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Genetics of ecdysteroid-regulated central nervous system metamorphosis in *Drosophila* (Diptera: Drosophilidae)

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***Broad-Complex*, *Deformed*, *IMP-E1*, transcriptional regulation, steroid hormones, homeodomain, metamorphosis, neurogenetics, ecdysone, *Drosophila*, zinc-finger protein, central nervous system**

Abstract. We are interested in identifying members of the genetic pathway through which 20-hydroxyecdysone (20HE) mediates reorganization of the central nervous system (CNS) during metamorphosis. Our entry point is the *Drosophila Broad-Complex* (*BR-C*), an early 20HE-inducible locus with three genetic subfunctions, each represented by a lethal complementation group. Our previous analysis of mutants demonstrated that all three *BR-C* subfunctions are necessary for CNS morphogenesis and one is essential for visual system organization. We believe the mutant phenotypes result from faulty expression of genes normally regulated by the BRC family of zinc-finger proteins. BRC target genes are predicted to have expression patterns and/or mutant phenotypes that partially overlap with those of the *BR-C*. We have examined two candidate genes, *IMP-E1* and *Deformed* (*Dfd*), to determine their positions relative to *BR-C* in the hormone-regulated pathway of CNS metamorphosis. Identified by Natzle and colleagues on the basis of 20HE-inducibility in imaginal discs, *IMP-E1* transcripts were also found in a subset of CNS glial cells. Our recent experiments show that *BR-C* expression is spatially and temporally poised to regulate *IMP-E1* induction by 20HE. We examined *IMP-E1* transcript accumulation in larval and prepupal CNS of *BR-C* lethal mutants representing each of the three complementation groups. In all three cases, *IMP-E1* induction in the CNS of *BR-C* mutants was comparable to that of wildtype and of genetic controls. Thus, activity of any individual *BR-C* subfunction is not essential for *IMP-E1* induction. *Dfd* is a homeotic selector gene in the Antennapedia complex whose larval CNS expression has been shown by others to be restricted to a subset of subesophageal ganglion cells. We have demonstrated that *Dfd* mutants manifest a defect in subesophageal ganglion metamorphosis, namely separation from the thoracic ganglion, indistinguishable from that of *BR-C* mutants. However, *Dfd* transcript accumulation in the CNS appears to be indifferent to 20HE levels in vivo or in vitro. Alternative models for the genetic pathways controlling CNS metamorphosis are discussed.

INTRODUCTION

Broad-Complex: A kingpin in the cascade of ecdysteroid-regulated gene activity

In *Drosophila*, as in other holometabolous insects, many tissues that are required during both larval and imaginal stages depend upon pulses of 20-hydroxyecdysone (20HE) to induce their transition from juvenile to adult form and function (Granger & Bollenbacher, 1981; Riddiford, 1985). These hormonal signals have profound effects on patterns of transcriptional activity (Ashburner et al., 1974; Richards, 1980; Riddiford, 1982; Andres & Thummel, 1992), shifting the developmental paths of many tissues. We are interested in the genetic bases of ecdysteroid action during metamorphosis, with a particular emphasis on central nervous system (CNS) reorganization. The *Drosophila Broad-Complex* (*BR-C*), a 20HE-inducible regulatory locus in the "early" puff at chromosomal location 2B3-5

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(Chao & Guild, 1986), has emerged as an essential mediator of hormone action on all tissues examined (Kiss et al., 1976, 1988; Restifo & White, 1991, 1992).

The *BR-C* encodes three genetic subfunctions, each represented by a lethal complementation group: *broad* (*br*), *reduced bristles on the palpus* (*rbp*), and *lethal(1)2Bc* (*2Bc*). Mutants lacking all *BR-C*$^+$ function die at the end of larval life after a period of prolonged wandering. The primary defect is not one of hormone deficiency, but rather results from an inability to transduce the systemic hormonal signal into the behavioral and anatomical hallmarks of entry into metamorphosis (Stewart et al., 1972; Kiss et al., 1976, 1978; Murphy et al., 1977; Fristrom et al., 1981). Mutants bearing lethal hypomorphic alleles die during metamorphosis, and manifest a syndrome of multi-organ system developmental failure, including defects in larval tissue histolysis and faulty maturation of adult tissues. *BR-C*$^+$ function is required in a cell-autonomous manner by the larval epidermis for tanning and sclerotization to form the pupal case at the onset of metamorphosis (Kiss et al., 1976). Adult epidermal development, including eversion and elongation of the imaginal discs, as well as fusion of individual disc derivatives to form a contiguous epithelium, is also *BR-C*$^+$-dependent (Kiss et al., 1988). Internal tissue phenotypes include persistence of larval salivary glands and midgut; degeneration of the dorsal-ventral indirect flight muscles; faulty morphogenesis of the proventriculus, adult salivary glands, retina and CNS; and neural disorganization in the central visual system (Restifo & White, 1991, 1992; Restifo & Merrill, 1994; Sandstrom & Restifo, 1994).

The *BR-C* encodes a family of DNA-binding transcriptional regulators (DiBello et al., 1991; Von Kalm et al., 1994), that control the qualitative and quantitative hormone-responsiveness of intermolt, early, and late puff genes (Belyaeva et al., 1981; Zhimulev et al., 1982; Crowley et al., 1984; Lepesant et al., 1986; VijayRaghavan et al., 1988; Guay & Guild, 1991; Karim et al., 1993). In particular, both the activation and repression of the salivary gland-specific glue protein genes and activation of the 71E late cluster require *BR-C*$^+$ function (Guay & Guild, 1991; Karim et al., 1993). In addition, the transcriptional patterns of several early genes (*E74A*, *E75A*, and *BR-C*), as well as *EcR* (the ecdysone receptor), are disrupted in *BR-C* mutants in vivo and/or in vitro. These observations place *BR-C* above at least some of the other early (primary response) genes in the regulatory hierarchy and suggest *BR-C* autoregulation (Karim et al., 1993). In the case of *Sgs4*, trans-regulation is mediated by binding of BRC proteins (Von Kalm et al., 1994).

Thus, we believe the mutant tissue phenotypes summarized above arise from improper regulation of BRC target genes. With respect to both tissue and molecular phenotypes, mutants of the three complementation groups differ. Some phenotypes are common to mutants of all three groups; the others are seen in mutants of two complementation groups, or are unique to mutants of a single complementation group (Kiss et al., 1988; Guay & Guild, 1991; Restifo & White, 1991, 1992; Karim et al., 1993). Thus, *rbp* mutants have a "phenotypic signature" consisting of the common phenotypes, plus several unique defects: larval salivary gland persistence, dorsal-ventral indirect flight muscle degeneration, and failure to activate the 71E late gene cluster (Restifo & White, 1991, 1992; Karim et al., 1993).

The link between phenotype and genotype most likely resides in the molecular features of BRC proteins. Spanning over 100 kb of genomic DNA, the *BR-C* uses multiple promoters and alternative splicing to produce a large array of transcripts which in turn encode a handful of protein isoforms (DiBello et al., 1991). BRC proteins all share a common

amino-terminal core domain (BRcore) and a "Z" domain containing one of four alternative pairs of zinc fingers (DiBello et al., 1991; Bayer and Fristrom, pers. comm.) that mediate DNA binding (Von Kalm et al., 1994). A growing body of circumstantial and experimental evidence supports the hypothesis of DiBello et al. (1991) that the subfunctions represented by *br*, *rbp*, and *2Bc* complementation groups reside in zinc-finger domains Z2, Z1, and Z3, respectively (Emery et al., 1994; K. Crossgrove & G. Guild, pers. comm.; C. Bayer & J. Fristrom, pers. comm.). Hence, our working model is that phentoypes in mutants of different complementation groups reflect the misregulation of distinct, but partially overlapping, subsets of BRC target genes.

CNS reorganization during metamorphosis in wildtype and mutants

Underlying the anatomical and behavioral differences between larval and adult stages in holometabolous insects are alterations in CNS structure and function (Edwards, 1969; Pipa, 1973). The role of ecdysteroids in regulating or modulating events of CNS metamorphosis, including axonal and dendritic reorganization, neurotransmitter phenotype, morphogenetic movements, and cell death, is well-documented, especially in Lepidoptera (Pipa, 1973; Truman, 1988; Weeks & Levine, 1990, 1992; Tublitz, 1993).

In flies, the larval CNS is remodeled through a combination of cell birth and differentiation (White & Kankel, 1978; Truman & Bate, 1988; Hofbauer & Campos-Ortega, 1990; Ito & Hotta, 1992), synaptogenesis and synaptic modification (e.g., Meinertzhagen & Hanson, 1993), complex 3-dimensional movements (Hertweck, 1931; White & Kankel, 1978), breakdown and re-formation of the blood-brain barrier (Lane & Swales, 1978) and relatively modest amounts of degeneration (Stocker et al., 1976; Technau & Heisenberg, 1982) and cell death (Fischbach & Technau, 1984; Hofbauer & Campos-Ortega, 1990; Kimura & Truman, 1990). These events are also coordinated with and influenced by the arrival of new afferent input, most notably in the visual system (reviewed in Meinertzhagen & Hanson, 1993). The resulting changes in the overall size and shape of the *Drosophila* CNS are depicted in Fig. 1. Several key morphogenetic features of wild-type CNS metamorphosis include: (i) separation of the subesophageal ganglion (SEG) from the thoracic ganglion (TG) with concomitant formation of the cervical connective; (ii) expansion of the three thoracic segments and condensation of the eight abdominal ones; (iii) fusion of the brain in the midline; and (iv) elaboration of a large and highly ordered visual system.

In support of the hypothesis that ecdysteroid-induced alterations in gene expression are essential for *Drosophila* CNS metamorphosis, we have shown multiple significant defects of CNS metamorphosis in mutants of the 20HE-regulated *BR-C* (Fig. 2; Restifo & White, 1991). Mutants of all three complementation groups manifest (i) failure of SEG-TG separation, resulting in a short or non-existent cervical connective (Fig. 2A); (ii) failure of midline fusion, leaving a gap between the right and left sides of the brain (Fig. 2B); and (iii) abnormal position of optic lobe neuropil, apparently due to faulty 3-D rearrangement (Fig. 2C). *2Bc* mutants show profound disorganization of optic lobe neuropil (Fig. 2D), especially of the lobula complex; *br* mutants occassionally have subtle optic lobe defects.

We interpret these data to mean that 20HE, acting through BRC transcription factors, is required for proper morphogenesis and neural reorganization during CNS metamorphosis. This interpretation is strengthened by our evidence that *BR-C* transcript accumulation is 20HE-inducible in the CNS (Restifo et al., 1993) and that EcR can be detected in all

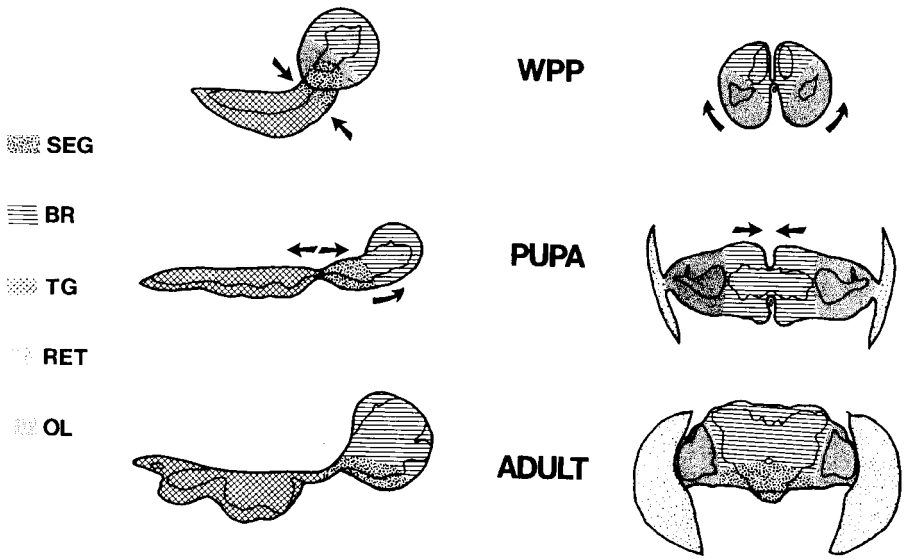

Fig. 1. Wild-type CNS metamorphosis, depicted in schematic form, as seen in lateral (left) and frontal (right) views. Cartoons were drawn from histological specimens from white prepupal (WPP; top), young pupal (P5; middle), and pharate adult (bottom) stages. The scale is the same throughout. The thin interior lines indicate the junction of the neuropil and neuronal cell body layer. The shading (code on far left) shows major anatomical regions: SEG – subesophageal ganglion; BR – brain; TG – thoracic ganglion; RET – retina; OL – optic lobes. The lateral view highlights formation of a constriction between the SEG and TG, followed by separation of SEG and TG, resulting in formation of the cervical connective. The frontal view highlights midline fusion of the brain and development of the visual system.

regions that are affected in the *BR-C* mutants (Truman et al., 1994). In addition, recent studies have demonstrated effects of 20HE on specific cellular features of *Drosophila* CNS reorganization, for example the modulation of neurogenesis rates (Awad & Truman, unpublished, summarized in Truman et al., 1993) and control of post-eclosion cell death (Robinow et al., 1993). It remains to be determined whether these ecdysteroid influences require the action of *BR-C*.

IMP-E1 and *Deformed*

One of several approaches we are taking to identify BRC target genes in the CNS is to consider candidate genes whose spatio-temporal expression patterns or mutant phenotypes suggest they might be regulated by the *BR-C*. Two such genes are the focus of this report: *Deformed*, a homeotic gene required for SEG maturation (Restifo & Merrill, 1994), and *IMP-E1*, a 20HE-inducible gene expressed in a subset of glial cells (Natzle et al., 1988).

IMP-E1 is a member of the class of primary-response ecdysterone-inducible genes that do not encode transcriptional regulators (see discussion in Andres & Thummel, 1992, and Natzle, 1993). Cloned on the basis of their inducibility in imaginal discs, *IMP-E1* transcripts are also hormone-inducible in the late larval CNS (Natzle et al., 1988). They are expressed in the perineurium, a glial sheath that forms the blood-brain barrier, and in the layer of large glial cells (interface glia) at the junction of the cellular rind and the neuropil

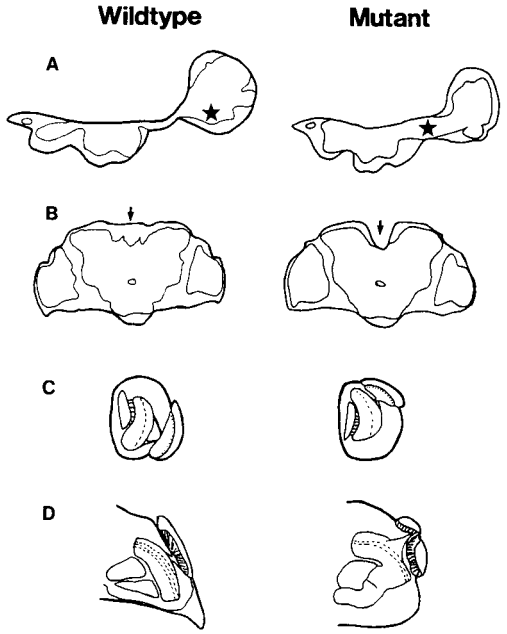

Fig. 2. Defects of CNS metamorphosis in *BR-C* mutants (right), as compared to wild-type animals (left). All cartoons were drawn from histological specimens. (A) Failure of separation of the subesophageal ganglion (marked with a star) from the thoracic ganglion, lateral view. This phenotype is also seen in *Dfd* mutants. (B) Failure of brain fusion in the dorsal midline (compare arrows), frontal view. (C) Abnormal optic lobe position, lateral view. (D) Disorganization of the optic lobe neuropil, horizontal view. Optic lobe position is also abnormal in (D).

(Natzle et al., 1988). *IMP-E1* transcripts are found on membrane-bound polysomes, indicative of encoding a secreted or membrane-associated protein. Although the function of *IMP-E1* is not yet known, it is tempting to speculate that its product is involved in the remodeling of the blood-brain barrier and glial scaffold, and thereby promotes normal CNS morphogenesis. Disruption of *IMP-E1* expression might then cause morphogenetic defects of the type seen in *BR-C* mutants. If *IMP-E1* is a BRC target gene, then we would expect their spatial expression patterns to overlap, and that *BR-C* induction might precede that of *IMP-E1*.

A member of the Antennapedia complex, *Deformed* (*Dfd*) is a homeotic selector gene required for segment identity in the embryonic and adult head (reviewed in McGinnis et al., 1990). *Dfd* transcripts and protein in the CNS are restricted to a subset of subesophageal ganglion cells (Harding et al., 1985; Wedeen et al., 1986; Martinez-Arias et al., 1987, Chadwick & McGinnis, 1987; Mahaffey et al., 1989; Diederich et al., 1991). We have shown that *Dfd* mutants manifest SEG-TG separation failure indistinguishable from that of *BR-C* mutants (Restifo & Merrill, 1994). In addition, *Dfd* and *BR-C* mutants share overlapping phenotypes of the maxillary palps and proboscis, ventral adult head structures that are innervated by the SEG, and *br* mutations act as enhancers of the *Dfd* maxillary palp phenotype (Restifo & Merrill, 1994). If *Dfd* is a target of BRC proteins, then one might expect (i) that changes in *Dfd* expression in vivo would follow peaks of *BR-C* expression, and (ii) that *Dfd* expression would be 20HE-inducible, perhaps with late-gene response characteristics.

MATERIALS AND METHODS

Strains and stocks

Mutant and wild-type (Canton-S) strains used in these experiments have been described previously (Restifo & White, 1991; Restifo & Merrill, 1994). All cultures were performed at 25°C, 40–70% relative humidity, using the corn flour/yeast/sugar/agar medium of Elgin (1978), and care was taken to ensure optimal larval density. In most cases, the medium was supplemented with 0.05% bromphenol blue, as described in Maroni & Stamey (1983), for the purpose of staging wandering third instar larvae with respect to gut contents. Under our laboratory conditions, newly wandering larvae with blue staining of the entire gut (including proventriculus) will pupariate approximately 9 hr later. These animals correspond to Puff Stage 1 (Ashburner & Berendes, 1978), prior to the premetamorphic pulse of 20HE (Huet et al., 1993). Older wandering larvae who have cleared the gut fully will pupariate approximately 1.5 hr later. We used clear-gutted animals only if, at the time of dissection, their salivary glands were bloated due to secretion of glue into their lumen, which is a 20HE-triggered event (Boyd & Ashburner, 1977). Staging of prepupae and pupae was done according to the criteria of Bainbridge & Bownes (1981), and notations were made of the time elapsed following puparium formation or head eversion (pupation).

To generate animals bearing overlapping deficiencies that remove all *BR-C* genomic DNA, we mated *Df(1)S39 cho²/FM6l* virgin females to *y lt242/y²YSz280* males, and selected male wandering larvae with cho Malpighian tubules. To generate the most severe loss of *rbp⁺* function, we mated *y rbp⁵/Binsn* virgin females to *y npr1³ w sn³/y²Y67g* males, and selected yellow female larvae or prepupae.

Tissue preparation and RNA extraction

Tissues used for immediate RNA analysis were dissected in Ikeda Ringers as described previously (Restifo & White, 1991). RNA extraction was done as described in Guild (1984). CNS samples were transferred, without carryover of dissection buffer, directly into extraction buffer: phenol: chloroform: : isoamyl alcohol (50 : 25 : 24 : 1) and vortexed. Tubes were kept on ice while additional samples were collected. For in vitro culture, blue-gutted larvae were selected just after wandering, rinsed in oxygenated Robb's saline (Robb, 1969), and inverted after removal of the posterior end of the animal. This procedure leaves the CNS, as well as most of the peripheral nerve connections to imaginal discs, viscera and body-wall, intact. After the culture period, the CNS was dissected from the other tissues and its RNA extracted.

In vitro organ culture

Larval organs were cultured at room temperature (23 ± 1°C) in oxygenated Robb's phosphate-buffered saline according to the method of Karim & Thummel (1992). All samples were incubated for the first hour without addition of hormone, and all were cultured for a total of nine hours with oxygenation. Samples were incubated in saline containing either 20HE (5×10^{-6}M) or 20HE and cycloheximide (7×10^{-5}M) for the final 2, 4, 6, or 8 hr of culture.

Preparation of radiolabeled probes

Single-stranded riboprobes were synthesized in vitro from bacteriophage promoters (SP6, T3, and T7) by the general method of Melton et al. (1984), and labeled with either ^{3}H-UTP or ^{32}P-UTP and ^{32}P-CTP (NEN). To detect all *BR-C* transcripts, the cDNA clone pqdm527 (provided by G. Guild, University of Pennsylvania), which includes both *BRcore* and *Z1* regions (DiBello et al., 1991), was linearized with HindIII, and T7 polymerase was used to generate a 3.1-kb antisense cRNA. A *Z1*-specific antisense riboprobe of 700 bp was generated by linearizing pqdm527 with BamHI. The *BR-C* sense-strand probe was synthesized, after linearization with NdeI, using T3 polymerase. The template for synthesis of *IMP-E1* riboprobes was a genomic clone (E1-8.0RI, provided by J. Natzle, University of California at Davis; Natzle et al., 1988) linearized with ApaI. The SP6 polymerase-synthesized full-length cRNA is 8 kb, but there was usually a range of sizes (2–8 kb), with an average of about 4 kb. The template for synthesis of *Dfd* riboprobes was a cDNA clone (pcDfd41, provided by N. McGinnis; Regulski et al., 1987) linearized with HindIII; SP6 polymerase synthesizes a 3-kb cRNA.

Random hexamer-primed ^{32}P-dUTP labeling of DNA for *rp49* probes (O'Connell & Rosbash, 1984) was done by the method of Feinberg & Vogelstein (1984), as modified by Boehringer Mannheim. The template was a genomic subclone (pHR0.6, provided by C. Thummel, University of Utah) with a 0.6-kb insert, of which the majority is exon 3. Since *rp49* transcript levels are relatively constant during the

developmental intervals examined in these studies (Andres et al., 1993), they provide a reliable indicator of sample loading and blotting efficiency.

In situ hybridization

In situ hybridization to 6-μm sections of paraffin-embedded tissues was done as described by Ingham et al. (1985), with the modifications of Robinow & White (1988).

RNA blot analysis

RNA samples were size-fractionated electrophoretically on a denaturing 2.2M formaldehyde 1% agarose gel and transferred to uncharged nylon membrane (Nytran, Schleicher & Schuell) overnight in 10X SSC. A ladder of RNA size markers (Promega) ranging in size from 360 nt to 9.5 kb, was included in one or more lanes of each gel. The RNA was bound to the membrane by UV crosslinking in a Stratalinker (Stratagene) on the "auto" setting. The membrane was cut in two at approximately the 900-bp position to allow parallel probing for *rp49* transcripts. For riboprobes, blots were prehybridized for 4–6 hr at 65°C (60°C for *IMP-E1*) in 50% formamide, 5X SSPE, 1% SDS, 1X Denhardt's, 10 μg/ml poly(A), 250 μg/ml denatured salmon sperm DNA, and 100 μg/ml denatured yeast tRNA. For random-primed probes, the blot was prehybridized for 4–6 hr at 42°C in 50% formamide, 5X SSC, 50 mM $NaPO_4$ (pH 6.5), 0.5% SDS, 1X Denhardt's, and 250 μg/ml denatured salmon sperm DNA. Hybridization was performed overnight in fresh hybridization fluid at the same temperature as for prehybridization, with the addition of the probe ($2–5 \times 10^6$ cpm/ml). In some cases 6.25% dextran sulfate was added to the hybridization fluid.

High stringency washes were performed at 65°C (except 60°C for *IMP-E1*) in 0.1X SSPE, 0.5% SDS for riboprobes, and in 0.1X SSC, 0.1% SDS for random-primed probes. At this stringency, no signal is obtained with any *BR-C* riboprobe hybridized to RNA from animals bearing overlapping deletions that remove all *BR-C* genomic sequences (data not shown). Prior to repeated hybridization of a blot with a different probe, the membrane was washed for 30 min at 100°C in 10 mM Tris-HCl (pH 8), 1 mM Na_2EDTA (pH 8), 1% SDS.

Relative transcript abundance was quantified by computer-assisted analysis of autoradiograms using Image 1.44 software (NIH). All quantitative comparisons of transcript levels are relative to *rp49* and/or rRNA.

RESULTS

Spatial distribution of *BRcore* transcripts in the larval CNS

We began our investigation of *BR-C* expression in the CNS using in situ hybridization to sections of paraffin-embedded tissues dissected from larvae shortly after wandering. The eye-antenna and leg imaginal discs were retained in the preparations, attached to the CNS. Tritiated riboprobes were prepared from a cDNA clone containing both *BRcore* and *Z1* sequences (see Materials and methods). The antisense probe, which is capable of hybridizing to any *BR-C* transcripts, localizes to all cellular regions of the optic lobes, brain, and ventral ganglion (Fig. 3), as well as the subesophageal ganglion (ventral to the plane of section shown in Fig. 3).

At higher magnification (data not shown), the hybridization signal was seen over several distinct classes of cells: (i) neuronal somata of all anatomical regions, (ii) the neuroblasts of the optic lobe proliferation centers, (iii) midline cells of the ventral ganglion; (iv) large cells located at the junction of the neuropil and the rind of neuronal somata (see arrowheads in Fig. 3). The latter are likely to represent the interface or perineuropilar glia (Strausfeld, 1976), suggesting that *IMP-E1* and *BR-C* transcripts co-localize in these cells. The resolution of this in situ hybridization method is insufficient to determine what fraction of cells in a given region or of a given type express *BR-C* transcripts. It was also difficult to assess whether the hybridization signal at the periphery of the CNS was overlying

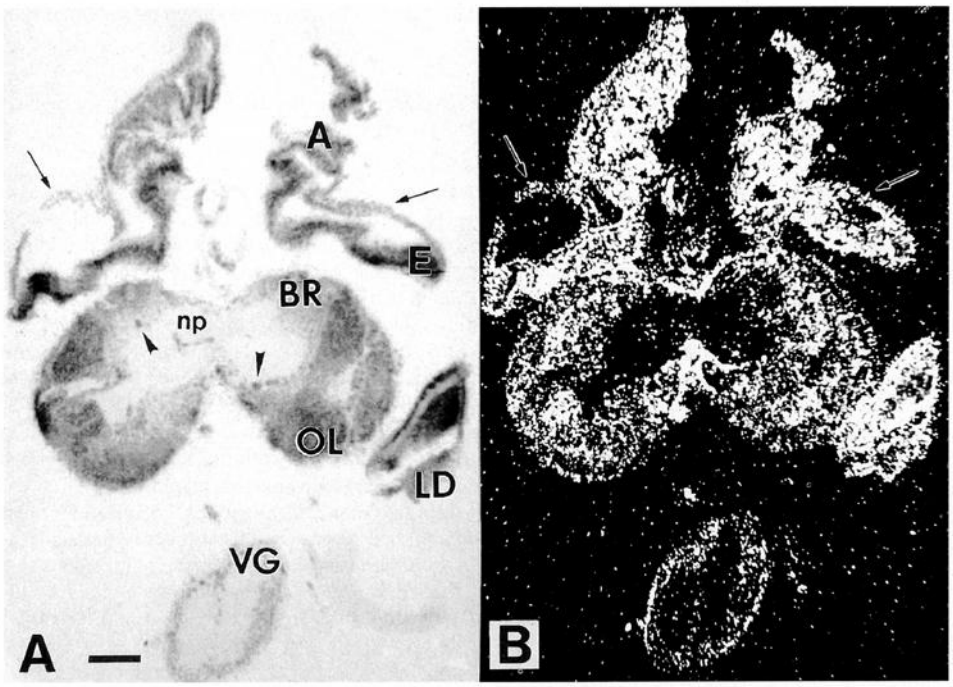

Fig. 3. *BR-C* transcripts in the larval CNS. Brightfield (A) and darkfield (B) images of a section throught the CNS and imaginal discs from a wandering third instar larva hybridized with a tritiated riboprobe made from a cDNA clone containing both *BRcore* and *Z1* regions (see Materials and methods). The section is horizontal but somewhat oblique, allowing brain and optic lobes as well as the distal ventral ganglion to be seen. All cellular regions (which appear dark in the brightfield image) show high concentrations of silver grain deposits, whereas the lightly-stained neuropil (np), which contains very few cell bodies, has very little signal. E and A – respective portions of the eye-antenna imaginal disc; OL – optic lobe; BR – brain; VG – ventral ganglion; LD – leg imaginal disc. Arrows indicate the peripodial membrane of the eye-antenna disc. Arrowheads point to large glial cells at the interface between the neuropil and neuronal cell body layer. Scale bar = 50 μm.

the perineurium, but immunostaining with an anti-BRcore antibody showed protein expression in nuclei of this structure (Restifo & H. Alonso-Pimentel, unpublished).

Hybridization signals from CNS samples immediately after wandering and 6–8 hr after wandering were indistinguishable (data not shown). A robust hybridization signal is also seen over the cells of the imaginal discs, including both the peripodial membrane (arrows in Fig. 3) and the folded columnar epithelium. Hybridization to sections of dissected tissues or whole larvae with the sense probe revealed no signal above background over any tissue (data not shown).

IMP-E1 expression in the CNS in vivo and in vitro

Natzle et al. (1988) demonstrated 20HE-inducible *IMP-E1* expression in the late larval CNS. To extend their analysis, we performed Northern blot experiments on CNS RNA from individuals ranging in age from foraging third instar larvae to young pupae. As seen

in Fig. 4, an *IMP-E1* transcript of approximately 6.0 kb is detectable in larval, prepupal, and pupal CNS RNA. Because we find a single transcript size class in whole animals, and the same sized transcript in the CNS, we believe this corresponds to the previously reported transcript of 7–7.5 kb (Natzle et al., 1988; Natzle, 1993 and pers. comm.). *IMP-E1* transcript levels are very low in foraging larvae and blue-gutted wandering larvae, but rise considerably in clear-gutted larvae. Quantitation of these and other comparable data (e.g., Fig. 6) indicates the average increase in *IMP-E1* CNS transcript abundance between the blue- and clear-gutted stages is approximately 5-fold (range 2–10 fold; 10 independent RNA preparations). This suggests the premetamorphic pulse of 20HE has induced accumulation of *IMP-E1* transcripts in the CNS, although such an effect need not be due to direct stimulation of transcription rate. *IMP-E1* transcript levels remain very high in white prepupae, sometimes exceeding those in clear-gutted larvae (see Fig. 6). Note that the magnitude (relative to *rp49*) and timing (clear-gutted larvae vs. white prepupae) of peak *IMP-E1* transcript accumulation varies among independent wild-type CNS RNA preparations, suggesting individual variation in these parameters. *IMP-E1* transcript levels decline during the prepupal period, remaining moderate in young pupae (P5). This CNS time-course parallels that reported for *IMP-E1* transcripts in whole animals (Natzle et al., 1992).

The in vivo CNS expression profile of *IMP-E1* contrasts with that of *BR-C Z1* transcripts (Fig. 4). Like *IMP-E1*, *BR-C Z1* expression is very low in the CNS of foraging animals. However, the 4.6-kb *BR-C Z1* transcripts are already present at greater-than-baseline levels in the CNS of blue-gutted wandering larvae, increasing less than two-fold by the clear-gutted stage (Restifo et al., 1993). Similar results are seen for the three *Z2* and three *Z3 BR-C* transcripts in the CNS, such that no *BR-C* transcript shows an accumulation pattern suggesting significant induction by the premetamorphic peak of 20HE (Restifo et al., 1993). Thus, *BR-C Z1* transcript accumulation precedes that of *IMP-E1* in the CNS. In addition, *BR-C Z1* transcripts undergo a less pronounced decline (relative to their own peak) during the prepupal period and early pupal period than do *IMP-E1* transcripts.

In vitro, *IMP-E1* transcripts in the CNS respond rapidly to 20HE, consistent with an early response to the hormone. But, as has been shown in imaginal discs (Natzle, 1993), peak *IMP-E1* induction is not enhanced significantly when protein synthesis is inhibited by cycloheximide (14-fold with 20HE alone vs. 17-fold with 20HE plus cycloheximide; Fig. 5). Under identical conditions, *BR-C Z1* transcript induction by 20HE is boosted from 16- to 45-fold (Estes & Restifo, unpublished). Thus, *IMP-E1* expression, in both the CNS and imaginal discs, fails to manifest the superinduction that typifies hormonal responses in the presence of cycloheximide of early genes of the transcriptional regulator class. The somewhat delayed accumulation of *IMP-E1* transcripts in the presence of 20HE and cycloheximide, compared to 20HE alone, may reflect their sensitivity to toxic effects of the protein synthesis inhibitor, as has been seen in imaginal discs (Natzle, 1993).

IMP-E1 induction in *BR-C* mutants

BR-C transcripts or proteins are spatially and temporally poised to regulate the response to 20HE of *IMP-E1* in the perineurium and interface glia. To test the hypothesis that induction of *IMP-E1* in the CNS requires *BR-C*$^+$ function, we examined CNS RNA from *BR-C* mutants of all three complementation groups at the larval-prepupal transition,

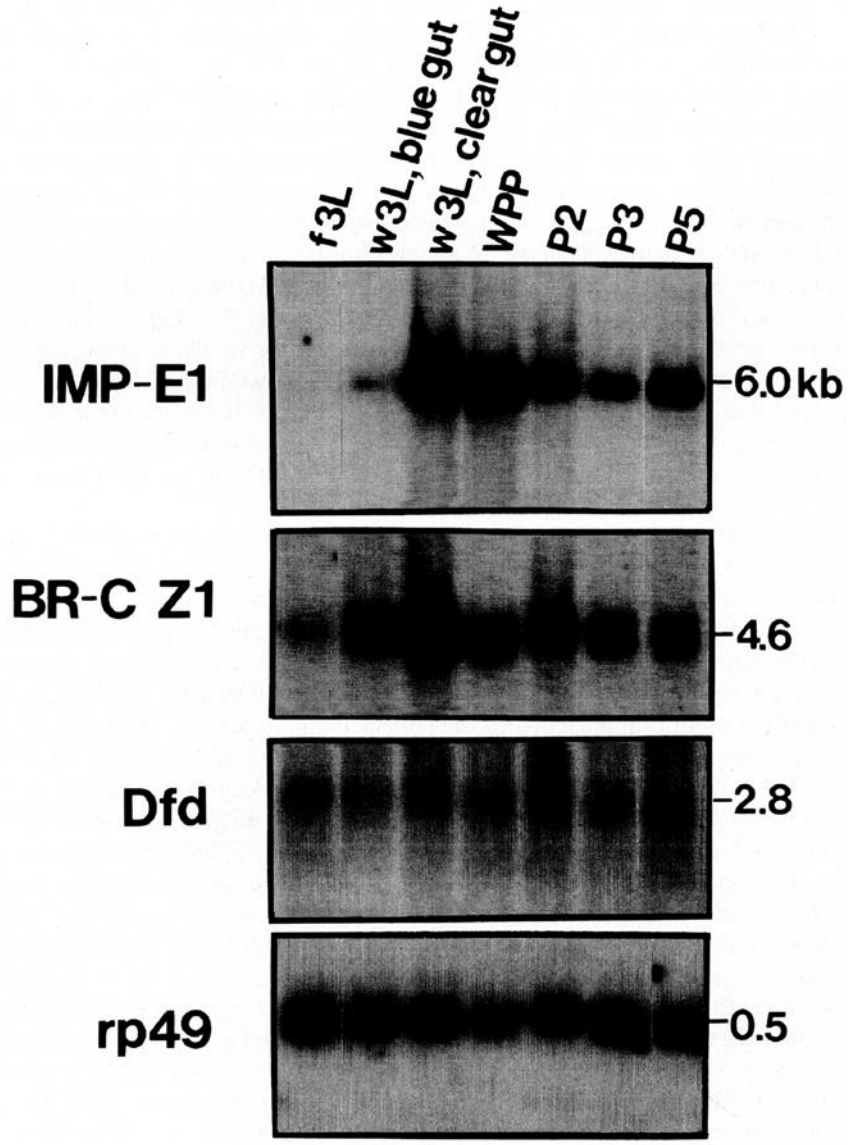

Fig. 4. In vivo transcript profiles during the larval-pupal transition. Each lane contains 10 μg total RNA of CNS dissected from full-sized foraging third instar larvae (f3L), blue-gutted wandering third instar larvae (w3L, blue gut), clear-gutted wandering third instar larvae (w3L, clear gut), white prepupae (WPP), brown prepupae (P2), bubble prepupae (P3), or young pupae 1–2 hr after head eversion (P5). The blot was probed serially for *IMP-E1*, *BR-C Z1* (using a Z1-specific probe), *Dfd*, and *rp49* transcripts. Relevant portions of the autoradiograms are shown.

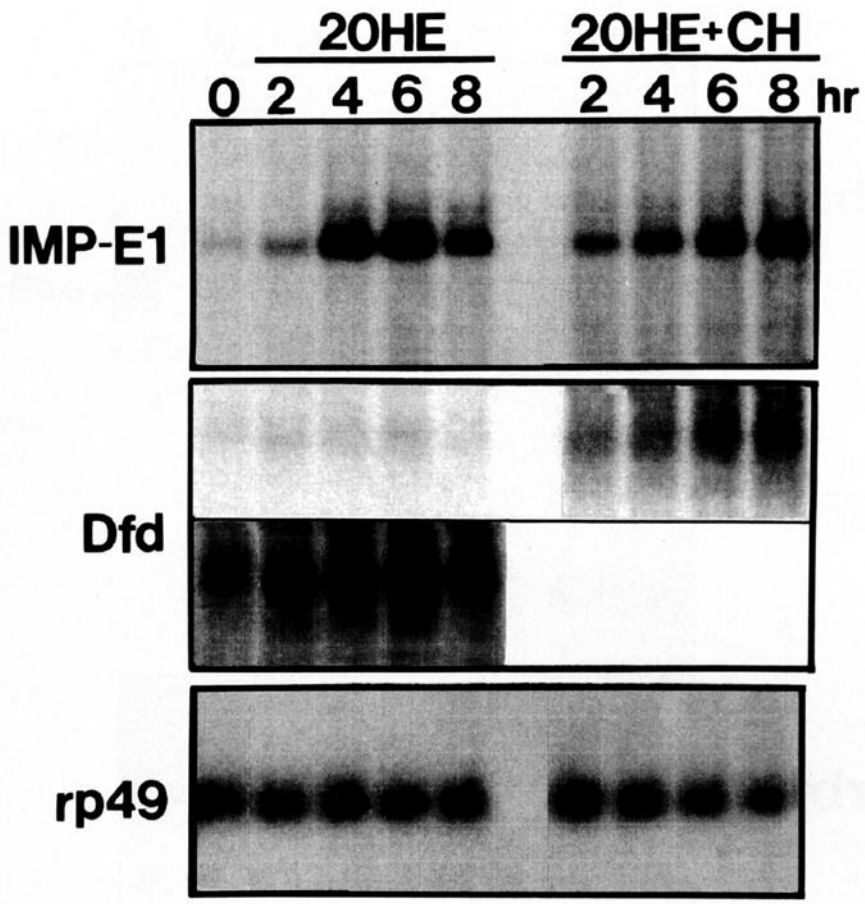

Fig. 5. Expression of *IMP-E1* and *Dfd* during in vitro culture with 20HE. Each lane contains 10 μg total RNA of CNS from larvae cultured in vitro for 9 hr, with either 20HE (5×10^{-6}M) or 20HE and cycloheximide (CH; 7×10^{-5}M) added during the indicated final hours of incubation (see Materials and methods for details). The blot was probed serially for *IMP-E1*, *Dfd*, and *rp49*. In the *Dfd* panel, the top part represents a relatively short exposure autoradiogram, and the bottom left part represents a longer exposure.

specifically at blue-gutted and clear-gutted wandering and at white prepupal stages. In each case we also examined samples from two types of *BR-C*$^{+}$ animals, a wild-type laboratory strain (Canton-S), and genetic control animals in which the mutation was covered by a wild-type copy of the *BR-C* region (see Materials and methods). The mutants used were the strongest available for each class. Amorphic alleles (*br*^{5} and *2Bc*^{1}) were used if available; for *rbp* (for which no amorphic allele exists), we generated animals bearing the strongest available allele (*rbp*^{5}) over a null allele for the entire locus (*npr1*^{3}). This

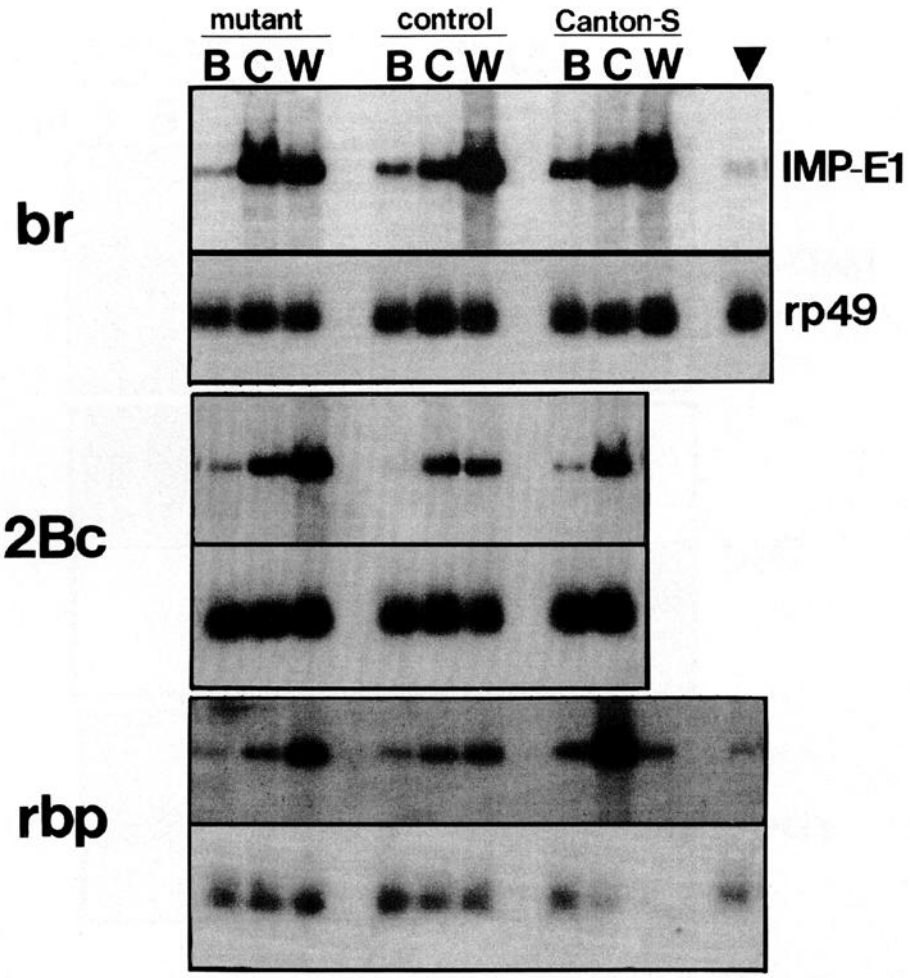

Fig. 6. *IMP-E1* induction in vivo in *BR-C* mutants. For each of three separate experiments, we compared *IMP-E1* transcript levels in the CNS (20 per lane) of blue-gutted wandering larvae (B), clear-gutted wandering larvae (C), and white prepupae (W). Canton-S CNS samples were included in each experiment to show the wild-type induction pattern. In each panel, the top portion shows the *IMP-E1* transcripts and the bottom shows *rp49* transcripts. To test the effect of *br*, we compared *y* br^5/Y (mutant) to *y* br^5/y^2Y67g (control). For *2Bc*, the mutants were *y* $2Bc^1/Y$ and the controls were *y* $2Bc^1/y^2Y67g$. In the case of *rbp*, the mutants were *y* rbp^5/y $npr1^3$ *w* sn^3 and the controls were their *y* $rbp^5/Binsn$ sibs. The solid triangle represents RNA from 20 CNS dissected from blue-gutted wandering larvae lacking *BR-C* genomic DNA.

effectively halved the dose of residual *rbp* function, relative to an rbp^5 homozygote, resulting in death at the time of pupation.

In all three cases, in vivo CNS *IMP-E1* induction is comparable in mutants and the controls (Fig. 6). Note that there is some variability among $BR\text{-}C^+$ animals in the timing of

peak transcript level, most often seen in white prepupae, but sometimes in clear-gutted third instar larvae (e.g., the *2Bc* control). However, there is no consistent difference between mutants and controls in the timing of the peak. Thus, function of any two *BR-C* complementation groups is sufficient for in vivo induction of *IMP-E1* in the CNS. Because of the possibility of functional redundancy among the *BR-C* complementation groups, additional experiments would be required to rule out some role of *BR-C* in *IMP-E1* induction in the CNS. Note that blue-gutted larvae lacking all *BR-C* genomic DNA (lanes marked with triangle in Fig. 6), do have low levels of *IMP-E1* CNS transcripts. However, these animals can not be used for in vivo experiments to examine the effect of complete loss of $BR\text{-}C^{+}$ function, because they die during a period of prolonged wandering with variable degrees and timing of gut evacuation (Restifo, unpublished observations).

Deformed expression in the CNS in vivo and in vitro

A prominant 2.8-kb *Dfd* transcript has been seen by Northern analysis of whole-animal poly(A)$^{+}$ RNA (Chadwick & McGinnis, 1987). A similarly sized transcript is detectable in the CNS of third instar larvae, prepupae and early pupae, with no notable change in abundance during that interval (Fig. 4). Thus, unlike *IMP-E1* and *BR-C*, *Dfd* transcript levels (i) do not increase between foraging and wandering third instar larval stages; and (ii) do not decline during the prepupal-pupal transition. In recent preliminary experiments, we have found wild-type levels of *Dfd* transcripts in the CNS of $BR\text{-}C^{-}$ animals (data not shown).

During in vitro culture, *Dfd* CNS transcript levels are relatively indifferent to exposure to 20HE (Fig. 5). In the presence of 20HE and cycloheximide, CNS *Dfd* transcript levels are somewhat boosted with respect to those seen with 20HE alone. This could reflect transcript stabilization due to the absence of newly synthesized proteins that normally enhance the turnover of *Dfd* transcripts. Alternatively, *Dfd* transcription may be negatively regulated by proteins with very short halflives. The relative constancy of *Dfd* transcript levels in vivo and in vitro has been seen in multiple independent experiments. Thus, we find no evidence in the larval or prepupal CNS of *Dfd* induction by 20HE.

DISCUSSION

The distribution of *BR-C* transcripts in the larval CNS is widespread, including all regions whose metamorphic development is disrupted in *BR-C* mutants (Fig. 3). This is consistent with an autonomous role of BRC target gene expression in the metamorphosis of optic lobes, brain, and subesophageal ganglion. However, genetic mosaic analysis will be required to test this hypothesis, and an influence of other tissues is not precluded by the data. Spatial distributions of the individual *BR-C* zinc-finger variants may shed some light on their regional functions. The spatial pattern of *BR-C* transcripts suggests they may co-localize with *IMP-E1* in a subset of CNS glia, and with *Dfd* in the subesophageal ganglion. Our primary goal was to investigate the positions of *IMP-E1* and *Dfd* relative to *BR-C* in the genetic pathway(s) responsible for CNS metamorphosis.

IMP-E1 expression in the CNS has two hallmarks of an early response to 20HE, namely rapid transcript accumulation upon exposure to hormone in vivo or in vitro, and induction in the presence of a protein synthesis inhibitor (Figs 4, 5). Nuclear run-off experiments

demonstrate that *IMP-E1* induction in mass-isolated imaginal discs occurs at the level of transcription initiation (Natzle, 1993), but such experiments have not been performed on CNS samples. Because of the methods used for its cloning, *IMP-E1* is expected to encode a secreted or membrane-associated protein (Natzle et al., 1988). Thus it is not surprising that in the CNS (Fig. 5) and imaginal discs (Natzle, 1993), inhibition of protein synthesis does not cause the superinduction typical of early genes that encode transcriptional regulators, e.g., *BR-C* (Chao & Guild, 1986; Estes & Restifo, unpublished), *E74* (Thummel et al., 1990) and *E75* (Segraves & Hogness, 1990).

A recent study of EcR localization in the CNS revealed EcR-B1 in the perineurium of wandering third instar larvae, but neither EcR-A nor EcR-B1 was detected in the interface glia until after pupariation (Truman et al., 1994). Thus, the induction of CNS *IMP-E1* transcripts, assesed by Northern analysis, may represent the response of the perineurial glia only. Molecular and genetic characterization of *IMP-E1* should help determine whether it functions in blood-brain barrier remodeling or in reorganization of the interface glial scaffold that segregates neuronal cell bodies from their processes.

The early puff genes differ in their in vitro sensitivities to 20HE and show a variety of temporal expression profiles in vivo (Karim & Thummel, 1992; Huet et al., 1993). *BR-CZ1* transcript accumulation in the CNS of young (blue-gutted) wandering larvae (Fig. 4) suggests a high-sensitivity response, which may reflect induction by the leading edge of the pre-metamorphic 20HE peak. Alternatively, CNS *BR-C* expression may respond to a low-amplitude 20HE pulse during larval wandering, analogous to the "commitment" pulse in *Manduca* (see Andres et al., 1993). Higher resolution analysis of *BR-C* expression during the third instar, currently underway, should allow us to distinguish between these possibilities.

We addressed the question of whether *BR-C*, which regulates the hormonal responsiveness of many classes of genes (see Introduction), is necessary for CNS *IMP-E1* induction at the larval-prepupal transition. Our analysis of mutants shows clearly that no single *BR-C* complementation group is absolutely required for the full hormonal induction of *IMP-E1* transcript accumulation in the CNS. (There is a minor caveat with respect to rbp^+ function, because it is not possible with the existing mutants to eliminate it completely while also retaining the other functions.) Before concluding that *BR-C* has no influence on *IMP-E1* response to 20HE, one must consider the possibility that the three complementation groups represent redundant or overlapping roles, such that elimination of any one of them is insufficient to disrupt the hormonal response. Indeed, the fact that mutants of the three complementation groups share CNS morphogenetic phenotypes, and show variable penetrance and expressivity of those phenotypes (Restifo & White, 1991), suggests that br^+, rbp^+, and $2Bc^+$ functions do overlap. So, to rule out a requirement of $BR\text{-}C^+$ function for *IMP-E1* induction, we are now assessing CNS *IMP-E1* transcript accumulation in *BR-C* null mutants cultured with 20HE in vitro. Our preliminary results demonstrate hormone inducibility of *IMP-E1* in $BR\text{-}C^-$ CNS.

It is possible that *IMP-E1*, as well as other primary response genes encoding "worker" proteins (e.g., *Eip28/29*; Cherbas, 1993), represent one or more *BR-C*-independent hormone-regulated genetic pathways. If so, these may contribute to the lethality of *BR-C* null mutants, whose death after a day or two of prolonged wandering contrasts with the 2–3-week prolongation of larval life in some ecdysterone-deficient mutants (Garen et al.,

1977; Schwartz et al., 1984; Holden et al., 1986). Perhaps the mutants are made sick by induction of BRC-independent early genes whose products in the cytoplasm, plasma membrane and extracellular space act in the absence of BRC-dependent late gene products. The net effect of this imbalance in multiple tissues might cause widespread pathology, leading to death of the animal in a relatively short period of time.

Our in vivo and in vitro analyses of *Dfd* expression in the CNS suggest that this homeoprotein-encoding gene is indifferent to 20HE levels (Figs 4, 5). This constitutive expression pattern makes it unlikely that *Dfd* is a BRC target gene during the larval-pupal transition. However, it does not rule out direct or indirect interaction of BRC and DFD proteins, such as overlapping target genes, during SEG maturation. The situation may be different in the epidermis where there is already one precedent for a DFD target not expressed in the CNS (Mahaffey et al., 1993). Both *BR-C* and *Dfd* transcripts are expressed in the antennal disc (Fig. 3 and Chadwick & McGinnis, 1987, respectively), from which maxillary palps and proboscis develop, and the br^{27} mutation acts as an enhancer of the *Dfd* palp phentoype (Restifo & Merrill, 1994). Thus, *Dfd* expression in the disc may require *BR-C*$^+$ function.

How can one reconcile the indistinguishable SEG phenotypes of *BR-C* and *Dfd* mutants with the distinct difference in hormone-responsiveness of the two genes? CNS maturation requires rapid elongation of axonal projections of ascending TG neurons and descending neurons of the optic lobes, brain, and SEG. Under normal circumstances this results in formation of the cervical connective. We propose that coordinated growth of this subset of CNS neurons requires at least: (i) positional or segment identity information (e.g., provided by *Dfd* in the SEG); (ii) a particular pattern of 20HE titer; and (iii) *BR-C*$^+$ function, acting via target genes to transduce the hormonal signal. BRC targets are predicted to include those encoding cytoskeletal components required in large quantities during rapid axonal growth. Involvement of cells other than CNS neurons, including glia, afferent inputs, and efferent targets may also play an essential regulatory role. For example, connections with the body wall may produce mechanical tension to which a subset of cells responds by rapid axon elongation, as has been shown for vertebrate neurons (Heidemann & Buxbaum, 1994). We predict that loss-of-function mutations in a variety of seemingly unrelated genes could give rise to an "SEG-TG separation" phenotype.

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