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The nicotinic acetylcholine receptor subunit *Mdα6* from *Musca domestica* is diversified via post-transcriptional modification

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Abstract

Recent studies showed that deletion of a nicotinic acetylcholine receptor (nAChR) subunit gene, *Dα6* in *Drosophila melanogaster* results in a strain that is resistant to spinosad, indicating that *Dα6* is important for the toxic action of this insecticide. To determine if spinosad resistance in house flies was due to a mutation(s) of *Mdα6* (the orthologue of *Dα6* from house flies), cDNAs were cloned and characterized from an insecticide-susceptible and a spinosad-resistant strain of the house fly, *Musca domestica*. The cDNAs contain a 1470-bp open reading frame encoding 490 amino acid residues, 415-bp 5′ untranslated region (UTR) and a polymorphic 3′-UTR of ~371 bp. The predicted mature protein possesses 468 amino acid residues, has the typical features of a nAChR α subunit and is 97% identical to *Dα6*. Quantitative real-time PCR analysis revealed that *Mdα6* was expressed in the head and the thorax at 1300- and 26-fold higher levels, respectively, than in the abdomen. There was no difference in the expression level of *Mdα6* between spinosad-resistant and susceptible strains. Ten isoforms arising from alternative splicing were characterized, with isoform II being most common. A-to-I RNA editing was examined and found at 12 sites: editing at 11 of these sites resulted in an amino acid substitution. *Mdα6* is linked to autosome 1 (spinosad resistance was previously shown to be linked to autosome 1). Single nucleotide polymorphisms, alternative splicing, mRNA levels and A-to-I RNA editing were compared between head and thorax and between insecticide-susceptible and spinosad-resistant strains. These comparisons indicate that *Mdα6* is not responsible for spinosad resistance in house flies.

Keywords: Nicotinic acetylcholine receptor, alternative splicing, RNA editing, real-time PCR, spinosad, house fly.

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Introduction

Nicotinic acetylcholine receptors (nAChR) belong to the Cys-loop superfamily of ligand-gated ion channels that include γ-aminobutyric acid (GABA)-gated Cl^- channels, glycine receptors, glutamate-gated Cl^- channels and 5-hydroxytryptamine type 3 (5-HT3) receptors (Lester *et al.*, 2004). The nAChRs play an essential role in the fast excitatory neurotransmission at cholinergic synapses in both vertebrates and insects (Karlin, 2002). However, nAChRs are confined to the central nervous system in insects, unlike in vertebrates where they are also found in neuromuscular junctions (Gundelfinger & Schulz, 2000).

The nAChRs are composed of five subunits, typically two αs and three non-αs, but receptors consisting of only α subunits are also known (Couturier *et al.*, 1990; Marshall *et al.*, 1990). The α subunits are characterized by the presence of two adjacent cysteine residues in loop C, while the non-α or β subunits lack this cysteine doublet (Gotti & Clementi, 2004; Sattelle *et al.*, 2005). Each subunit possesses a large N-terminal extracellular domain that includes components forming the acetylcholine (ACh) binding site and four hydrophobic transmembrane domains (TM1–4), with TM2 contributing the majority of the amino acids that line the ion channel (Karlin, 2002). The ACh binding site is located at the interface of two subunits and is possibly contributed by three loops (loops A–C) of an α subunit and by three (loops D–F) of another subunit (Grutter & Changeux, 2001). In addition to multiple subunit genes, at least some of nAChRs are additionally diversified through post-transcriptional modifications, such as RNA editing and alternative splicing (Sattelle *et al.*, 2005).

Nicotinic AChRs are the target site for two relatively new insecticides: neonicotinoids and spinosad. The neonicotinoids (imidacloprid, thiamethoxam, etc.) (Narahashi, 1996; Tomizawa *et al.*, 1999) are nAChR activators and are effective

tools used to control a variety of insects, especially pests that have developed resistance to other classes of insecticides. However, resistance to neonicotinoids has been detected in field populations of whiteflies (*Bemisia tabaci*), plant hoppers (*Nilaparvata lugens* and *Laodelphax striatellus*), Colorado potato beetles (*Leptinotarsa decemlineata*) and aphids (*Myzus persicae*, *Aphis gossypii*, *Nasonovia ribisnigri* and *Phorodon humuli*) (Liu *et al*., 2005; Mota-Sanchez *et al*., 2005; Nauen & Denholm, 2005). In the case of *N. lugens*, imidacloprid resistance appears to be the result of a change in two nAChR subunit genes (Liu *et al*., 2005). Imidacloprid was registered for house fly (*Musca domestica*) control in the USA in 2004. Imidacloprid resistance in house flies has not yet been reported, although having the sequence of nAChR subunit genes from this pest may be an important first step in the design of molecular assays for resistance detection. Spinosad is a relatively new insecticide derived from the bacterium *Saccharopolyspora spinosa* and is used to control a wide range of insects. The primary target of spinosad appears to be nAChRs with a secondary site of attack being GABA-gated Cl^- channels (Salgado & Sparks, 2005). It was recently shown that deletion of *Dα6* results in a strain of *Drosophila melanogaster* that is resistant to spinosad (Orr *et al*., 2006; Perry *et al*., 2007). Resistance to spinosad has been reported from field populations of diamondback moth, *Plutella xylostella*, from the USA (Zhao *et al*., 2002) and beet armyworm, *Spodoptera exigua*, from the USA and Thailand (Moulton *et al*., 2000), although the molecular basis for these cases of resistance are not known. Spinosad was available for house fly control in the USA in 2005.

A strain of the house fly that is > 150-fold resistant to spinosad was selected from a field population (Shono & Scott, 2003). The resistance could not be overcome with insecticide synergists, suggesting that it was a result of target site insensitivity. Resistance is conferred by a recessive gene on autosome 1 (Shono & Scott, 2003). The characteristics of resistance in house flies are similar to those in diamondback moth (Zhao *et al*., 2002) and tobacco budworm, *Heliothis virescens* (Salgado & Sparks, 2005). Herein, we report the cloning and characterization of the nicotinic acetylcholine receptor alpha subunit 6 (*Mdα6*) gene from house flies, as well as its alternative splicing, RNA editing, expression and linkage analysis in spinosad-susceptible and resistant strains of house flies. Our results indicate that *Mdα6* is not responsible for spinosad resistance in house flies.

Results and discussion

Isolation of Mdα6 *cDNA*

A 428-bp fragment was amplified from a cDNA preparation of aabys house fly heads using the degenerate primers MaF2 and MaR1 (Table 1). This fragment included cDNAs of three closely related nAChR subunit genes most similar to *Drosophila melanogaster Dα5*, *Dα6* and *Dα7* (BLASTP search, www.ncbi.nlm.nih.gov). Specific primers for the *Dα6*-like gene were designed and used for 5′- and 3′-rapid amplification of cDNA ends (RACE). The two RACE products were cloned and sequenced. Finally, PCR amplifications were conducted using the primers Ma6F and Ma6R (Table 1), to amplify a 1601-bp fragment containing the entire open reading frame (ORF) of the *Mdα6* gene.

Table 1. Sequences of primers used in this study

Name	Sequence (5′–3′)
MaF2:	ATGAARTTYGGNWSNTGGACNTAYGA
MaR1:	GCNACCATRAACATDATRCARTTRAA
Ma5RaceR1	TCCACATACGGTTCCGGACAGCAGGCA
Ma5RaceR2	TACGATGGTATTCTTTTTGCCCGGC
Ma3RaceF3	TGCCTGCTGTCCGGAACCGTATGTGGA
Ma3RaceF1	CCGTGTGTGCTGATATCCTCAATGGCTT
Ma6F	CGCACCAACCAACCAACTCAGC
Ma6R	AGGTTTCTTGCATTGTTGTTGTTGTCTCA
gM6VF1	GCTGATGAGGGATTCGATGGCACGT
gM6VR1	TTCCATCATAAGTCCAACTACCGAATTTC
qM6F1	TCCTCGGTGGTCCTGACGGT
qM6R1	GCAGCCATTGTAGAAATACGGAACG
gM6F5	CAAGGCCGATGATGAGGCTGAGCT
gM6R4	CAGAACTGTCACTGTGGCTATTATTG

The *Mdα6* cDNA contains a 1470-bp ORF which encodes 490 amino acid residues (Fig. 1), 415-bp of 5′-untranslated region (UTR) that contains in-frame stop codons, and ~371-bp of 3′-UTR that has a polyadenylation signal (AATAAA) 311 bp downstream of the stop codon. Several polymorphisms were identified in the 3′-UTR in some clones (data not shown). There were 17 and 12 synonymous single nucleotide polymorphisms (SNPs) in the ORF in the susceptible aabys and spinosad resistant rspin strains, respectively (Table 2). There were an additional 13 and 21 non-synonymous SNPs in the susceptible (aabys) and spinosad-resistant (rspin) strains, respectively (Table 3). Among the non-synonymous SNPs, at least eight were because of A-to-I RNA editing (discussed below). Both strains had transcripts in common; however, two polymorphic sites were unique to aabys and 10 were unique to rspin (Table 3). If changes in *Mdα6* were responsible for spinosad resistance, we would expect all transcripts in the rspin strain to be different from aabys. However, our results show no evidence of a selective sweep associated with the *Mdα6* locus in rspin (i.e. rspin contained transcripts also found in aabys).

The *Mdα6* cDNA encodes a 490-amino acid peptide that possesses a 22-residue long signal peptide (predicted by SignalP 3.0; Bendtsen *et al*., 2004). Mdα6 possesses typical characteristics of nAChR α subunits, including a signal peptide, a long N-terminal extracellular domain, four hydrophobic transmembrane domains (TM1–4), and the

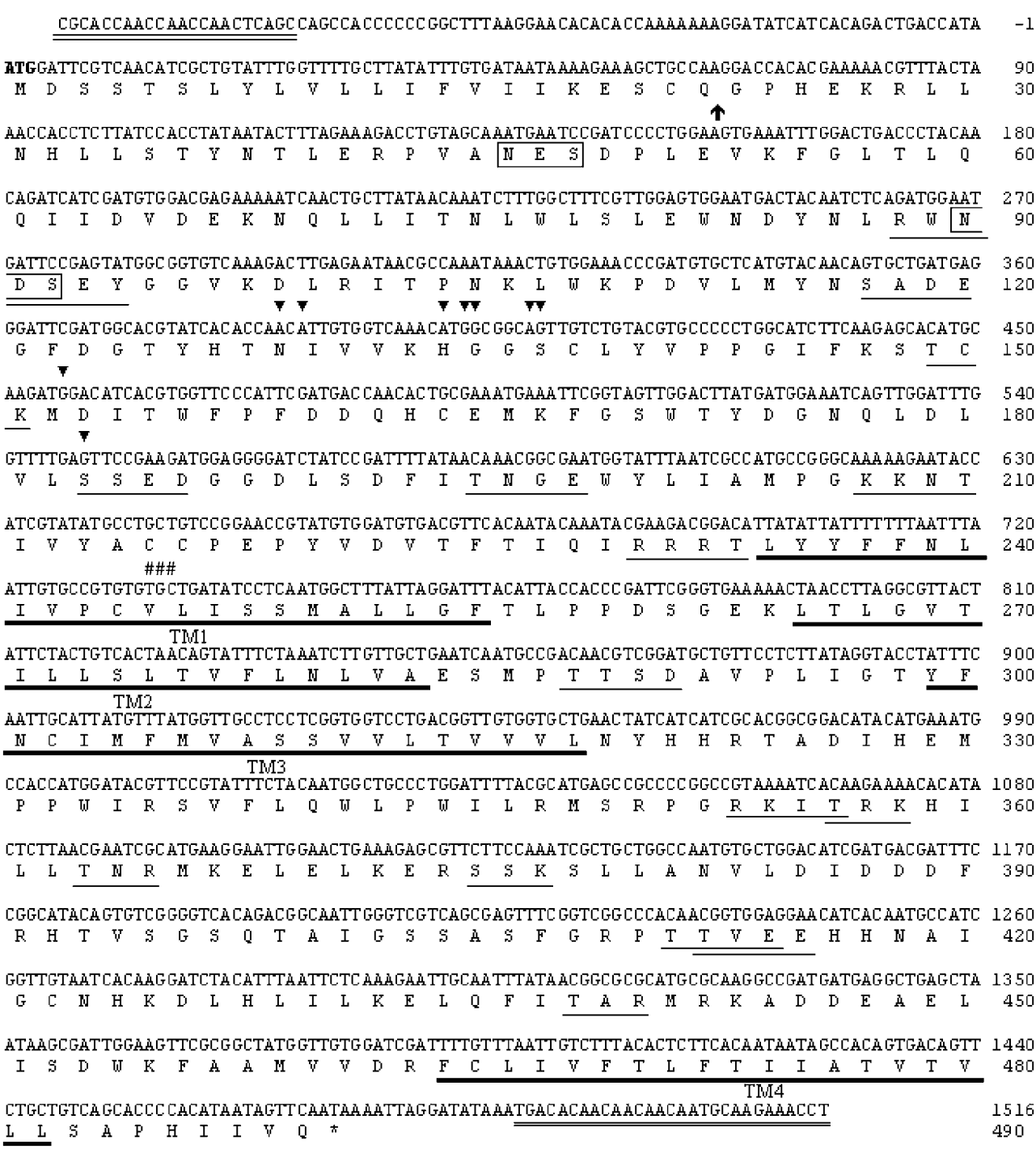

Figure 1. Nucleotide and deduced amino acid sequences of *Mdα6* cDNA (isoform II) from *Musca domestica* (GenBank accession no. DQ498130). The translation start codon, ATG, is shown in bold. The stop codon, TAA, is indicated by a star. The signal peptide cleavage site is marked with a vertical arrow. Potential N-linked glycosylation sites are boxed. The potential phosphorylation sites are underlined. The primers used to amplify the ORF are double underlined. Transmembrane domains (TM1–4) are underlined with a thick line. The cysteine doublet (characteristic of nAChR α subunits) is marked as '###'. A-to-I editing sites are marked with triangles. Only partial 5′- and 3′-sequences are shown.

YXCC motif in the N-terminal extracellular domain (Fig. 1). The N-terminal domain possesses the Cys loop consisting of two cysteines separated by 13 residues, a motif found in all ligand-gated ion channels (Karlin, 2002) and the acetylcholine binding site forming regions (loops A–F) (Grutter & Changeux, 2001). The protein contains two potential N-glycosylation sites in the N-terminal extracellular domain and 15 potential phosphorylation sites for protein kinase C,

Table 2. Single nucleotide polymorphisms resulting in synonymous mutations in *Mdα6* cDNAs of spinosad-susceptible (aabys) and resistant (rspin) strains of *Musca domestica*

	Site of polymorphism																
Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
aabys																	
nt	A/T	A/G	A/G	A/G	T/C	A/C	A/G	A/C	A/G*	T/G	T/C	T/C	A/C	A/G	A/G	A/G	A/G
position	51	183	309	327	432	636	669	691	864	945	978	1050	1143	1212	1227	1245	1365
aa	I	Q	T	K	G	V	V	R	Q	V	D	R	A	S	R	E	K
position	17	61	103	109	144	212	223	241	288	315	326	350	381	204	409	415	455
rspin																	
nt	A/T	T/C	A/C	A/C	A/G	A/G*	T/C	A/G	T/C	T/C	A/G	A/G					
position	429	432	636	691	855	864	978	993	1050	1083	1191	1212					
aa	P	G	V	R	S	Q	D	P	R	L	S	S					
position	143	144	212	241	285	288	326	331	350	361	397	404					

*Found in transcripts containing exon 8b.

Table 3. Single nucleotide polymorphisms resulting in non-synonymous mutations in *Mdα6* cDNAs of spinosad-susceptible (aabys) and resistant (rspin) strains of *Musca domestica*

	Site of polymorphism																				
Strain	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21
aabys																					
nt	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G*	A/G*								
position	106	386	388	401	403	404	409	410	456	548	623	851	863								
aa	T/A	N/S	I/V	H/R	N/S/D/G	N/S/D/G	N/S/D/G	N/S/D/G	I/M	N/S	K/R	E/G	Q/R								
position	36	129	130	134	135	135	137	137	152	183	208	284	288								
rspin																					
nt	T/C	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G	A/G*	A/G*	T/C	C/T	A/G	A/G
position	44	74	251	386	388	401	403	404	409	410	456	548	574	593	796	851	863	938	950	1198	1420
aa	V/A	H/R	D/G	N/S	I/V	H/R	N/S/D/G	N/S/D/G	N/S/D/G	N/S/D/G	I/M	N/S	N/D	E/G	T/A	E/G	Q/R	P/L	A/V	T/A	T/A
position	15	25	84	129	130	134	135	135	137	137	152	183	192	208	266	284	288	313	317	340	473

*Found only in transcripts with exon 8b.

casein kinase II, tyrosine kinase and cAMP- and cGMP-dependent protein kinase, seven of which are located in the N-terminal extracellular domain, one is located in the extracellular TM2–3 linker, and seven are located in the cytoplasmic TM3–4 linker (Fig. 1). Glycosylation and phosphorylation are important in regulation of receptor assembly, ligand binding, altering receptor desensitization and its recovery, and ion permeability (Chen *et al.*, 1998; Fenster *et al.*, 1999; Wecker *et al.*, 2001; Courjaret *et al.*, 2003; Nishizaki, 2003; Charpantier *et al.*, 2005; Wanamaker & Green, 2005). The results from BLASTP searches (NCBI database), using the mature protein sequence, showed that Mdα6 has 97, 89, 84 and 73% identity with Dα6 of *D. melanogaster* (AAM13393), Agamα6 of *Anopheles gambiae* (AAU12509), α7-2 of *H. virescens* (AAD32698) and α7-2 of *M. persicae* (CAI54102), respectively.

Transcriptional variants of Mdα6

The genomic structure of *Mdα6* orthologues in *D. melanogaster* (*Dα6*) and *An. gambiae* (*Agamα6*) have been determined, using the completed genome sequences (Grauso *et al.*, 2002; Jones *et al.*, 2005). Both contain 12 exons with alternative exons 3 (3a and 3b) and 8 (8a, 8b and 8c in *D. melanogaster* and 8b and 8c in *An. gambiae*). Five splice variants were identified in *D. melanogaster* (Grauso *et al.*, 2002). A fragment containing the entire *Mdα6* ORF was amplified with the primers Ma6F and Ma6R (Table 1) and cloned. Sequencing of clones containing the entire *Mdα6* ORF revealed alternative exon use (Table 4) in *Mdα6*.

Table 4. Alternative exons 3 and 8 of *Mdα6* of *Musca domestica*

Alternatives	Amino acid sequence*	Amino acid position
Exon 3a	DEKNQ**I**L**T**TN**A**WL**N**L	66–80
Exon 3b	DEKNQ**LL**I**T**N**L**WL**S**L	66–80
Exon 8a	GVTILLS**L**TVF**LN**LV**AESMPTT**S**D**AVPL**I**	268–296
Exon 8b	GVTILLS**L**TVF**LN**LV**AETLPQV**S**D**AIPL**L**	268–296
Exon 8c	GVTILLS**Q**TVF**SL**LV**GNVITKT**S**E**AVPL**L**	268–296

*Amino acids that differed among alternative exons are shown in bold. Sequence for exon 8b does not show changes caused by A-to-I editing.

Table 5. Relative expression of *Mdα6* isoforms in spinosad-susceptible (aabys) and resistant (rspin) strains of *Musca domestica*

		Isoform frequency (%)*				
Strain	Tagma	I	II	III	IV	VI
aabys						
	head	10.3 ± 0.6	54.7 ± 9.2	10.7 ± 11.0	0.0	24.0 ± 5.3
	thorax	13.3 ± 5.8	43.3 ± 11.5	20.0 ± 0	3.3 ± 5.8	20.0 ± 17.3
rspin						
	head	14.0 ± 6.9	69.0 ± 10.1	3.3 ± 5.7	0.0	13.7 ± 5.5
	thorax	23.3 ± 15.3	36.7 ± 5.8†	33.3 ± 5.8†	0.0	6.7 ± 5.8

*Three individual flies were used for each strain. Ten clones were analysed per fly per body region. Values represent the mean ± SD (n = 3). Isoform VII was not detected.
†Significantly different from the head of rspin (Student's t-test, $P < 0.05$).

Most of these were readily identified because they were nearly identical to *Dα6* transcripts. Based on the alternative use of exons 3 and 8 in *Dα6*, and the classification criteria of Grauso *et al*. (2002), six splicing variants were characterized from *Mdα6*: isoform I (3a + 8b) (GenBank accession no. DQ498129), isoform II (3b + 8a) (DQ498130), isoform III (3b + 8b) (DQ498131), isoform IV (3a + 3b + 8a) (DQ498132), isoform VI (3a + 8a) (DQ498134) and isoform VII (3a + 8c) (DQ498133). The relative expression of *Mdα6* isoforms in the PCR-amplified transcriptome from the head and thorax of individual flies is shown in Table 5. Isoform II was most common. Although isoform VII was found in the initial sequencing and cloning studies (see above), it was not detected in these individual flies. The percentages of the isoforms were not significantly different between the head and thorax of individual flies from the same strain, or between heads or thoraces of aabys and rspin house flies, except for isoforms II and III. Isoform II was significantly lower and III was significantly higher in thorax than in the head in rspin house flies ($P < 0.05$), but it was not significantly different from aabys house flies. Therefore, alternative splicing does not appear to be involved in spinosad resistance.

In addition, other splice variants resulting from possibly incorrect or imprecise splicing were also identified. Isoform VIII (DQ498135, identified from one clone) is identical to isoform II, except that it contains intron 9. This generates a stop codon 18 bp down stream of the end of exon 9, and thus a truncated protein. Isoform IX (DQ498136, identified from two clones) lacked exons 7 and 8. In isoform X (DQ498137, identified from one clone), exons 5 and 6 were replaced by a 14-amino-acid insertion. In isoform XI (DQ498138, identified from one clone) exons 5–8 were replaced by a 12-amino-acid insertion. Isoforms VIII–XI lack either the acetylcholine binding site or the two conserved TM1 and TM2 transmembrane domains. Therefore, they would not form functional nAChRs (Sattelle *et al*., 2005) and their role is thus unknown.

RNA editing

A-to-I RNA editing catalysed by adenosine deaminases acting on RNA (ADARs) is a pre-mRNA processing event that converts adenosine (A) to inosine (I) within short (approximately 9–15 bp), double-stranded regions of RNA molecules (Seeburg, 2002). Because the inosine is recognized as guanosine (G) by the cellular machinery, this editing functions as A-to-G conversion. Therefore, A-to-I editing may result in amino acid substitutions that can change protein function or alter gene expression by affecting RNA splicing, stability and localization (Sattelle *et al*., 2005). Unambiguous identification of A-to-I editing sites requires both genomic and cDNA sequences. Grauso *et al*. (2002) identified seven A-to-I RNA editing sites in *Dα6* (six in exon 5 and one in exon 6). The editing sites in *Mdα6* exon 5 were investigated in this study by examining the PCR-amplified genomic sequence and 10 cDNA sequences (clones) from the same individuals (n = 3 individuals and 30 clones). The nucleotide (nt) A was found in all of the *Mdα6* homologous positions in the genomic sequences, and G or A was found at eight sites in the cDNA sequences at a similar frequency to *Dα6* (Table 6). Sites 1 and 2 were considered to be adult-specific editing in *Dα6* (Grauso *et al*., 2002). Two new A-to-I editing sites in exon 5 of *Mdα6* were identified in this study and are located at nt 409 and 410. They are edited with a frequency of 3–10% and 58–73%, respectively. They resulted in amino acid substitutions from N to D or G or S in loop E. A putative A-to-I editing site at nt 548 in *Mdα6* (= site 7 in *D. melanogaster*) was also examined by comparing only cDNA sequences. This site appears to be edited at a high frequency (65–79%) and results in an N to S substitution in loop F. In exon 8b, three additional A/G polymorphic sites (putative A-to-I editing sites) were also identified at nt 851, 863 and 864, resulting in E to G and/or Q to R amino acid changes. These amino acid substitutions occurred in the important ligand binding domain and could alter the N-glycosylation of the protein. Our results showed that the RNA editing was not significantly different either

Table 6. Frequency of A-to-I RNA editing of *Mdα6* in spinosad-susceptible (aabys) and resistant (rspin) strains of *Musca domestica*

		Percentage of transcripts with RNA editing* at eight different sites (amino acid position)							
Strain	Tegma	1(386)†	2(388)	3(401)	4(403)	5(404)	6(409)	7(410)	8(456)
aabys									
	head	14.3 ± 16.9	28.0 ± 13.9	0.0	61.3 ± 18.0	65.3 ± 12.9	3.7 ± 6.4	58.0 ± 19.3	71.3 ± 28.0
	thorax	23.3 ± 15.3	33.3 ± 20.8	3.3 ± 5.8	76.7 ± 11.5	76.7 ± 11.5	10.0 ± 10.0	70.0 ± 20.0	80.0 ± 17.3
rspin									
	head	10.0 ± 10.0	16.7 ± 11.5	6.7 ± 11.5	80.0 ± 17.3	86.7 ± 15.3	6.7 ± 5.8	73.3 ± 5.8	83.3 ± 5.8
	thorax	16.7 ± 20.8	32.7 ± 23.7	3.3 ± 5.8	81.0 ± 8.5	81.0 ± 8.5	3.3 ± 5.8	71.7 ± 17.6	81.0 ± 8.5

*Three individual flies were used for each strain. Ten clones were analysed per fly per body region. Values represent the mean ± SD ($n = 3$).
†Sites of editing were numbered for convenience. Nucleotide numbers are given in parentheses.

Table 7. Different transcripts produced by A-to-I RNA editing of *Musca domestica Mdα6*

Transcript	*n* (aabys)*	*n* (rspin)*	Sites edited†
1	6H 6T	3H 4T	none
2	7H 9T	11H 10T	4,5,7,8
3	4H 3T	1H 6T	1,2,4,5,7,8
4	3H 3T	2H 4T	2,4,5,7,8
5	1H 1T	2H 2T	4,5,8
6	2H	1T	8
7	2T	1H	1,2,4,5,6,7,8
8	1T	2H	5,7,8
9	–	2H	3,5,7,8
10	–	2H	4,5
11	1H 1T	–	4,7,8
12	1H 1T	–	5,8
13	1H	–	2,4,5,6,7,8
14	1H	–	5
15	1T	1H	4
16	1T	1H	1,4,5,7,8
17	1T	–	2,4,5,6,8
18	1T	–	1,2,3,4,5,7,8
19	–	1H	2,4,5,8
20	–	1H	4,5
21	–	1H 1T	4,5,6,7,8
22	–	1T	3,4,5,7,8

*Number of transcripts found in head (H) and thorax (T).
†Sites numbered as in Table 6.

between head and thorax or between aabys and rspin strains ($P > 0.05$) (Tables 6 and 7) and led us to exclude A-to-I RNA editing as the cause of spinosad resistance in house flies. Other putative A-to-I editing sites (outside exon 5 and one site in exon 6) were not investigated because of their proximity to intron/exon boundaries and the large size of the introns (for which we have no sequence information).

The A-to-I RNA editing in nAChR α6 subunit genes varies among species of insects. The greatest number of edited sites and the highest frequency of editing are found in the house fly and in *D. melanogaster*. At the other extreme of the scale is *An. gambiae*, where RNA editing was not detected in *Agamα6* (Jones *et al.*, 2005). An intermediate species appears to be *H. virescens* (Grauso *et al.*, 2002). While there was some editing detected in *H. virescens*, no editing was detected at sites 1, 2 and 7 in the α7-2 gene, even though site 7 was edited at a high frequency in *M. domestica* and in *D. melanogaster* (Grauso *et al.*, 2002). A-to-I RNA editing has also been reported for other nAChR subunits of *D. melanogaster* (Hoopengardner *et al.*, 2003).

Expression of Mdα6

Expression of *Mdα6* in the head, thorax and abdomen of aabys adult flies was investigated using quantitative real-time PCR. The *Mdα6*-specific primers were located at the C-terminal of TM3 and the linker of TM3 and TM4, so we could detect all the isoforms characterized in this study. The detected copy number was 21,536 ± 3470 (mean ± SE), 445 ± 60 and 17 ± 3 in the head, thorax and abdomen, respectively (Fig. 2A). *Mdα6* expression was 1300- and 26-fold higher in the head and thorax, respectively, relative to the abdomen. This pattern is consistent with the idea that *Mdα6* is expressed in the central nervous system (CNS) of house flies because the CNS of adult house flies is highly specialized, consisting of a cephalic ganglion (a complex of brain and suboesophageal ganglia) in the head, and a thoracic compound ganglion (a fusion of all the thoracic and abdominal ganglia) in the thorax and no ganglion in the abdomen (Hewitt, 1914). This expression pattern is greatly similar to that of house fly *Mdα2* (Gao *et al.*, 2007). There was no significant difference in the expression of *Mdα6* between spinosad-susceptible and resistant strains (Fig. 2B), suggesting that differences in expression of *Mdα6* are not responsible for resistance.

Expression of nAChRs changes considerably during development and aging (Gotti & Clementi, 2004). We did not examine the temporal expression of *Mdα6* in the present study. However, the expression of its orthologue, *Dα6* was investigated in different developmental stages of *D. melanogaster* and found to be most abundant in embryos (100%) and then to decrease to 40% in both the larvae and adults (Grauso *et al.*, 2002). A similar temporal expression pattern was also found in rats and humans (Gotti & Clementi, 2004).

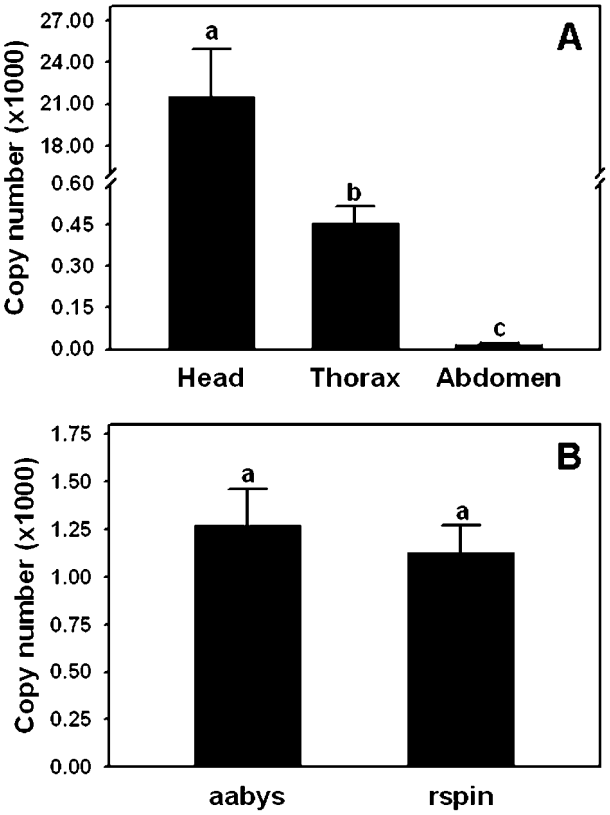

Figure 2. Expression of *Mdα6* in different tagmata (A) and whole bodies (B) of adult house flies measured by quantitative real-time PCR (qRT-PCR). Copy number is per 360 ng of total RNA (amount used in each reaction for the qRT-PCR). Error bars represent standard error of the means of three replicates. Different letters with the bars indicate that the means are significantly different ($P < 0.05$) in Tukey's test.

Linkage analysis

A 188-bp PCR product containing intron 10 of *Mdα6* was amplified from genomic DNA of individual flies (17 aabys and nine OCR). Three alleles were found and denoted as A (accession no. DQ498139), B (DQ498140) and C (DQ498141). Alleles A and B were found only in aabys, and allele C only in OCR. Genotyping the backcross [aabys female × F_1 male (aabys female × OCR male)] offspring showed that allele C was present (as a heterozygote) only in individuals that were wild-type for autosome 1 (+/*ac*; *ar/ar*; *bwb/bwb*; *ye/ye*; *snp/snp*) (Table 8). Thus, unambiguously, the *Mdα6* gene resides on autosome 1. This determination is supported by *D. melanogaster*/*M. domestica* homology maps (Foster *et al.*, 1981).

Conclusions

It was recently shown that deletion of *Dα6* results in a strain of *D. melanogaster* that is resistant to spinosad (Orr *et al.*, 2006; Perry *et al.*, 2007). Thus, *Dα6* is clearly necessary for the toxic action of spinosad to be manifest. The rspin strain of house fly is highly resistant to spinosad. This resistance is monofactorial, linked to autosome 1, highly recessive and could not be overcome with synergists, leading to conclusion that the resistance is because of target site insensitivity (Shono & Scott, 2003). If changes in *Mdα6* were responsible for spinosad resistance, we would expect all transcripts in the rspin strain to be different from aabys. However, our results show no evidence of a selective sweep associated with the *Mdα6* locus in rspin. Thus, we conclude that changes in *Mdα6* are not responsible for spinosad resistance in the house fly. *D. melanogaster*/*M. domestica* homology maps (Foster *et al.*, 1981) suggest that *α5* and *β3* orthologous genes should be found on autosome 1 in the house fly (i.e. the same linkage group as spinosad resistance). Comparison of these two genes between resistant and susceptible strains may provide insight as to the molecular basis of spinosad resistance in the house fly.

Table 8. Linkage analysis of *Mdα6* in the backcross progeny [aabys female × F_1 male (aabys female × OCR male)]

Genotype	Individuals heterozygous for allele C/total*
+/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ye*/*ye*; *snp*/*snp*	3/3
ac/*ac*; +/*ar*; *bwb*/*bwb*; *ye*/*ye*; *snp*/*snp*	0/3
ac/*ac*; *ar*/*ar*; +/*bwb*; *ye*/*ye*; *snp*/*snp*	0/3
ac/*ac*; *ar*/*ar*; *bwb*/*bwb*; +/*ye*; *snp*/*snp*	0/5
ac/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ye*/*ye*; +/*snp*	0/6

*The *Mdα6* allele C was found in the OCR, but not in the aabys strain.

Experimental procedures

House flies

Three strains of house flies were used: aabys (insecticide susceptible, with the recessive morphological markers *ali-curve* (*ac*), *aristapedia* (*ar*), *brown body* (*bwb*), *yellow eyes* (*ye*) and *snipped wings* (*snp*) on autosomes 1, 2, 3, 4 and 5, respectively), OCR (cyclodiene resistant) and rspin (spinosad resistant with the same five markers as aabys). The rspin strain is genetically similar to the aabys strain, except for the spinosad resistance locus on autosome 1 (Shono & Scott, 2003). Flies were maintained in the laboratory as previously described (Scott *et al.*, 2000).

mRNA isolation and RT-PCR

Heads of (< 1-day-old) aabys and rspin flies were used for isolation of mRNA using a QuickPrep™ Micro mRNA purification kit (Amersham Biosciences, Piscataway, NJ, USA). First strand cDNA was synthesized from 450 ng of mRNA by priming with oligo(dT) using SuperScript™ III first strand synthesis system for RT PCR kit (Invitrogen, Carlsbad, CA, USA). The degenerate primers MaF2 and MaR1 (Table 1) were designed based on the conserved regions (MKFGSWTYD and FNCIMFMVA, respectively) of previously reported insect nAChR α subunit genes. A 428-bp fragment was amplified from aabys flies using Advantage® 2 polymerase Mix

(BD Biosciences Clontech Laboratory, Mountain View, CA, USA) in a total volume of 25 μl. The PCR thermal programme consists of one cycle of 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 55 °C for 30 s and 68 °C for 1 min and a final extension at 68 °C for 10 min. PCR products were directly cloned into a pCR®2.1-TOPO® vector and transformed into TOP10 cells using a TOPO-TA Cloning® kit (Invitrogen). DNA sequencing was performed using an ABI PRISM 3730 DNA analyser (Applied Biosystems, Foster City, CA, USA) at the Biotechnology Resource Center at Cornell University, Ithaca, NY.

Rapid amplification of cDNA ends (RACE) and cloning of the open reading frame (ORF)

A SMART™ RACE cDNA amplification kit (BD Biosciences, Palo Alto, CA, USA) was used for 3′- and 5′-RACE. The 3′- and 5′-RACE-ready cDNA was synthesized with 1 μg of mRNA. A seminested PCR strategy was used for both 5′- and 3′-RACE. The primers Ma5RaceR1 and Ma3RaceF3 (Table 1) were used in the primary PCR using the Advantage® 2 PCR system provided in the kit for 5′- and 3′-RACE, respectively. PCR products were analysed on a 1% agarose gel. The target bands were excised and DNA was purified with a QIAEX®II gel extraction kit (Qiagen Sciences, Valencia, CA, USA). Purified PCR product (1 μl) and Ma5RaceR2 or Ma3RaceF1 (Table 1) were used in the second seminested PCR using 2× Reddy Mix™ PCR master mix (ABgene House, Epsom, Surrey, UK). The following thermal programmes were used: for 5′-RACE, one cycle of 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 66 °C for 30 s and 72 °C for 2 min and a final extension at 72 °C for 10 min for the primary PCR and one cycle of 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 1.5 min and a final extension at 72 °C for 10 min for the seminested PCR. For 3′-RACE, one cycle of 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 3 min and a final extension at 72 °C for 10 min for the primary PCR and one cycle of 95 °C for 1 min, 35 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 2 min and a final extension at 72 °C for 10 min for the seminested PCR. PCR product purification, cloning and sequencing were performed as described above.

A fragment containing the ORF of *Mdα6* was amplified from 5′-RACE-ready cDNA with the primers Ma6F and Ma6R (Table 1, Fig. 1) using Advantage® 2 polymerase mix (BD Biosciences, Mountain View). The PCR programme was one cycle of 95 °C for 1 min, 30 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 2 min and a final extension at 72 °C for 10 min. PCR products were directly cloned into the pCR®2.1-TOPO® vector (Invitrogen). Transformation and sequencing were performed as above.

Sequence analysis

N-glycosylation and phosphorylation sites were predicted using Prosite (Hulo *et al*., 2006). Transmembrane domains were predicted by PRED-TMR (Pasquier *et al*., 1999). The signal peptide was predicted using SignalP 3.0 (Bendtsen *et al*., 2004). Multiple sequence alignment was performed using CLUSTAL W using the Gonnet matrix (Megalign program, DNASTAR Inc., Madison, WI, USA).

RNA editing and alternative splicing in different body parts

Total RNA and genomic DNA extraction from a head and thorax was performed using TRIzol® reagent (Invitrogen). DNA contamination in the total RNA was eliminated by treating the samples with TURBO DNA-*free*™ (Ambion, Austin, TX, USA). All procedures were carried out according to the manufacturer's instructions in a final volume of 22 μl for RNA and 50 μl for DNA.

PCR fragments were amplified from RNA using Titanium™ one-step RT-PCR kit (BD Biosciences, Mountain View) using the primers Ma6F and Ma6R (Table 1) and 2 μl RNA in a total volume of 25 μl and the following thermal cycler programme: 50 °C for 60 min followed by 94 °C 5 min, 35 cycles of 94 °C for 30 s, 64 °C for 30 s and 68 °C for 2.5 min and a final extension at 68 °C for 10 min. The PCR product was purified using a QIAEXII gel purification kit (Qiagen, Valencia, CA, USA), cloned and sequenced as described above. The exon V genomic fragment was amplified using Advantage® 2 polymerase mix (BD Biosciences, Mountain View) with primers gM6VF1 and gM6VR1 (Table 1) and the following thermal cycler programme: 95 °C for 1 min, and 32 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s and a final extension of 72 °C for 10 min. The PCR product was purified using a QIAEXII gel purification kit (Qiagen, Valencia) and sequenced directly. The RT-PCR products were cloned and sequenced as described above. Three flies were used for each strain and 10 clones from each head and thorax were sequenced. Sequences were analysed using Lasergene 6 software (DNASTAR, Inc.) and were also inspected manually. Exons were identified based on the *Mdα6* orthologue from *D. melanogaster*, *Dα6*. The expression of each isoform (%) was determined in 10 clones for each fly. Each percentage was converted to an arcsin value and analysed by Student's *t*-test.

Real-time PCR quantification

Total RNA was isolated from heads, thoraces and abdomens, respectively, of aabys flies (< 1-day-old), or from whole bodies of aabys and rspin flies (< 1-day-old) (for comparison between strains) using TRIzol® reagent (Invitrogen) and treated with DNase (Ambion). First strand cDNA was synthesized in a total 100 μl reaction volume using 18 μg of the DNase-treated RNA with TaqMan Reverse transcription reagents using random hexamers (Applied Biosystems). PCR of 20 μl per reaction was performed using 2 μl of the cDNA samples, power SYBR® Green PCR master mix (Applied Biosystems) and the specific primers qM6F1 and qM6R1 (Table 1) for *Mdα6* using an ABI PRISM 7900 HT Sequence Detection System with Sequence Detection software (version 2.1) (Applied Biosystems). All procedures were conducted according to the manufacturer's instructions. The PCR programme consisted of 50 °C for 2 min and 95 °C for 10 min for initiation, 40 cycles of 95 °C for 15 s and 60 °C for 1 min, followed by 95 °C for 15 s, 60 °C for 15 s and 95 °C for 15 s for melting curve analysis. The PCR-specific amplification was assessed by the melting curve analysis and electrophoresis of the PCR products on 1.8% agarose gel. External standard curves were constructed using six 10-fold serial dilutions of plasmids (pCR®2.1-TOPO® vector containing the *Mdα6* ORF) starting from 0.04 ng/μl. This analysis was replicated three times. Gene expression levels were analysed with a randomized complete block design using one-way ANOVA. Gene copy numbers were transformed to log values and significant differences were determined using Tukey's test at $P = 0.05$ (PROC GLM, SAS Institute, 2001).

Linkage analysis

Linkage analysis was performed by the association of *Mdα6* polymorphisms (identified in the aabys and OCR strains) with the

five recessive markers of the aabys strain (Kozaki *et al*., 2002). Female aabys were crossed with male OCR to produce F_1 flies heterozygous for all five autosomes. F_1 males were then backcrossed to aabys females and the offspring were sorted according to phenotype. Five phenotypes were used to conduct the linkage analysis. These phenotypes were heterozygous at only one chromosome, as indicated by the absence of a recessive morphological marker. Flies that were heterozygous for each of autosomes one through five were denoted as +/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ye*/*ye*; *snp*/*snp*, *ac*/*ac*; +/*ar*; *bwb*/*bwb*; *ye*/*ye*; *snp*/*snp*, *ac*/*ac*; *ar*/*ar*; +/*bwb*; *ye*/*ye*; *snp*/*snp*, *ac*/*ac*; *ar*/*ar*; *bwb*/*bwb*; +/*ye*; *snp*/*snp* and *ac*/*ac*; *ar*/*ar*; *bwb*/*bwb*; *ye*/*ye*; +/*snp*, respectively.

Genomic DNA was extracted from 17 aabys and nine OCR parental individuals, 11 F_1, and at least three individuals from each previously stated backcross phenotype. A genomic DNA fragment (188-bp in aabys and 192-bp in OCR) was amplified using the 2× ReddyMix™ PCR master mix (ABgene House) with the primers gM6F5 and gM6R4 (Table 1) using the following thermal cycler programme: one cycle of 95 °C for 1 min, 30 cycles of 95 °C for 30 s, 64 °C for 30 s and 72 °C for 30 s, and a final extension at 72 °C for 7 min. The PCR product was purified using a QIAquick PCR purification kit (Qiagen) and then sequenced as mentioned above.

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