DOI: 10.1534/genetics.105.046631

Interaction Between Hormonal Signaling Pathways in *Drosophila melanogaster* as Revealed by Genetic Interaction Between *Methoprene-tolerant* and *Broad-Complex*

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Manuscript received June 9, 2005
Accepted for publication September 20, 2005

ABSTRACT

Juvenile hormone (JH) regulates insect development by a poorly understood mechanism. Application of JH agonist insecticides to *Drosophila melanogaster* during the ecdysone-driven onset of metamorphosis results in lethality and specific morphogenetic defects, some of which resemble those in mutants of the ecdysone-regulated *Broad-Complex* (*BR-C*). The *Methoprene-tolerant* (*Met*) bHLH–PAS gene mediates JH action, and *Met* mutations protect against the lethality and defects. To explore relationships among these two genes and JH, double mutants were constructed between *Met* alleles and alleles of each of the *BR-C* complementation groups: *broad* (*br*), *reduced bristles on palpus* (*rbp*), and *2Bc*. Defects in viability and oogenesis were consistently more severe in *rbp Met* or *br Met* double mutants than would be expected if these genes act independently. Additionally, complementation between *BR-C* mutant alleles often failed when MET was absent. Patterns of BRC protein accumulation during metamorphosis revealed essentially no difference between wild-type and *Met*-null individuals. JH agonist treatment did not block accumulation of BRC proteins. We propose that MET and BRC interact to control transcription of one or more downstream effector genes, which can be disrupted either by mutations in *Met* or *BR-C* or by application of JH/JH agonist, which alters MET interaction with BRC.

HORMONAL regulation of insect development involves the relatively well-understood steroid 20-hydroxyecdysone (20E) (Henrich and Brown 1995; Riddiford *et al.* 2000; Spindler *et al.* 2001; Thummel and Chory 2002) and the more enigmatic sesquiterpenoid juvenile hormone (JH). 20E orchestrates molting and metamorphosis and regulates reproduction (Wyatt and Davey 1996; Riddiford *et al.* 2000). Its dimeric receptor ECR/USP is a 20E-dependent transcription factor that regulates the expression of target genes, including both primary response (early) genes, such as *Broad-Complex* (*BR-C*), that are directly and rapidly induced by 20E and secondary response (late) genes that are regulated by the primary response gene products (Ashburner *et al.* 1974; Richards 1997). JH action during larval development, at least in lepidopteran and hemimetabolous insects, is to maintain the "status quo," probably to allow proper larval molting and prevent premature metamorphosis (Williams 1961; Zhou and Riddiford 2002). At the end of larval development, circulating JH is degraded, enabling 20E to trigger metamorphosis (Riddiford 1996). JH reappears in many adult insects to control both oogenesis and male accessory gland function (Wyatt and Davey 1996). Neither the JH receptor nor its molecular mechanism of action is well understood (Gilbert *et al.* 2000), although JH, as well as JH agonists (JHA), can regulate gene expression (Jones 1995; Dubrovsky *et al.* 2000; Sempere *et al.* 2002). The 20E and JH signaling pathways interact during development. In hemipteran and lepidopteran larvae, withdrawal of JH resulted in 20E induction of precocious metamorphosis, whereas application of JH during the last larval instar resulted in a supernumerary instar or larval-pupal intermediate (Riddiford 1994; Gilbert *et al.* 2000). These two hormones also interact in adults to control oogenesis (Soller *et al.* 1999).

In *Drosophila melanogaster*, metamorphosis is controlled by several waves of 20E secretion: the first at the end of the third larval instar driving pupararium formation, the second 10–12 hr after pupararium formation (APF) triggering pupation, and finally a large wave beginning 25–30 hr APF (Handler 1982; Riddiford

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1993). JH has been detected in Drosophila larvae but not pupae (Bownes and Rembold 1987; Sliter *et al.* 1987) when its absence is thought to permit 20E control of metamorphosis. Application of exogenous JH or JH analog insecticides, such as methoprene and pyriproxyfen which act as JHA (Staal 1975; Wilson 2004), does not block puparium formation or pupation. Rather, it results in disruptions of the adult abdominal epidermis, male genitalia orientation, and many internal tissues, resulting in death during the late pupal (pharate adult) stage (Ashburner 1970; Madhavan 1973; Postlethwait 1974; Wilson and Fabian 1986; Riddiford and Ashburner 1991; Restifo and Wilson 1998).

The primary response gene *BR-C* is composed of three lethal complementation groups: *broad* (*br*), *reduced bristles on the palpus* (*rbp*), and *2Bc*, mutations of which cause death during prepupal or pupal stages (Belyaeva *et al.* 1980; Kiss *et al.* 1988). Null alleles of the entire gene, called *nonpupariating1* (*npr1*), cause death in late third-instar larvae (Kiss *et al.* 1988; Gonzy *et al.* 2002), demonstrating that *BR-C* function is essential for metamorphic onset. *BR-C* uses alternative splicing to encode a small family of transcription factors with amino-terminal BTB–POZ domains linked to one of four possible C_2H_2 zinc-finger domains: BRC–Z1, BRC–Z2, BRC–Z3, and BRC–Z4 (Dibello *et al.* 1991; Bayer *et al.* 1996). During metamorphosis BRC isoforms regulate numerous downstream effector genes (Guay and Guild 1991; Karim *et al.* 1993; von Kalm *et al.* 1994; Hodgetts *et al.* 1995; Bayer *et al.* 1996, 1997; Crossgrove *et al.* 1996; Liu and Restifo 1998; Mugat *et al.* 2000; Dubrovsky *et al.* 2001; Dunne *et al.* 2002; Sempere *et al.* 2003). Although there is some functional redundancy among the isoforms, in general, BRC–Z1 provides the function for *rbp*, BRC–Z2 for *br*, and BRC–Z3 for *2Bc* (Crossgrove *et al.* 1996; Bayer *et al.* 1997; Sandstrom *et al.* 1997; Liu and Restifo 1998; Consoulas *et al.* 2005).

BR-C metamorphosis phenotypes include failures of larval tissue histolysis (Lee and Baehrecke 2001; Kucharova-Mahmood *et al.* 2002), epidermal morphogenesis (Kiss *et al.* 1988), and internal tissue remodeling (Restifo and White 1991, 1992; Sandstrom *et al.* 1997; Consoulas *et al.* 2005). Several phenotypes, such as a split-brain abnormality (Restifo and White 1991) were common to all *BR-C* mutants, while others, such as failure of thoracic muscle attachment or persistence of larval salivary glands (Restifo and White 1992) were restricted to one or two complementation groups. This suggested that BRC proteins operate in two pathways, "common," requiring all three functions and "restricted," requiring a subset of them (Restifo and White 1991, 1992; Restifo and Wilson 1998). Following metamorphosis *BR-C* is expressed in egg chamber follicle cells to function during oogenesis (Huang and Orr 1992; Deng and Bownes 1997; Tzolovsky *et al.* 1999).

Our previous work showed that lethal pharate adults developing from wild-type methoprene-treated larvae showed a selective *BR-C* phenocopy, including disruptions of the central nervous system and salivary glands (Restifo and Wilson 1998). The methoprene syndrome was striking in that it included none of the restricted-pathway defects, which is inconsistent with methoprene simply blocking the 20E induction of *BR-C* expression during the larval-to-pupal transition. We interpreted the pathological effects of methoprene to reflect dysfunction of *BR-C* and probably additional primary response genes during metamorphosis, resulting in aberrant expression of secondary response genes. In contrast, other investigators found that the JHA pyriproxyfen caused abnormal pupal cuticle gene expression, apparently due to abnormal *BR-C* expression (Zhou and Riddiford 2002).

Methoprene-tolerant (*Met*) is essential for the manifestation of the toxic and morphogenetic effects of JH/JHA in *D. melanogaster* (Wilson and Fabian 1986; Riddiford and Ashburner 1991; Wilson 1996; Restifo and Wilson 1998). *Met* mutants are resistant to these effects of methoprene (Wilson and Fabian 1986). MET can bind JH III with specificity and nanomolar affinity (Shemshedini and Wilson 1990; Miura *et al.* 2005), suggesting that it is a component of a JH receptor. *Met* encodes a bHLH–PAS transcriptional regulator family member (Ashok *et al.* 1998) and MET can activate a reporter gene in transfected Drosophila S-2 cells (Miura *et al.* 2005).

We hypothesize that *BR-C* and *Met* function together in one or more aspects of development. If methoprene disrupts 20E-mediated metamorphic development or oogenesis by acting on *BR-C* or its downstream genes, one would expect double mutants to show synergistic genetic interaction, such as synthetic lethality of viable alleles, shifts in lethal phase, or enhanced oogenesis defects. We found that animals carrying both *Met* and *BR-C* mutations showed just such synergistic interactions. However, we did not detect any major disruption in BRC protein accumulation following methoprene treatment, suggesting that the MET and BRC interact to regulate expression of downstream effector gene(s).

MATERIALS AND METHODS

Stocks: *BR-C* mutant alleles used in this study were previously used to investigate internal tissue metamorphosis (Restifo and White 1991, 1992; Restifo and Merrill 1994; Sandstrom *et al.* 1997; Consoulas *et al.* 2005). *BR-C* mutant progeny were identified by visible markers *yellow*, *white*, and/or *singed*. Wild-type *BR-C* function was provided by second- and/or third-chromosome *BRC–Z1*, *BRC–Z2*, or *BRC–Z3* cDNA transgenes with *hsp70* promoters (Bayer *et al.* 1997). The third-chromosome *hsBRC-Z3* transgene, provided by C. Bayer (University of Central Florida, Orlando, FL) is leaky in a temperature-sensitive manner (R. Spokony, H. J. Clark, and L. L. Restifo, unpublished data). Survival to adult eclosion of $2Bc^{l}$*/Y; hsBRC-Z3/+* varied with rearing temperature: 9% at 22–23°, 12% at 25°, and 29% at 29°. Most of the *Met* alleles used in this study were isolated from a methoprene-susceptible isogenic *vermilion* (*v*) strain as previously described (Wilson and Fabian 1987). After isolation, each *Met* allele was backcrossed to

v for 5–7 generations to minimize background genome differences that might impact comparison of phenotypes. A functional copy of Met^+ was provided by a second-chromosome genomic transgene (Ashok *et al.* 1998; Wilson and Ashok 1998). The Y-borne duplications $y^2Y67g\ 19.1$ (hereafter termed y^2Y67g), bearing $BR\text{-}C^+$ (Belyaeva *et al.* 1980; Lindsley and Zimm 1992) and $y^+Yv^+B^{S-}$ (hereafter termed $Y\text{-}Met^+$), bearing Met^+ (Lindsley and Zimm 1992) were used to cover *BR-C* and *Met* mutations, respectively.

Double mutants were constructed by genetic recombination, and each was maintained heterozygous with an *FM7* balancer chromosome. Each double-mutant chromosome was tested to verify the presence of each mutation and the absence of inadvertently introduced lethal mutations.

***D. melanogaster* culture and methoprene treatment:** Stocks and crosses were cultured on one of three standard media with mold inhibitors, at 25° with a 12:12 L:D photoperiod unless otherwise specified. For experiments comparing phenotypes, the same culture medium and other conditions were used for all genotypes. For progeny phenotype analysis, cultures were performed in glass vials (Capital Vial) without larval crowding, and all progeny were censused and examined, either as adults or as uneclosed pupae.

Cultures were tested for methoprene resistance on diagnostic doses given in Table 5 of ZR-2008, the biologically active isomer of methoprene [isopropyl-(2E,4E)-11-methoxy-3,7,11-trimethyl-2,4-dodecadienonate], as previously described (Wilson 1996; Wilson and Ashok 1998). At lethal concentrations, mortality occurs in Met^+ typically during the pharate adult stage. Eclosing survivors were examined for methoprene-induced morphogenetic defects of malrotated male genitalia and defective sternal bristles, particularly on the posterior sternites. For analysis of BRC proteins, 100–120 *OreRC* or $v\ Met^{27}$ eggs were transferred to glass bottles containing Drosophila Instant Food (Carolina Biologicals) with either a high-lethal dose of methoprene or vehicle (acetone) alone, as described in Restifo and Wilson (1998). Control and methoprene-treated cultures were reared in parallel.

***BR-C* phenotype examination:** Well-described *BR-C* phenotypes, including lethality and epidermal defects of wing and maxillary palpus morphology (Kiss *et al.* 1988) were examined. Lethality was assigned to the prepupal, pupal, or pharate adult stage on the basis of external appearance (Bainbridge and Bownes 1981). Homozygous or hemizygous Met^{27} pupae can be identified by a slightly elongated pupal case (T. G. Wilson, unpublished data). For quantitative lethal-phase analysis, white or very young brown prepupae were transferred to moistened ashless filter paper (Whatman no. 42) in small glass petri dishes and allowed to continue developing in a humid chamber. Developmental stage was monitored daily until eclosion or death was evident. We found some variability (<5%) in the survival rates from pupariation to eclosion of $Met\ br^1$ and $Met\ rbp^2$ in different genetic backgrounds. To accurately compare the phenotypes of the various allele combinations, crosses were standardized using *FM7*-balanced mothers whenever possible.

Ovipositional rate and ovary examination: Females isolated within 4–6 hr after eclosion were provided with wild-type (*OreRC*) males in food vials sprinkled with baker's yeast. Egg counts were made at 2-day intervals when the medium was changed, and fertility of the eggs was noted. Oogenesis was assessed by dissecting ovaries from females at several times after eclosion and examining for the presence of stages 8–14 vitellogenic oocytes as previously described (Wilson and Ashok 1998).

Analysis of BRC protein accumulation by immunoblotting: White prepupae (WPP) were collected and either homogenized immediately or placed in humid chambers for further development. In experiments with late-pupal stages, animals were resynchronized at head eversion. Protein extraction was based on the method of Emery *et al.* (1994). For each time point, 5–10 animals were homogenized in 50–100 μl sample buffer with a Teflon pestle in a microcentrifuge tube. Sample buffer consisted of 75 mm Tris-HCl, pH 6.8, 6% SDS, 15% glycerol, 10% β-mercaptoethanol and protease inhibitors (0.1 μg/μl pepstatin A, 0.5 μg/μl leupeptin, and 10 mm PMSF, Sigma). Following centrifugation for 10 min at 14,000 rpm (Eppendorf 5415C), the supernatant was used immediately for electrophoresis or stored at −80° for up to 3 weeks, which did not compromise BRC protein stability (data not shown).

Extracts representing 0.25- or 0.5-animal equivalents were heated for 5 min at 90°, quick chilled on ice for 10 min, and separated by SDS–PAGE (Towbin *et al.* 1979). For optimal band separation and size assessment, we used large (16 × 18 cm) 10% acrylamide gels on a Hoefer SE 600 Ruby electrophoresis apparatus at constant current (30 mA) for 5 hr. Otherwise, 12% acrylamide gels (7 × 8 cm) were run on a Bio-Rad mini-PROTEAN II apparatus at constant voltage (195 V) for 50 min. Proteins were transferred to nitrocellulose membranes (Protran, 0.45 μm, Schleicher & Schuell) by electroblotting at 4° overnight. Overall protein pattern was detected by staining the membrane with 0.5% Ponceau-S (Sigma, St. Louis).

Nonspecific binding sites were blocked with phosphate-buffered saline (PBS) plus 0.1% Tween 20 (TPBS) and 5% w/v nonfat dry milk powder (Bio-Rad) for 90 min at room temperature. Blots were probed with either anti-BRcore (mAb25E9) or anti-Z1 (mAb3C11) mouse monoclonal antibodies (Emery *et al.* 1994), diluted 1:2,500 or 1:100, respectively, in PBS + 5% w/v milk powder, for 2 hr at room temperature or overnight at 4°. After three 10-min washes in TPBS, the blots were incubated with HRP-conjugated goat anti-mouse IgG (Cappel, ICN) at 1:10,000 (with anti-BRcore) or 1:2,500 (with anti-Z1) for 1 hr at room temperature. After another three 10-min washes, the signal was revealed by chemilumiscent detection of HRP (ECL detection kit; Amersham-Pharmacia) and exposure to X-ray film (Kodak X-OMAT AR). To evaluate lane loading, the blot was stripped (by serial 10-min washes in water, 0.2N NaOH, water, and TPBS), reblocked, and reprobed with anti-actin (mAb1501; Chemicon) at 1:20,000 (Mackler and Reist 2001).

RESULTS

Interaction between *Met* and *rbp* alleles: We first investigated the impact of reduced *Met* function on *rbp* mutants with marginal viability. Heteroallelic mutants carrying the viable allele rbp^2 with the *BRC*-null allele $npr1^3$ showed ~60% eclosion (Figure 1). Careful examination of $rbp^2/npr1^3$ mutants, starting at puparium formation and continuing throughout metamorphosis, showed that 100% of them were able to pupate, after which ~15% died as early pupae (Figure 1). Eighty percent of the original cohort survived to late pharate adult stage, with ~20% failing to eclose. In contrast, when the hypomorphic-viable allele Met^3 was crossed onto each of the *BR-C* mutant chromosomes, the survival curve of the resulting heteroallelic double mutants was shifted (Figure 1), showing a dramatic reduction in viability.

We next examined survival in double mutants carrying Met^{27}, a *bono fide* null allele (Wilson and Ashok 1998), with each of two alleles of *rbp*: rbp^2, a weak allele,

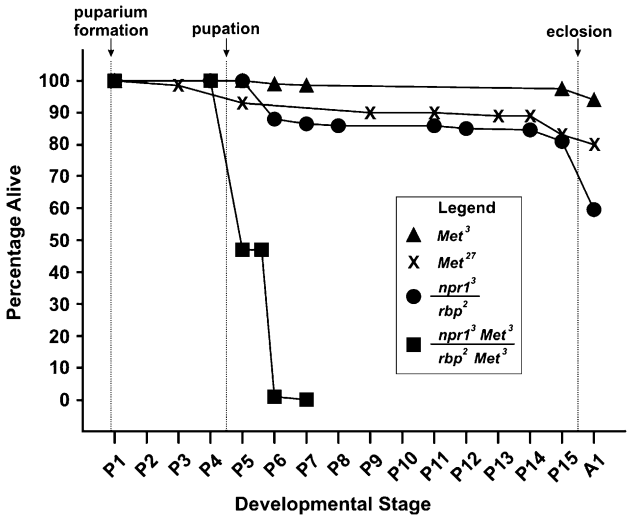

FIGURE 1.—*BRC* and *Met* mutations interact to cause synergistic enhancement of lethality during metamorphosis. Survival curves were obtained by selecting individuals as very young prepupae, culturing them at 25° in a humid chamber, and evaluating their developmental progress, with staging based on BAINBRIDGE and BOWNES (1981). The *x*-axis is not strictly proportional to time because the stages are of unequal duration and mutants of different genotypes develop at different rates. Single-mutant *BRC* progeny, *y rbp*^{2} *w/y npr1*^{3} *w sn*^{3} ($n = 156$) were generated by crossing *y npr1*^{3} *w sn*^{3}*/Binsn* females to *y rbp*^{2} *w/y*^{2}*Y67g* males. Control siblings from the same cross, *y rbp*^{2} *w/Binsn* ($n = 157$) showed 100% eclosion. Double mutants, *y rbp*^{2} *w v Met*^{3}*/y npr1*^{3} *w v Met*^{3} ($n = 115$) were generated by crossing *y npr1*^{3} *w v Met*^{3}*/FM7, y*^{31d} *B v* females to *y rbp*^{2} *w v Met*^{3}*/Y* males. Sibling controls from that cross, *y rbp*^{2} *w v Met*^{3}*/FM7, y*^{31d} *B v* ($n = 114$) had 100% eclosion. Data for *v Met*^{3} ($n = 284$) and *v Met*^{27} ($n = 125$) include similar numbers of hemizygous male and homozygous female progeny from the respective inter-sib matings.

and *rbp*^{1}, a severe allele (BELYAEVA *et al.* 1980; KISS *et al.* 1988). *Met*^{27} flies show good survival, with ≤15% mortality during pupal development (Figure 1). Flies homozygous for *rbp*^{2} and carrying one copy of *Met*^{27} showed good survival to adults (Table 1). In contrast, survival of *rbp*^{2} *Met*^{27} homozygotes to the adult stage was poor, dying typically during the pharate adult stage (Table 1).

Double mutants homozygous for *Met*^{27} and *rbp*^{1} did not survive to adulthood, as expected (Table 1). The lethal phase was shifted from the readily discernable pharate adult stage seen in *rbp*^{1} pupae (BELYAEVA *et al.* 1980; KISS *et al.* 1988) to an earlier, less well-defined prepupal/early pupal stage, judging from visual observation of the pupae. Heteroallelic *rbp*^{2} *Met*^{27}*/rbp*^{1} *Met*^{27} individuals, in contrast to surviving *rbp*^{2} *Met*^{27}*/rbp*^{1} individuals, were also prepupal/early pupal lethals (Table 1). Therefore, loss of *Met*$^+$ gene product resulted in pharate adult lethality in *rbp*^{2} and shifted the lethal phase of *rbp*^{1} to an earlier stage.

Lethality in *rbp*^{2} *Met*^{27} homozygotes or *rbp*^{2} *Met*^{27}*/rbp*^{1} *Met*^{27} heteroallelic pupae could be rescued by one copy of *Met*$^+$ as the *p[EN71]* transgene (Table 1).

TABLE 1

Survival to adulthood of females carrying various combinations of *BR-C* and *Met* alleles

Mutant progeny of interest	*N*	Estimated survival of mutant (%)
	rbp alleles	
rbp^2 *Met*^{27}*/rbp*^{2} *Met*^{27}	189	<2
rbp^2 *Met*^{27}*/rbp*^{2}	498	86
rbp^2 *Met*^{27}*/rbp*^{2} *Met*^{27}*; p[Met*$^+$*]/+*	298	84
rbp^1 *Met*^{27}*/rbp*^{1} *Met*^{27}	204	0
rbp^1 *Met*^{27}*/rbp*^{1}	350	0
rbp^1 *Met*^{27}*/rbp*^{2} *Met*^{27}	278	0
rbp^2 *Met*^{27}*/rbp*^{1}	265	100
rbp^2 *Met*^{27}*/rbp*^{1} *Met*^{27}*; p[Met*$^+$*]/+*	384	80
	br alleles	
br^1 *Met*^{27} *br*^{1} *Met*^{27}	238	<2
br^1*/br*^{1} *Met*^{27}	195	84
br^1 *Met*^{27}*/br*^{1}*Met*^{27}*; p[Met*$^+$*]*	277	64
br^5 *Met*^{27}*/br*^{1} *Met*^{27}	135	0
br^5 *Met*^{27}*/br*^{1}	247	54
br^5 *Met*^{27}*/rbp*^{1} *Met*^{27}	239	0
br^5 *Met*^{27}*/rbp*^{1}	271	38
br^5 *Met*^{27}*/rbp*^{2} *Met*^{27}	294	10
br^5 *Met*^{27}*/rbp*^{2}	125	82
br^1 *Met*^{27}*/rbp*^{1} *Met*^{27}	182	8
br^1 *Met*^{27}*/rbp*^{1}	242	88
	2Bc alleles	
2Bc^1 *Met*^{27}*/2Bc*^{1} *Met*^{27}	119	0
2Bc^1 *Met*^{27}*/2Bc*^{1}	247	0
2Bc^1 *Met*^{27}*/rbp*^{2} *Met*^{27}	360	78
2Bc^1 *Met*^{27}*/rbp*^{1} *Met*^{27}	133	0
2Bc^1*/rbp*^{1} *Met*^{27}	238	92
2Bc^1 *Met*^{27}*/br*^{1} *Met*^{27}	350	82
2Bc^1 *Met*^{27}*/br*^{5} *Met*^{27}	208	24
2Bc^1*/br*^{5} *Met*^{27}	178	104

Flies were generated from *FM7*-bearing females carrying the first chromosome listed in the above genotypes. *N* is the total female progeny examined from a particular cross, and estimated survival is the percentage of non-*FM7* females of interest divided by 0.5. Homozygous *Met*^{27} female survival is presented first, followed by the corresponding heterozygous *Met*^{27} female survival, and finally representative *p[Met*$^+$*]* transgenic survival.

Interaction between *Met* and *br* alleles: We next tested for interaction between *Met* and *br*. A viable *br* allele, *br*^{1}, exists (MORGAN *et al.* 1925; KISS *et al.* 1988). Individuals homozygous for *br*^{1} *Met*^{27} were usually lethal, but escaper adults eclosed in small numbers (Table 1), and hemizygotes showed slightly higher viability (Table 3) and were fertile. Examination of *br*^{1} *Met*^{27} pupae showed lethality in both pupal and especially pharate adult stages. Transgenic *br*^{1} *Met*^{27}; p[EN71]/+ flies readily survived (Table 1). Individuals homozygous or hemizygous for the severe *br*^{5} allele were lethal in early pupal development (KISS *et al.* 1988) and double mutants of *Met*^{27} with *br*^{5} were likewise lethal in prepupal/early pupal development. Heteroallelic *br*^{5} *Met*^{27}*/br*^{1} pupae

TABLE 2

Survival of br^1 hemizygotes and oviposition by homozygotes carrying various *Met* alleles

Genotype of *X* chromosome	Hemizygotes (% of F_1 adults)	Oviposition (eggs/female/10 days)
br^1	27	880 ± 50
Met^3	25	773 ± 81
br^1 *Met*	19	179 ± 25
br^1 Met^3	25	134 ± 9
br^1 Met^{A3}	5.4	16 ± 7
br^1 Met^{E1}	26	987 ± 79
br^1 Met^{I28}	14	26 ± 8
br^1 Met^{253}	15	12 ± 3
br^1 Met^{W3}	7.8	27 ± 7
br^1 Met^{27}	2.8	3.4 ± 0.8
br^1 Met^{D29}	21	32 ± 5

For each chromosome, F_1 progeny were generated from at least three separate cultures of *FM7*-balanced females × *FM7/Y* or br^1 *Met*/y^2*Y67g* males, the latter to generate homozygous females for oviposition determination. Survival is expressed as the percentage of adult hemizygotes for the indicated *X* chromosome present among the F_1 progeny. Oviposition (eggs laid ± SEM) was determined over a 10-day period beginning at 2–4 day post eclosion for females that were homozygous for the indicated genotype. When >100 eggs were laid during a 2-day period on the food surface, the total oviposition was estimated by extrapolating from one counted quadrant of the food surface. The oviposition rate of Met^3 was similar to that of the other *Met* alleles, except for the lower rate of Met^{27} Wilson and Ashok (1998).

TABLE 3

Oviposition by females of various *BR-C* and *Met* genotypes

Genotype	*N*	Eggs laid/female/2-day period: day after eclosion 2	4	6	8
br^1 *v* Met^{27}/br^1 *v* Met^{27}	63	0	1.1	<1	<1
br^1 *v* Met^{27}/br^1	20	6.6	30.9	42.6	38.0
$\frac{br^1 v\ Met^{27}}{br^1 v\ Met^{27}}$; *p*[$Met^+$]/ +	20	1.0	13.0	14.0	14.6
v/*v*	30	9.3	76.6	129	136
$\frac{y\ rbp^2\ w\ v\ Met^{27}}{y\ rbp^2\ w\ v\ Met^{27}}$	43	0	0	<1	<1
$\frac{y\ rbp^2\ w\ v\ Met^{27}}{y\ rbp^2\ w}$	20	4.8	17.8	17.6	16.6
$\frac{y\ rbp^2\ w\ v\ Met^{27}}{y\ rbp^2\ w\ v\ Met^{27}}$; *p*[$Met^+$]/ +	30	4.1	29.8	34.0	32.3

Each value is the mean of egg counts from females of the indicated genotypes isolated from at least two cultures. Due to low preadult survival, 5–7 cultures were required to produce the indicated numbers of the exceptional double mutant homozygotes.

survived well, but br^5 Met^{27}/br^1 Met^{27} heteroallelic individuals were lethal in prepupal/early pupal development (Table 1), an effect of Met^{27} similar to that seen in rbp^2 Met^{27}/rbp^1 Met^{27}.

Interaction between br^1 and other *Met* alleles: It is possible that the effects of Met^{27} in the double mutants are allele specific. To determine if other alleles of *Met* also show an interaction with br^1, double mutants were constructed and the resultant hemizygotes examined. Table 2 shows hemizygote survival values for each allele combination. Examination of the pupae showed the lethal phase generally to be pharate adult, as found for br^1 Met^{27}. The only *Met*-weak allele recovered to date is Met^{E1}, and the double mutant showed good survival. The remaining alleles resulted from mutagenesis screens employing a variety of mutagens (Wilson and Fabian 1987; Ashok *et al.* 1998) and generally show poorer survival with br^1. Therefore, the interaction between *Met* and br^1 is not allele specific for Met^{27}.

Interaction between *Met* and *2Bc* alleles: Finally, we tested for interaction between *Met* and *2Bc*. Both alleles of *2Bc*, $2Bc^1$ and $2Bc^2$, have a similar phenotype of prepupal lethality (Belyaeva *et al.* 1980; Kiss *et al.* 1988). Individuals homozygous for $2Bc^1$ Met^{27} (Table 1) or $2Bc^2$ Met^{27} (data not shown) were also lethal as expected, and visual examination of the pupae showed the stage of prepupal lethality to be indistinguishable from that of *2Bc* individuals. Therefore, either *Met* does not interact with *2Bc* or the prepupal/early pupal-lethal phase cannot be shifted to an earlier stage in individuals carrying Met^{27}.

Altered BR-C complementation patterns in the absence of MET: *BR-C* complementation group mutants complement one another to varying extents, with *rbp* partially complementing *br*, and *2BC* fully complementing both *rbp* and *br* (Belyaeva *et al.* 1980; Kiss *et al.* 1988). To determine if Met^{27} influences complementation for viability, double mutants for both the weak and severe alleles of *br* and *rbp* and for *2Bc* were crossed and survival of the F_1 examined. The absence of Met^+ resulted in failure of br^5 and rbp^1 to complement one another as well as reduced complementation between br^5 and rbp^2 (Table 1). Complementation was reduced between $2Bc^1$ and either br^5 or rbp^1. However, complementation in the Met^{27} double mutants continued to be robust between either of the weak alleles br^1 or rbp^2 with $2Bc^1$ (Table 1). Therefore, an absence of MET reduced complementation to varying degrees, depending on the alleles examined, being less evident with the weak alleles and with the $2Bc^1$ mutation.

Oogenesis in *BR-C Met* double mutants: Both of these genes are involved in oogenesis in Drosophila (Huang and Orr 1992; Wilson and Ashok 1998; Tzolovsky *et al.* 1999). Normally, vitellogenic oocyte development begins within 12 hr following eclosion, and mature eggs begin to be oviposited within 48 hr (King 1970; Wilson and Ashok 1998). *BR-C* homozygotes for the weak alleles br^1 and rbp^2 showed good oviposition, although below that of *v*/*v* females (Table 3). Met^{27} females show oogenesis reduced to ~20% of wild type (Wilson and

Ashok 1998). To determine if interaction between *BR-C* and *Met* is apparent during this process in adults, oviposition was examined in *BR-C Met* homozygotes at 2-day intervals following eclosion. Homozygous br^1 Met^{27} and rbp^2 Met^{27} females occasionally survived to adulthood, and survivors showed strong reductions in oviposition: both br^1 Met^{27} and rbp^2 Met^{27} homozygotes laid only a few eggs during an 8-day examination period, and casual examination of these females for another week showed no change in the ovipositional pattern.

To determine if the ovipositional failure was due to a defect in oogenesis or in oviposition, both br^1 Met^{27} and rbp^2 Met^{27} females were dissected at 5–7 days following eclosion and their vitellogenic oocytes staged and censused. Despite having access to ample yeast and courting wild-type males, there were few (usually 0–3) vitellogenic oocytes in the ovaries of these females, showing that the defect resides in oogenesis, not oviposition. However, no degenerating oocytes were present, a condition suggestive of hormonal disruption (Wilson 1982; Soller *et al.* 1999).

Oviposition was also measured in the br^1 *Met* double-mutant combinations of the various *Met* alleles. Some of the allele combinations resulted in severe disruption of oviposition (Table 2), presumably due to defects in oogenesis, as seen for Met^{27}. Other combinations resulted in ovipositional rates higher than those of br^1 Met^{27}. In summary, the severe depression in oviposition seen for some *Met* allele combinations represents a strong allele interaction with br^1 and is not specific for the Met^{27} allele.

The link between *BRC* and methoprene: We addressed the enigmatic relationship between *BR-C* and methoprene in three contexts: (i) the overlap between methoprene-induced defects and *BR-C* common phenotypes, (ii) the influence of *BR-C* function on sensitivity to methoprene, and (iii) the effect of methoprene on *BR-C* expression.

The BR-C common phenotypes include malrotation of male genitalia: In our previous study, we showed that methoprene treatment of wild type causes a specific partial phenocopy of *BRC*-associated internal defects (Restifo and Wilson 1998). Here, we tested whether *BR-C* mutants of each complementation group show the well-known methoprene-induced malrotation of the male genitalia (Postlethwait 1974; Wilson and Fabian 1986). In some cases, this required combining strong mutations with moderate wild-type transgene activity, an established method for revealing late developmental functions (Hall and Thummel 1998). The malrotation phenotype is of particular interest because a genetic interaction between *Met* and *spin*, a *Fas2* mutation with a malrotation phenotype, has recently been described (Adam *et al.* 2003).

For *rbp*, we found that 100% of rbp^1/Y hemizygotes have malrotated genitalia (Table 4). This fully penetrant phenotype was rescued by a *BR-C*$^+$ *Y*-borne duplication y^2Y67g and uncovered by the Y-borne duplication with an interstitial deletion $y^2YSz280$ that lacks all *BR-C* sequences, confirming that it maps to the *BR-C* region. To generate *br* mutant males that die as pharate adults (when the genitalia are pigmented), we partially rescued *br*-null mutants br^5/Y using two transgenic copies of heat-shock-inducible *BRC–Z2*. Optimal heat-shock protocols rescue lethality (Bayer *et al.* 1997), gene expression (Liu and Restifo 1998), and CNS morphogenesis (R. F. Spokony and L. L. Restifo, unpublished data). To obtain partial rescue, we heat-shocked unsynchronized third-instar larvae once (37°, 1 hr), and then twice more 18 and 23 hr later. This resulted in very small numbers of pharate adults, 93% of which (14/15) had malrotated genitalia (Table 4). For *2Bc*, we first observed malrotation in two very rare, late-dying $2Bc^2/Y$ mutants (Restifo and White 1991, 1992; Consoulas *et al.* 2005). To examine larger numbers, we used a "leaky" *BRC–Z3* transgene whose expression is dependent on temperature (see materials and methods). We found malrotated genitalia, inversely related to rearing temperature: 14% at 25° and 40% at 22–23° (Table 4).

Neither ubiquitous expression of *BRC–Z2* nor *BRC–Z3* in wild type caused malrotation. Genetic controls, br^5/y^2Y67g or $2Bc^1/y^2Y67g$, exposed to the corresponding temperature protocol showed only very rare malrotation (4 or <1%, respectively), confirming the mapping of the phenotype to *BR-C* and suggesting the possibility of a very small heat-shock effect. In summary, *BR-C* mutants of all three complementation groups have malrotated male genitalia, which adds this methoprene-induced defect to the list of *BR-C* common phenotypes.

TABLE 4

Malrotation of the male genitalia is a developmental phenotype of all *BR-C* complementation groups

Genotype	Culture conditions	Phenotype: % with malrotation
	reduced bristles on palps	
rbp^1/Y	25° standard	100 ($n = 31$)
rbp^1/y^2Y67g	25° standard	0 ($n = 128$)
$rbp^1/y^2YSz280$	25° standard	100 ($n = 23$)
	broad	
br^5/Y; *hsZ2/+*; *hsZ2/+*	37° heat shocks; partial rescue	93 ($n = 15$)
y w sn^3/Y; *hsZ2/+*; *hsZ2/+*	37° heat shocks	0 ($n = 16$)
br^5/y^2Y67g	37° heat shocks	4 ($n = 48$)
	lethal(1)2Bc	
$2Bc^2/Y$	25° standard	100 ($n = 2$)
$2Bc^1/Y$; *hsZ3/+*	25°; modest partial rescue	14 ($n = 28$)
$2Bc^1/Y$; *hsZ3/+*	22–23°; modest partial rescue	40 (n =53)
$2Bc^1/y^2Y67g$	22–23°	0.8 ($n = 261$)
yw/Y; *hsZ3/+*	22–23°	0 (n =582)

TABLE 5

Survival and morphological defects in various *BR-C* and *Met* flies following treatment with methoprene

	Methoprene dose			
Genotype	0.05	0.01	0.005	0.001
Oregon-RC	0	0	11 (100)	62 (69)
rbp²/rbp²/Y	0	0	7.6 (100)	70 (63)
br¹/br¹/Y	0	0	13 (89)	75 (74)
2Bc¹/FM7	0	0	3.1 (100)	58 (48)
npr1³/FM7	ND	0	14 (100)	67 (72)
Met³/Met³/Y	45 (0)	67 (0)	92 (0)	ND
br¹ Met³/br¹ Met³/Y	40 (0)	74 (0)	81 (0)	ND

Mean $N = 63$, range 40–79, individuals were evaluated at each methoprene dose applied to at least triplicate cultures of 30 individual larvae. Survival is expressed as percentage of individuals of the indicated genotype surviving to adulthood. Numbers in parentheses represent the percentage of survivors having abnormal sternite bristle/male genitalia. *Met³* was used as the *Met* allele because of high viability with *br¹*. ND, not determined.

Sensitivity of BR-C mutants to methoprene: Met mutations confer semidominant resistance to both the toxic and morphogenetic effects of methoprene (Wilson and Fabian 1986; Restifo and Wilson 1998). To determine if *BR-C* mutations, either singly or in double-mutant combination with *Met*, affect the response to methoprene treatment, larvae were raised in the presence of one of four diagnostic concentrations of methoprene and evaluated for survival and the external morphology of surviving adults. Viable *BR-C* mutations do not change the susceptibility to methoprene from that of wild type (Table 5). Similarly, reducing the dose of *2Bc⁺* or *BR-C⁺* by 50% (*2Bc¹/FM7* or *npr1³/FM7*, respectively) did not shift the sensitivity to methoprene. Moreover, in the double mutant, *br¹* did not affect the resistance conferred by *Met³*. Hence, *BR-C⁺* function does not appear to impact the MET-dependent methoprene-sensitivity mechanism.

Effect of Met and methoprene on BRC protein accumulation: We investigated BRC protein expression for two reasons. First, a plausible mechanistic explanation for the genetic interaction between *Met* and *BR-C* would be that *Met⁺* upregulates *BR-C* expression, and that reduced BRC levels in *Met* mutants would enhance the lethality of partial-loss-of-function *BR-C* genotypes. Second, reported effects on *BR-C* transcript levels caused by JH/JHA treatment (Zhou *et al.* 1998; Zhou and Riddiford 2002) have failed to provide an explanation for the methoprene phenocopy of *BR-C* common defects, especially those involving internal structures of the head and thorax (Restifo and Wilson 1998).

The BRC family of proteins, which migrate as three size groups (Emery *et al.*,1994; Mugat *et al.* 2000), is readily detected by immunoblotting of proteins extracted from whole white prepupae (Figure 2A). The largest group, Emery's p118, is thought to represent BRC–Z4; the middle group, Emery's p91 and p81 contains BRC–Z1 and –Z3; the smallest group, Emery's p64 and p57 contains BRC–Z2. Over the subsequent 24 hr, especially after head eversion (~12 hr APF), BRC protein levels declined (Figure 2B). In *Met²⁷* mutants, the pattern and relative abundance of BRC isoforms detected over this first day of metamorphosis was indistinguishable from those of wild type (Figure 2B). Likewise, methoprene treatment of wild-type animals did not change the overall quantities and isoform patterns of BRC proteins (Figure 2C).

In independent experiments, BRC proteins were evaluated over a 3-day interval, representing ~75% of metamorphosis, during which animals were resynchronized at head eversion. At 24 hr APF (12 hr after head evesion), BRC proteins were detectable in the methoprene-treated animals, but not in the controls (Figure 3A). Similarly, methoprene-treated animals showed mild persistence of BRC–Z1 during midpupal stages, following a peak in accumulation at the normal time, 8 hr APF. There was no reappearance of BRC–Z1 or any

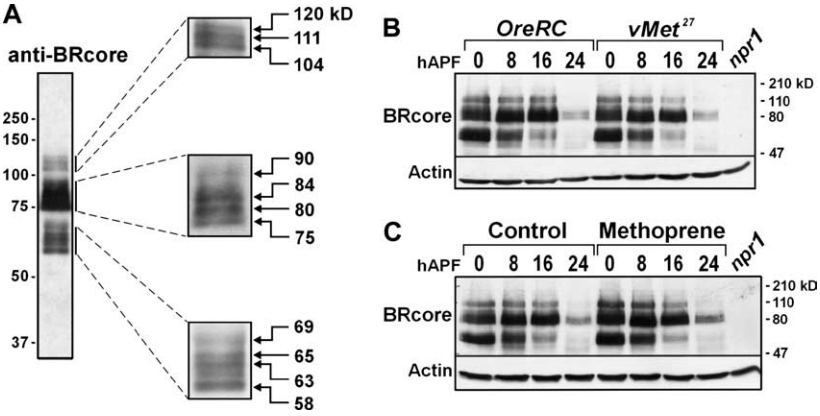

Figure 2.—BRC protein accumulation during the first day of metamorphosis is not affected by lack of MET or by treatment with methoprene. Immunoblotting of whole-body protein extracts with anti-BRcore; 0.5-animal equivalents per lane. (A) Wild-type (*OreRC*) white prepupae. The migration positions of molecular weight markers are shown on the far left. The individual boxes on the right show optimized images of each group of BRC proteins, obtained by changing exposure times or amounts of protein loaded. The indicated molecular weight estimates are averages based on three or more independent experiments. (B and C) Time course of BRC accumulation in animals collected at puparium formation and sampled every 8 hr. The rightmost lanes contain protein from *BR-C*-null mutant (*npr1³/Y*) wandering third-instar larvae as a negative control to verify the specificity of the antibody. Each blot was reprobed for actin as an indicator of protein loading. hAPF, hours after puparium formation. (B) Wild-type (*OreRC*) and *Met*-null mutant (*v Met²⁷*). The BRC protein profiles are qualitatively and quantitatively indistinguishable. (C) Control and methoprene-treated *OreRC*. The BRC protein profiles are very similar.

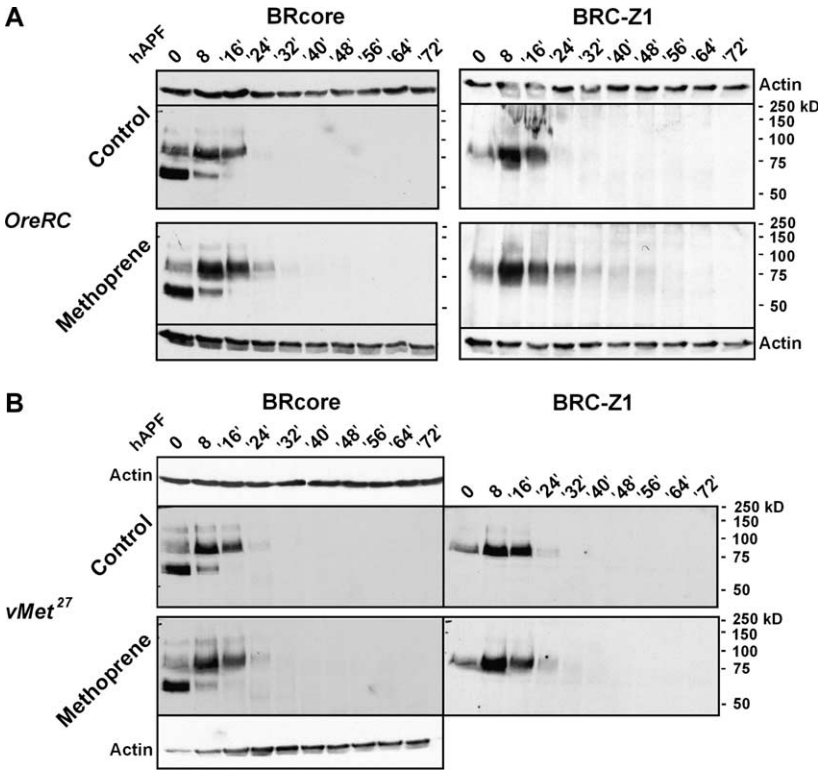

FIGURE 3.—BRC protein accumulation is mildly prolonged by methoprene treatment. Animals were collected at puparium formation, sampled at 0 and 8 hr, resynchronized at head eversion (~12 hr APF in control wild-type animals), and then sampled 4 hr after head eversion (= 16 hr APF) and at 8-hr intervals thereafter. Immunoblotting with anti-BRcore and anti-Z1; 0.5-animal equivalents per lane. In each experiment, control and methoprene-treated animals were reared and processed in parallel, the gels were run simultaneously in the same apparatus, and the antibody labeling and detection were performed in parallel. Each of the blots was reprobed for actin as an indicator of protein loading. (A) Wild type (*OreRC*). Two independent immunoblotting experiments are shown. Treatment with a lethal dose of methoprene causes a mild prolongation of BRC protein accumulation, especially of the abundant 75–85-kDa bands, which contain primarily BRC–Z1. Methoprene does not cause reappearance of BRC in the midpupal period (40– 64 hr APF). (B) *Met*-null mutant (Met^{27}). Sequential immunoblotting for BRC–Z1 and BRcore. As in wild type, BRC accumulation is mildly enhanced by treatment with a lethal dose of methoprene.

other BRC isoforms during mid-to-late pupal stages (Figure 3A), even on very long exposures of the immunoblots (data not shown). In summary, chronic larval treatment with lethal doses of the JH agonist and mimic methoprene does not block BRC protein accumulation during the first day of metamorphosis, a developmental window in which *BR-C* function is essential.

Over the 3-day interval, BRC protein profiles in Met^{27} mutants were normal, in both the presence and absence of methoprene (Figure 3B). These data demonstrate that the accumulation of BRC proteins at the onset of metamorphosis is MET independent. Hence, altered BRC expression, at least at the level of the whole-body protein accumulation, cannot explain the genetic interactions between *BR-C* and *Met* mutations.

DISCUSSION

In this work, we have examined two genes required for signaling by 20E (*BR-C*) and JH (*Met*) to probe for interaction between these pathways. We have found evidence for interaction between *Met* and *BR-C* as reflected by synergistically reduced viability and oogenesis seen in double mutants. Consistent results were seen with different combinations of *Met* and *br* or *Met* and *rbp* alleles (Figure 1; Tables 1 and 2), indicating that the interactions are not allele specific in either direction.

Met interacted with both the weak-viable alleles br^1 and rbp^2 and with the severe alleles br^5 and rbp^1 (Table 1) during pupal development. Each of the weak alleles possesses sufficiently functional gene product to permit completion of pupal development; but this amount is insufficient when MET is absent or defective. The more severe rbp^1 homogygotes are pupal lethal, but only at late metamorphosis, in the pharate adult stage (BELYAEVA *et al.* 1980). Lethality was shifted in rbp^1 Met^{27} pupae to prepupal/early pupal development, suggesting that MET absence causes the residual rbp^1 function to be insufficient during these earlier stages in pupal development. Homozygotes of br^5 and *2Bc* die in the early and late prepupal stage, respectively (KISS *et al.* 1988), and the double mutants with Met^{27} showed a similar phenotype, demonstrating that the interaction cannot shift lethality to an earlier stage, late third-instar larvae. Our observations are consistent with the interaction between *BR-C* and *Met* beginning in prepupal or early pupal development. While we interpret the *Met–BR-C* interaction as enhancing the lethality of *br* and *rbp* mutations, it is also possible that *Met* becomes an essential gene when *BR-C* function is reduced, or that the interaction is mutual, such that both mutations become more severe in phenotype when they are present together.

Genetic interaction became strikingly evident when complementation failures between mutant alleles from different *BR-C* complementation groups occurred in the presence of Met^{27} (Table 1). Without MET, developing animals may be less able to make use of the partial functional redundancy among BRC isoforms that has been documented previously (BAYER *et al.* 1997).

The interaction between mutant alleles of *BR-C* and *Met* was also evident in the adult stage when oogenesis was examined. Both the rate of oviposition and the paucity of vitellogenic oocytes in ovaries of br^{1} Met^{27} and rbp^{2} Met^{27} females reflects almost complete failure of oogenesis, with only a few eggs oviposited during the lifetime of the female.

Previous studies have also detected *BR-C* interaction with other genes. Double mutants of *BR-C* with another primary response gene *E74* show interaction for some but not all of the phenotypic characters (Fletcher and Thummel 1995). In addition to interactions among transcription regulators of the ecdysone cascade, *br* alleles interact with genes involved in imaginal disc morphogenesis, including those encoding an atypical serine protease, *Stubble-stubboid* (Beaton *et al.* 1988; Appel *et al.* 1993), nonmuscle myosin II heavy chain (*zipper*), the Drosophila serum response factor transcription factor [*blistered*: (Gotwals and Fristrom 1991)], the small GTPase Rho1, cytoplasmic tropomyosin, and 22 others (Ward *et al.* 2003).

Although *BR-C* expression and function overlap the JH/JHA-sensitive period, data presented above demonstrate that methoprene treatment does *not* block BRC expression in either wild-type or *Met*-null mutants (Figures 2 and 3). Furthermore, the methoprene phenocopy, which excludes complementation group-specific defects (*e.g.*, larval salivary gland persistence, which is *rbp*-restricted), is not consistent with methoprene simply reducing BRC expression (Restifo and Wilson 1998). We propose that JH application results in abnormal *function* of BRC proteins, thus phenocopying certain characteristics common to all *BR-C* mutants. Therefore, the link between *BR-C* mutant phenotypes and JH-induced defects could be abnormal regulation of target genes, resulting in the phenotypic characteristics observed (Figure 4). Previously, we suggested several possibilities to explain methoprene pathology and *BR-C* phenocopy, including BRC interaction with an unidentified partner, perhaps MET (Restifo and Wilson 1998). We believe that the *Met–BR-C* genetic interaction reported in this work can be explained best by this hypothesized protein–protein interaction between MET and BRC to regulate one or more target genes. Supporting our hypothesis are the following findings: first, both proteins are located in the nucleus (Emery *et al.* 1994; Restifo and Hauglum 1998; Pursley *et al.* 2000; Renault *et al.* 2001), so there is no compartmental barrier to interaction. Second, both proteins appear to be transcription factors: BRC isoforms bind specific DNA sequences (von Kalm *et al.* 1994; Dubrovsky *et al.* 2001) and regulate transcription (Dubrovsky *et al.* 1994; Hodgetts *et al.* 1995; Crossgrove *et al.* 1996; Mugat *et al.* 2000). *BR-C* mutants have misexpressed secondary-response and other target genes (Guay and Guild 1991; Karim *et al.* 1993; Mugat *et al.* 2000; Renault *et al.* 2001; Dunne *et al.* 2002; Sempere *et al.* 2003). MET is a member of the bHLH–PAS family of transcription factors (Ashok *et al.* 1998) and was recently shown to act as one (Miura *et al.* 2005). Third, both are found at common times during development, such as prepupae (Karim *et al.* 1993; Ashok *et al.* 1998) and during vitellogenic oocyte development (Tzolovsky *et al.* 1999; Pursley *et al.* 2000). Finally, PAS domains in bHLH–PAS proteins are thought to promote protein–protein interaction (Heery *et al.* 1997), either with other PAS proteins or as coactivators with nuclear receptor proteins (Xu *et al.* 1999), and the BTB/POZ domain of BRC has been implicated in protein–protein interaction (Zollman *et al.* 1994; Melnick *et al.* 2002).

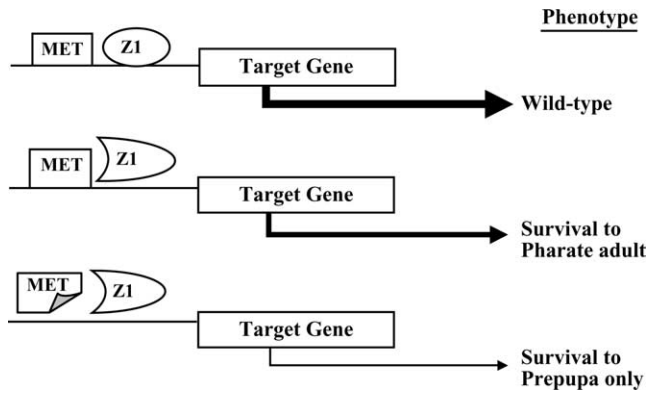

Figure 4.—Proposed scheme for regulation by MET and BRC–Z1 of a target gene necessary for pupal viability. Illustrated are three hypothesized transcriptional situations for *rbp*: top, wild type; center, in the presence of wild-type MET, strong hypomorphic *rbp* alleles cause lethality in the pharate adult stage; bottom, in *rbp Met* double mutants, lethality is shifted to the prepupal stage. No interaction between MET and Z1 is shown, but formation of heterodimers is possible. Likewise, each is shown binding DNA when the protein is wild type but not when mutant, although DNA binding by lesioned protein is possible. The presumed level of target-gene transcription is reflected by arrow thickness.

In Met^{27} mutants, BRC protein accumulation profiles are normal (Figures 2 and 3). Since metamorphosis is not derailed in Met^{27} pupae, BRC^{+} function in these pupae does not seem to be adversely affected. The fly may be protected from absence of MET by functional redundancy (Wilson and Ashok 1998). A candidate for the redundant substitute is the PAS gene *germ cell expressed* (*gce*), a gene with high (~70% amino acid identity) homology to *Met* (Moore *et al.* 2000) that could substitute for MET to rescue larval and/or pupal development. However, this substitute does not appear to be satisfactory if BR-C is mutant. When a *gce* mutant becomes available, its phenotype could help evaluate this hypothesis.

How does the application of exogenous JH act to phenocopy *BR-C*? It is clear that the action of these compounds occurs through MET, probably acting as a JH receptor component (Wilson and Fabian 1986; Shemshedini *et al.* 1990; Shemshedini and Wilson

1990; Ashok *et al.* 1998; Miura *et al.* 2005). JH is present during larval development when it presumably acts to prevent premature metamorphosis resulting from each wave of 20E secretion that triggers a molt. This failsafe mechanism may occur by JH binding by and conformational change of MET, resulting in regulation of genes necessary for molting or perhaps simply blocking expression of metamorphic genes. Studies with Drosophila S-2 cells have implicated the transcription factor E75A in promoting JH regulation of larval development (Dubrovsky *et al.* 2004). At metamorphosis, when little or no JH is present (Bownes and Rembold 1987; Sliter *et al.* 1987), *BR-C* is expressed, and we propose that BRC dimerizes with the nonliganded MET protein to regulate a different set of target genes, promoting the initiation of metamorphosis. If exogenous JH is present during this time, it binds to MET and results in a more larval conformation, resulting in inappropriate binding to BRC and leading to a change in target-gene expression patterns consequently seen as defects characteristic of *BR-C* mutants.

Other work has implicated *BR-C* in the action of the JH agonist pyriproxyfen during metamorphic disruption. Zhou and Riddiford (2002) showed that application of this compound to white prepupae resulted in re-expression of BRC–Z1 in the abdomen during late pupal development, which in turn caused abnormal development of abdominal epidermis, including bristle disturbances. Those findings differ from ours with methoprene in two significant ways. First, a lethal dose of methoprene caused a mild enhancement and prolongation of BRC protein accumulation in young pupae, but no re-expression at later times (Figure 3A). Second, the modest effect of methoprene on BRC protein profiles cannot mediate the developmental effects of this JHA because the same mild persistence of BRC was seen in Met^{27} mutants (Figure 3B), which are protected against methoprene-induced defects. It is not clear what underlies the difference in response of *BR-C* to methoprene and pyriproxyfen. We note that pyriproxyfen is a more powerful JH agonist than methoprene (Riddiford and Ashburner 1991), but qualitative differences in the actions of the two compounds may exist as well.

In summary, our results provide genetic evidence that supports other studies implicating *BR-C* as a focal point for interaction of JH and 20E signaling pathways, and they suggest that BRC and MET interact to regulate expression of one or more effector genes involved in metamorphic development.

We thank Cynthia Bayer for stocks and for pointing out the malrotated genitalia in partially rescued *br* males. T.G.W. carried out some of this work in the laboratory of Mary Bownes at the University of Edinburgh. L.L.R. thanks Hannah V. Foster and H. Jolene Clark for help with crosses, lethal phase determination, and male genitalia phenotype scoring. This work was supported by grants to T.G.W. (National Science Foundation grant IBN 0322136 and National Institutes of Health grant AI052290) and L.L.R. (Flinn Foundation Interdisciplinary Genetics Research Grant and National Institutes of Health HD038363). D.M.D. was supported by a Flinn Foundation Genetics Training Grant.

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Communicating editor: L. Harshman