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Authors: Pelletier, Matthew, French, Laura Leigh, Trinidad, Stephen, and Vandock, Kurt

Source: International Journal of Insect Science, 4(1)

Published By: SAGE Publishing

URL: https://doi.org/10.1177/IJIS.S9358

International Journal of Insect Science

SHORT REPORT

Sequence and Structure of the Tobacco Hornworm, *Manduca sexta,* Transhydrogenase Gene

Matthew Pelletier[1], Laura Leigh French[1], Stephen Trinidad[1] and Kurt Vandock[1,2]

[1]Department of Biology, Houghton College, Houghton, NY 14744. [2]United States Army Reserve, Army Reserve Medical Command APMC, Forest Park, GA 30297. Corresponding author email: kurt.vandock@houghton.edu

Abstract: The reversible mitochondrial transhydrogenase in *Manduca sexta* has been characterized and is involved with endocrine-mediated post-embryonic larval development in this model insect. While biochemical in vivo and in vitro studies have been accomplished, robust molecular studies of the transhydrogenase have not been possible due to deficient genomic data. In the present study, using a combination of degenerate oligonucleotide primers and raw genomic data, we have determined the structure and sequence of the transhydrogenase gene from the model insect *Manduca sexta*. The encoded protein is highly similar to other transhydrogenase proteins and this sequence is the first lepidopteran sequence reported to date.

Keywords: mitochondrial transhydrogenase, cDNA, lepidopteran

International Journal of Insect Science 2012:4 1–6

doi: 10.4137/IJIS.S9358

This article is available from http://www.la-press.com.

Introduction

The conversion of ecdysone to the physiologically active molting hormone 20 hydroxyecdysone (20-HE) is a critical conversion that is necessary for insect postembryonic development. This crucial conversion is catalyzed by ecdysone 20 monooxygenase (E20-M) in specific insect tissues, viz. fatbody, midgut and Malpighian tubules.[1–4] Further study of the E20-M system using the model insect, *Manduca sexta* revealed a predominant mitochondrial association of this enzyme.[5] Additionally, a marked preference for NADPH as the source of reducing equivalents is displayed for *M. sexta* mitochondrial E20-M's.[3] Recent molecular studies established that E20-M is indeed a P450-dependent steroid hydroxylase which is highly regulated and encoded by the gene *shade*.[6] Further evidence demonstrating the physiological importance of E20-M-mediated ecdysone conversion is evident in the identification of several different ecdysteroid cellular receptors which show over 1000 fold greater affinity for 20-HE then ecdysone.[7] In the mitochondrial E20-M-catalyzed conversion of ecdysone to 20-HE, the need for NADPH is apparent and this reductant could arise from the NADPH-forming insect transhydrogenation.

Biochemical and developmental characterization of midgut and fatbody tissues from *M sexta* have lead to the identification of a mitochondrial transhydrogenase (E.C. 1.6.1.1)[8–10] that catalyzes the following reaction:

$$NADPH + NAD^+ \leftrightarrow NADP^+ + NADH$$

Initial biochemical characterization assessed the transhydrogenase for reversibility, energy-linkages, pH optima, stability to dialysis/heat denaturation, transmembrane proton translocation, localization and phospholipid dependence.[9,10] The developmental significance of these transhydrogenations and the role(s) they may play in postembryonic development was evaluated.[8] This transhydrogenase appears to be physiologically linked to the developmentally necessary NADPH-requiring mitochondrial E20-M.

While *Drosophila* is the predominant genetic model insect, it does have its limitations with respect to physiological and biochemical studies.[11] As a result, the lepidopteran *M. sexta* has become an important model insect for biochemically- and physiologically-based studies. Most notably, the large size, ability to precisely stage, the large amount of data relating to its endocrine system, as well as the numerous studies with tissue homogenates relating to developmental variations in E20M activity[3,4,12] make *M. sexta* a suitable candidate for molecular study of the insect transhydrogenase. Taken together, previous studies suggest a physiological role of mitochondrial transhydrogenase in *M. sexta* post-embryonic development and set the framework for future studies relating to the expression of this gene and its product.

Materials and Methods

Rearing and staging animals

M. sexta were reared on an artificial diet under a non-diapausing photoperiod (L:D, 16:8) at 26 °C, day, 24 °C, night and ~60% relative humidity. Animals were defined and staged by morphological and behavioral (wandering) markers.[13] Midguts were isolated by dissection as described previously and stored in RNAlater (Qiagen) at 4 degrees for 2–4 weeks before isolating RNA.[14]

RNA isolation and cDNA synthesis

Midguts isolated as described above were weighed on an analytical scale and then macerated into small pieces with a sterile scalpel. Next, the macerated tissue was placed in buffer RLT (Qiagen; 30 µL per milligram of fresh weight) and passed through a 20 gauge needle for initial homogenization. It was then passed through a 25 gauge needle several times until no significant chunks of tissue remained. Total RNA was isolated using a RNEasy Mini kit (Qiagen) according to the manufacturer's recommendations. The resulting RNA was quantified with a Nanodrop spectrophotometer prior to cDNA synthesis. Complementary DNA was made using an iScript cDNA synthesis kit (BioRad) using either oligodT or random primers, according to the manufacturer's recommendations. For all reactions, 1 µg of total RNA was used per 20 µL cDNA synthesis.

Amplification of the transhydrogenase gene

Degenerate oligonucleotide primers were designed based on conserved amino acid sequences found in several transhydrogenase proteins (Fig. 1). PCR was carried out using 2 µL of cDNA synthesis product and

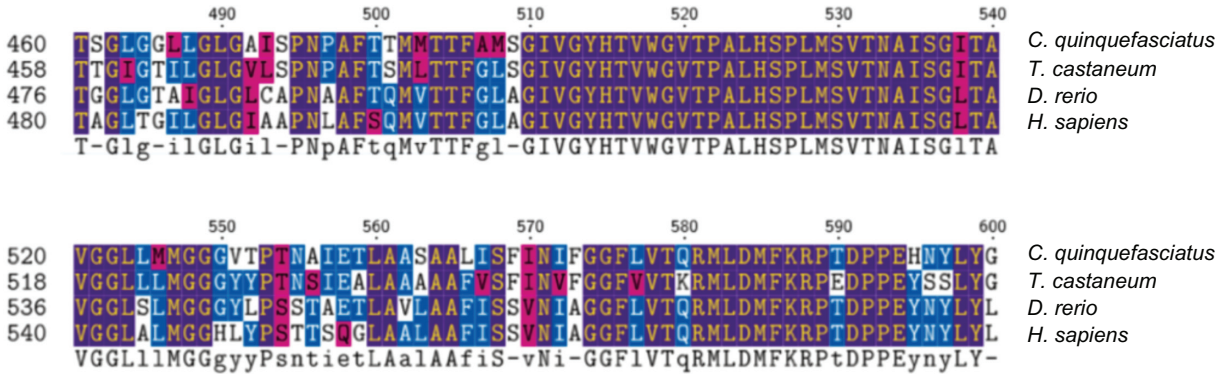

Figure 1. Alignment of transshydrogenase peptide sequences from divergent species.
Notes: The numbers at the sides and tops indicate amino acid positions in the alignment, and the consensus sequences YHTVWG (aa514 to 519 in the human sequence and RMLDMF (aa581–586 in the human sequence) were used to design degenerate primers.

BioMix™ (BioLine) according to the manufacturer's recommendations. Two degenerate primers with the sequences 5′ TAYCAYCANGTNTGGGGN3′ and 5′ RAACATRTCNARCATNCK3 were used at a final concentration of each primer of 10 µM during PCR. Thirty-five cycles of PCR were carried out at 94 °C for 30 seconds, 56 °C for 30 seconds, and extension at 72 °C for 45 seconds. A final extension at 72 °C for 10 minutes was also carried out. The PCR product obtained was cloned into the pCR8 vector using the TA topo cloning kit, using the protocol specified by the manufacturer (Invitrogen). Topo cloning products were used to transform *E. coli* via electroporation and transformants were selected on LB plates with spectinomycin at 100 µg/mL. The presence of the insert was confirmed by digestion with *Eco*RI. Plasmid DNA was then isolated using a standard mini-prep kit (Qiagen) and sequenced using the BigDye 3.1 sequencing kit according to the manufacturer's recommendations (Applied Biosystems).

Amplification of several other overlapping fragments which collectively span the entire coding region of transhydrogenase was performed using Accuzyme™ (Bioline) (Table 1). When using gene-specific primers, a final concentration of 1 µM was used in PCR. A standard reaction utilized an initial denaturation at 95 °C for 3 minutes, followed by 35 cycles of a 94 °C step for 30 seconds, annealing for 30 seconds at the T_m of the primer with the lower T_m (as calculated based on 4 °C for a G or C and 2 °C for an A or T in the primer), and extension at 72 °C for 2 minutes per kilobase pair of target DNA being amplified. When amplifying DNA fragments corresponding to amino acids 196 to 550 of the human protein, 5% DMSO was added to the PCR.

Electrophoresis and DNA sequencing

The sizes of PCR products were estimated by using 1.2% Flashgels (Lonza) or standard 1.2%–1.5% agarose/TBE gels. In some cases, PCR products were not specific, in which case they were run on a 1.2%–1.5% agarose/TBE gel, and then purified

Table 1. Primers utilized for amplification of *M. sexta* transhydrogenase.

Primer name	Sequence (5′ to 3′)
5′UTR	CGTACCCCTAGTCGCTAGT
Start	ATGTGTGGGTGTTTAAGAATATTG
196F	CACTCAGCTCGATGGCGAA
204F	TTATTGAGGCAGCAGCACAC
300F	GTGGATACGCTAAGGAAATGA
335F	GAGGATGCTGTACGGGACAT
526F	CACAGACATGAGAGGAGA
537F	CATAAGCAGCAATCCGCC
550F	TAGCAAGCACAGCGGCTT
580F	CGGATGCTTGACATGTTCAAG
750F	CTCGGTACATACATCGGTG
870F	GGATAGCTCCAGAACTGC
196R	TTCGCCATCGAGCTGAGTG
300R	TCATTTCCTTAGCGTATCCAC
405R	CATCCGTACCCAAGCTGAAT
519R	GCCCCACACTGTATGGTA
537R	GGCGGATTGCTGCTTATG
540R	GACGGCAGTGATACCTGACA
550R	AAGCCGCTGTGCTTGCTA
580R	CTTGAACATGTCAAGCATCCG
750R	CTCGGTACATACATCGGTG
870R	GGATAGCTCCAGAACTGC
Stop	TTACGCTGCAAGATGCTTGA
3′UTR	GGCTGTTAATTTTACTAAGTAGA

from the gel using the a Gel Extraction Kit (Qiagen). Sequencing of PCR products was carried out using BigDye Terminator 3.1 sequencing kit (Applied Biosystems) according to the manufacturer's recommendations.

Results

Transhydrogenase (E.C. 1.6.1.1) catalyzes the following reversible reaction:

$$NADPH + NAD^+ \leftrightarrow NADP^+ + NADH$$

In the current study, a strategy was utilized to clone and sequence the *M. sexta* transhydrogenase cDNA. Initially, a small fragment of the cDNA was amplified by PCR using degenerate primers based on conserved amino acid sequences in the protein (Fig. 1). Two conserved short peptide sequences were chosen for design of primers, based upon the fact that degeneracy of this primer pair was only 256-fold.

Total RNA was isolated from the midguts of animals at day five (wandering stage) of development from the fifth larval instar. This stage was chosen because previous research has shown the biochemical activity and de novo synthesis of transhydrogenase to be highest at this particular stage of fifth larval instar development in midgut.[8] After cDNA synthesis using random or oligo dT primers, PCR was carried out, electrophoresis of the resulting fragments showed a product between 200 and 300 bp (Fig. 2). This fragment was then cloned into a pCR8 vector and sequenced using vector-specific primers.

A BLAST search using the sequence obtained from this clone showed the DNA sequence of the fragment was 71% identical to transhydrogenase from *Anopheles gambiae* (**XM_312859.4)**, indicating successful amplification of a transhydrogenase fragment. After the cloning and sequencing of the 219 bp sequence, data from the ongoing genome project of *M. sexta* made it possible to download a non-annotated draft version of the genome useful in the design of *M. sexta* gene-specific primers to conserved transhydrogenase peptide sequences. By searching the draft sequence for the 219 bp sequence described above, it was possible to translate the surrounding DNA into all 3 reading frames and then align those amino acid sequences to existing transhydrogenase proteins. Utilizing this strategy, a set of overlapping fragments which appear to span the entire coding region of the gene was amplified by PCR (Figs. 3 and 4). Each PCR product was sequenced directly on both strands, using either the same primers used to produce the fragments or internal sequencing primers (Table 1).

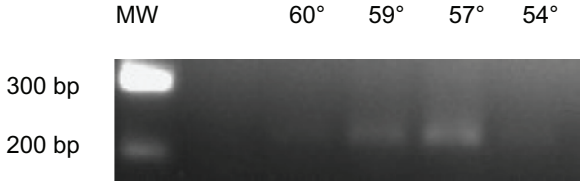

Figure 2. Agarose gel of PCR products of *M. sexta* transhydrogenase fragment using degenerate primers.
Notes: Degenereate primers (5′ TAYCAYCANGTNTGGGGN3′ and 5′ RAACATRTCNARCATNCK3) designed against the conserved polypeptide sequences YHTVWG and RMLDMFK (Fig. 1) were used to amplify a 219 base pair fragment at different annealing temperatures, using cDNA derived from day five fifth larval instar animals.

The experimentally-determined sequence of the cDNA was then compared to the draft genomic data to determine the structure of the gene (Fig. 4) aided by the help of the SIM 4 software package.[15] The transhydrogenase coding region of the genomic DNA spans a region of 15.8 kilobase pairs, of which only 3,141 base pairs is exon sequence. Comparison of the draft genomic sequence and the experimentally-determined cDNA indicates the gene has 22 exons.

Discussion

In comparing the structure of the transhydrogenase gene from *M. sexta* to other insects, it is clear that the distribution of exons and introns varies substantially (Table 2) among taxa. For instance, in the dipterans and coleopterans the gene is relatively compact with few and small introns, while in the Phthirapteran *P. humanus corporis* the gene is divided among many more exons. Unfortunately, an annotated genomic clone for the gene from another lepidopteran is not available for comparison. The amino acid sequence of the transhydrogenase of *M. sexta* is highly conserved, with 61% identity to even the human form of the protein.

Considering the biochemical characterization of the transhydrogenations catalyzed by *M. sexta* midgut mitochondria, an important physiological role of the transhydrogenase in larval development is evident.[8–10] Indeed, careful experimentation has shown that fatbody and midgut tissues display increased levels

ATGTGTGGGTGTTTAAGAATATTGAAGGCATATAAGCCAGTGCCGCGGTCCTGGCACTGCAGGCTCTCTAGCAGCTCTGCAGCTGCCCCTCCTAC
CCCAGGAGTGCCTTACACCAAACTTAGTATTGGAGTGCCTAAAGAGATATGGCAAGATGAAAGAAGAGTTGCTGTAGTACCTGCTGTTGTTAGC
AAATTAGTAAAAAAAGGTTTCAGTGTCAATGTAGAAGAGAATGCTGGGTTCTTAGCAAATTTTAATAACCAACTTTATGAAGAGGCTGGGGCCA
AAGTAACTAGCTTAAAGGATACATTCCAGTCTGATATTATTTTAAAAGTCCGTCCAATAGTAGACAATGAAGTGCCACATTTAAGGGATGAAGG
AACACTTATATCATTTCTATATCCAGCTCAAAATCAAGATTTAATTAAAAAGATGGCTGATAGGAAAATGAATGCATTTGCTATGGATTGTATCC
CTAGGATTAGTCGCGCCCAGGCTTTCGACGCACTCAGCTCGATGGCGAACGTAGCCGGGTACCGTGCAGTTATTGAGGCAGCAGCACACTTCCC
CAGATTCTTCTCAGGTCAGATGACGGCGGCGGGTCGCGTGCCGCCGTGCCGCGTGCTGGTGGTGGGCGGCGGCGTGGCGGGGCTGGCGGCGGCT
GCGCAGGCGCGCTGCATGGGCGCCGCCGTGCGCGCCTTCGACACGCGCCCCGCCGTGCGCGAGCAGATCGAGAGCCTCGGCGCCACCTTCGTCA
CCATGGACATGAAGGAAGAGGGAGCCGGGGAAGGTGGATACGCTAAGGAAATGAGTCAAGAGTTCCTGGACGCTGAGCGGGCGCTCCTGGGCA
AGGAAGCGAGGACCTCTGACGTGGTCATCAGCACCGCGCTCATCCCCGGCAAACCTGCCCCACTTCTTATCTTAGAGGATGCTGTACGGGACAT
GGCCCCTGGTAGCGTTATTGTTGACCTCGCAGCGGAGATGGGCGGAAACGTTGAAACAACAAAGAAGGGCCAAATCACAAAAACACACGGCGT
CACACACATCGGTCTCACGGACTTGCCGAGTCGCATGCCGGCACACGCCTCCACTCTCTACGCCAATAATATCTCCGGATTTCTATTCAGTTTGG
GTACGGATGATCACTTCCACATTAACCTGGAGGACGAGGTGACGCGCGGCGCGGTGGTGCTGAAGGCGGGCGAGCTGCTGTGGCCGCCGCCGCC
GCCGCCCAGCATGGCGCCTGCGCAGGCGGCCGCCAAGCCCACCGCCGTCAAGGCCGAACCACCCAATCCATTCAACGAGACGCTCAAAGATGCC
TTCTTATATTCTACTGGTTTAGCCTCTCTGATTGGTTTGGGAATAGCGTCACCCAACCCGGCATTCACAACAATGACTACCACCTTGGCTTTGGCA
GGTGTTGTGGGATACCATACAGTGTGGGGCGTGGTGCCGGCGCTGCACTCTCCTCTCATGTCTGTGACTAACGCCGTGTCAGGTATCACTGCCGT
CGGCGGATTGCTGCTTATGGGCGGCGGATACGTGCCCGAAACACCAGTGCAGTGGTTAGCAAGCACAGCGGCTTTAATCTCATTTGTTAACGTGT
TCGGTGGATTTATGGTGACGCAACGGATGCTTGACATGTTCAAGAGGCCCGGTGACCCACCAGAATACGGCTATTTGTACGCGATTCCGGCTGCT
GCCCTCATGGGCGGGTATATTACCACAGCAATGCAGGGTTACCCTGAGGTACATCAAATGGCGTATCTAGCCGCCTCATTGTGTTGTGTCGGCGC
GCTTGCAGGACTTAGCTCGCAGGCGACAGCCCGCAAAGGAAATTACCTAGGCATGATCGGCGTGGCGGGCGGCATCGCGGCGACGCTGGGCGC
GCTGACGCCGAGCGCGGAGGTGCTCGCGCAGATGCTGGGCGTGGCCGGCGTCGGCGGCCTCATCGGCGGAACCATCGCCAAGAAAATCGAGAT
CACTGATCTGCCTCAGTTGGTCGCCGGTTTTCACAGTTTGGTCGGAATGGCAGCCGTGCTGACGTGTCTCGCGACGTACATGCACGACTTCCCGG
CGATGGCGATGGATCCCACGGCGGCCACGCTCAAAACATCTCTCTTCCTCGGTACATACATCGGTGGAATTACATTTACTGGATCCCTAGTGGCT
TACGGCAAGCTGCAAGGCTCGCTATCATCGGCGCCACTGTTGTTGCCTGGCCGTCACGCCATCAATGCTGGCCTGTTGGCTGGCTCGCTAGGCTG
CGGCGGAGCTCTCTTAGCGTTCCCTGAAGCCCCGGGCTTGCCCTTGCTGTCTGCTGCGGCCGTGCTTAGTGGCATCCAGGGTCTGACGCTCACTG
CCGCTATTGGAGGAGCGGACATGCCGGTCGTTATAACTGTACTGAACAGCTACTCCGGCTGGGCGCTGTGTGCCGAAGGCTTCATGCTCAACAA
CTCTTTGATGACAATCGTCGGCGCTCTCATCGGCAGTTCTGGAGCTATCCTTTCCTACATTATGTGCAAGGCGATGAACCGATCGCTGCCCAACGT
GATCTTGGGTGGGTACGGCGTGACGAGCGGCGGCACCGCGCGCCCCGCCGGCGCCACGCACACTGAACTCAACGTAGACTCCGTCGCCGATCTC
ATTTACCGCGCCTCCAACATCATTATTACACCAGGTTATGGTCTATGCGTGGCGAAAGCGCAATATCCCATTGCTGAACTAGTCGAGTTATTAAA
ATCTGCCGGAAAGAAAGTGCGATTTGCCATACATCCTGTAGCCGGTCGTATGCCCGGCCAGCTTAATGTGTTGCTCGCCGAGGCCGGTGTTCCTT
ATGATGATGTGTATGAAATGGAGGAAATCAATGAGGAGTTCCCTGAAACAGATCTCGTACTTGTTATTGGTGCTAACGACACGGTGAACAGTGC
AGCTGAAGACGATCCCAATTCGCCCATTGCGGGAATGCCCGTACTCAAAGTATGGAAGTCAAATCAGGTGGTTGTTATGAAGAGGTCAATGGGC
GTGGGTTACGCCGCTGTGGACAACCCTATCTTCTACAATCCAAATACAGCCATGTTACTCGGCGACGCCAAGAAGACTTGCGACGCCTTGCTTGA
AAGAGTCAAGCATCTTGCAGCGTAA

Figure 3. cDNA sequence of the *M. sexta* transhydrogenase gene.

Figure 4. Structure of the *M. sexta* transhydrogenase genomic clone.
Notes: Dark-shaded rectangles represent exons while rectangles with no fill color represent introns. The figure is drawn to scale based on the relative sizes of the introns and exons. Only the portion of the representing the coding region is shown.

of all transhydrogenase activities during the crucial wandering stage of the fifth larval instar. Furthermore, it has been shown that these increases in activity are the result of de novo enzyme synthesis and coincide with increases in both E20-M activity and its encoding gene, *shade*.[7,12]

Research detailing the role of the cytochrome P-450 steroid hydroxylase E-20M has established its role in the NADPH-dependent conversion of the molting hormone, ecdysone, to its physiologically active form, 20-HE.[3,16–18] Because this activity requires NADPH, and is predominantly associated with the mitochondria of the ecdysone target tissues such as midgut, fatbody, and Malphighian tubules[5,19] an association with the reversible transhydrogenase seems likely. In view of the coincidental peaks in

Table 2. Gene structure of transhydrogenase in various taxa.

Species	NCBI reference sequence	Percent identity[a]	Exons	cDNA length[b]	Total genomic[c]
M. sexta	NA	NA	22	3150	15.8
A. gambiae	XP_312859.4	70	7	3198	4.7
P. humanus corporis	XP_002428630.1	68	19	3204	4.7
T. castaneum	XP_970382.1	68	7	3192	3.7
A. aegypti	XP_001662741.1	70	8	3198	13.1
H. sapiens	NP_892022.2	61	21	3258	102.9
C. quinquefasciatus	XP_001845031.1	69	7	3195	5.4

Notes: [a]Percentage of amino acids conserved between the *M. sexta* transhydrogenase and various other transhydrogenase proteins; [b]Base pairs of DNA; [c]The genomic region, in kilobase pairs, spanned from the translational start to stop codon.

ecdysone 20-monooxygenase activity and transhydrogenase activity, a developmental relationship between these mitochondrial proteins has been suggested.[8]

With the sequence and structure of the *M. sexta* transhydrogenase gene established, further study of how this mitochondrial protein regulates insect development can be completed. Certainly, a clear association between the endocrine-mediated events during post-embryonic larval development and *M. sexta* mitochondrial transhydrogenase is evident. Considered collectively, the data from this study and previous biochemical characterizations set the framework for future molecular studies that can further elucidate the role of transhydrogenase in *M. sexta* larval development.

Acknowledgments

The authors would like to thank Houghton College for funding through the Summer Research Institute. K.P. Vandock was supported by the United States Army Reserve, APMC. Preliminary *Manduca sexta* sequence data were obtained from Gary Blissard, Boyce Thompson Institute at Cornell University, Michael Kanost, Kansas State University, and the Baylor College of Medicine Human Genome Sequencing Center. Funding for the sequencing was provided by a DARPA grant to Gary Blissard and an NIH grant to Michael Kanost.

Author Contributions

All work was shared equally. All authors agree with manuscript results and conclusions, and have reviewed and approved of the final manuscript.

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