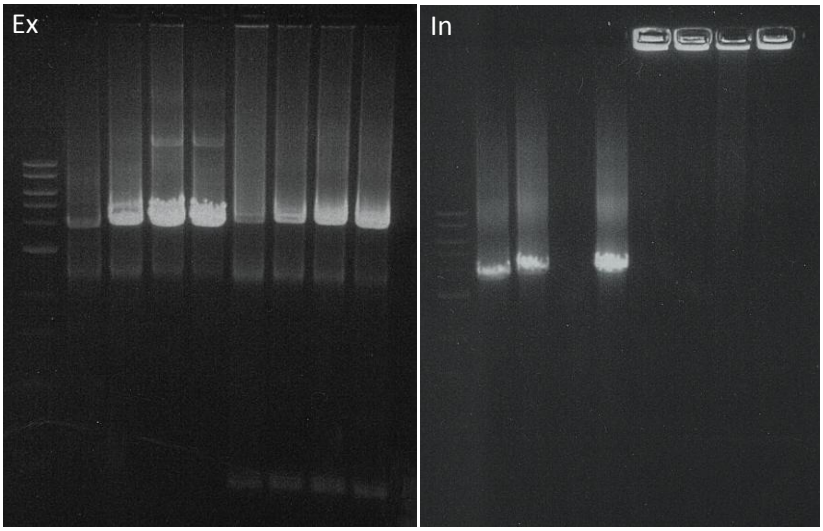

Figure 6. Nested PCR Amplification of the 5' and 3' Ends of the EST's GR921028.1 and GR921488.1

Lane 1 contains a NEB 1 kb ladder, lanes 2-5 contain the PCR reactions for GR921028.1 and lanes 6-9 contain the PCR reactions for GR921488.1. Lanes 2-5 and 6-9 were setup using a decreasing gradient of the amount of the library template supplied to the reaction (10, 8, 6, and 4 µL of template in a total reaction volume of 20 µL). The internal gel (on the right) shows that the internal reaction for GR921488 failed; therefore the product from the external GR921488.1 reaction was used to continue in the following experiments.

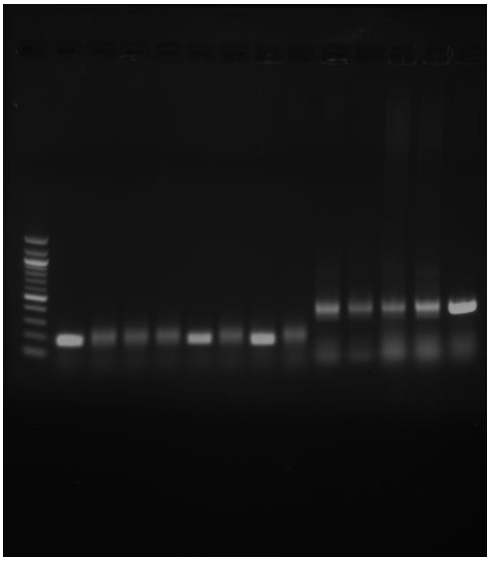

Figure 7. Initial Colony PCR of the 5’ and 3’ Regions of the GR921028.1 and GR921488.1 EST Sequences.

Colony PCR was performed using the 3’ internal primer from the nested PCR reaction and the M13-20 universal primer. Lane 1 contains the NEB 100 BP ladder. Lanes 2-9 contain colonies from the GR921028.1 EST and lanes 10-14 contain colonies from the GR921488.1 EST. All lanes show a positive result band between 200 and 500 bp.

Discussion

The original hypothesis that *M. sexta* may have multiple JHEH-related proteins, was based on the discussion in Seino *et al*. (2010) that while most lepidopteran species (i.e.: *M. sexta* and *B. mori*) only have one primary JHEH, most of these species also possess several related proteins. Therefore, there was the expectation that the orthologues of these related proteins would be found in the *M. sexta* EST library. Because of this, the discovery that the MT010B2B02 gene has no transmembrane domain is quite interesting due to the fact that no cytosolic Juvenile Hormone Epoxide Hydrolase has yet been discovered, meaning that this could be a completely novel protein with properties and functions yet to be studied by our peers in the field of Juvenile Hormone research. It would be reasonable to speculate that this cytosolic JHEH could simply be a standard detoxification epoxide hydrolase like those found in many organisms; however, the fact that the top result for a BLAST search with the MT010B2B02 sequence is the primary JHEH from *Bombyx mori* (Figure 2) lends credibility that this gene is most likely specific for Juvenile Hormone rather than just being a standard cytosolic epoxide hydrolase. It is interesting that this gene aligns so strongly with the JHEH gene from *B. mori* due to the fact that *B. mori* is closely related to *M. sexta* on the tree of life (data not included). Future research into the structure and function of MT010B2B02 will be needed to confirm these preliminary results and determine its substrate structure specificity.

Conversely, the BLAST results for MT010A2D07 tell a different story. Due to the high similarity between MT010A2D07 and the sequences for two general epoxide hydrolases from other distantly related species (Figure 1), it is suspected that this gene's protein is a general detoxification protein.

The attempt to isolate potential genes from the Malpighian Tubule EST library using touchdown nested PCR with degenerate primers was quite unsuccessful; however it did reveal several things. The first was that our EST library was too large a mix of different DNA to obtain good results using degenerate primers. Having 256 possible primers in the degenerate mix and using this degenerate mix on an EST Library allowed for a great statistical probability of false priming occurring. The choice to use nested PCR was aimed at getting around this inherent chance of false priming because the nature of nested PCR is to have higher background in the external run and then a greatly decreased amount of background in the internal PCR. This decrease in background is achieved because in order for background DNA to be amplified it would have to false prime twice; once in the external round and once in the internal round. In order to increase the precision of the nested PCR to an even greater level, we performed the external PCR using a so-called "touch-down" method in which the annealing temperature is decreased by a degree at a time over a period of ten cycles so as to increase the chances of the mix of degenerate primers amplifying all possible genes of interest in the external round. While this is sound in theory, in practice it was seen that even with unwanted background being decreased greatly (Figure 3), too many segments of DNA were amplified in both the external and internal rounds in the library screening lanes (Figure 4). This resulted in a smear of DNA from approximately 1 kb down to 500 bp. This leads into the second conclusion that can be drawn from this procedure: there are multiple genes with at least a basic similarity to JHEH. Meaning, that a more refined approach to the experiment in the future may lead to the ability to isolate potential genes directly from the EST library.

Seeing as how attempting to directly discover and isolate unknown genes from the EST library was not working, a search was performed to find other *M. sexta* EST libraries publicly

available in the GenBank database. This yielded several published EST libraries from which the EST sequences of GR921488.1, GR921028.1, and FK816866.1 were found. From figure 5 it can be seen that both GR921488.1 and GR921028.1 are expressed in the Malpighian Tubules. Due to these positive results we were able to begin isolating both of these potential JHEH-like genes. The challenge with this isolation is that the known sequence has the potential of only being a portion of the actual cDNA of its original full length gene because of how EST libraries are constructed. An EST library may contain multiple copies of the same gene all with differing lengths at the 3' and 5' ends. Therefore, a method was devised in which primers were designed in the known region facing out towards the plasmid vector that contained the gene in the EST library. By amplifying the entire vector from this known point outward, any unknown region at the 5' and 3' ends would be amplified also and could then be sequenced to find exact primers to isolate the entire gene. Preliminary results from a colony PCR of the amplified vector containing only the 5' and 3'ends of the gene are promising (Figure 7). Future experimentation will yield both genes in their entirety.

The overall results of this research show that multiple JHEH-like genes do exist in the *Manduca sexta* Malpighian Tubules. This confirms the original hypothesis that multiple genes would exist based on the fact that several closely related species also exhibit multiple isoforms of JHEH. While it is expected that these new isoforms are not as active or abundant as the primary Wojtasek enzyme, they could potentially provide key knowledge into the complete metabolic pathway of JH regulation in the Malpighian Tubules and could possible act as targets for future pesticide development.

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