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Chitin synthase genes in *Manduca sexta*: characterization of a gut-specific transcript and differential tissue expression of alternately spliced mRNAs during development

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Abstract

Chitin, the linear homopolymer of β-1,4-linked *N*-acetylglucosamine, is produced by the enzyme chitin synthase (CHS). In general, this insoluble polysaccharide is found in two major extracellular structures in insects, the cuticle that overlays the epidermis and the peritrophic membrane (PM) that lines the midgut. Based on amino acid sequence similarities, insect CHSs are divided into two classes, A and B, and to date no more than two CHS genes have been identified in any single insect species. In species where both CHSs have been identified, one class A CHS and one class B CHS are always present. This finding suggests that these two genes may encode enzymes that synthesize chitin in different epithelial tissues. In our laboratory, we previously characterized transcripts for a class A CHS gene (*MsCHS1*) from the tobacco hornworm, *Manduca sexta*. We observed the expression of this gene in the larval epidermis, suggesting that the encoded enzyme functions to synthesize cuticular chitin. In this paper, we characterize a second chitin synthase gene (*MsCHS2*) belonging to class B and its cDNA from *Manduca* and show that it is expressed only in the midgut. This cDNA contains an open reading frame of 4575 nucleotides, which encodes a conceptual protein that is 1524 amino acids in length and is predicted to contain 16 transmembrane spans. Northern blot analysis of RNA isolated from anterior, medial, and posterior sections of the midgut from feeding larvae indicate that *MsCHS2* is primarily expressed in the anterior midgut, with transcript levels tapering off in the medial and posterior midgut. Analysis of the *MsCHS2* gene sequence indicates the absence of an alternate exon in contrast to the *MsCHS1* gene, which yields two transcripts, *MsCHS1a* and *MsCHS1b*. RT-PCR analysis of the differential expression of these alternately spliced transcripts reveals that both splice variants are present in the epidermis. However, the ratio of the two alternately spliced transcripts varies during development, with *MsCHS1a* being generally more predominant. Southern blot analysis using a probe specific for CHS indicated that *Manduca* has only two CHS genes, akin to other insect species. Results from an analysis of expression of both genes in different tissues and developmental times indicate that the MsCHS1 enzyme is used for the synthesis of chitin in the cuticle and tracheae, whereas MsCHS2 is utilized exclusively for the synthesis of PM-associated chitin in the midgut.

Keywords: Chitin synthase; Glycosyltransferase; Chitin; *Manduca sexta*; Tobacco hornworm; Insect; Alternate exon splicing; Epidermis; Cuticle; Peritrophic membrane; Midgut; Tracheae; Transmembrane enzyme

Abbreviations: CHS, chitin synthase; Ms, *Manduca sexta*; PM, Peritrophic membrane; RT, reverse transcriptase; PCR, polymerase chain reaction; UDP, uridine-5′-diphosphate; GlcNAc, *N*-acetylglucosamine; SDS, sodium dodecyl sulfate; RpS3, Ribosomal protein S3; ORF; open reading frame.

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1. Introduction

Chitin, a linear homopolymer of β-1,4-linked *N*-acetylglucosamine (GlcNAc), is the second most abundant biopolymer next to cellulose. It is the major structural polysaccharide present in the insect exoskeleton and gut lining (reviewed in Merzendorfer and Zimoch, 2003). Despite its biological significance, relatively little information is known about the chitin biosynthetic pathway in insects or other invertebrates. Chitin synthase (CHS, UDP-GlcNAc: chitin 4-β-*N*-acetylglucosaminyltransferase, EC 2.4.1.16) catalyzes the polymerization of chitin from UDP-*N*-acetylglucosamine (UDP-GlcNAc) monomers. CHSs are large enzymes located in the plasma membrane, enabling the newly synthesized chitin to be extruded from the cell into extracellular locations (Cohen, 1991). CHSs belong to the GT2 family of glycosyltransferases, which also includes cellulose synthases (Coutinho et al., 2003).

CHSs have been extensively studied in fungi (Valdivieso et al., 1999). A large family of genes encodes fungal CHSs and as many as eight different CHS genes have been identified in a single species (Munro and Gow, 2001). Fungal CHSs have been found to have different roles in cell wall and septum biosynthesis and are expressed at different developmental stages (Specht et al., 1996; Merz et al., 1999; Valdivieso et al., 1999; Munro and Gow, 2001; Roncero, 2002). Nematodes, however, have fewer CHS genes than fungi. Two CHS genes were identified in *Caenorhabditis elegans* following genome sequencing (Veronico et al., 2001). Furthermore, CHS genes have been reported in several other nematodes including *Brugia malayi*, *Meloidogyne artiellia*, and *Dirofilaria immitis* (Harris et al., 2000; Veronico et al., 2001; Harris and Fuhrman, 2002).

Insect CHSs have only recently begun to be characterized with a cDNA sequence reported for a CHS from *Lucilia cuprina* (Tellam et al., 2000). Since then, CHS cDNAs/genes from several other insect species including *Aedes aegypti, Drosophila melanogaster, Anopheles gambiae, Tribolium castaneum* and *Manduca sexta* have been characterized (reviewed in Kramer and Muthukrishnan, 2004). Based on relative amino acid sequence similarities, fungal CHSs are divided into six classes (Munro and Gow, 2001). Insect CHSs, however, are divided into only two classes, classes A and B, based on the limited number of insect CHS amino acid sequences available (Fig. 1). After completion of sequencing of the genomes of *D. melanogaster* and *A. gambiae*, only two putative CHS genes were found in each species, one belonging to each class. In addition, only two putative CHS genes, one in class A and one in class B, were found in *T. castaneum* by screening a BAC library of genomic DNA and no additional genes were identified by Southern blot analysis of genomic DNA (Arakane et al., 2004). The characterization of CHS genes from several different insect species representing three orders (Diptera, Coleoptera, and Lepidoptera) suggests that insects encode only two CHS genes, one belonging to each class.

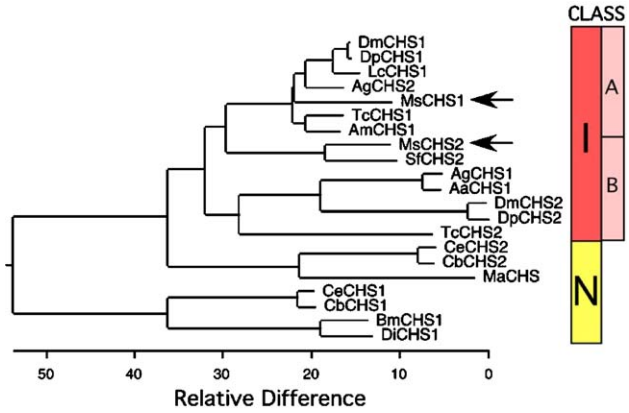


Fig. 1. Classification of insect CHSs based on relative amino acid sequence similarities. Sequences from CATMWHET to WGTRE were aligned by ClustalW (PAM250) to generate the phylogenetic tree. This is a conserved region about 500 amino acids in length in each sequence and includes the catalytic domain with high sequence similarity and proceeds into a more variable region that defines five transmembrane spans. CHSs were from *Drosophila melanogaster* (Dm), *Drosophila pseudoobscura* (Dp), *Lucilia cuprina* (Lc), *Anopheles gambiae* (Ag), *Manduca sexta* (Ms), *Tribolium castaneum* (Tc), *Apis mellifera* (Am), *Spodoptera frugiperda* (Sf), *Aedes aegypti* (Aa), *Caenorhabditis elegans* (Ce), *Caenorhabditis briggsae* (Cb), *Meloidogyne artiellia* (Ma), *Brugia malayi* (Bm), and *Dirofilaria immitis* (Di). Genbank Accession Numbers (DNA) are as follows: *DmCHS1* (NM_206430), *DmCHS2* (NM_079485), *DpCHS1* (AADE01001241), *DpCHS2* (AADE01000881), *LcCHS1* (AF221067), *AgCHS1* (AY056833), *AgCHS2* (XM_321336), *MsCHS1* (AY062175), *MsCHS2* (AY821560), *TcCHS1* (AY291475), *TcCHS2* (AY291477), *AmCHS1* (XM_395677), *SfCHS2* (AY525599), *AaCHS1* (AF223577), *CeCHS1* (T25G3.2), *CeCHS2* (F48A11.1), *CbCHS1* (CAE66574), *CbCHS2* (CAE62792), *MaCHS* (AY013285), *BmCHS1* (AF274311), and *DiCHS1* (AF288618). Arrows indicate the two *Manduca sexta* CHSs.

In general, chitin deposition in insects occurs in two major extracellular structures. This polysaccharide is used in the assembly of the cuticle (exoskeleton) and the peritrophic membrane (PM) of the midgut. The division of insect CHSs into two classes may be of functional relevance. In *L. cuprina*, studies of tissue specificity showed that the class A CHS is expressed in the epidermis and tracheal epithelial cells but not in the midgut (Tellam et al., 2000). Furthermore, the presence of an alternate exon has been identified in genes encoding each class A CHS, but to date, no alternate exon has been identified in those encoding class B CHSs (Arakane et al., 2004). *TcCHS2*, the class B CHS gene in *T. castaneum*, is expressed during periods when the insect is actively feeding (Arakane et al., 2004). Furthermore, the class B CHS gene in *A. aegypti* is expressed in the midgut, and the level of expression increases following blood feeding in females (Ibrahim et al., 2000). Therefore, it appears that class A CHSs

specialize in cuticle chitin production, whereas class B CHSs specialize in chitin production for the PM. In this paper, we provide conclusive experimental evidence for specialization of CHS genes for the synthesis of chitin in specific tissues of the tobacco hornworm, *M. sexta*.

2. Materials and methods

2.1. Insect cultures

M. sexta larvae were reared at 27 °C using an artificial diet as described previously (Bell and Joachim, 1976).

2.2. MsCHS2 cDNA sequencing

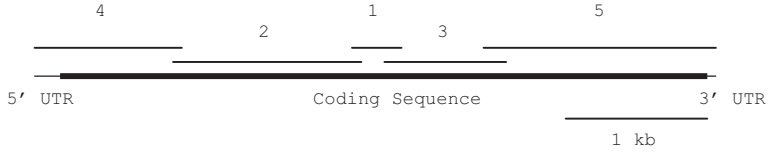
Total RNA was isolated from dissected fifth instar midgut tissues using the RNeasy® Protect Mini Kit (Qiagen). Superscript II® reverse transcriptase (Invitrogen) was used to prepare cDNA according to the manufacturer's instructions. Pairs of gene-specific and degenerate primers, designed from conserved regions of insect CHSs, were used to obtain overlapping PCR products using the cDNA as the template (Fig. 2). The 5′- and 3′-ends of the transcript were obtained using 5′- and 3′-RACE, respectively. These PCR products were cloned into the pCR-II TOPO® vector (Invitrogen) and transformed into TOP10 chemically competent cells (Invitrogen). Following plasmid DNA purification, the sequence of each construct was determined using an automated sequencer (ABI Prism 3700, DNA Sequencing Facility, Kansas State University, Manhattan, KS) and the resulting overlapping sequences were assembled to obtain the full-length *MsCHS2* cDNA sequence.

2.3. Genomic DNA sequencing

Genomic DNA was extracted from dissected epidermal tissue from 20 fourth instar larvae after homogenization in 1.7 mM PIPES buffer (pH 6.5). Following phenol/chloroform/isoamyl alcohol (25:24:1, v/v/v) and chloroform/isoamyl alcohol (24:1, v/v) extractions, the DNA was ethanol precipitated in 0.3 M sodium acetate overnight and redissolved in water.

Overlapping PCR fragments were obtained using pairs of gene-specific primers designed from the corresponding cDNA sequence of *MsCHS2* using genomic DNA as a template. Sequencing of these PCR products was carried out as described in Section 2.2.

The majority of the *MsCHS1* gene organization at the 5′ end was previously determined (Zhu et al., 2002). The remaining downstream sequences of the 3′ end including the alternate exon encoded in the *MsCHS1* gene were sequenced following PCR amplification using gene-specific primers designed from the published cDNA sequence.



PCR Fragment	Size (bp)	Primer		
		Direction[1]	Type[2]	Sequence (5′-3′)
1	359	F	D	tggwsncargtnatgtayatg
		R	D	tcrtccatnarngcrttncc
2	1333	F	D	ggntggtgggaraa
		R	G	gatccacggatattggcagg
3	869	F	G	cctggtaccagatgttcgag
		R	D	acytcnckngtncccca
4	1049	F	5′ RACE Adapter	ggccacgcgtcgactagtac
		R	G	cgtagttcctgcagcaccg
5	1648	F	G	cactgcggcaacatttgg
		R	3′ RACE Adapter	gaccacgcgtatcgatgtcga

[1] F: Forward, R: Reverse
[2] D: degenerate primer, G: gene specific primer

Fig. 2. *MsCHS2* cDNA cloning strategy. The full-length *MsCHS2* cDNA sequence was determined by sequencing five overlapping PCR fragments using midgut cDNA as a template. PCR fragments 4 and 5 were obtained using 5′- and 3′-RACE, respectively.

2.4. DNA and protein sequence analyses

The amino acid sequence of *MsCHS2* was deduced following translation of the corresponding cDNA sequence using the translation tool at the ExPASy Proteomics website (http://us.expasy.org/tools/dna.html). Other protein sequence analysis tools used in this study, including MW, pI, and topology prediction, were obtained from the ExPASy Proteomics website (http://us.expasy.org/). The deduced amino acid sequences of CHSs from *Anopheles* (XM_321951), *Drosophila* (NM_079485), and *Tribolium* (AY291477) were obtained as described previously (Arakane et al., 2004). The sequence of *MsCHS1* was obtained from the published data (Zhu et al., 2002). Multiple sequence alignments of deduced amino acid sequences were made using Multiple Alignment software (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html). Alignments of nucleotide sequences were made using ClustalW software (PAM250).

2.5. Northern blot analysis of CHS transcripts

For expression analysis in midgut, tracheal, and epidermal tissues, northern blots were performed as described previously (Merzendorfer et al., 1997). In brief, RNA purification and synthesis of hybridization probes was carried out as follows. Purification of poly(A) RNA was performed using the Quickprep Micro mRNA Purification Kit (Amersham) according to the manufacturer's instructions. Digoxigenin-labeled RNA probes were generated by *in vitro* transcription using the Dig RNA-labeling Kit (Roche) and SP6 or T7 RNA polymerase. In order to obtain the template for *in vitro* transcription, pGEM-Teasy vectors (Promega) containing *MsCHS1* and *MsCHS2* fragments (nucleotide positions 4333–4588 and 4241–4477, respectively) were linearized with either *Nco*I or *Nde*I, respectively. Following electrophoresis in a 2% formaldehyde/agarose gel, the RNA was stained with Radiant Red (BioRad) and documented with the Fluor-S Multi-Imager (BioRad). After transferring the RNA to a nylon membrane (Hybond^{TM}-N^{+}, Amersham Biosciences), hybridization was carried out with the *MsCHS1*- and *MsCHS2*-specific RNA probes followed by washing at 55 °C for *MsCHS1* and at 60 °C for *MsCHS2*, respectively. Detection of RNA bands via anti-DIG antibodies (Fab fragments, Roche) conjugated with alkaline phosphatase was performed with CSPD® (Roche) as a chemiluminescence substrate. Membranes were exposed to an X-ray film at room temperature (Kodak X-omat AR).

For expression analysis of CHS transcripts during development, total RNA was isolated from dissected midgut and epidermal tissues from various stages using the RNeasy® Protect Mini Kit (Qiagen) according to the manufacturer's instructions. Following ammonium acetate precipitation in isopropanol, the concentrated RNA samples were redissolved in water. Approximately 10 μg of total RNA from each sample was fractionated in a 1.5% formaldehyde/agarose gel. The RNA was transferred in 20 X SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate pH 8.0) to a nylon membrane (Hybond^{TM}-N^{+}, Amersham Biosciences). Probes specific for *MsCHS1* and *MsCHS2* were designed from dissimilar sequences corresponding to nucleotide positions 4333–4588 and 4241–4477, respectively. The DNA probes corresponding to these regions were prepared using Ready-To-Go^{TM} DNA Labeling Beads (-dCTP, Amersham) and radiolabeled with (α-^{32}P) dCTP (Perkin Elmer). Hybridization and washing were performed under high stringency conditions: 0.5 M phosphate buffer, pH 7.2 with 7% SDS, 1 mM EDTA, (Church and Gilbert, 1984), 62 °C overnight, followed by washing with 1 X SSC, 0.1% SDS at 65 °C. Following detection of the *MsCHS2* transcript by autoradiography, the membrane was stripped by boiling in 0.1% SDS and then re-probed with a mixture of the *MsCHS1* probe and a probe specific for the constitutively expressed housekeeping gene, ribosomal protein S3, RpS3 (Jiang et al., 1996).

2.6. RT-PCR analysis of MsCHS1/MsCHS2 expression and alternate exon usage

Total RNA was isolated from dissected epidermal and anterior midgut tissues from various stages of development using the RNeasy® Protect Mini Kit (Qiagen) according to the manufacturer's instructions. Two micrograms of total RNA were utilized as templates for cDNA synthesis using an oligo-(dT) primer. This cDNA then served as a template for the subsequent PCR reactions. To minimize variations in primer annealing in the *MsCHS1*/*MsCHS2* expression analysis, a common forward primer was used that was designed from a region where the sequences of both transcripts are identical. The sequence of this primer was 5′-GAAAGGCGCTCATGGACG-3′, which spans positions 2432–2449 in *MsCHS1* and 2398–2415 in *MsCHS2*. To allow for the simultaneous analysis of *MsCHS1*/*MsCHS2* expression, two gene-specific reverse primers were designed to produce different size PCR products when used in conjunction with the common forward primer. The sequences of these primers were 5′-TGAAGGAAGCCCAAGAGAG-3′ (spanning positions 3275–3293) for *MsCHS1*, and 5′-ACGTTGTTCAAATTGCATAGG-3′ (spanning positions 3120–3140) for *MsCHS2*. These primers were designed to have similar melting temperatures, enabling both PCR reactions to be carried out in the same vessel. The same strategy was used to examine the alternate exon usage of *MsCHS1*. Here, a common reverse primer was

designed downstream of the alternate exon with the sequence 5′-TTCGTTATTAGCACCTAGGG-3′ (spanning positions 4466–4485). A forward primer specific for the *MsCHS1a* alternate exon with the sequence 5′-TGAAAGAATTGAGAGACTCG-3′ (located near the 5′ end of the alternate exon and spanning positions 3788–3807) and another forward primer specific for the *MsCHS1b* alternate exon with the sequence 5′-ATTACCTACATCGAGGAGAC-3′ (located near the 3′ end of the alternate exon and spanning positions 3919–3938) were used. For controls, primers for the constitutively expressed housekeeping gene RpS3 were also utilized. A series of PCR reactions of different cycle numbers were carried out in order to determine the appropriate cycle number to be used in the analysis. All PCR reactions used in the expression analysis were conducted using the following conditions: denaturation at 94 °C for 30 s, annealing at 58 °C for 45 s. and polymerization at 72 °C for 1 min. for 20 cycles.

2.7. Southern blot analysis of M. sexta genomic DNA for CHS sequences

Three different restriction enzymes, *Eco*RV, *Eco*RI, and *Sal*I, were used to digest 10 μg of *Manduca* genomic DNA using one endonuclease in each reaction. The resulting digests were subjected to electrophoresis in a 0.9% agarose gel and transferred to a nylon membrane (Hybond^{TM}-N^{+}, Amersham Biosciences) in 0.4 M NaOH. The *MsCHS1* probe was designed from a highly conserved region of the gene that is 88% identical to the *MsCHS2* gene, allowing for the simultaneous detection of both CHSs. This probe was radiolabeled with α^{32}P-dCTP (Ready-To-Go^{TM} DNA Labeling Beads, Amersham) and corresponded to nucleotide positions 2315–2515 of the *MsCHS1* cDNA. To demonstrate the ability of the probe to detect even distantly related CHS genes from another species, digestions (10 μg each) of genomic DNA from *Drosophila* were carried out in parallel using the restriction enzymes *Hin*dIII and *Bam*HI. For controls, non-radioactive PCR fragments (200 pg each) corresponding to the *MsCHS1* and *MsCHS2* probes were also analyzed in parallel. Hybridization was carried out at 50 °C followed by washing at 37 °C with 1 X SSC/0.1% SDS. The membrane was exposed to X-ray film for 7 days.

3. Results

3.1. Sequence analysis of the MsCHS2 cDNA

The sequence of the entire cDNA (AY821560) corresponding to the *MsCHS2* gene was obtained by sequencing DNA fragments obtained by PCR reactions using cDNA prepared from midgut tissue as the template and degenerate primers covering most of the ORF as well as by 5′- and 3′-RACE (Fig. 2). In total, 4821 nucleotides of this transcript were sequenced, which contained an open reading frame of 4575 nucleotides. The encoded protein is 1524 amino acids in length with a predicted MW of approximately 175 kDa (Fig. 3). The predicted pI of MsCHS2 is pH 6.06, which is slightly more acidic than the predicted pI of pH 6.56 for MsCHS1. The catalytic domain of CHS is probably located on the cytoplasmic side of the membrane (Tellam et al., 2000). Located near the C-terminal end of the putative catalytic domain (box in Fig. 3) of MsCHS2 is the QRRRW 'signature sequence' of CHSs (Nagahashi et al., 1995). The CHS 'signature sequence', QRRRW, may correspond to the QXXRW motif found in cellulose synthases (Saxena et al., 2001). Using the topology prediction software, TMHMM, MsCHS2 is predicted to be an integral membrane protein with 16 transmembrane helical spans (data not shown). Five transmembrane spans (5-TMS) are predicted to be located immediately following the putative central catalytic domain. This topology is similar to that of cellulose synthases and has been predicted to constitute a pore in the membrane through which the newly synthesized carbohydrate polymers may be extruded (Richmond, 2000). Class A CHSs have two unique characteristics, a transmembrane segment encoded by an exon that is alternately spliced and a coiled-coil domain (Arakane et al., 2004). Genes encoding class B CHSs, however, do not contain an alternate exon and the coiled-coil domain is typically not predicted in these proteins. Consistent with other class B CHSs, MsCHS2 is not predicted to have a coiled-coil domain immediately following the cluster of five transmembrane segments (Paircoil Program, Berger et al., 1995). Alignment of MsCHS2 with other class B insect CHSs shows a high degree of conservation, particularly in the middle of the putative catalytic domain (Fig. 3). The superior alignment of MsCHS2 with other class B insect CHSs, as opposed to class A CHSs, demonstrates that this protein is very probably an enzyme of the B class.

3.2. Sequencing of MsCHS2 and MsCHS1 genes

The *MsCHS2* gene (AY821561) was sequenced using several sets of primers that were designed from the corresponding cDNA sequence to PCR amplify overlapping genomic DNA fragments (Fig. 4). Genomic DNA encoding *MsCHS2* is 18,525 bp including the 5′- and 3′-UTRs and contains 24 short exons ranging in size from 98 to 331 nucleotides, which are separated by 23 introns of varying lengths. In contrast to the gene sequences of class B CHSs from other insects, the *MsCHS2* gene is considerably larger and interspersed with a greater number of introns. This observation may

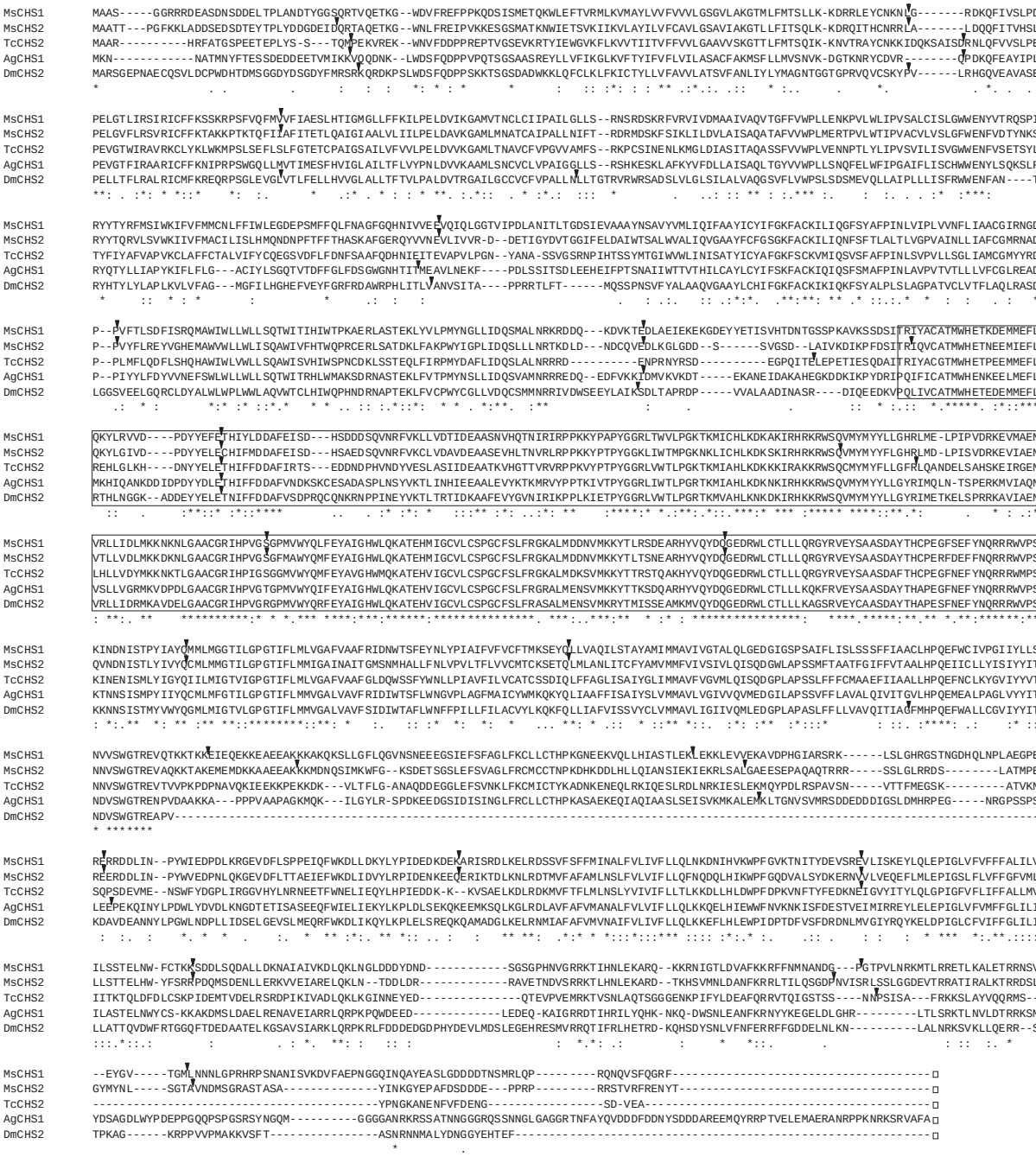


Fig. 3. Alignment of the deduced amino acid sequences of MsCHS1, MsCHS2, TcCHS2, AgCHS1, and DmCHS2 using Multiple Alignment software. The putative catalytic domain is boxed. The positions in the protein sequences where coding regions are interrupted by introns are indicated by shaded arrowheads. Symbols below the aligned amino acid sequences indicate identical (*), highly conserved (:), and conserved residues (.).

be a general characteristic of the *Manduca* genome, as the pattern of numerous introns interspersed in the gene is also observed in *MsCHS1* and other sequenced *Manduca* genes. The 5′ UTR of *MsCHS2* is interrupted by a single intron, whereas the short 3′ UTR contains no introns. A careful analysis of the *MsCHS2* gene sequence and comparison with the corresponding cDNA sequence indicate that no alternate exon is present in this gene, as is the case for other class B CHS genes (Arakane et al., 2004). However, following the completion of the *MsCHS1* gene sequence, an alternate exon was identified that corresponds to exon 20 in the *MsCHS2* gene. Within this region of the *MsCHS1* gene sequence (equivalent to the region between exons 19 and 21 of *MsCHS2*), the sizes and positions of the exons in *MsCHS1* and *MsCHS2* perfectly match, with differences observed only in the intron sizes and in the presence of the alternate exon

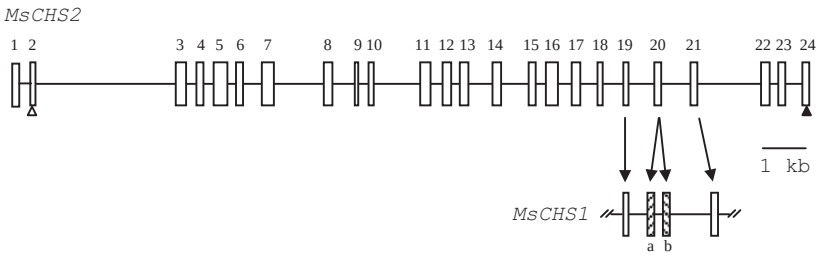


Fig. 4. Schematic diagram of the exon-intron organization of the *MsCHS2* gene and a portion of the *MsCHS1* gene. Boxes indicate exons and lines indicate introns. The location of the ATG start codon and TAA stop codon are represented by open and shaded triangles, respectively. The crosshatched exons, a and b, represent the alternative exons in the *MsCHS1* gene that correspond to exon 20 in the *MsCHS2* gene. The majority of the *MsCHS1* gene organization at the 5′ end was previously determined (Zhu et al., 2002).

(corresponding to exon 20 of *MsCHS2*) in *MsCHS1*. Furthermore, the intron positions are conserved between the two CHS genes in 10 additional positions for a total of 14 identical positions (Fig. 3). The alternate exons of *MsCHS1* are equal in length (177 nucleotides) and encode 59-amino acid-long segments composed of a putative transmembrane span located in the middle of the exon sequence and two short flanking sequences, one facing the cytosolic side and the other the extracellular side. The corresponding exon in *MsCHS2*, exon 20, also encodes a topologically similar peptide sequence. In all, the presence of an alternate exon in the *MsCHS1* gene demonstrates a higher degree of complexity of organization compared to the *MsCHS2* gene.

3.3. Expression of MsCHS1 and MsCHS2 in various tissues

The expression of CHS genes in *Manduca* has been analyzed in the midgut, epidermal, and tracheal tissues by northern blot analysis (Fig. 5). Expression of *MsCHS2* is restricted to the midgut, with no detectable expression in the tracheal and epidermal tissues, where *MsCHS1* is expressed. The *MsCHS2* transcript has also been determined to be absent in numerous other tissues including Malpighian tubules, fat body, and hemocytes (data not shown). Therefore, MsCHS2 is a midgut-specific CHS. As predicted from the cDNA sequences, the sizes of the two transcripts are different, with *MsCHS1* being slightly larger (5.1 kb) than the *MsCHS2* transcript (4.8 kb).

3.4. Localization of MsCHS2 in the midgut

Northern blot analysis of midguts from the feeding stage that were divided into three equal segments corresponding to the anterior, medial, and posterior sections of the midgut shows the highest level of *MsCHS2* expression in the anterior midgut with expression levels tapering off in the medial and posterior midguts (Fig. 6, right). The drop in the level of *MsCHS2* expression is drastic between the anterior and the medial midgut sections, indicating that the anterior midgut contributes most of the MsCHS2.

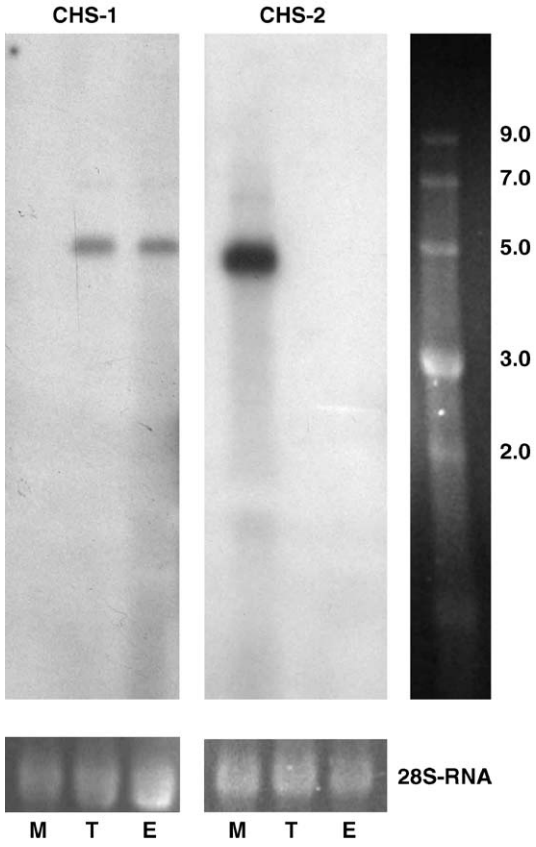


Fig. 5. Expression of the CHS genes *MsCHS1* and *MsCHS2* in different tissues. Poly (A) RNA of fifth instar larvae was isolated either from abdominal tracheae (T), the epidermis (E) or from midguts (M) from which tracheae had been removed as much as possible. After gel electrophoresis and northern blotting, hybridization was performed under highly stringent conditions with digoxigenin labeled ssRNA probes complementary to either *MsCHS1* or *MsCHS2*. Detection was carried out with anti-digoxigenin antibodies conjugated with alkaline phosphatase using CSPD as a chemiluminescence substrate. To control loading and to determine fragment lengths, ribosomal RNA (28S RNA) and a RNA standard with fragment lengths indicated in kb were stained after gel electrophoresis with Radiant Red.

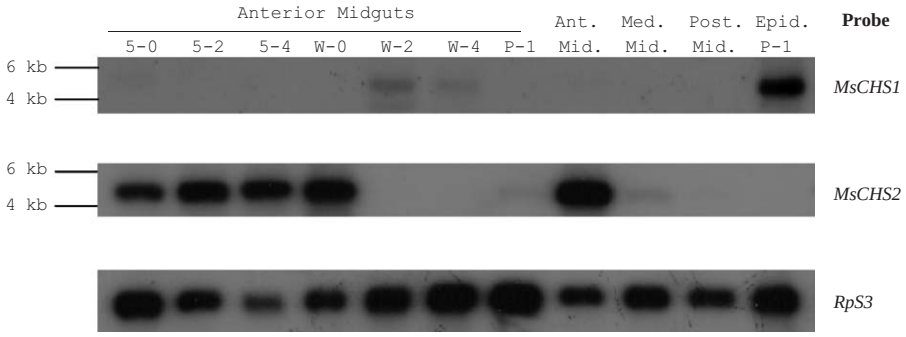


Fig. 6. Northern blot analysis of chitin synthase transcripts in different sections of midgut during development. Total RNA was extracted from anterior midguts at various stages during development: fifth instar days 0, 2, 4; Wandering days 0, 2, 4; Pupa day 1. Probes specific for *MsCHS1*, *MsCHS2*, and *RpS3* were radiolabeled using (α-^{32}P) dCTP. Following hybridization and detection by autoradiography using the *MsCHS2* probe, the membrane was stripped by boiling in 0.1% SDS. The membrane was then re-probed with both the *MsCHS1* and *RpS3* probes simultaneously. Abbreviations are as follows: Ant. Mid., anterior midgut; Med. Mid., medial midgut; Post. Mid., posterior midgut; Epid. P-1, epidermis pupa day 1. Anterior, medial, and posterior midgut samples were obtained from dissected midgut tissues during the fifth larval instar.

3.5. Expression of MsCHS1 and MsCHS2 in the epidermis and anterior midgut during development

The tissue-specificity of expression of the two genes, *MsCHS1* and *MsCHS2*, has been determined during development. Analysis of the *MsCHS2* expression pattern (Fig. 6) indicates that this CHS is expressed throughout the feeding stage (fifth instar days 0, 2, and 4) as well as on the first day of the wandering stage (W-0). Following the onset of the wandering stage, the level of *MsCHS2* transcripts drops dramatically with no significant quantity of mRNA being detected past the second day of this stage. Low levels of *MsCHS1* transcripts are detected on days 2 and 4 of the wandering stage, a time when there are no detectable *MsCHS2* transcripts in the midgut (Fig. 6). The presence of only minor amounts of *MsCHS1* transcripts in the anterior midgut samples (on wandering days 2 and 4) is also confirmed by the RT-PCR analysis described below (Fig. 7).

3.6. RT-PCR analysis of the accumulation of alternately spliced transcripts from MsCHS1

The utilization of the two alternate exons of *MsCHS1* for generation of alternately spliced transcripts was investigated using RT-PCR (Fig. 7). At all time points when *MsCHS1* is expressed in epidermal tissues, both splice variants are present. However, the relative amounts of the two transcripts vary substantially during development. The ratio of *MsCHS1a* /*MsCHS1b* is high during the larval feeding and pupal stages in the epidermis. In contrast, the ratio of the *MsCHS1b*/*MsCHS1a* transcripts in the epidermis is the highest during the prepupal stage (lane W-4). Interestingly, in the anterior midgut also, this ratio is the highest at the same time. Because *MsCHS2* is virtually undetectable in the midgut at this stage, we investigated the possibility that the *MsCHS1* expression in the midgut is due to a tissue other than the gut epithelium. Zimoch et al. (2005) have shown that tracheae isolated from the midgut tissue at this time point have the same high ratio of *MsCHS1b*/*MsCHS1a* transcripts. Therefore, by extrapolation, it is likely that some of the *MsCHS1b* transcripts that appear in the epidermis (which is invariably contaminated with tracheae) may have originated from tracheae.

3.7. Southern blot analysis of CHS genes in M. sexta

Southern blot analysis of genomic DNA supports there are only two CHS genes in *M. sexta* (Fig. 8). A probe designed to be specific for CHS genes was designed from the *MsCHS1* gene around a conserved region where its sequence is 88% identical to the *MsCHS2* gene. This probe was capable of hybridizing to both CHS genes in *Manduca* as shown by cross-hybridization of the radioactive probe with unlabeled probe DNAs containing either CHS gene. Furthermore, the ability of this probe to detect other CHS genes was demonstrated by its ability to recognize CHS gene sequences in digests of genomic DNA from a distantly related insect species, *D. melanogaster*. The CHS probe was able to recognize genes encoding both class A and class B CHSs in *Drosophila*, namely *DmCHS1* and *DmCHS2*, respectively. Because the probe was able to recognize both CHS genes from a distantly related insect, it is unlikely that this probe would have been unable to recognize another CHS gene(s) in *M. sexta*. The restriction enzymes were selected, after analysis of the gene sequences, because of their ability to produce differently sized DNA fragments from the two CHS genes. Only two bands are visible in the *Eco*RI and *Sal*I digestions and the sizes of these fragments correspond to the predicted sizes of the digestion products of *MsCHS1* and *MsCHS2* (expected to be detected by this probe) as

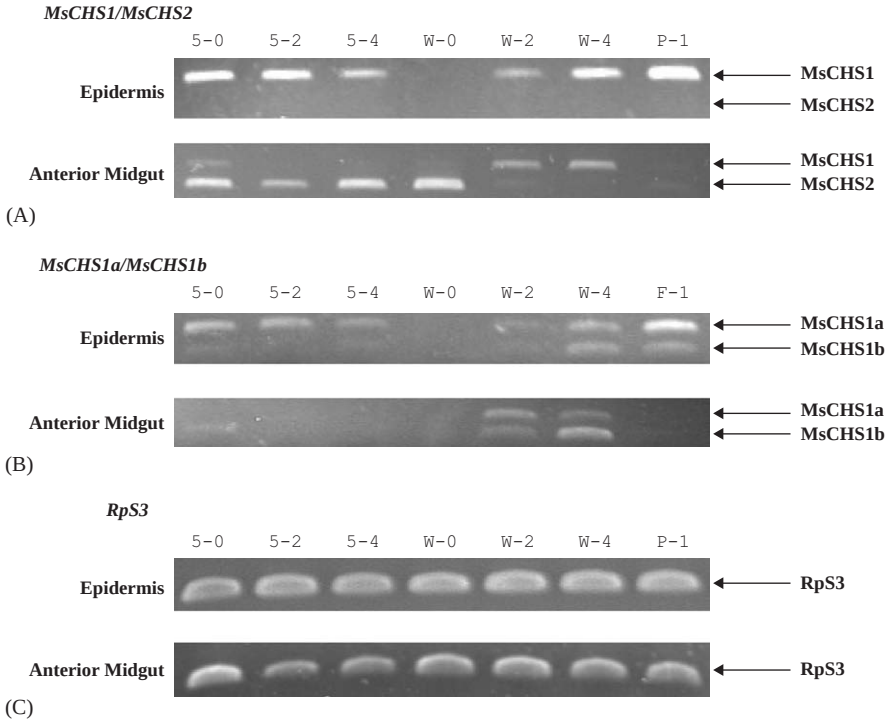


Fig. 7. RT-PCR analysis of the expression of chitin synthase genes in the epidermis and anterior midgut of *M. sexta* during development. The cDNAs used for PCR analysis were prepared from total RNA extracted from dissected epidermal and anterior midgut tissues at various stages during development: Fifth instar days 0, 2, 4; Wandering days 0, 2, 4; Pupal day 1. Two sets of gene-specific primers were used to study the relative expression of *MsCHS1/MsCHS2* in panel A, and *MsCHS1a/MsCHS1b* in panel B. These primers were designed to produce different size PCR products to allow for simultaneous analysis. For controls, primers for the constitutively expressed housekeeping gene RpS3 were used in panel C.

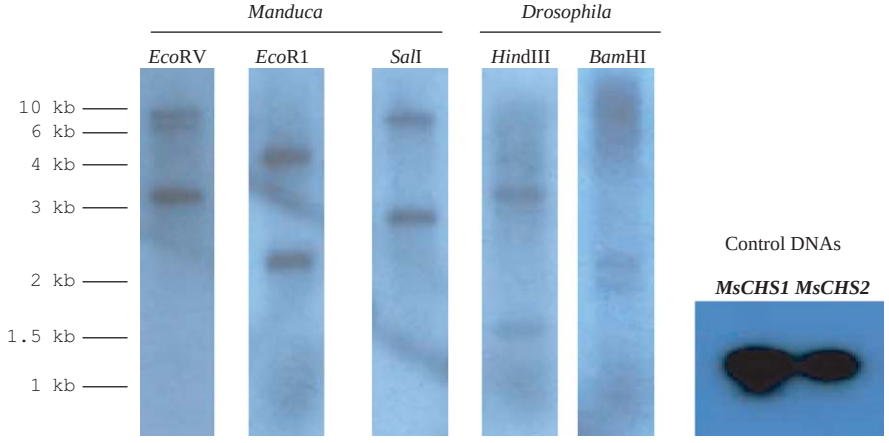


Fig. 8. Southern blot analysis of *Manduca sexta* and *Drosophila melanogaster* genomic DNA. *Manduca* DNA (10-μg per lane) was digested with the following restriction enzymes: *Eco*RV, *Eco*RI, and *Sal*I. *Drosophila* DNA (10 μg per lane) was digested with *Hin*dIII and *Bam*HI restriction enzymes. A ^{32}P-radiolabeled probe corresponding to nucleotide positions 2315 to 2515 of *MsCHS1* was used in the hybridization as described in Section 2. For controls, non-radioactive PCR fragments (200 pg each) corresponding to *MsCHS1* and *MsCHS2* probes were also assayed in parallel.

deduced from the genomic DNA sequences. The *Eco*RV digestion lane shows a single band of approximately 3 kb (corresponding to the *MsCHS1* gene) and two closely spaced bands of approximately 7 kb. The sizes of the two larger bands correspond to two possible fragments (6933 and 7831 bp) in *MsCHS2* that would be produced as a result of either incomplete digestion and/or methylation of the restriction enzyme cleavage sites. We conclude that like other insects, *M. sexta* has only two CHS genes, namely *MsCHS1* and *MsCHS2*.

4. Discussion

We recently reported the cloning of the first *Manduca sexta* CHS gene (*MsCHS1*; Zhu et al., 2002). The

previous manuscript described a single CHS transcript and part of the corresponding genomic sequence. In the present work, we have fully characterized a second *M. sexta* CHS gene, *MsCHS2*, and completed the missing genomic sequences for the 3′ end of *MsCHS1*. Southern blot analyses suggest the existence of only these two CHS genes in the *M. sexta* genome. The presence of only two distinct CHS genes in the lepidopteran *Manduca*, the coleopteran *Tribolium*, and the dipterans *Drosophila* and *Anopheles* (Arakane et al., 2004) has led to the hypothesis that these enzymes may have distinct functions and may be responsible for deposition of chitin associated with different chitinous structures within insects. Since the major chitin-containing structures found in insects are the cuticle and the PM that lines the midgut, it has been hypothesized that one CHS is utilized for the synthesis of chitin in the cuticle, whereas the other enzyme functions exclusively to synthesize chitin in the PM. However, until now, this hypothesis has never been tested in a single insect species by analyzing the expression of the two CHS genes in several tissues at different developmental stages. The large size and well-characterized life cycle of *M. sexta* make it a suitable model organism for this study. Because of its size, dissection of epidermal, tracheal, and midgut tissues with minimal contamination from other tissues was possible in *Manduca*, allowing for straightforward analysis of CHS expression in various tissues. Furthermore, sections of anterior, medial, and posterior midgut samples were easily obtained to further study the differential expression of CHS throughout the midgut.

From the northern blot and RT-PCR analyses, it is clear that *MsCHS1* is expressed in the epidermis and tracheae, while *MsCHS2* is expressed exclusively in the midgut. The low amounts of *MsCHS1* transcripts that are detected in midgut tissues (at times when *MsCHS2* expression is low or undetectable) are attributable to tracheal contamination (Fig. 5; Zimoch et al., 2005). Thus, there is strict tissue specificity of expression of the two CHS genes. The observed difference in the developmental patterns of expression of the two CHS genes also supports their distinct biological roles. *MsCHS1* expression is observed during periods of growth such as during the larval-larval and larval-pupal intermolts when the insect is actively synthesizing new cuticle and forming new tracheae, which requires an increase in the amount of exoskeletal and tracheal chitin. On the other hand, *MsCHS2*, is not expressed during molting (Zimoch et al., 2005) but is expressed only during larval (and adult) feeding periods when synthesis and elaboration of a new PM occurs. Expression of this gut-specific CHS is observed throughout the feeding stage in the fifth instar and transcripts are still seen at the point when the insect enters the wandering stage. However, only trace amounts of *MsCHS2* transcripts are detected on the second day of the wandering stage and beyond, suggesting a rapid down-regulation of synthesis and/or turnover of these transcripts during the late wandering stages. These abrupt changes in levels of *MsCHS2* transcripts may reflect changes in the synthesis/turnover rate of this transcript at this stage when rapid changes in hormonal levels are known to occur (Riddiford, 1994). These observations are in agreement with the hypothesis that *MsCHS2* functions to synthesize PM-associated chitin and is required only when the PM is being synthesized. The absence of *MsCHS2* transcripts in other tissues also supports this hypothesis.

As discussed above, *MsCHS2* is gut-specific and functions to synthesize PM-associated chitin in the midgut. However, trace amounts of *MsCHS1* transcripts were observed in midgut preparations at the following stages; 5–0, W-2, W-4 (Figs. 6 and 7). Nevertheless, we consider that *MsCHS1* has no role in the synthesis of PM for the following reasons. A comparison of the developmental profile of expression of the two CHS genes in the midgut is clearly different, peaking at different times. In fact, on day 0 of the wandering stage, when *MsCHS2* expression is at a high level, there is no detectable *MsCHS1* expression in the midgut. Conversely, on day 4 of the wandering stage, when *MsCHS1* levels in the midgut are maximal, there are no detectable *MsCHS2* transcripts. These results suggest that the *MsCHS1* expression in the midgut is under a different control mechanism from that of *MsCHS2* and transcripts for *MsCHS1* found in the midgut may originate from a cell type other than the midgut columnar cells, which express only *MsCHS2*. The possibility of significant epidermal contamination of the midgut is low because of the size of the insect at the time of dissection and the meticulous care taken during the isolation of the midgut. Therefore, the source of *MsCHS1* transcripts must be from a non-epidermal tissue that is intimately associated with the midgut. We have ruled out the possibility that contamination of our midgut preparations with foregut or hindgut tissues was the source of *MsCHS1* transcripts by carefully dissecting the midgut to avoid such contamination. The most likely candidate is tracheal tissue, which is closely associated with the midgut columnar cells. Previous immunolocalization studies have shown that tracheae indeed have CHS protein (Zimoch and Merzendorfer, 2002). However, the nature of the CHS gene that was expressed in the tracheae was not established in that study. The present study addresses this issue by using poly A RNA isolated directly from tracheae (free of other midgut tissue) and gene-specific probes. Transcripts for *MsCHS1*, but not those for *MsCHS2*, were detected in tracheae (Fig. 5). Therefore, tracheae appear to express *MsCHS1* exclusively. Direct evidence for the synthesis of *MsCHS1* by tracheae has also been obtained by Zimoch et al. (2005). The trace amounts

of this transcript detected in midgut preparations during the feeding stage and somewhat larger amounts during the wandering stage are probably due to tracheae being a minor component of the midgut tissue and the different timing of expression of the CHS genes in these two tissues. Further analyses including in situ hybridization with gene-specific probes or immunolocalization studies using antibodies specific for each CHS are required to more definitively address this issue.

The presence in the *MsCHS1* gene of two alternate exons that correspond to exon 20 in the *MsCHS2* gene was revealed in this study. This region of the gene was not sequenced in our previous study (Zhu et al., 2002). Classification of the *MsCHS1* and *MsCHS2* genes based on amino acid sequence similarities of the encoded proteins indicates that *MsCHS1* encodes a class A CHS, whereas *MsCHS2* codes for a class B CHS (Fig. 1). The presence of alternate exons in genes for class A CHSs has been reported in several other insect species including *Tribolium*, *Drosophila*, and *Anopheles* (Arakane et al., 2004). However, an alternate exon has not been identified in genes encoding any insect class B CHS sequenced to date, including *MsCHS2*. Although class A CHSs of most insect species are designated as being products of CHS1 genes, the naming of *A. gambiae* CHS genes are reversed, with *AgCHS2* encoding the class A CHS and *AgCHS1* encoding the class B CHS. Similarly, the naming of *Aedes aegypti* CHSs is also reversed. Thus, all CHS genes characterized so far encoding class A CHSs from insects belonging to the lepidopteran, coleopteran, and dipteran orders have alternate exons. The presence of the alternate exons may prove to be a distinguishing feature of class A CHSs in all insect species.

The tissue specificity of expression of the alternately spliced transcripts, *MsCHS1a* and *MsCHS1b*, has provided some interesting insights into the biological importance of the two isoforms of the enzymes generated by alternate splicing. In the epidermis, the *MsCHS1a* transcript is preferentially accumulated over the *MsCHS1b* transcript, particularly during the feeding and pupal stages. However, the preferential accumulation of the *MsCHS1b* transcript in tracheae (Zimoch et al., 2005) isolated from the midgut (prepupal stage) at a time when *MsCHS2* is not expressed, suggests an important role for the encoded enzyme in tracheal development. Although we have not isolated tracheae from epidermal tissue, it is likely that tracheae embedded in the epidermis will have the same preference for the *MsCHS1b* isoform as the tracheae associated with midgut tissue. In fact, tracheal contamination probably contributes to some (or all) of the *MsCHS1b* transcript levels observed in the epidermis, and therefore the preference for the *MsCHS1a* isoform in epidermal tissue may be even greater.

A comparison of the deduced amino acid sequences of the segments encoded by the two alternate exons of *MsCHS1* reveals a high degree of sequence similarity. However, a significant difference between the two isoforms is the presence of a potential *N*-glycosylation site, NKSV, in *MsCHS1b*, which is absent in *MsCHS1a* (replaced by the sequence DSSV). This potential *N*-glycosylation site is also present in the alternate exon homologous to *MsCHS1b* of each class A CHS currently sequenced from other insects (Arakane et al., 2004). Perhaps the function of the alternate exon is to provide a site for *N*-glycosylation in the *MsCHS1b* isoform. This isoform could therefore have different enzymatic properties and/or be differentially regulated and serve a slightly different function than its *MsCHS1a* counterpart. An analysis of the alternate exon usage of *MsCHS1* in the midgut indicates that the *MsCHS1b* isoform is preferentially expressed over *MsCHS1a* during the prepupal stage (wandering, day 4). At this stage, the tracheae undergo a major structural rearrangement and actively synthesize chitin (Zimoch et al., 2005). It will be interesting to determine whether the presence of the *MsCHS1b* isoform has any influence on the rate of chitin synthesis and/or chitin deposition in the tracheae.

The existence of at least two CHS genes in *Manduca* was previously determined, but the exact number of genes remained unclear (Zimoch and Merzendorfer, 2002). Using Southern blot analysis, only two CHS genes were identified in *M. sexta* (Fig. 8). To date, no more than two CHS genes have been identified in any insect species. Analysis of the completed genome sequences of *D. melanogaster* and *A. gambiae* indicates that only two putative CHS genes are present in those species. The *Tribolium* genome project is currently underway, but it is unlikely that more than two CHS genes will be found since Southern blot analysis and extensive BAC library screening also indicated the presence of only two CHS genes (Arakane et al., 2004). Even though it appears that there are only two CHS genes, three isoforms of CHS are possible because of the utilization of the two alternate exons of *MsCHS1*, a common characteristic of all genes encoding class A CHSs analyzed thus far. The function of *MsCHS2*, which is made exclusively by the midgut, is clearly the formation of PM by the midgut cells as shown by this study in which the expression of both genes was followed during different developmental stages. Gene knockout or RNA interference studies can provide direct evidence for the distinct role of these two CHS genes.

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References

Arakane, Y., Hogenkamp, D.G., Zhu, Y.C., Kramer, K.J., Specht, C.A., Beeman, R.W., Kanost, M.R., Muthukrishnan, S., 2004. Characterization of two chitin synthase genes of the red flour beetle, *Tribolium castaneum*, and alternate exon usage in one of the genes during development. Insect Biochem. Molec. Biol. 34, 291–304.

Bell, R.A., Joachim, F.G., 1976. Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms. Ann. Entomol. Soc. Am. 69, 365–373.

Berger, B., Wilson, D.B., Wolf, E., Tonchev, T., Milla, M., Kim, P.S., 1995. Predicting Coiled Coils by Use of pairwise Residue Correlations. Proc. Natl. Acad. Sci. USA 92, 8259–8263.

Church, G.M., Gilbert, W., 1984. Genomic Sequence. Proc. Natl. Acad. Sci. USA 81, 1991–1995.

Cohen, E., 1991. Chitin biochemistry. In: Binnington, K., Retnakaran, A. (Eds.), Physiology of the Insect Epidermis. CSIRO Publications, East Melbourne Victoria, Australia, pp. 94–112.

Coutinho, P.M., Deleury, E., Davies, G.J., Henrissat, B., 2003. An evolving hierarchical family classification for glycosyltransferases. J. Mol. Biol. 328, 307–317.

Harris, M.T., Fuhrman, J.A., 2002. Structure and expression of chitin synthase in the parasitic nematode *Dirofilaria immitis*. Mol. Biochem. Parasitol. 122, 231–234.

Harris, M.T., Lai, K., Arnold, K., Martinex, H.F., Specht, C.A., Fuhrman, J.A., 2000. Chitin synthase in the filarial parasite, *Brugia malayi*. Mol. Biochem. Parasitol. 111, 351–362.

Ibrahim, G.H., Smartt, C.T., Kiley, L.M., Christensen, B.M., 2000. Cloning and characterization of a chitin synthase cDNA from the mosquito *Aedes aegypti*. Insect Biochem. Molec. Biol. 30, 1213–1222.

Jiang, H., Wang, Y., Kanost, M.R., 1996. Primary structure of ribosomal proteins S3 and S7 from *Manduca sexta*. Insect Mol. Biol. 5, 31–38.

Kramer, K.J., Muthukrishnan, S., 2004. Chitin metabolism in insects: a revisit. In: Gilbert, L.I., Iatrou, K., Gill, S. (Eds.), Comprehensive Molecular Insect Science, vol. 4. Biochemistry and Molecular Biology. Elsevier Press, Oxford, UK, (Chapter 3).

Merz, R.A., Horsch, M., Nyhlen, L.E., Rast, D.M., 1999. Biochemistry of chitin synthase. In: Jollès, P., Muzzarelli, R.A.A. (Eds.), Chitin and Chitinases. Birkhäuser Verlag, Berlin, Germany, pp. 9–37.

Merzendorfer, H., Harvey, W.R., Wieczorek, H., 1997. Sense and antisense RNA for the membrane associated 40 kDa subunit M40 of the insect V-ATPase. FEBS Lett 411, 239–244.

Merzendorfer, H., Zimoch, L., 2003. Chitin metabolism in insects: structure, function and regulation of chitin synthases and chitinases. J. Exp. Biol. 206, 4393–4412.

Munro, C.A., Gow, N.A., 2001. Chitin synthesis in human pathogenic fungi. Med. Mycol. 39 (Suppl. 1), 41–53.

Nagahashi, S., Sudoh, M., Ono, N., Sawada, R., Yamaguchi, E., Uchida, Y., Mio, T., Takagi, M., Arisawa, M., Yamada-Okabe, H., 1995. Characterization of chitin synthase 2 of *Saccharomyces cerevisiae*. Implication of two highly conserved domains as possible catalytic sites. J. Biol. Chem. 270, 13961–13967.

Richmond, T., 2000. Higher plant cellulose synthases. Genome Biol. REVIEWS3001.1-3001.6.

Riddiford, L.M., 1994. Cellular and molecular actions of juvenile hormone I. General considerations and premetamorphic actions. Adv. Insect Physiol. 24, 213–274.

Roncero, C., 2002. The genetic complexity of chitin synthesis in fungi. Curr. Genet. 41, 367–378.

Saxena, I.M., Brown Jr., R.M., Dandekar, T., 2001. Structure-function characterization of cellulose synthase: relationship to other glycosyltransferases. Phytochemistry 57, 1135–1148.

Specht, C.A., Liu, Y., Robbins, P.W., Bulawa, C.E., Iartchouk, N., Winter, K.R., Riggle, P.J., Rhodes, J.C., Dodge, C.L., Culp, D.W., Borgio, P.T., 1996. The *chsD* and *chsE* genes of *Aspergillus nidulans* and their roles in chitin synthesis. Fungal Genet. Biol. 20, 153–167.

Tellam, R.L., Vuocolo, T., Johnson, S.E., Jarmey, J., Pearson, R.D., 2000. Insect chitin synthase: cDNA sequence, gene organization and expression. Eur. J. Biochem. 267, 6025–6042.

Valdivieso, M.H., Duran, A., Roncero, C., 1999. Chitin synthases in yeast and fungi. EXS 87, 55–69.

Veronico, P., Gray, L.J., Jones, J.T., Bazzicalupo, P., Arbucci, S., Cortese, M.R., Di Vito, M., De Giorgi, C., 2001. Nematode chitin synthases; gene structure, expression and function in *Caenorhabditis elegans* and the plant parasitic nematode *Meloidogyne artiellia*. Mol. Genet. Genomics 266, 28–34.

Zhu, Y.C., Muthukrishnan, S., Specht, C.A., Dittmer, N., Kanost, M.R., Kramer, K.J., 2002. Sequence of a cDNA and expression of the gene encoding a putative epidermal chitin synthase of *Manduca sexta*. Insect Biochem. Molec. Biol. 32, 1497–1506.

Zimoch, L., Hogenkamp, D., Kramer, K., Muthukrishnan, S., Merzendorfer, H., 2005. Regulation of chitin synthesis in the larval midgut of *Manduca sexta*. Insect Biochem. Molec. Biol., in press, doi:10.1016/j.ibmb.2005.01.008.

Zimoch, L., Merzendorfer, H., 2002. Immunolocalization of chitin synthase in the tobacco hornworm. Cell Tissue Res. 308, 287–297.