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Aedes aegypti dopa decarboxylase: gene structure and regulation

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Abstract

Dopa 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, indicating an indirect regulatory role for this hormone. Clusters of putative ecdysone-responsive elements and zinc-finger binding domains for the products of *Broad-Complex* gene family are identified in the 5′-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

Dopa 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.*, 1998). 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 larvae 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; Hiruma *et al.*, 1995) and several regulatory elements required for tissue-specific expression have been identified (Bray & Kafatos,

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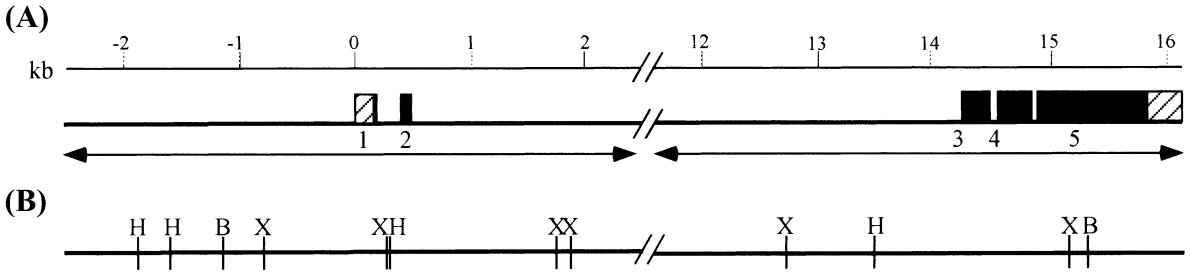

Figure 1. Organization of the *Ae. aegypti Ddc* gene. (A) Intron/exon organization. (B) Restriction sites occurring in the sequenced portion of the *Ae. aegypti Ddc* gene (H = *Hin*dIII, B = *Bam*HI, X = *Xba*I). Hatched regions identify untranslated exon sequences. Black boxes denote translated regions. 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.

1991; Scholnick *et al*., 1986). 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),

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.

Exon		Junction sequences		Intron	
No.	Size	5′-donor	3′-donor	No.	Size
1	179	CTCGT**gt**aat	aac**ag**ACCGA	1	231
2	85	GATCG**gt**aag	tac**ag**CCGTG	2	≈ 14000
3	234	CATGG**gt**aag	tgc**ag**ATTGC	3	60
4	310	ATCTA**gt**aag	cct**ag**ATCAA	4	61
5	1245				

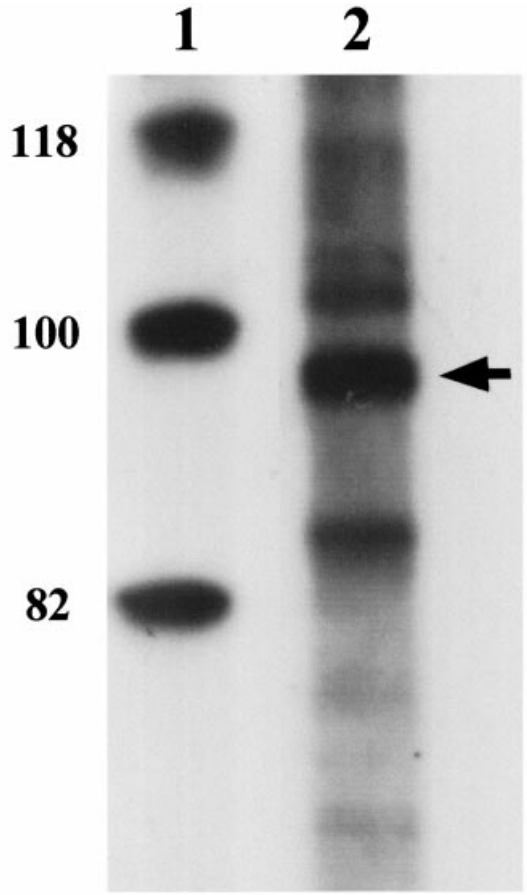

Figure 2. Autoradiograph of primer extension reaction using total RNA template extracted from ovaries of blood-fed mosquitoes. Lane 1 was loaded with end-labelled dephosporylated ØX174 digested with *Hin*fI. Lane 2 contains the primer extension reaction. The arrow denotes the predominant 97-bp primer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

Figure 3. Sequence analysis of 2.1 kb from the 5′ promoter region and first exon of the the *Aedes aegypti Ddc* gene. The start of transcription is at position +1 and is indicated in bold with a double underline. Two potential capsite initiator consensus sequences are marked by bold underlines. A consensus TATA box at –84 has been boxed. Nucleic acid sequence representing the first exon is in uppercase. The gt 5′-splice site of the first intron and the start codon are in bold. The proposed EcRE and EcRE-half site are italicized and indicated with large dash underlines at positions –333 and –1940, respectively. All potential *BR-C*-gene family zinc-finger binding sites are underlined; overlapping BR-C transcription factor binding sites have been double-underlined (see Table 2). The priming site for primer extension reactions is indicated with a dash–dot underline beginning at position +75. The sequence data presented here has been updated under the original cDNA GenBank Accession number, U27581.

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). The level of *Ddc* message

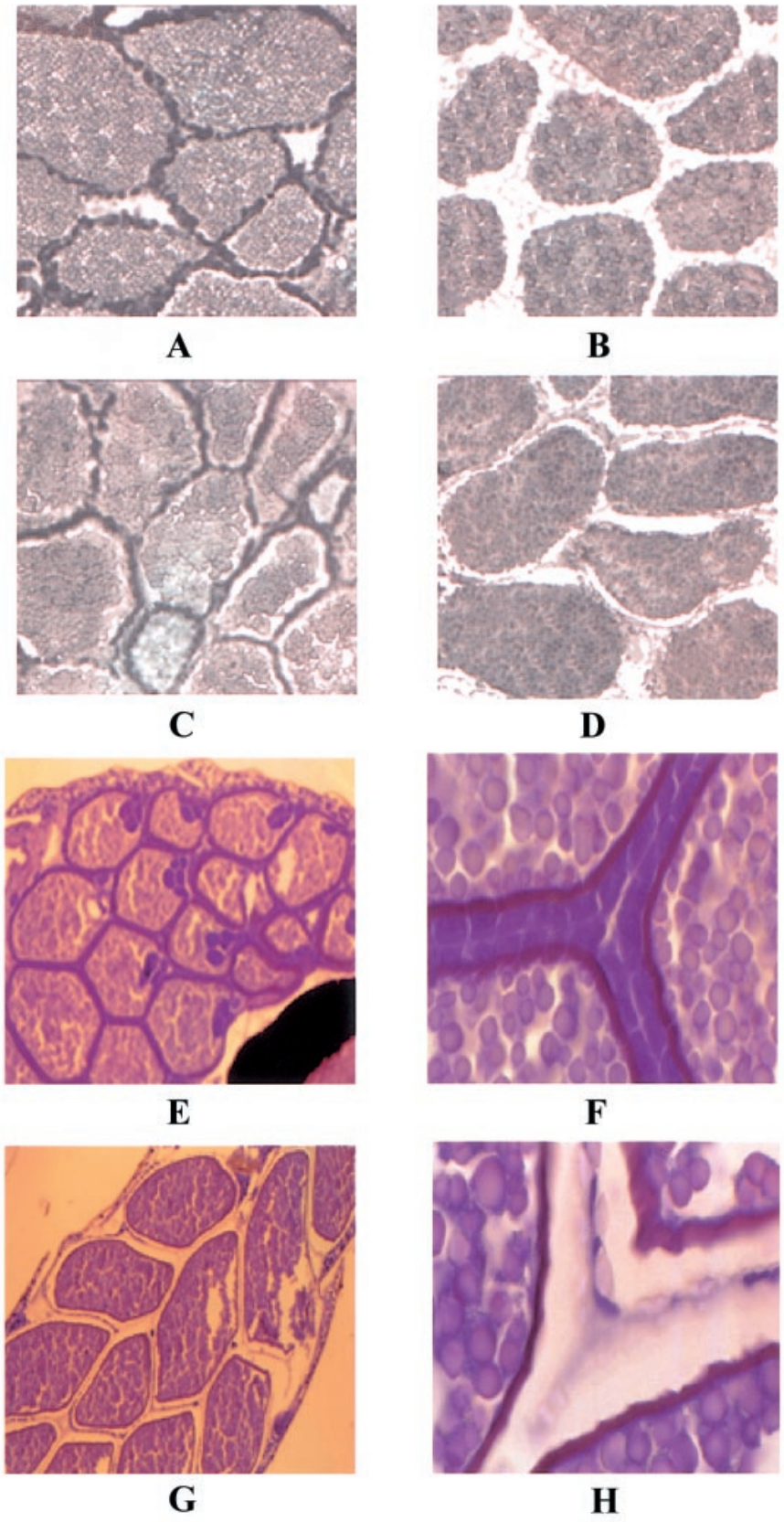

Figure 4. Cellular localization of *Ddc* mRNA in developing ovaries. (A, B) Thirty-six-hour post-blood-fed (pbf) ovary sections hybridized with a biotin-labelled probe specific to *Ddc and* a non-specific adenovirus probe, respectively. (C, D) Forty-eight- and 60-h pbf ovary sections hybridized with 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.

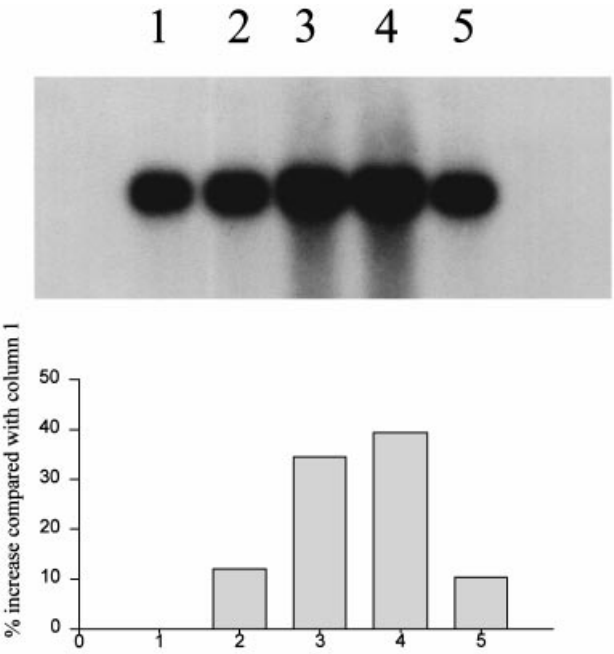

Figure 5. Autoradiograph of a Northern blot demonstrating the effect of 20E injection into blood-fed, decapitated female *Ae. aegypti* on ovarian *Ddc* transcription. Each lane contains two ovary pair-equivalents of total RNA: Lane 1, sugar fed controls; Lane 2, blood-fed, decapitated, saline-injected; 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).

Table 2. Potential regulatory motifs observed in the 5′-promoter and untranscribed 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, RGKTSA-NTGMMYY (Antoniewski *et al*., 1993). The EcRE half site, TGACCT was reported by Cherbas *et al*. (1991). Two clusters of *BR-C*-gene family zinc-finger consensus binding sites, Z1, Z2 and Z4 (YWATTANY, ACTATYAW and WNTAAYTA), respectively (Hodgetts *et al*., 1995) are included and are grouped according to the clusters in which they occur.

Motif	Sequence	% Similarity	Position	Strand
Aedes aegypti EcRE	AGGGCAAGGAATT	100	–333	plus
	GGGTGGTGGATTG	100	2601	plus
	AGTACATTGTTTT	92	12522	plus
Drosophila EcRE	AGGGCAAGGAATT	84	–333	plus
	GGGTGGTGGATTG	69	2601	plus
	AGTACATTGTTTT	77	12522	plus
EcRE-$^{1}/_{2}$ site	AGGTCA	100	–1940	minus
	TGACCT	100	1208	plus
	AGGTCA	100	13090	minus
BR-C Z1	AGTAATTA	100	–173	minus
	TTATTATT	100	–134	plus
BR-C Z4	AGTAATTA	100	–173	plus
	TAATTATA	100	–171	minus
BR-C Z1	TTATTATC	100	–1307	plus
	TTATTATT	100	–1138	plus
	TTATTAAT	100	–1135	plus
BR-C Z2	TTAATAGT	100	–1384	minus

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 *Aedes* 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* transcritpion 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 suggested regulatory elements that occur in this proximal upstream region of *Ae. aegypti Ddc*. Several additional sequence similarities were identified in intron 2 (Table 2). Two clusters of putative *BR-C* transcription factor binding motifs, each consisting of four sites, were present in the upstream sequence (Table 2). Fifteen additional *BR-C* consensus binding sites were identified throughout the remaining mosquito sequence, representing binding domains for each of the four zinc-finger isoforms (Z1-Z4) used in footprinting studies by Hodgetts *et al*. (1995). All but one of these sites occurred in the second intron, within 300 bp of at lease one other *BR-C* binding motif. Searches for other insect motifs involved in regulation of *Ddc* in *D. melanogaster* and *M. sexta* were negative. Additionally, sequence comparisons of the known insect *Ddc cis* promoter regions from *D. melanogaster* and *M. sexta* revealed no significant identities. Likewise, comparisons of upstream regions of another *Ae. aegypti* gene, VgA1 (Romans *et al*., 1995), known to be transcriptionally upregulated for ovary development following blood feeding, identified no significant sequence relationships.

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. melanogaster* 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*s cannot accommodate a similar splicing mechanism to 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 are genes 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 decapitation and subsequent saline inoculation, resulted in a slight induction of *Ddc* transcription above non-blood-fed control levels, however injection of 20E immediately following decapitation 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 decapitation/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 regulation of *Ddc* expression in third-instar *D. melanogaster* larvae recently was shown to require the binding of a mediating transcription factor from the 20E-induced *Broad Complex* gene family in close proximity to concensus EcREs (Hodgetts *et al.*, 1995). Evidence from *Drosophila* studies, patterns observed for other *Aedes* genes induced by blood meal, and the observation that cycloheximide inhibits 20E induction of *Ddc* provided a context in which to search for possible regulatory motifs, particulary EcREs and *BR-C*-gene transcription factor binding domains.

Analysis of the *Ddc* 5′-flanking DNA and introns identified several potential regulatory motifs (Fig. 3, Table 2). Sequence similarities to short, partially ambiguous sequences typical of experimentally determined binding domains will be expected to occur randomly, however several features of our findings suggest homologies. The consensus *Ae. aegypti* EcRE displayed in Table 2 was deduced by alignment of putative *Ae. aegypti* EcREs described from three vitelline envelope genes whose regulation is directly controlled by binding of the ecdysone receptor complex (Edwards *et al.*, 1998). The *Aedes* EcRE at position –333 provides the best congruency with experimentally derived data from *D. melanogaster*, and is considerably less likely to occur by chance than the other possible motifs presented (Table 2). Recombinant BR-C proteins carrying four different zinc-finger isoforms, Z1–4 were used in footprinting analysis of *Drosophila Ddc* to identify four consensus *BR-C*-gene encoded zinc-finger-binding sequences (BR-C Z1–4). Two clusters of these consensus binding sites were identified in the *cis* regulatory region (Table 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 ecdysone-responsive transcription factors.

This work defines an indirect role for 20E in blood meal-induced ovarian 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 cartons 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 paraffin wax prior to injection of decapitated females (Wheelock *et al.*, 1988). For some experiments, the translational inhibitor, cycloheximide, was coinjected into the mosquito heamocoel 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 *Eco*RI, followed by column purification using Wizard DNA Preps (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 ovary 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 V 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′-ATTGATTTTCTGGGGCTATTGAG-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-^{32}P]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*I (Lab-Tek Products) and sectioned at –18 °C. The ovary sections (6–8 μm) were mounted on super frost plus microscope slides (Fisher Scientific) and fixed immediately in ethanol and acetic acid (3 : 1) for 5 min. *In situ* hybridization and detection was performed as described in the kit instructions (Gibco BRL). The *Ddc* probe used in this study was a 631-bp cDNA fragment. The negative control probe was derived from adenovirus and was supplied with the kit. The *Ddc* probe was labelled with biotin by irradiation with UV light. Following incubation in proteinase K (40 μg/ml), tissue sections were hybridized with the probe for 4 h. Tissue sections were then overlaid with blocking solution and working conjugate solution that 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 incubated in NBT/BCIP solution for colour development, and then examined microscopically and photographed.

Sequence analysis

DNA sequence data were analysed using the Sequence Analysis Software Package from the Genetics Computer Group (GCG) (Devereaux *et al.*, 1984). GCG 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, U27581.

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