Aedes aegypti dopa decarboxylase: gene structure and regulation

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Abstract

Dopamine decarboxylase converts L-dopa to dopamine, a precursor molecule for diverse biological activities in insects including neurotransmission and a variety of tanning reactions required for development, reproduction and defence against parasites. Herein, we report the cloning and sequencing of the Aedes aegypti Ddc gene, including 2.1 kb of the upstream promoter region. The transcribed region of the gene spans more than 16 kb and contains five exons. In situ hybridization localizes the blood-meal-induced ovarian transcription of this gene to the follicular epithelial cells surrounding individual oocytes. Ovary tissue transcription of Ddc is increased in response to injection of 20-hydroxyecdysone to levels equal to those observed for blood-fed controls, however coinjection with the translational inhibitor cycloheximide negates the effect, observed for blood-fed controls, however coinjection with 20-hydroxyecdysone to levels equal to those observed for blood-fed controls. Therefore, the effect of 20-hydroxyecdysone on the transcription of Ddc is dependent on translational activity. The transcriptional upregulation of Ddc in ovaries of female mosquitoes following ingestion of a blood meal has been attributed to induction by the insect hormone 20-hydroxyecdysone (20E) that is released as a result of the hormonal cascade initiated by blood feeding (Schlaeger & Fuchs, 1974a, b).

In Drosophila melanogaster, production of 20E in the haemolymph peaks five times during larval moulting and pupariation, and each increase coincides with high levels of DDC enzyme activity, indicative of transcriptional control of expression. Several ecdysone response elements (EcREs) have been empirically characterized for D. melanogaster genes (Antoniewski et al., 1993; Vögtli et al., 1996). Additionally, Wang et al. (1998) studied the capacity of the Ae. aegypti ecdysone receptor complex, a heterodimer of ecdysone receptor and Ultraspiracle, to bind a variety of possible EcREs derived from the ecdysone-responsive consensus half-site sequence, AGGTCA. In D. melanogaster, larval epidermal tissue rapidly responds to the addition of 20E, however recent studies indicate that hormonal induction of Ddc in third-instar larva is mediated by a member of the Broad-Complex (BR-C) early gene transcription factor family (Hodgetts et al., 1995; Bayer et al., 1997).

The differential, tissue-specific regulation of Ddc in D. melanogaster and Manduca sexta has been studied by experimental promoter analysis (Scholnick et al., 1986; Konrad & Marsh, 1987; Hodgetts et al., 1994; Hruma et al., 1995) and several regulatory elements required for tissue-specific expression have been identified (Bray & Kafatos, 1993). Aedes aegypti Ddc contains five exons. The transcribed region of the gene spans more than 16 kb and includes 2.1 kb of the upstream promoter region. These elements are discussed in the context of common insect Ddc regulatory mechanisms.

Keywords: Aedes aegypti, dopa decarboxylase, gene structure, gene expression, ovary development.

Introduction

Dopamine decarboxylase (DDC; EC 4.1.1.28) contributes to diverse physiological events in insects. The product of this enzyme’s activity, dopamine, acts as an intermediate substrate for various tanning and melanization reactions, and also is a neurotransmitter (Eveleth et al., 1986; Scholnick et al., 1986). In mosquitoes the biochemical pathway of tanning and melanization reactions has been well characterized, and likely is initiated with the action of phenol oxidase (PO) on the substrate tyrosine to produce L-dopa (3,4-dihydroxyphenylalanine) that subsequently is converted by DDC to dopamine (Li & Christensen, 1993). Studies using the yellow fever mosquito, Aedes aegypti, demonstrated the requirement for DDC activity in tanning of the egg chorion, an event that is initiated by the ingestion of a blood meal in anautogenous mosquitoes (Li & Christensen, 1993; Li, 1994). Following oviposition, the chorion blackens to form a protective outer layer that allows the eggs to withstand an indeterminate desiccation period required in this species’ life cycle. We previously reported that the Ddc gene is transcriptionally upregulated in the ovaries of female mosquitoes following ingestion of a blood meal (Ferdig et al., 1996). This increased transcription correlates with a burst of DDC activity in the ovaries and has been attributed to induction by the insect hormone 20-hydroxyecdysone (20E) that is released as a result of the hormonal cascade initiated by blood feeding (Schlaeger & Fuchs, 1974a, b).

In Drosophila melanogaster, production of 20E in the haemolymph peaks five times during larval moulting and pupariation, and each increase coincides with high levels of DDC enzyme activity, indicative of transcriptional control of expression (Clark et al., 1986). Several ecdysone response elements (EcREs) have been empirically characterized for D. melanogaster genes (Antoniewski et al., 1993; Vögtli et al., 1996). Additionally, Wang et al. (1998) studied the capacity of the Ae. aegypti ecdysone receptor complex, a heterodimer of ecdysone receptor and Ultraspiracle, to bind a variety of possible EcREs derived from the ecdysone-responsive consensus half-site sequence, AGGTCA. In D. melanogaster, larval epidermal tissue rapidly responds to the addition of 20E, however recent studies indicate that hormonal induction of Ddc in third-instar larva is mediated by a member of the Broad-Complex (BR-C) early gene transcription factor family (Hodgetts et al., 1995; Bayer et al., 1997).

The differential, tissue-specific regulation of Ddc in D. melanogaster and Manduca sexta has been studied by experimental promoter analysis (Scholnick et al., 1986; Konrad & Marsh, 1987; Hodgetts et al., 1994; Hruma et al., 1995) and several regulatory elements required for tissue-specific expression have been identified (Bray & Kafatos, 1993). Aedes aegypti Ddc contains five exons. The transcribed region of the gene spans more than 16 kb and includes 2.1 kb of the upstream promoter region. These elements are discussed in the context of common insect Ddc regulatory mechanisms.

Keywords: Aedes aegypti, dopa decarboxylase, gene structure, gene expression, ovary development.
In each case, the regulation of this enzyme seems to occur at the level of transcription. The *Drosophila Ddc* gene produces two different transcripts, one in the epidermis and another in the central nervous system (CNS), which can be accounted for by an alternative splicing mechanism in which all four exons are involved in neuronal transcription, but the second exon is spliced out of the epidermal message (Eveleth et al., 1986; Morgan et al., 1986).

Catecholamine metabolism in mosquitoes also plays a role in the melanotic encapsulation defence response against parasites. Both PO and DDC activities are increased in haemolymph of mosquitoes undergoing defence reactions wherein a darkened, catecholamine-derived capsule is formed around filarial parasites in the haemocoel of resistant individuals (Chen & Laurence, 1987; Nappi et al., 1992; Zhao et al., 1995). Understanding the control of the catecholamine metabolizing pathways in these diverse biological activities in mosquitoes requires knowledge of how the requisite enzymes are regulated. In order to begin to elucidate the basic mechanisms underlying DDC activity in mosquitoes, we have isolated and characterized the complete *Ddc* gene including the upstream sequence. Additionally, we have tested the role of 20E in regulating *Ddc* transcription.

### Results

#### Gene organization

A single 41-kb cosmid clone, 112.3G2, was isolated from an *Ae. aegypti* genomic library by polymerase chain reaction (PCR) screening using primers specific to the previously described *Ae. aegypti Ddc* cDNA (Ferdig et al., 1996). Sequence data derived from this clone allowed us to determine that it contains the entire *Ddc* gene as well as extensive 5' flanking DNA. The transcriptional unit spans approximately 16 kb and consists of five exons (Fig. 1). The *Ddc* gene contains four introns; three small introns (1, 3 and 4) that were completely sequenced (Table 1).

![Figure 1](image1.png)

**Figure 1.** Organization of the *Ae. aegypti Ddc* gene. (A) Intron/exon organization. (B) Restriction sites occurring in the sequenced portion of the *Ae. aegypti* Ddc gene (H = HindIII, B = BamHI, X = XbaI). Hatched regions identify untranslated exon sequences. Black boxes denote translated regions. Arrows under the schematic identify the 9100 bases that were sequenced. The length of intron 2 is approximately 14 kb as determined by sequencing, polymerase chain reactions and restriction digests.

![Figure 2](image2.png)

**Figure 2.** Autoradiograph of primer extension reaction using total RNA template extracted from ovaries of blood-fed mosquitoes. Lane 1 was loaded with end-labelled dephosphorylated ØX174 digested with HindIII. Lane 2 contains the primer extension reaction. The arrow denotes the predominant 97-bp primer extension product observed in four separate experiments.

### Table 1. Sizes of exons and introns and splice junction sequences of the *Aedes aegypti Ddc* gene. Exon sequences are in capital letters, and intron sequences are in lower case.

<table>
<thead>
<tr>
<th>Exon</th>
<th>Junction sequences</th>
<th>Intron</th>
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and one extremely large intron (2; > 14 kb). Approximately 3.5 kb from each end of intron 2 was sequenced (Fig. 1). PCR, optimized for long-range amplification, along with restriction digests and Southern blotting were used to confirm the size of the remaining unsequenced region of the second intron.

Four repeats of the primer extension procedure generated a prominent product at 97 bp (Fig. 2). Reactions under a variety of optimization conditions produced several other bands that incorporated less label, including two possible secondary starts of transcription at 85 and 104 bp (Fig. 3). However, the larger of these potential secondary sites was not present in messenger RNA (mRNA) from various tissues as determined by Northern blot analysis using a probe specific for the segment immediately 5′ to the primary transcription start site. The major primer extension product specifies a start of transcription that is 85 bp downstream of a standard TATA box and 27 and 5 bp from two consensus cap site initiator sequences (Fig. 3; Cherbas & Cherbas, 1993). The 5′-untranslated region extends 102 bp 5′ of the initiation codon. The mRNA size deduced from this proposed start of transcription is 1.88 kb, somewhat smaller than the 2.1 kb size estimated from Northern blots of ovary-derived transcripts, a discrepancy that could be accounted for by the presence of a poly(A) tail (Ferdig et al., 1996). The positions of introns 1 and 2 are conserved among insects as determined by comparisons with gene structure for D. melanogaster and M. sexta (Eveleth et al., 1986; Hiruma et al., 1995), however the first intron for both Ae. aegypti and M. sexta corresponds to intron 2 in D. melanogaster.

Transcriptional studies

In situ hybridization was used to localize ovary tissue transcription to the cells comprising the follicular epithelium (Fig. 4A, E and F).
Figure 4. Cellular localization of Ddc mRNA in developing ovaries. (A, B) Thirty-six-hour post-blood-fed (pbf) ovary sections hybridized with a biotin-labelled probe specific to Ddc and a non-specific adenovirus probe, respectively. (C, D) Forty-eight- and 60-h pbf ovary sections hybridized with a Ddc-specific probe, and illustrate the decrease in the number of transcripts in the later stages of oocyte development. (E–H) Two magnifications of ovary sections taken from 36- (E–F) and 72-h pbf (G–H) and stained with haematoxylin/eosin. Panels E and F should be compared with panel A to identify the layer of follicular epithelial cells transcribing this gene. Panels G and H show the degeneration of the follicular epithelium in the late stages of egg-batch maturation.
been inoculated with either extract from blood-fed, decapitated females that had for levels of transcription. Co-inoculation of 20E with cycloheximide determined whether this increased activity was a result of female mosquitoes resulted in a threefold increase of DDC activity in ovary extracts (Schlaeger & Fuchs, 1974c). To determine whether this increased activity was a result of transcriptional activation of the Ddc gene, total RNA was extracted from blood-fed, decapitated, saline-injected; Lane 3, blood-fed, decapitated, injected with 0.5 μl containing 1 μg 20E; Lane 4, 24 h blood-fed controls; and Lane 5, coinjection of cycloheximide with 20E. The average pixel number in each treatment was determined using NIH Image program and presented below the Northern blot as the percentage increase in DDC transcription as compared with sugar fed controls; Lane 2, blood-fed, decapitated, saline-sugar-fed control mosquitoes (Lane 1).

Figure 5. Autoradiograph of a Northern blot demonstrating the effect of 20E injection into blood fed, decapitated female Ae. aegypti on ovarian Ddc transcription. Each lane contains two oocyte pair-equivalents of total RNA: Lane 1, sugar fed controls; Lane 2, blood fed, decapitated, saline-injected; Lane 3, blood fed, decapitated, injected with 0.5 μl containing 1 μg 20E; Lane 4, 24 h blood fed controls; and Lane 5, coinjection of cycloheximide with 20E. The average pixel number in each treatment was determined using NIH Image program and presented below the Northern blot as the percentage increase in DDC transcription as compared with sugar-fed control mosquitoes (Lane 1).

decreases through 60 h from its peak at 36 h, coinciding with the deterioration of the follicle cells in the maturing, post-blood-fed oocytes (Fig. 4C, D, G and H). Previous work demonstrated that injection of 20E into female mosquitoes resulted in a threefold increase of DDC activity in ovary extracts (Schlaeger & Fuchs, 1974c). To determine whether this increased activity was a result of transcriptional activation of the Ddc gene, total RNA was extracted from blood-fed, decapitated females that had been inoculated with either Ae. aegypti physiological saline or 20E (Fig. 5). Ovary tissue from saline-injected individuals transcribed this gene at levels slightly higher than non-blood-fed controls. Transcription levels from 20E inoculated mosquitoes were similar to those of blood-fed controls. Five repetitions of this experiment confirmed this pattern of transcription. Co-inoculation of 20E with cycloheximide resulted in Ddc message intensity similar to that observed for levels of Ddc transcription in non-blood-fed and saline-inoculated controls (Fig. 5).

Sequence analysis
Approximately 2.1 kb of DNA 5′ to the start of transcription was sequenced (Fig. 3). A search for known regulatory motifs was conducted on this upstream region and on the sequenced regions of the four introns. Figure 3 denotes the regions of the four introns. Figure 3 denotes the region of interest. The sequence from -3 to -300 bp of the start of transcription was determined. Sequences were analyzed with respect to the EcRE half-site, TGACCT, and the WRKY motif, WNYTAA. The results show a significant enrichment for WRKY sites in the upstream region of Ddc.

Table 2. Potential regulatory motifs observed in the 5′ promoter and untranslated regions of Ae. aegypti Ddc. The Ae. aegypti ecdysone responsive element (EcRE) (RGKKSNNNGNNYK) was inferred from alignment of consensus Ae. aegypti EcREs from three vitelline envelope genes known to be directly regulated by 20E (Edwards et al., 1998). The identified Ae. aegypti sequences are presented (5′–3′, plus strand) along with the percentage similarity to the query motif and position with respect to the start of transcription. These same Ae. aegypti sequences are compared to the reported D. melanogaster EcRE consensus, ROKTSA-NTGMY (Antoniwski et al., 1993). The EcRE half-site, TGACCT was reported by Charbas et al. (1991). Two clusters of BR-C-gene family zinc-finger consensus binding sites, Z1, Z2 and Z4 (YWATIANY, ACTAYAW and WNTAAYTA, respectively (Hodgetts et al., 1995) are included and are grouped according to the clusters in which they occur.

<table>
<thead>
<tr>
<th>Motif</th>
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<th>% Similarity</th>
<th>Position</th>
<th>Strand</th>
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<td>–333</td>
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Discussion

Aedes aegypti Ddc gene spans 16 kb and consists of five exons (Fig. 1, Table 1). The major primer extension product defines a start of transcription that is consistent with the observed mature transcript size (Ferdig et al., 1996), but is 78 bp downstream of a putative TATA element (Fig. 3). The absence of a TATA box at –30 could indicate that the Ae. aegypti Ddc gene uses a TATA-less promoter (Huber et al., 1998; Santoni et al., 1998). Two consensus arthropod cap site initiator sequences (Cherbas & Cherbas, 1993) are located just upstream of the proposed start of transcription, one at position –27 and the other at –5, 50 and 72 bp downstream from the putative TATA box, respectively. The position of the upstream cap site sequence is 10–20 bp farther from the TATA box than typically separates these two sequences (Cherbas & Cherbas, 1993).

The positions of the first two introns in the Aedes Ddc gene are shared with intron positions of the two other described insect Ddc genes from D. melanogaster and M. sexta (Eveleth et al., 1986; Hiruma et al., 1995), however these introns correspond to the second and third introns in Drosophila. The 5’ region of D. melanogaster Ddc is organized such that the second exon is spliced out for expression in epidermal tissue (Morgan et al., 1986; Scholnick et al., 1986). Embryonic developmental profiles using Northern analyses of total RNA from D. mela-

logaster identified several different sized bands resulting from high levels of intron-containing RNAs of unusually long-lived splicing intermediates (Morgan et al., 1986). The Ae. aegypti intron/exon organization resembles that of M. sexta, and the upstream intron/exon junctions of the Ae. aegypti and M. sexta Ddc genes cannot accommodate a similar splicing mechanism to that of the fruit fly (Fig. 1, Table 1; Hiruma et al., 1995). In addition, Hiruma et al. (1995) demonstrated that a single transcript is produced in M. sexta, regardless of tissue and timing of transcription. We could observe no obvious transcript size variation on Northern blots of mRNA prepared from various Ae. aegypti tissues, including RNA associated with the larval epidermis, neurological tissue and egg chorion (data not shown). However, because comparison of lanes on a Northern blot does not provide proof of a single-sized transcript, it can only be postulated that transcriptional processing in Ae. aegypti is more analogous to that of M. sexta than D. melanogaster.

Ddc belongs to a group of mosquito genes whose transcription is upregulated in either the fat body or the ovaries immediately after ingestion of a blood meal. Specifically, these genes are involved in vitellogenesis and vitelline envelope formation (Lin et al., 1993). Understanding the regulation of these genes requires specific information as to the cellular site of transcript production in the complex ovary tissue. Figure 4 identifies the spatial distribution of Ddc transcription in 36–60 h post-blood-fed ovaries, and localizes the mRNA to the follicular epithelial cell layer that surrounds each developing oocyte. This cell type has been previously described to produce blood meal-inducible transcripts (Lin et al., 1993). These authors reported that 20E directly regulates transcription of a gene related to the D. melanogaster vitelline membrane protein genes. More recently, work on these same genes identified an anterior–posterior specific transcription pattern in the follicle. This pattern is not evident for Ddc transcript distribution, appearing instead as a uniform layer surrounding the oocyte (Edwards et al., 1998).

Because previous work demonstrated that signals from the mosquito head were required for ovarian development, and because injection of the insect hormone 20E into the haemocoel of Ae. aegypti individuals resulted in increased DDC activity (Schlaeger & Fuchs, 1974a,b,c), we evaluated whether this increased activity is attributable to transcriptional activation. Our studies differed from those of Schlaeger and Fuchs in that our experimental mosquitoes were blood-fed and decapitated prior to injection of saline or 20E. This approach provides a closer approximation of the physiological events associated with blood feeding and eliminates the possibility that other head factors could be induced by the introduction of exogenous ecdysone. We also used a tenfold lower concentration of hormone in a manner consistent with studies of the role of 20E in regulating egg development (Lin et al., 1993). Figure 5 demonstrates that blood feeding, followed immediately by decapsulation and subsequent saline inoculation, resulted in a slight induction of Ddc transcription above non-blood-fed control levels, however injection of 20E immediately following decapsulation stimulated transcription similar to the level observed for the blood-fed control mosquitoes. These studies indicate that maximal Ddc transcription in response to blood feeding requires the action of 20E, however egg development genes in mosquitoes have been shown to be under both direct and indirect hormonal control. For instance, studies by Lin et al. (1993) showed that injection of 20E into blood-fed, decapitated females resulted in transcription of the vitelline membrane genes by the ovary, and that this transcriptional activation was not inhibited by cycloheximide, demonstrating that no intervening protein production is required for this hormonal regulation. Conversely, ecdysone was unable to induce transcription of the yolk protein genes, Vg and VCP, in cultured fat body when the translational inhibitor was included (Deitsch et al., 1995). This result suggests that ecdysone induces expression of other transcription factor(s) that, in turn, upregulate yolk protein expression. Consequently, we conducted our decapsulation/injection experiments in the presence of coinjected cycloheximide. In the presence of this translational inhibitor, Ddc transcript levels do not undergo the same increase observed when hormone is injected alone, thereby demonstrating that the ovarian response to 20E by Ddc is indirect (Fig. 5).
Expression of Ddc in the epidermis and neurones of D. melanogaster is controlled by independent cis regulatory elements, because deletion analyses have identified regions that affect the specificity of tissue expression (Scholnick et al., 1986; Bray & Kafatos, 1991). For example, alteration of 2 bp in a cis element proximal to the start of transcription (~57 to ~72) results in epidermal expression. The association of larval epidermal Ddc expression with ecdysteroid levels has been the focus of studies on hormonal regulation of late-stage differentiation events (Kraminsky et al., 1980; Clark et al., 1986). This positive and temporally precise transcriptional regulation of Ddc expression in third-instar D. melanogaster larvae recently was shown to require the binding of a medialing transcription factor from the 20E-induced Broad Complex gene family in close proximity to consensus EcREs (Hodgetts et al., 1995). Evidence from Drosophila studies, (Clark et al., 1986). This positive and temporally precise C- gene encoded zinc-finger-binding sequences (BR-C late-stage differentiation events (Kraminsky et al., 1980; Clark et al., 1986). This positive and temporally precise zinc-finger isoforms, Z1– 4 were used in footprinting analysis of Drosophila Ddc to identify four consensus BR- (T able 2). Recombinant BR-C proteins carrying four different zinc-finger isoforms, Z1– 4). T wo clusters of these consensus binding sites were identified in the cis  regulatory region (T able 2). Four additional groups, consisting of a total of fourteen sites, were present in the intronic sequences. Clustering of sites and the presence of a proximal EcRE, as reported here, also characterize the empirically defined BR-C-responsive regions in Drosophila (Hodgetts et al., 1995), consequently the authors proposed that binding of the BR-C-gene family members relaxes the DNA around the EcRE and optimal transcription requires binding of these additional edcsyne–responsive transcription factors.

This work defines an indirect role for 20E in blood meal-induced ovariian transcription of Ddc by the follicular epithelial cells. Although specific understanding of Ddc regulatory mechanisms awaits detailed promoter analysis and binding assays, data presented here describe the organization of the Aedes Ddc gene and propose potential regulatory events that could direct its expression. Knowledge of this gene’s transcriptional control will provide insight into critical pathways by which Ae. aegypti mosquitoes tan their eggs and kill filarial parasites.

Experimental procedures

Animals

Mosquitoes (black-eyed Liverpool strain of Ae. aegypti) used in this study were reared according to the methods described by Christensen & Sutherland (1984). Blood feeding required for RNA expression analysis was accomplished by providing an anaesthetized mouse to 3–5-day-old females housed in cartoon covered with marquisette. For experiments requiring hormone treatments, 1 μg of 20E in 0.5 μl of Aedes saline containing penicillin and streptomycin was injected into the thorax of 3–5-day-old females as described by Lu & Hagedorn (1986). The neck wound was sealed with parafilm wax prior to injection of decapitated females (Wheelock et al., 1988). For some experiments, the translational inhibitor, cycloheximide, was conjeced into the mosquito haemocoel by including an additional 0.2 μl of 10−2 M cycloheximide prepared in Aedes saline, as described by Bownes et al. (1987), to the inoculum. The inoculation experiments followed by Northern blotting were repeated three times.

Library screening and sequencing

The Ae. aegypti SuperCos (Stratagene) cosmid library was provided by D. W. Severson (University of Notre Dame). Primers designed to the 5′-most coding sequence of Ae. aegypti cDNA (Ferdig et al., 1996) were used to screen, via PCR, sequentially less-inclusive pools of DNA representing the genomic library, to identify a final ninety-six-well plate containing the positive clone for Ddc. This plate was replicated to a membrane for probing with Ddc to identify the well containing the positive clone. Cosmid DNA was isolated using a large-scale alkaline lysis preparation. The sequencing template was prepared by limited digestion of the cosmid with EcoRI, followed by column purification using Wizard DNA Prep (Promega). Sequencing reactions were performed using the Dye Terminator Cycle Sequencing Kit (ABI, Perkin Elmer). Analysis was performed using the Sequencing Software Module provided with the ABI Prism DNA Sequencer. Primers were designed using the Sequence Assembly and Contig Management Software (Lasergene Sequence Analysis System, DNASTar Inc.).

RNA analysis

Total RNA was prepared using the single step acid guanidinium thiocyanate–phenol–chloroform extraction method (Chomczynski & Sacchi, 1987). For Northern blotting, total RNA was separated in formaldehyde agarose gels as previously described (Sambrook et al., 1989). Concentration of RNA for gel loading was determined by spectrophotometry (OD 260). Total RNA was loaded by ovariace pair equivalents for Fig 5. Blotting and hybridization procedures are described by Ferdig et al. (1996). Ddc message was detected using a 520-bp probe prepared from the 5′ end of cDNA that spanned the first three spliced exons. Gels containing
total RNA from different tissues were run for 16 h at 30 °C prior to blotting in order to maximize the ability to detect subtle size variation in tissue-specific transcripts. The autoradiographs were scanned into Adobe Photoshop and the average pixel value for each lane was calculated using NIH Image software. These values were compared as percentage increase in DDC transcription as compared with sugar-fed controls.

**Primer extension**

Primer extension experiments were performed following methods adapted from Sambrook et al. (1989) using the AMV Reverse Transcriptase Primer Extension System (Promega). A 23-nucleotide long primer (5′-AT TGCATTCTGGGCGCAT TGGAS-3′) was designed to the 5′-terminus of the Ddc-cDNA clone sequence for use in primer extension reactions (Fig. 2). End-labelling with [γ-^32P]ATP (3000 Ci/mmol, 10 mCi/ml) was performed using T4 polynucleotide kinase (Promega). The radiolabelled primer was annealed at 60 °C for 1 h to 30 μg of total RNA isolated from 24 h post-blood-fed ovaries. Extension was carried out at 41.5 °C for 35 min with Superscript RNase H^- Reverse Transcriptase ( Gibco BRL). The products were separated on a denaturing polyacrylamide gel and visualized by autoradiography.

In situ hybridization

Female mosquitoes were anaesthetized on ice at various times following blood feeding, and ovaries were collected by a previously described method (Li & Christensen, 1993) and frozen immediately in liquid nitrogen. The frozen ovaries were embedded in Tissue-Tek II (Lab-Tek Products) and sectioned at –18 °C. The ovary sections contained streptavidin–alkaline phosphatase conjugate and conjugate dilution buffer, respectively. After washing in phosphate-buffered saline (PBS) and in alkaline–substrate buffer, the ovary sections on slides were hybridized with the probe for 4 h. Tissue sections were then overlaid with blocking solution and working conjugate solution that was incubated in NBT/BCIP solution for colour development, washed in PBS, and then examined microscopically and photographed.

**Sequence analysis**

DNA sequence data were analysed using the Sequence Analysis Software Package from the Genetics Computer Group (GCC) (Devereaux et al., 1984). GCC programs used for evaluating the Ddc gene include Map, Gap, Bestfit, and Findpatterns.

**GenBank accession number**

The sequence data presented here has been updated under the original cDNA GenBank Accession number, U271581.

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**References**


