Hormonal induction of Dopa decarboxylase in the epidermis of Drosophila is mediated by the Broad-Complex

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SUMMARY

The 2B5 early puff locus corresponds to the Broad-Complex (BR-C) and encodes a family of transcription factors whose members are induced by the molting hormone ecdysone. Mutations in the br subcomplementation group substantially reduce the levels of Dopa decarboxylase (DDC) in the epidermis of mature third instar larvae but not in mature second instar organisms. Enzyme levels are normal in the central nervous system of the two mutants examined. The specificity of these effects suggests that a product of the BR-C locus mediates the rapid appearance of DDC in mature third instar larvae experiencing an elevated titer of ecdysone. The likely identity of this protein has been confirmed by pursuing the observation that the br28 allele is caused by the insertion of a P element into the Z2 DNA-binding domain. Both the transcript and a protein carrying this domain are present in the epidermis and a BR-C recombinant protein carrying the Z2 finger binds to the first intron of the Ddc gene. Five binding sites have been identified within the intron by DNase I footprinting and a core consensus sequence has been derived which shares some identity with the consensus binding site of the Z2 protein to the Sgs-4 regulatory region. Our demonstration that Ddc is a target of BR-C in the epidermis is the first direct evidence of a role for this early gene in a tissue other than the salivary glands. The data reinforce the idea that BR-C, which clearly mediates a salivary gland-specific response to ecdysone, may play a widespread role in the hormone’s activation of gene cascades in other target tissues.

Key words: Dopa decarboxylase, Drosophila, Broad-Complex, CNS, nervous system

INTRODUCTION

Studies on the insect moulting hormone began 60 years ago with the classic work of Fraenkel (1935) which demonstrated the release of the metamorphic hormone near the end of the larval stage in the blow-fly Calliphora. Subsequent work by Clever, Karlson, Sekeris and their colleagues (for review, see Sekeris, 1991) led to the identification of 20-OH-ecdysone (herein referred to as ecdysone) as the molting hormone and established the ecdysone system as a useful model for understanding the mechanisms of vertebrate steroid hormone action. The enzyme Dopa decarboxylase (DDC) was the first well-defined gene product whose appearance during development appeared to be regulated by ecdysone (Karlson and Sekeris, 1962). DDC catalyses the conversion of Dopa to dopamine that is required to tan and harden the newly moulted cuticle of diptera. A conceptual framework describing how Ddc gene regulation might occur was provided by Ashburner and Richards from their studies on the induction of chromosome puffing patterns in explanted salivary glands cultured under a variety of ecdysone regimes (Ashburner et al., 1974). Approximately six ‘early genes’, visualized as the corresponding set of early puffs, is directly induced by an ecdysone-receptor complex at the end of the third instar period just prior to puparium formation. These genes act as autoregulators, eventually shutting off their own transcription and that of the intermolt genes some of which encode the salivary gland glue proteins. They also induce the large set of ‘late genes’, which comprise the tissue-specific cascade seen in the puffing studies. Recent studies in Drosophila have confirmed the general features of the model. The gene that encodes the ecdysone receptor (EcR) has been cloned and the majority of the EcR-binding sites on the salivary gland chromosomes correspond to early and late puff loci (Koelle et al., 1991). The three early genes that have been characterized to date all encode families of transcription factors (Burris et al., 1990; Segraves and Hogness, 1990; DiBello et al., 1991). In two cases, E74A and E75A, the protein products have been shown to bind to both the early and late target puff loci (Urness and Thummel, 1990; Hill et al., 1993). A central role in the regulatory hierarchy controlled by ecdysone is played by the Broad-Complex (BR-C), the early gene which resides at puff locus 2B5 on the X-chromosome. Mutations in BR-C affect not only intermolt and late gene expression (Guay and Guild, 1991) but also E74A, E75A
and BR-C early gene induction, which has prompted a revision of the original model (Karim et al., 1993).

In fact, the Ashburner model fails to convey adequately the complexity of responses to the variations in hormone titer that occur in late larval stages and through the prepupal period. The late gene class has been differentiated into early-late and late-late genes on the basis of hormone withdrawal experiments (Ashburner and Richards, 1976). The early-late genes share with the classic early genes a requirement for the presence of edcsynone for their continued expression. They are similar to the late genes in that the appearance of puffs at early-late loci is dependent upon continued protein synthesis. The E78 early-late gene has now been cloned and shown to encode two nested transcription units E78A and E78B (Stone and Thummel, 1993). Both transcripts appear during the pupal stage but the E78B transcript profile in vivo shows maximal expression at pupariation. However, unlike its puff at 78C, the E78B transcript is induced by edcsynone in the presence of cycloheximide and the locus is therefore more properly viewed as a variant of the early gene class (Stone and Thummel, 1993; Huet et al., 1995).

The hormonal control of epidermal Ddc appears to be very similar to that of E78B in several respects. The Ddc transcript reaches maximum levels at pupariation (Andres et al., 1993) and Ddc mRNA is rapidly induced in hormonally naive epidermis following exposure to exogenous edcsynone (Kraminsky et al., 1980). Also, although inhibitors of protein synthesis reduce they do not eliminate Ddc mRNA accumulation (Clark et al., 1986). Since the inhibition of protein synthesis does reduce Ddc levels substantially from control levels, the full induction of Ddc must involve a protein product synthesized after the addition of exogenous edcsynone. The extensive collection of BR-C mutants facilitated the genetic test of a possible interaction between this early gene and Ddc. We demonstrate the existence of such an interaction and have pursued the analysis to the point of showing that a recombiant protein encoded by BR-C binds to the first intron of the Ddc gene. The sequences to which binding occurs are included within a cis-acting region that Shen and Hirsh (1994) have shown controls the tissue specificity of the splicing of the primary Ddc transcript. This raises the interesting possibility that the BR-C might participate in regulating the Ddc splicing pathway in the epidermis.

MATERIALS AND METHODS

Drosophila stocks

BR-C mutations were maintained over BinSn, an X-chromosome balancer carrying the markers Bar and singed (Lindsley and Zimm, 1992). The mutations were carried on a chromosome marked with yellow and were isolated by Kiss et al. (1988), Belyaeva et al. (1980) and C. Bayer (personal communication). All are recessive lethals with the classic early genes a requirement for the presence of edcsynone. The mutations were carried on a chromosome marked with yellow (exper-imental group) separated from wild type (controls) based on the pigmentation of the denticle belts and mouth parts.

Purification of BR-C recombinant proteins

The expression plasmids from which we obtained the BR-C proteins were constructed by cloning cDNA fragments into the vector pDS-MCS (Schindler et al., 1992) as described elsewhere (von Kalm et al., 1994). The fragments contain the core region and one of the possible zinc finger motifs encoded at the 3’ end of the gene (DiBello et al., 1991; Bayer and Fristrom, personal communication). The BR-C protein is designated as BRcore-Q1-Z1 since it contains a glutamine-rich (Q) region and the BR-C protein is designated as BRcore-Q1-Z3 since it contains an arginine and serine-rich (NS domain (DiBello et al., 1991). The recombinant proteins were purified by Ni2+NTA column chromatography (Hochuli, 1990) as in von Kalm et al. (1994). Fractions containing the recombinant protein were identified by SDS acrylamide gel electrophoresis and pooled. The denatured protein was dialysed successively for 2 hour periods at 4°C against a buffer containing 10 mM Hapes (pH 7.9), 80 mM KCl, 1 mM DDT, 0.1% Triton X-100, 10 μM ZnCl2, 5% glycerol and (a) 1 M urea, (b) 0.1 M urea and (c) no urea and 20% glycerol. In the final dialysis step for Z2 isoflorm purification, a white flocculent precipitate developed but it disappered upon further dialysis into 8 M urea, 0.1 M NaHPO4, 0.1 M Tris-HCl (pH 8.0) and did not reappear when the protein was again renatured by carrying out the dialysis steps above. Purified Z2 and Z3 proteins were stored in 10 mM Hapes (pH 7.9), 80 mM KCl, 1 mM DDT, 0.1% Triton X-100, 10 μM ZnCl2, 20% glycerol. Z1 and Z4 were stored in the same buffer containing 1 M urea, since they aggregated and precipitated at lower urea concentrations. The recombinant BR-C proteins all have a POZ domain at their N terminus; this domain (also known as the BTB domain; Zollman et al., 1994) facilitates protein-protein interactions in both homomorphic and heteromorphic molecules depending on the particular protein involved (Bardwell and Treisman, 1994). This might explain the propensity of the BR-C recombinant proteins to aggregate during renaturation.

Filter binding assay

Binding of the purified Z2 recombinant protein to a 7.6 kb restriction fragment that encodes the entire Ddc locus (Scholnick et al., 1983; Chen and Hodggets, 1987) was carried out using nitrocellulose filters according to Papoulas (1988). An appropriate restriction digest of the fragment was end labelled using the Klenow fragment of E. coli DNA polymerase I (Sambrook et al., 1989) and binding to the BR-C recombinant protein carried out in a 50 μl reaction containing: 2x10^5 cts/minute labelled fragments, 1-5 μl protein (50 μg/ml), 1 μg BSA, 50 mM KCl, 10% glycerol, 0.1 mM DTT, 0.1 mM PMSE and 20 mM Tris-HCl (pH 7.5). The bound fragments were eluted, precipitated and fractionated on a 2% agarose gel on which an equal quantity of the input DNA was loaded so that preferential retention of the bound DNA could be visualized on the subsequent autoradiograph of the dried gel.

Gel retardation assay

Mobility shift assays with recombinant BR-C protein were performed on Ddc DNA fragments as described by Chodosh (1988), with minor modifications. A plasmid containing the Ecorl-SalI Ddc region of interest (Fig. 1) was linearized and end-labelled with γ-32P-ATP and polyadenylate kinase. The end-labelled fragment was released from the vector by restriction, subjected to agarose gel electrophoresis and purified from the gel using Geneclean II (Bio101, Inc). Binding was carried out for 60 minutes at 4°C with 4-6x10^5 cts/minute of the labelled fragment, 4-8 μg poly(dI-dC)-(dI-dC) and up to 8 μg BR-C protein in a 20 μl reaction volume containing 20 mM Tris-HCl (pH 7.5), 10% glycerol and 50 mM KCl. The binding reaction was fractionated on a 4% acrylamide gel which was dried and subjected to...
autoradiography. The conditions used to demonstrate binding to the 217 bp \textit{DraI-TaqI Ddc} DNA fragment (Fig. 1) differed somewhat from those just described. The fragment that was subcloned into pUC19 was released from the vector by EcoRI-HindIII digestion, labelled by end-filling the restriction sites using Klenow and purified from an acrylamide gel. The binding reaction contained 0.5 μg of poly(dI-dC).

**DNAase I footprinting analysis**

The 217 bp \textit{DraI-TaqI Ddc} DNA fragment (Fig. 1) was end-labelled by polynucleotide kinase at the \textit{DraI} (transcribed strand) or \textit{EcoRI} (non-transcribed strand) sites flanking the insert and then released from the vector by appropriate restriction. DNAase I (Worthington) footprinting was carried out as we have described elsewhere (von Kalm et al., 1994). The detection of RNA extraction and RT-PCR

The detection of \textit{BR-C} transcript levels in the epidermis was carried out using RT-PCR, as described elsewhere (O’Keefe et al., 1995). The epidermis of wandering third instar larvae was dissected free of other tissue in 0.75% saline and total RNA obtained (O’Keefe et al., 1995). RNA was also obtained for amplification from whole other tissue in 0.75% saline and total RNA obtained (O’Keefe et al., 1995). The 217 bp DNA fragment (Fig. 1) was end-labelled with \textit{EcoRI}-HindIII digestion, labelled by polynucleotide kinase at the HindIII (transcribed strand) or EcoRI (non-transcribed strand) sites flanking the insert and then released from the vector by appropriate restriction. DNAase I (Worthington) footprinting was carried out as we have described elsewhere (von Kalm et al., 1994).

**RNA extraction and RT-PCR**

The detection of \textit{BR-C} transcript levels in the epidermis was carried out using RT-PCR, as described elsewhere (O’Keefe et al., 1995). The epidermis of wandering third instar larvae was dissected free of all other tissue in 0.75% saline and total RNA obtained (O’Keefe et al., 1995). RNA was also obtained for amplification from whole organisms, collected within a 1 hour period following the clearing of the dye which was monitored 1.5 hours later. Mutant and wild-type organisms whose guts had cleared of dye within this period were returned to the food for 2 hours and then harvested for analysis. The time of clearing of the dye in wild-type animals was determined to occur 2.5 hours before pupariation (data not shown). In presenting the results, DDC activity of mutant males is expressed as a percentage of the activity of the wild-type sibs segregating in the stock. Enzyme levels in whole organisms reflect predominately epidermal levels since the contribution of neural DDC to the total is less than 10% (Scholnick et al., 1983). Two and sometimes three independent collections of organisms were assayed at the two stages and the results are shown in Table 1. Organisms assayed at the second moult showed no significant difference among mutant strains. However, an analysis of variance (not shown) showed significant (P<0.01) relative differences in DDC levels among the mutant strains at pupariation. Duncan’s multiple range test revealed three different groups of mutant strains. The first group contained strains whose enzyme levels were equivalent to the wild type: \textit{2Bc}¹, \textit{2Bc}², \textit{rbp}³, \textit{br}³\textit{ab}, \textit{2Bab}¹, \textit{2Bab}³ and \textit{2Bab}⁵. The second group contained strain \textit{br}³ with about half the normal enzyme level and the third group was comprised of \textit{br}³, \textit{br}³\textit{m}, \textit{npr}³⁷ and \textit{2Bab}⁵ all of which exhibited DDC levels less than one third normal. There is a strong correlation between mutations in the \textit{br} subcomplementation group and reduced DDC activity. In fact the only other mutants that affected DDC activity were \textit{npr}³⁷ and \textit{2Bab}³. The former fails to complement any of the \textit{BR-C} mutations while the latter

<table>
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<th>Sample 2</th>
<th>Sample 3</th>
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<td>–</td>
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<td>\textit{rbp}³</td>
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<td>101</td>
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(b) pupariation

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<td>107</td>
<td>101</td>
<td>–</td>
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</table>

The values in the table are enzyme levels in male organisms expressed as a percentage of the activity in wild-type sibs segregating in the same cross. Each sample represents a separate collection of organisms and the data on 3 determinations of DDC activity were averaged for each extract. Since \textit{npr}³⁷ organisms do not pupariate, these mutants in (b) were staged along with their sib controls using the dye clearing technique described in the text. No significant differences were revealed when the means in (a) were subjected to one way analysis of variance of the Duncan’s multiple-range test (Snedecor and Cochrane, 1980).

**RESULTS**

**DDC enzyme levels are under control of the Broad-Complex**

Organisms were collected within a 1 hour period following the second moult and again as white prepupae with the exception of \textit{npr}³⁷ which fails to pupariate. In this case, wandering third instar larvae were collected from food that had been supplemented with 0.05% bromophenol blue (Andres and Thummel, 1994). Those whose guts were blue were returned to the food to allow clearing of the dye which was monitored 1.5 hours later. Mutant and wild-type organisms whose guts had cleared
belongs to the 2Bab subcomplementation group, whose lethal alleles also fail to complement the br group (Belyaeva et al., 1980; Kiss et al., 1988). The observation that only one of the four 2Bab alleles tested reduced DDC activity is somewhat surprising although the 2Bab<sup>5</sup> allele is the strongest of the four that we examined (C. Bayer, personal communication).

**Ddc** transcript levels in a subset of these mutants have been determined by quantitative RT-PCR (O’Keefe et al., 1995). In every case where there were substantial reductions in DDC activity levels, there were comparable reductions in the steady state levels of the Ddc transcript pool. In the *rbp<sup>4</sup>* mutant where the enzyme levels were approximately normal, transcript levels were also normal.

In order to test the effect of **BR-C** mutations on neural DDC activity, enzyme activity was assayed in the dissected brains of mature larvae from two of the mutants strains showing the largest reductions in total DDC levels. The neural complex comprising the optic lobes and the ventral ganglion from hemizygous *npr<sup>17</sup>* wandering male larvae was collected and the DDC determinations expressed as a percentage of the values obtained in the brains of wild-type organisms segregating in the same cross. The mean value of the three extracts was 120% in contrast to the value of 22% in whole organisms (Table 1), which reflects the epidermal level predominately. One assay on brains collected from *br<sup>5</sup>* mutant males yielded 111% of the activity of brains from wild-type males. These data make it clear that, although allele-specific reductions in epidermal levels of DDC occur in mature larvae, the enzyme activity in the brains of these organisms is not reduced by the **BR-C** mutations.

**BR-C proteins can bind to the Ddc genomic region**

The data in Table 1 strongly suggest that mutations in the *br* subcomplementation group affect Ddc gene activation at pupariation. The fact that one of these mutations, *br<sup>23b</sup>* is caused by a P element insertion into a genomic region encoding the BR-C Z2 isoform (DiBello et al., 1991; Emery et al., 1994), led us to ask directly whether the Z2 isoform can bind to Ddc genomic DNA. Distinct tissue-specific Ddc transcripts, localized to the central nervous system and the epidermis respectively, are derived from this gene (Eveth et al., 1986; Morgan et al., 1986). They are illustrated in Fig. 1A along with a restriction map of the gene. The recombinant Z2 isoform was prepared as described in the Materials and Methods and the filter binding assay was carried out on different restriction digests of the **Ddc** genomic DNA fragment (Fig. 1A) known to include all regulatory elements required for proper temporal and spatial Ddc expression (Scholnick et al., 1983; Marsh et al., 1985; Chen and Hodgetts, 1987). A HindIII-SstI digest of a plasmid carrying this fragment produces 9 products (Fig. 1A), two of which were preferentially retained on the filter by the Z2 protein (Fig. 1B). The bound 2.7 kb DNA molecule is derived exclusively from the vector. The retained 1.3 kb HindIII fragment includes much of the first intron of the
epidermal transcript (Fig. 1A). Further digestion with SalI cleaved the 1.3 kb fragment into two fragments of 720 and 624 bp. Using the triple digest in the filter binding assay (Fig. 1C) clearly shows Z2 binding to only the 720 bp HindIII-SalI region which lies almost entirely within the first intron of the epidermal transcript (Fig. 1A). The final localization of the binding region is shown in Fig. 1D. The 736 bp EcoRI-SalI fragment was isolated, end-labelled and restricted with DraI which produces two fragments of 249 and 487 bp respectively (Fig. 1A). The Z2 isoform binds almost exclusively to the larger fragment, which is located predominately within the first intron of the epidermal Ddc gene but includes the second exon of the neural transcript.

The 736 kb EcoRI-SalI fragment and subfragments within it (see Fig. 1A) were subjected to mobility shift experiments with the recombinant Z2 protein as described in the Materials and Methods. The full-length EcoRI-SalI fragment shows extensive retardation by Z2 and the binding proceeds through one or more intermediates at non-saturating levels of protein (Fig. 2A). The non-discreet nature of these intermediates may result from the rather large size of this fragment. The binding to the EcoRI-SalI piece is eliminated in the presence of a 75-fold excess of unlabelled fragment (Fig. 2B). Additional binding studies (data not shown) revealed that little binding occurred to either the 249 bp EcoRI-DraI or the 272 bp TaqI-Sall intervals on the 736 bp EcoRI-SalI fragment. These results suggested that all of the Z2 binding was localized to the central 217 bp DraI-TaqI fragment (see Fig. 1A). When this fragment was isolated, labelled and subjected to mobility shift analysis, extensive retardation was seen (Fig. 2C).

The DraI-TaqI fragment was subjected to DNase I footprinting using the recombinant Z2 protein (Fig. 3). Four obvious footprints, each associated with a DNase I hypersensitive site, were resolved on the transcribed strand (Fig. 3A). These footprints were numbered beginning at the promoter proximal site. The extent of each footprint is shown in Fig. 3A. These footprints were numbered beginning at the promoter proximal site. The extent of each footprint is shown in Fig. 3A. These footprints were numbered beginning at the promoter proximal site. The extent of each footprint is shown in Fig. 3A. These footprints were numbered beginning at the promoter proximal site.

The BR-C is expressed in the epidermis prior to maximal Ddc expression

A reverse transcriptase-polymerase chain reaction (RT-PCR) approach was used to test whether BR-C transcripts are present in epidermal preparations that express DDC enzyme activity. Total RNA was isolated from hand-dissected epidermal preparations of wandering larvae, amplified and the products visualized on an agarose gel (Fig. 5). The products detected in whole organisms of the same stage are included for comparison. All four of the zinc finger classes could be detected and the sizes of the amplified bands (0.78 kb (Z1), 0.32 kb (Z2), 0.78 kb (Z3) and 1.1 kb (Z4)) were those expected for the BRcore-Q1-Z1, BRcore-Z2, BRcore-Z3 and BRcore-Z4 isoforms, respectively (DiBello et al., 1991; C. Bayer, personal communication). The two isoforms of Z1, Q1 and Q2, differ by only 51 nucleotides (DiBello et al., 1991) and may not have been resolved on our gels. The 'TNT' variants of the Q1-Z1 isoform (DiBello et al., 1991) could have been resolved but were never observed. In every case, the epidermal products that we did detect were indistinguishable from those detected in whole organisms.

Identification of epidermal BR-C proteins by western blotting

Protein extracts from epidermal preparations of wild-type and

![Fig. 2. Mobility shift experiments using Z2. The 736 bp EcoRI-SalI fragment or the 217 bp DraI-TaqI subfragment within it (Fig. 1A) were end-labelled and subjected to mobility shift analyses as described in the Materials and Methods. (A) Binding to the EcoRI-SalI fragment was carried out in the presence of 0, 0.5, 1, 2, 4 and 8 μg of Z2 protein, respectively. (B) Binding of Z2 to the EcoRI-SalI fragment was carried out with 0 (lane 1) and 6 μg of Z2 (lanes 2 and 3). In lane 3 the binding was carried out in the presence of a 75-fold excess of unlabelled fragment. Some of the retarded DNA did not enter the gel in lane 2 and forms an apparent band at the origin. (C) Binding of Z2 to the DraI-TaqI fragment in the presence of 0, 0.5, 1 and 4 μg respectively of protein.](image-url)
The presence of the Z2 isoform transcript and the predominant BR-C proteins at all time points.

**DISCUSSION**

The first comprehensive intragenic complementation map of the BR-C (Belyaeva et al., 1980) indicated that rbp, br, 2Bc and 2Bd belonged to independent subgroups. Kiss et al. (1988) subsequently suggested that br and rbp be grouped together with 2Bd. However, both Guay and Guild (1991) and Karim et al. (1993) showed that whereas rbp+ function was essential for the transcription of the glue genes Sgs-3, Sgs-4 and Sgs-5, mutants in the br group had no effect on glue gene expression suggesting that the rbp and br functions do not overlap. This conclusion is confirmed by our data which show, conversely, that mutations in the br group strongly reduce DDC enzyme and mRNA levels (O’Keefe et al., 1995), whereas an rbp mutant is without effect.

The pronounced reduction in Ddc mRNA levels in nprl7 is expected on the basis of its inability to complement any other BR-C mutant (Belyaeva et al., 1980). The situation with the 2Bab mutants is more complex. These mutants fail to complement only the br and rbp mutants according to Belyaeva et al. (1980) although Kiss et al. (1988) were inclined to group these mutants with the nprl non-complementing class. Our data suggest that the 2Bab mutants are a heterogeneous group since only one of the four alleles that we examined had an effect on DDC activity (Table 1).

There is a strong correlation between the br+ function and the Z2 isoform because the br28 mutation is known to disrupt the Z2 zinc finger pair (DiBello et al., 1991; Schouls, 1993; Emery et al., 1994; C. Bayer, pers. comm.). The strongest Z2 protein-binding sites on the entire 7.6 kb genomic region known to be sufficient for correct developmental expression of Ddc (Scholnick et al., 1983; Chen and Hodgetts, 1987) are confined to a 217 bp region in the first intron (Fig. 3). The five AT-rich binding sites all share a common core sequence, CTAT, which is also present in the consensus binding site of Z2 to the Sgs-4 regulatory domain (von Kalm et al., 1994; Fig. 4B). The data of von Kalm et al. (1994) and Emery et al. (1994) suggest that rbp+ function acts through the Z1 domain to control Sgs-4 expression in the salivary glands and our data suggest that the br+ function acts through the Z2 domain to control Ddc expression in the epidermis. Since it is clear that rbp+ function (and by inference the Z1 isoform) controls additional genes expressed exclusively in the late larval and prepupal salivary gland (Guay and Guild, 1991; Karim et al., 1993), it will be interesting to determine whether the Z2 isoform also controls additional target genes in the epidermis.

We hypothesise that the binding of a BR-C Z2 isoform is required for full activation of Ddc transcription and failure to produce wild-type levels of this isoform in br mutants explains the genetic interaction between the BR-C and Ddc loci reported here. The presence of the Z2 isoform transcript and the predominance of its protein in the epidermis (Fig. 5, 6) is consis-
tent with this regulatory interaction. We have recently obtained
in vivo evidence that confirms the significance of the interac-
tion revealed by the binding studies. Epidermal \textit{Ddc} transcript
levels are about 5-fold lower in \textit{br5} mutant larvae than in wild-
type organisms (O’Keefe et al., 1995). However, when high
levels of \textit{Z2} protein are synthesized from a transgene under the
control of a heat-shock promoter in a \textit{br5} mutant background,
\textit{Ddc} transcripts accumulate to normal levels. In addition, heat-
shock expression of \textit{Z2}, but no other \textit{BR-C} protein, rescues
tanning in \textit{br5} prepupae (C. Bayer, LvK and J.
Fristrom, unpublished).

Since several mutants in the \textit{Ddc} structural gene exhibit reduced levels of
tanning of the puparium (Wright, 1987), this
suggests the \textit{br5} mutant’s inability to tan is a
specific effect of a failure to activate the \textit{Ddc} gene.

Despite the requirement for a \textit{Z2} product, we do not
believe that induction of the \textit{BR-C} is a suffi-
cient condition for maximum \textit{Ddc} transcript ac-
cumulation at pupariation. In the first place, none of the
mutants eliminates epidermal \textit{Ddc} expression
entirely (Table 1). Secondly, we have shown that
\textit{Ddc} mRNA levels do increase substantially in the
absence of protein synthesis at this stage (Clark et al.,
1986). This suggests a role for a protein
product, present before the rise in the late third
instar ecdysone titer, in \textit{Ddc} expression. It is
possible that the \textit{BR-C} \textit{Z2} isoform works in
concert with other regulators to control \textit{Ddc}
expression. We note the presence of two potential
ecdysone receptor response element
sequences (EcREs, Cherbas et al., 1991) within the first intron of \textit{Ddc}.
One lies about 300 bp upstream of the first \textit{Z2}-
binding site and the second lies between sites \textit{Z2}3,4
(Fig. 4A) which suggests that an interaction
between the \textit{Z2} isoform and the ecdysone receptor
complex could promote full induction of \textit{Ddc}
at pupariation. In the absence of the \textit{br5} function, the
receptor complex alone might elicit limited \textit{Ddc}
induction. This could explain the lower levels of
\textit{Ddc} induction seen in naive epidermis following
the addition of ecdysone and inhibitors of protein
synthesis (Clark et al., 1986).

The existence of binding sites on the \textit{DraI-TaqI}
fragment for all four of the \textit{BR-C} recombinant
proteins is still consistent with our suggestion that
the \textit{Z2} isoform mediates full \textit{Ddc} induction
at pupariation. The \textit{Z2} binding-sites fall into three
classes that can be distinguished by their overlap
with the other \textit{BR-C} binding sites (Figs 3, 4A).
The \textit{Z2}5, sites5 overlap with \textit{Z1}-, \textit{Z3}- and \textit{Z4}-

binding sites; sites \textit{Z2}3,5 overlap with \textit{Z1}- and \textit{Z4}-

binding sites and \textit{Z2}1 overlaps with a \textit{Z3}-binding
site. Binding of the other \textit{BR-C} isoforms members
in close proximity to the \textit{Z2} sites could indicate
that \textit{BR-C} plays a role in repressing \textit{Ddc}
expression in tissues other than the epidermis.
The occupancy state of the \textit{Z2} sites could be deter-
mined by the stochiometric ratios of the four \textit{BR-
C} proteins and, in turn, regulate \textit{Ddc} expression.
Since a \textit{Z2} isoform is the most abundant species
in the epidermis, it might occupy the \textit{Z2}-binding

sites causing the exclusion of some or all of the other \textit{BR-C}
proteins from these sites. In the salivary glands, an analogous
situation might prevail at \textit{Sgs-4}. Although all four \textit{BR-C}
isoforms can footprint on the critical element III of the regu-
laratory region (von Kalm et al., 1994), only the \textit{Z1} isoform
accumulates to substantial levels in this tissue (Emery et al.,
1994 and unpublished data) suggesting that this isoform
provides the \textit{rbp}+ function by acting through element III to
direct late larval expression of \textit{Sgs-4} (von Kalm et al., 1994).

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{(A) The six \textit{BR-C}-binding sites revealed by the footprinting data are shown
below the line and the putative ecdysone receptor-binding site (EcR) above the line
that represents the 217 bp \textit{DraI-TaqI} fragment of the first intron (Fig. 1A). The
various isoforms that bind at or near these sites are shown below each site. (B) The
DNA sequences of the 17 \textit{BR-C} isoform-binding sites identified by the footprinting
are summarized below the sequence of the non-transcribed strand of the \textit{DraI-TaqI}
fragment that was taken from Eveleth et al. (1986). Since this sequence differed in
one place from that published in Morgan et al. (1986), we confirmed our data in
Eveleth et al. (1986) using Sanger’s method and note further that the A+G ladders
used for the footprinting (Fig. 3) are consistent with the sequence shown. The
footprint sequences are all shown on the non-transcribed strand with the exception of \textit{Z4}.
Absolutely conserved core regions in the binding sites are underlined. The \textit{Sgs-4}
consensus sequences are taken from von Kalm et al. (1994). Y=either pyrimidine; N=any
nucleotide; W=A or T; M=A or C.}
\end{figure}
The lack of any mutant effects on DDC activity at the molt into the third instar period indicates that BR-C does not play any direct role in the appearance of the DDC peak that occurs at this time (Kraminsky et al., 1980). This is not surprising given that the maximum ecdysone titer occurs nearly 12 hours before the molt (Kraminsky et al., 1980) and transcript levels of BR-C are very low at the second to third instar molt (Andres et al., 1993). In fact, Hiruma and Riddiford (1990) have shown that, in cultured epidermis from fourth instar larvae of Manduca sexta, Ddc expression required the addition of hormone and then its removal, a result similar to that reported for the Drosophila Ddc in cultured imaginal discs (Clark et al., 1986). We have suggested that Ddc induction in the larval epidermis involves at least two very different stage-specific mechanisms (Hodgetts et al., 1986). At pupariation, ecdysone induction of the metamorphic-specific BR-C leads to Ddc activation in the larval epidermis whereas at the end of embryogenesis, during the first two larval instars and following disc evagination, BR-C-independent mechanisms may lead to Ddc expression. A protein(s), induced by ecdysone, has recently been identified in Manduca sexta, whose presence appears to be responsible for repressing Ddc during non-metamorphic stages when ecdysone levels are high (Hiruma et al., 1995).

Decay of this protein following the ecdysone peaks could lead to the delayed appearance of DDC that we have noted in Drosophila at stages other than pupariation (Kraminsky et al., 1980; Clark et al., 1986).

The lack of br mutant effects on DDC levels in the CNS of mature larvae suggests an ecdysone-independent regulation of the complex spatial distribution of neural DDC (Beall and Hirsh, 1987; Konrad and Marsh, 1987). Recent evidence suggests that the CNS-specific splicing pattern (Fig. 1A) is a default pathway and that cis-acting sequences located within the first intron interact with an epidermal repressor to block the 3’-acceptor site of the neural-specific exon B (Shen and Hirsh, 1994). Although it is tempting to postulate that the BR-C encodes this repressor, the prediction that this would lead to an enrichment of the CNS splice form in the epidermis of br mutants is not substantiated by our analysis of Ddc transcripts using RT-PCR (O’Keefe et al., 1995). However, no systematic attempt was made in that study to assess the relative levels of the different splice forms and a confirmation of a role for the BR-C in Ddc splicing awaits further investigation.

Ddc induction clearly shares the major features of early-late gene induction in the salivary glands: induction occurs rapidly following a rise in ecdysone titer (Kraminsky et al., 1980) and full activation is dependent on BR-C function (Karim et al., 1993) and continued protein synthesis (Clark et al., 1986). Cascades of ecdysone-induced gene expression can be
Hormonal induction of Dopa decarboxylase in Drosophila


REFERENCES


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