The Function of the *Broad-Complex* During *Drosophila melanogaster* Oogenesis

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ABSTRACT

The *Broad-Complex* (*BR-C*) is an early ecdysone response gene that functions during metamorphosis and encodes a family of zinc-finger transcription factors. It is expressed in a dynamic pattern during oogenesis. Its late expression in the lateral-dorsal-anterior follicle cells is related to the morphogenesis of the chorionic appendages. All four zinc-finger isoforms are expressed in oogenesis, which is consistent with the abnormal appendage phenotypes resulting from their ectopic expression. We investigated the mechanism by which the *BR-C* affects chorion deposition by using BrdU to follow the effects of *BR-C* misexpression on DNA replication and *in situ* hybridization to ovarian mRNA to evaluate chorion gene expression. Ectopic *BR-C* expression leads to prolonged endoreplication and to additional amplification of genes, besides the chorion genes, at other sites in the genome. The pattern of chorion gene expression is not affected along the anterior-posterior axis, but the follicle cells at the anterior of the oocyte fail to migrate correctly in an anterior direction when *BR-C* is misexpressed. We conclude that the target genes of the *BR-C* in oogenesis include a protein essential for endoreplication and chorion gene amplification. This may provide a link between steroid hormones and the control of DNA replication during oogenesis.

THE regulation of the expression of structural genes is critical in morphogenesis. This requires differential expression of transcription factors, which in turn regulate the tissue-specific expression of structural genes. Drosophila oogenesis is ideal for the study of developmental gene regulation as it takes a fairly short time to develop from a stage-1 egg chamber to a mature egg and all the stages are morphologically well defined. Further, egg chambers, the developmental units of oogenesis, contain only the somatically derived follicle cells and the germline cells. The former undergo dramatic morphogenetic movements and eventually synthesize the yolk and then the eggshell, as well as interact with the germline cells to generate the two major axes of the egg and embryo.

The *Broad-Complex* (*BR-C*), a gene encoding a family of zinc-finger transcription factors (DiBello *et al.* 1991; Bayer *et al.* 1996), has been shown to be expressed in the follicle cells in a dynamic pattern, the late pattern being defined by two groups of dorsal-anterior follicle cells at stage 10B of oogenesis (Deng and Bownes 1997; for the staging of oogenesis, refer to Spradling 1993). This dorsal-anterior expression pattern is specified by the Grk-DER and decapentaplegic (DPP) signaling pathways along the two major axes, and is associated with the function of the *BR-C* in dorsal appendage formation. The involvement of *BR-C* in dorsal appendage morphogenesis was shown by mutational analysis of *BR-C* partial "loss-of-function" mutants, and was supported by ectopic expression of *BR-C* "transgenes" during oogenesis. It is proposed that the *BR-C* may provide a link between pattern formation and cell behavior in morphogenesis (Deng and Bownes 1997).

The *BR-C* has been previously identified as a key gene required for Drosophila metamorphosis. It is among the early ecdysone responsive genes, which are directly activated by the ecdysone receptor and coordinate the subsequent transcription of the tissue-specific "late genes" (Ashburner 1974; and for reviews see Kiss *et al.* 1988; Karim *et al.* 1993; Zhimulev *et al.* 1995; Bayer *et al.* 1996a). The *BR-C* is located at chromosomal region 2B5. Genetically, the *BR-C* locus has three fully complementing functions: *br* (*broad*), *rbp* (reduced bristle number on palpus), and *2Bc*, as well as a noncomplementing *npr* (nonpupariating) class (Figure 1C; Belyaeva *et al.* 1980). Additionally, a number of *BR-C* alleles have been categorized to the *2Bab* group. These alleles do not complement *br* or *rbp* mutations, but do complement *2Bc* mutations (Belyaeva *et al.* 1980). The nonpupariating mutations are probably null mutations, because alleles in this class fail to complement mutations in each of the three complementing groups. These mutants are also phenotypically indistinguishable from deletions of the locus. It has been shown by genetic analysis that *BR-C* is essential for the morphogenesis of imaginal discs. br^+ function is primarily required in the elonga-

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tion and eversion of appendages from imaginal discs as well as tanning and hardening of the larval cuticle. rbp^+ function, on the other hand, is essential for muscle and bristle development. Additionally, both rbp^+ and $2Bc^+$ functions are vital for histolysis of the larval tissues and gut morphogenesis. $2Bc^+$ function was shown to be essential in the fusion of discs to form a continuous adult epidermis (Kiss *et al.* 1988). All three functions are also required for the reorganization of the central nervous system (CNS) (Kiss *et al.* 1988; Emery *et al.* 1994).

The *BR-C* encodes a family of C_2H_2 zinc-finger proteins (Z1, Z2, Z3, and Z4), which share a common amino-terminal (the *BR-C* "core") domain but differ in zinc-finger domains (DiBello *et al.* 1991; Bayer *et al.* 1996b). The core contains a highly conserved amino-terminal motif, called the BTB or POZ domain, which appears to be involved in protein-protein interactions and is widely distributed among metazoans (DiBello *et al.* 1991; Bardwell and Treisman 1994; Zollman *et al.* 1994). The core is alternatively spliced to one of the four zinc-finger domains (Figure 1), generating four classes of proteins, the Z1, Z2, Z3, and Z4 isoforms. Additionally, three variants of the Z1 isoform have been identified. They differ in the linker region between the core motif and the zinc-finger domain.

Some genetic studies suggest a one-to-one link be-

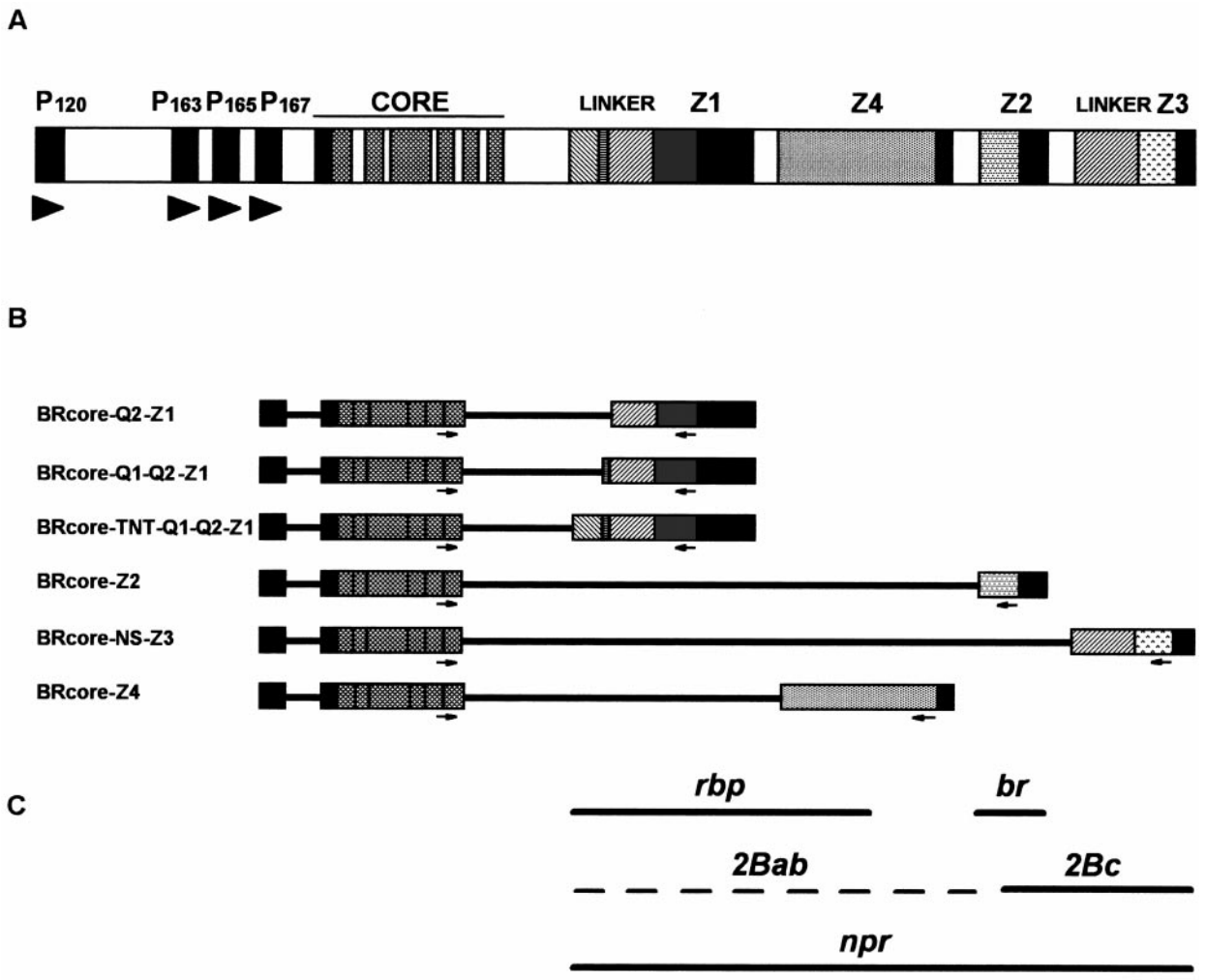

Figure 1.—Organization of the *BR-C* gene. (A) Molecular organization of the *BR-C* gene, which maps to the 2B5 region (adapted from Chao and Guild 1986). For simplicity and clarity the upstream half of *BR-C*, including the first exon, is not shown in the figure. Shaded boxes represent open reading frames; solid boxes represent untranslated regions of the *BR-C* transcripts. Two putative promoters have been previously described: P distal at nucleotide 120; and P proximal at nucleotides 163, 165, and 167 (DiBello *et al.* 1991; Bayer *et al.* 1996b). The Z1–Z2 transcripts can be synthesized from either P_{120} or P_{165}, while Z2 and Z4 initiate only at P_{165} and P_{163}, respectively (Bayer *et al.* 1996b). The linker regions for Z1 and Z3 domains are contiguous in the respective exon. (B) Organization of zinc-finger isoforms. Differentially spliced *BR-C* transcripts share a common core domain linked to one of the four (Z1–Z4) different pairs of C2H2 zinc-finger domains. The TNT, Q_1, and Q_2 linker sequences found in Z1 transcripts are contiguous in the Z1 exon, and generate by alternatively splicing three Z1 isoforms (Bayer *et al.* 1997). Arrows indicate the primer pairs used in RT-PCR analysis. (C) Complementation map of the *BR-C* based on Bayer *et al.* (1997).

tween the specific complementing genetic functions and protein isoforms. However, other data suggest that the relationships between the complementing groups and protein isoforms are more complicated. For example, in the br^{28} mutant, Z3 transcripts and protein levels are reduced and all Z1 isoforms are truncated. Clearer data on these relationships were provided by Bayer *et al.* (1997), who showed that lethality associated with each of the complementing groups was rescued using heat-inducible transgenes. It was found that br^+ function is only provided by the Z2 isoform. Despite this, there may be functional redundancy or regulatory dependency associated with rbp^+ and $2Bc^+$ functions. It was found that Z1 transgenes provide full rbp^+ function, while Z4 provides partial function. The *2Bc* lethality is fully rescued by Z3 protein expression, and partially rescued by Z2 protein expression.

The two clusters of chorion genes on the X-chromosome and third chromosome, which are responsible for the production of large amounts of chorion protein in the follicle cells at very precisely defined points in late oogenesis, are selectively amplified above the level of the remainder of the follicle cell genome, which also endoreplicates to produce polyploid cells (Spradling and Mahowald 1980; Orr-Weaver 1991). The third chromosome cluster is amplified 60- to 80-fold and the X-chromosome group 15- to 20-fold above the rest of the genome. This specific amplification depends on *cis*-acting sequences among the chorion genes (Orr-Weaver and Spradling 1986; Delidakis and Kafatos 1989).

We know that the *BR-C* expression in the lateral-dorsal-anterior follicle cells during oogenesis is related to its function in dorsal appendage formation. However, we do not know what the early function of the *BR-C* is, when it is expressed in all follicle cells at stage 6 of oogenesis. Since the chorion genes encode major eggshell components, and rbp^+ function has been reported to be necessary for chorion gene amplification during oogenesis (Orr *et al.* 1989; Huang and Orr 1992), we investigate in this article the relationship between the *BR-C* and chorion gene amplification and expression.

MATERIALS AND METHODS

Drosophila strains: The following *Drosophila melanogaster* strains were used: Oregon R, br^1, br^5, br^6, rbp^1, rbp^2, $2Bc^1$, $2Bc^2$, npr^6 (Kiss *et al.* 1988); br^{A47} (Deng and Bownes 1997); w^{1118}, *hs.Z1* (527-5; 708-1), *hs.Z2* (CD5-1), *hs.Z3* (797-3), *hs.Z4* (Z4-11) (Bayer *et al.* 1997). The rbp^2 and Br^1 alleles are viable and were maintained as homozygotes. All other *BR-C* mutations were maintained over *Binsn*, an X-chromosome balancer carrying the markers *Bar* and *singed*. All stocks were maintained on standard cornmeal food at 26°.

Antibody staining of ovaries: Ovaries were dissected from yeasted flies in Ringer's solution. The anterior parts of the ovaries were torn apart to facilitate antibody penetration. The ovaries were transferred to a microfuge tube containing 2% *p*-formaldehyde (in 1 × PBS) and fixed for 30 min at room temperature. The fixative was carefully removed and ovaries were washed in 1 ml of PTW [1.5% (v/v) Tween-20 in PBS] for 5 min. Then the ovaries were incubated in 1% (w/v) bovine serum albumin (Sigma, St. Louis) in PTW for 1 hr. Blocking was accomplished by incubation of the ovaries in PTW-NGS [5% (v/v) normal goat serum in PTW] for 2 hr at room temperature. The first antibody was added at 1:200 dilution in PTW and the incubation was carried out overnight at 4°. Residual antibody was washed away with three changes of PTW with 30 min of incubation per change. The HRP-conjugated secondary antibody (Promega, Madison, WI) was then added to the ovaries at 1:500 dilution and incubated for 2 hr at room temperature or overnight at 4°. Excess secondary antibody was removed with three PTW washes at 30-min intervals. Diaminobenzidine (DAB) staining solution (Sigma) was added and the staining was allowed to proceed for 10–30 min before washing with several changes of PBS to stop the reaction. Stained ovaries were mounted in PBS/glycerol (1:4) to allow microscopy.

Hoechst staining: Ovaries were dissected in PBS and fixed in 4% *p*-formaldehyde (w/v in 1 × PBS) for 20 min. This was followed by washing in 1 × PBT [1% (v/v) Triton-X100 in PBS] for 30 min. The ovaries were then washed for 30 min in PBS and stained for 5 min in 1 μg/ml Hoechst 33258 (Sigma; dissolved in PBS). After washing in PBS for 2 hr to overnight, the ovaries were mounted in PBS/glycerol (1:4) and examined under a fluorescent microscope.

Preparation of the eggshell for dark-field microscopy: Freshly laid eggs were collected from the apple juice plate and placed in a drop of Hoyer's mountant (Hoyer's mounting medium:lactic acid = 1:1) on a glass slide and covered by a coverslip. After an overnight incubation at 65° the slides were ready for dark-field microscopy.

RNA extraction and RT-PCR: The *BR-C* transcript levels in ovaries were detected by reverse transcriptase (RT)-PCR as described previously (Hodgetts *et al.* 1995). Total RNA from ovaries and larvae (control RNA) was isolated using RNAeasy-Total RNA Kit (QIAGEN, Chatsworth, CA, no. 74104). The RNA (5 μg) was primed with oligo-p$(dT)_{15}$ and reverse transcribed using Superscript II (Gibco BRL, Gaithersburg, MD) following the supplier's protocol. For the subsequent DNA amplification, 5% of the first-strand reaction mix was used. To amplify each of the zinc-finger domains, appropriate primer pairs were added to the PCR mixture: a common primer for the core domain was combined with one of the four primers for the respective zinc-finger motif. The sequence data for the primers were obtained from DiBello *et al.* (1991) and Hodgetts *et al.* (1995): core, 5′-ACAAGATGTTCCATG CAGCC-3′; Z1, 5′-TGCTGGTGCTGCTGGTGATA-3′; Z2, 5′-TCATCTCCATTTCGCCGGGA-3′; Z3, 5′-GATGGCGGTCGT CTTAAGCA-3′; Z4, 5′-GTGGTTGTTCAGCGAGTTCA-3′. In the PCR reaction QIAGEN Taq Polymerase and the protocol designed for use with Q-Solution was used. The PCR reaction was carried out as follows: one cycle at 94° for 4 min; 35 cycles, step one at 94° for 30 sec, step two at 60° for 30 sec, step three at 72° for 1.5 min; one cycle at 72° for 7 min.

BrdU labeling: Ovaries were dissected at room temperature in 1 × Grace's medium (Flow Laboratories, no. 2700049) and incubated for 1 hr in 15 μm BrdU (Sigma) in Grace's medium (Lilly and Spradling 1996). After washing in EBR (Ephrussi Beadle Ringer) the ovaries were fixed for 20 min in 37% formaldehyde/buffer B/dH_2O (1:1:4; Lin and Spradling 1993), followed by 1 hr denaturing in 2 N HCl and neutralizing for 2 min in 100 mm Na tetraborate. The tissue was rinsed several times in PBT (PBS + 0.1% Triton X-100) and blocked for 1 hr in 5% NGS in PBT. After overnight incubation in 1:20 dilution of anti-BrdU antibody (Becton-Dickinson, San Jose, CA, no. 347580) detection was carried out with HRP-

conjugated secondary antibody (1:25 dilution). Sigma Fast DAB peroxidase substrate (no. 4168) was used in the peroxidase color reaction. The latter was enhanced with 10 μl 1 m Ni SO_4 per 1 ml staining solution.

***In situ* hybridization to mRNA in ovaries:** The protocol is based on the procedure previously described (Tautz and Pfeifle 1998) and modified as follows. The ovaries were dissected in Ringer's solution and fixed for 20 min in 4% *p*-formaldehyde in PBS. After rinsing the tissue in PBT it was treated for 10 min in methanol/0.5 m EGTA, pH 8 (9:1). The ovaries can then be stored in methanol at −20° for several months. The stored ovaries were rehydrated in PBT. The prehybridization was carried out for 1 hr at 45° in DNA Hybrix (50% deionized formamide, 5× SSC, 100 μg/ml sonicated salmon sperm DNA, 50 μg/ml Heparin, 0.1% Tween 20). The ovaries were hybridized overnight at 45° in DNA Hybrix containing digoxigenin-labeled probe (DIG DNA labeling and detection kit, Boehringer Mannheim, Indianapolis). For detection a 1:1000 dilution of anti-DIG-AP-conjugated Ab was used. The staining reaction was performed in 100 mm Tris pH 9.5, 50 mm MgCl, 10 mm NaCl, 0.2% Tween 20, 8 mm levamisole, 4.5 μl/ml NBT, and 3.5 μl/ml X-phosphate (Boehringer Mannheim). Anti-DIG-AP conjugate was preabsorbed with postfixed wild-type (Oregon R) ovaries at 4° overnight. The ovaries were mounted in a mixture of PBS/glycerol (1:4) for microscopy.

RESULTS

The *BR-C* protein distribution pattern during oogenesis: In a previous article (Deng and Bownes 1997), we reported that *BR-C* mRNA is expressed in follicle cells in a dynamic pattern. Its expression is first detected in all follicle cells at stage 6. During stage 10A, all the columnar cells, except the dorsal anterior follicle cells, contain the *BR-C* transcript. However, only two groups of dorsal-lateral-anterior follicle cells express the *BR-C* mRNA during stage 10B, marking the dorsal appendage secreting cells.

To investigate the function of the *BR-C* we need to establish whether or not the protein is distributed in a similar pattern to the mRNA during oogenesis. Antibodies that recognize the BR-core, Z1, or Z3 domains, respectively, were used to stain the whole-mount ovaries. Antibodies to the Z2 and Z4 isoforms have not been generated, so we were unable to check their expression pattern. Both the Z1 and BR-core antibodies exhibited similar staining patterns, while the Z3 antibody showed no staining during oogenesis. These observations are consistent with the results shown by RNA *in situ* hybridization; Z1 is the only zinc-finger isoform with expression at levels significantly high to be detected by *in situ* hybridization techniques during oogenesis.

The distribution pattern of the *BR-C* protein appears to be similar to that of its mRNA during stages 6–8 of oogenesis, when all follicle cells stain (Figure 2, A1). The protein is also detected in all columnar follicle cells except the dorsal anterior cells at stage 10 (Figure 2, A1–A3), similar to the pattern of mRNA distribution (Deng and Bownes 1997). However, the follicle cells at the posterior pole appear to be stained at this stage (Figure 2, A1), which differs from the mRNA distribution pattern. The late distribution pattern of the *BR-C* protein and mRNA differs. A very strong signal is observed in two groups of the lateral-dorsal follicle cells at stages 11 and 12, but the posterior and ventral follicle cells are still stained (Figure 2B). The signal in the posterior and ventral region disappears around late stage 13, leaving only the dorsal-appendage-associated

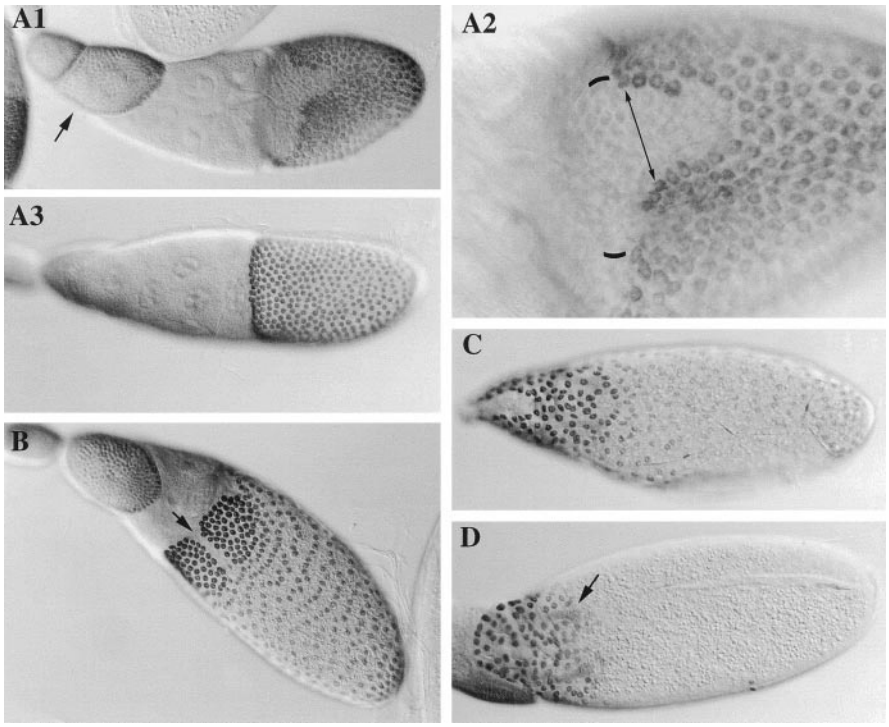

Figure 2.—The distribution pattern of the *BR-C* protein during oogenesis. (A1) Using BR-core antibody to stain the ovaries, signals are initially detected in all follicle cells at around stage 6 (arrowhead). Staining is also observed in all follicle cells at stage 7. During stage 10, columnar follicle cells over the oocyte are stained. However, staining is not seen at the dorsal anterior region. (A2) A closer look at the dorsal region. The dorsal gap is marked by a double-headed arrow, while the anterior gap is labeled by two curved lines. (A3) A ventral view of the same egg chamber. It appears that all ventral columnar cells are stained. (B) During stage 12, two groups of lateral-dorsal-anterior follicle cells are heavily stained, while the posterior and the ventral follicle cells are weakly stained. The dorsal gap between the two groups of the lateral-dorsal cells still exists (arrow). Expression in the posterior follicle cells becomes gradually weaker (C), until it disappears at approximately stage 13 (D). Expression is only detectable in the dorsal-appendage-associated follicle cells. The arrow shows a growing dorsal appendage.

follicle cells stained (Figure 2, C and D). The differences between the distribution patterns of the protein and mRNA presumably reflect the fact that the half-life of the protein is much longer than that of the mRNA. By the time late *BR-C* transcription occurs at the lateral-dorsal-anterior follicle cells, the protein translated from the early *BR-C* transcripts remains at the posterior and ventral side, while the mRNA has been degraded. Thus, the early and late protein distribution patterns overlap to form a gradient-like pattern at stages 11 and 12. The same reasoning could also be used to explain why the protein, but not the mRNA, is detected in the follicle cells at the posterior pole during stage 10.

Another feature of the *BR-C* protein distribution is that it only appears in the nuclei of the follicle cells (Figure 2), which is consistent with the fact that the *BR-C* encodes transcription factors.

***rbp*$^+$ function is required for dorsal appendage formation:** The genetic organization of the *BR-C* is shown in Figure 1. Bayer *et al.* (1997) reported that Z1 provides the full *rbp*$^+$ function. Since the Z1 zinc-finger isoform is expressed during oogenesis and is detectable by *in situ* hybridization, it is predicted that the *rbp* functional domain will be required. To test this, female homozygous viable *rbp*^{1} and *rbp*^{2} mutants were dissected to examine the eggshell phenotype. It was found that the dorsal appendages were abnormal, being shorter and rougher than the wild type, and the eggshells were much more fragile (Figure 3). Shortening of the dorsal appendages was observed in *rbp*^{2} homozygous mutants.

To test if *rbp* is the major functional domain involved in dorsal appendage formation we did a genetic analysis using the *npr* allele in crosses with *br*, *rbp*, and *2Bc* alleles (Table 1). The cross between *rbp*^{1} and *npr*^{6} produced only two males and no viable female heteroallelic mutants. Two *rbp*^{2} / *npr*^{6} females were produced in the cross with *rbp*^{2} flies. They lived for 2 days without laying any eggs. Then they were dissected to examine the ovarian phenotype. It was found that the ovaries were not completely developed and the few late stage oocytes formed had no appendages. Thus we were unable to examine *rbp* function by this method. The combination *2Bc* / *npr*^{6} was found to be completely lethal, and as a result we could not establish if there is a function encoded by the Z3 (2Bc) isoform.

The function of Z2 was investigated by further genetic crosses. The cross between *br*^{5} and *npr*^{6} produced no viable heteroallelic flies, while the cross between *br*^{6} and *npr*^{6} generated 22 heteroallelic males, but no females that could be examined. It was observed that eggs produced by *br*^{1} / *br*^{A47} and *br*^{1} / *npr*^{6} mothers have reduced dorsal appendages (Deng and Bownes 1997), suggesting that the *br* functional domain is likely to be required for dorsal appendage formation. To test this hypothesis, eggs produced by *br*^{5} / *br*^{1} and *br*^{6} / *br*^{1} females were examined, and found to have normal dorsal appendages (data not shown). This observation, along with the fact that the *br*^{del2} is actually an *rbp* allele (Huang and Orr 1992), suggests that the *br* functional domain, and hence Z2, is not involved in dorsal appendage formation.

How can the phenotype of *br*^{1} / *br*^{A47} and *br*^{1} / *npr*^{6} eggshells be explained if *br* is not the functional domain required for dorsal appendage formation? This could be understood if the *br*^{1} mutant not only affected *br* function, but also affected *rbp* function. To test this possibility, the eggshell phenotype of eggs laid from *br*^{1} / *rbp*^{1} mutants was examined. It was shown that eggs produced by the *br*^{1} / *rbp*^{1} mothers have reduced dorsal appendages, similar to those produced by the *br*^{1} / *br*^{A47} females. This indicates, therefore, that the *br*^{1} is in fact a weak *2Bc* or *2Bab* allele, which fails to complement either *rbp* or *br* function. This suggests that *rbp* (which encodes Z1 and Z4) is a functional domain involved in dorsal appendage formation during oogenesis; however, we cannot rule out the involvement of Z3 from these experiments due to the failure of these crosses to generate adult females due to early lethality.

Ectopic *BR-C* expression induces ectopic dorsal appendage material: Is *BR-C* function sufficient to direct

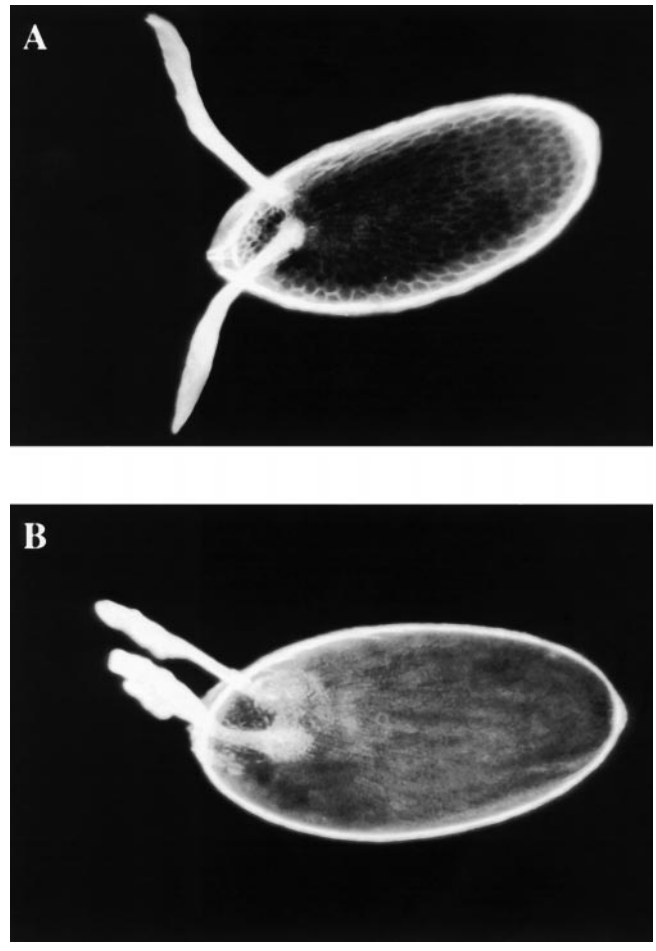

Figure 3.—Chorion defects in *rbp* mutants. (A) Wild-type chorionic appendages. (B) Appendages that are shorter and irregular in shape, produced by *rbp*^{1} females.

TABLE 1

Survival frequencies of *BR-C* mutants

Cross	Progeny: Heterozygotes[a] + Homozygous balancer[b]	Progeny: Heteroallelic mutants[c] ♂	Progeny: Heteroallelic mutants[c] ♀	Lethal period
$\frac{br^1}{br^1} \times \frac{npr^6}{Binsn}$	216	21	9	Not observed
$\frac{br^5}{Binsn} \times \frac{npr^6}{Binsn}$	158	0	0	III instar larva
$\frac{br^6}{Binsn} \times \frac{npr^6}{Binsn}$	36	22	0	Pupal
$\frac{rbp^1}{Binsn} \times \frac{npr^6}{Binsn}$	140	2	0	Pupal
$\frac{rbp^2}{rbp^2} \times \frac{npr^6}{Binsn}$	130	11	2	Pupal
$\frac{2Bc^1}{Binsn} \times \frac{npr^6}{Binsn}$	73	0	0	II–III instar larva
$\frac{2Bc^2}{Binsn} \times \frac{npr^6}{Binsn}$	215	0	0	Pupal

[a] Heterozygotes, flies carrying a *Br-C* mutant chromosome and a balancer chromosome (*br*/*Binsn*, *rbp*/*Binsn*, *2Bc*/*Binsn*, and *npr*/*Binsn*).

[b] Homozygous balancer, flies homozygous with respect to the balancer chromosome (*Binsn*/*Binsn*).

[c] Heteroallelic mutants, flies carrying different combinations of *Br-C* alleles (*br*/*npr*, *rbp*/*npr*, and *2Bc*/*npr*).

the formation of the dorsal appendages? To address this, heat-inducible Z1 transgenic flies (*hsp70*/Z1) were used to examine the effect of ectopic *BR-C* expression during oogenesis. Following standard heatshock (37°, 30 min) and incubation at 26° for 2–48 hr, ovaries of the *hsp70*/Z1 females were analyzed (Table 2). Flies were dissected to examine the effect of the heatshock at 2, 3, 5, 9, 24, and 48 hr after heat treatment. The first abnormalities in the egg chambers were observed 3 hr after the heatshock. The eggs laid during the first 3 hr following heatshock also have a very high hatch rate, presumably being sufficiently differentiated at the time of the heatshock for ectopic *BR-C* expression to have no effect. The results over this period do not differ significantly for the control heatshocks (Table 2). The strongest effect was observed between 4 and 6 hr. It was observed that extra dorsal appendage material was produced in the dorsal-anterior region of the eggshells (Figure 4, B–F). In most cases, dorsal appendage material appeared in the dorsal gap between the two appendages. It condensed at the base of the dorsal appendages and less material was deposited in the appendages themselves. The dorsal appendages did not elongate properly (Figure 4, B–D), presumably due to a failed migration of the follicle cells. Different phenotypes have been observed, depending on the stage of the egg chamber at the time of heatshock (Figures 4 and 5). Heatshock at the time of dorsal appendage formation, stage 11, leads to the "appendageless" phenotype or to small fused appendages (Figure 4, B and C). Appendages with abnormal shapes and/or different lengths have been observed on eggs at stages 12 and 13 at the time of heatshock (Figure 4, D–F). These observations indicate that ectopic Z1 can induce formation of ectopic dorsal appendage material. Nevertheless, the ectopic dorsal appendage material is restricted to the dorsal anterior eggshell, suggesting that the fate of the follicle cells is predetermined along the two major axes prior to the requirement for *BR-C* function in this process. It was hypothesized that the lack of *BR-C* expression in the dorsal-most follicle cells is due to high levels of expression of *pointed* (*pnt*) in those cells (Deng and Bownes 1997). The data shown here indirectly support this hypothesis. In the dorsalmost follicle cells, there could be competition between the expression of Pnt and *BR-C*. When high levels of Pnt are expressed, *BR-C* expression is inhibited in these cells. However, in heatshock lines, the *BR-C* expression would overcome the inhibition by Pnt. Thus, dorsal appendage material can be synthesized by these cells.

Although Z1 seemed to be the sole *BR-C* zinc-finger isoform expressed at high levels during oogenesis when analyzed by *in situ* hybridization, we tested Z2, Z3, and Z4 to determine if they exhibit a similar phenotype when ectopically expressed during oogenesis. Thus, *hsp70*/Z2, *hsp70*/Z3, and *hsp70*/Z4 flies were heat-

TABLE 2

Effect of heatshock on Z1–Z4 transgenic flies

Experiment	Fly stock	No. of eggs laid	Abnormal eggs (%)	Eggs hatched (%)
0–3 hr	Z1	172	5.8	94
	Z2	144	2.3	96
	Z3	134	1.5	98
	Z4	80	5.0	96
	w^{1118}	364	1.6	98
	OrR	94	2.1	99
3–5 hr	Z1	27	74	2
	Z2	68	55	8
	Z3	4	0	25
	Z4	23	43	4
	w^{1118}	137	2.5	97
	OrR	117	2.7	96
5–24 hr	Z1	10	89	6
	Z2	361	78	12
	Z3	27	42	18
	Z4	186	77	15
	w^{1118}	505	4.4	94
	OrR	363	3.6	98
24–48 hr	Z1	9	62	72
	Z2	670	33	93
	Z3	72	24	90
	Z4	390	18	97
	w^{1118}	50	1.3	100
	OrR	370	1.1	99

Z1–Z4, transgenic flies carrying constructs to misexpress the various zinc-finger isoforms of the *BR-C* in response to heatshock; w^{1118}, the host line used for the construction of the transgenic lines used as a control. OrR (wild-type) flies were also used as a control. Approximately 100 flies (50 males and 50 females) from each stock were used in the heatshock experiments.

shocked and the eggshell phenotype was examined (Table 2). It was found that ectopic dorsal appendage material is produced in the dorsal-anterior region of the eggshells by all three transgenic lines. This phenotype is similar to that exhibited by eggs of the *hsp70/Z1* flies after heatshock, suggesting that all of the four zinc-finger isoforms could be functional in dorsal appendage formation during oogenesis.

It is apparent from Table 2 that heatshock has the strongest effect on chorion morphology and egg viability in *hsp70*/Z1 flies. Z2–Z4 recovered viability to ~95% in 2 days, while Z1 recovered only to 72% during that time period. It was also found that heatshocked Z1, Z2, and Z4 flies lay abnormal eggs (Table 2). The ectopic expression of *BR-C* in Z3 flies was found to disrupt the process of egg development soon after the heatshock. We observed that some 20% of all laid eggs have aberrant micropyles, due to excess chorion formation. This could prevent the sperm entering the egg and hence subsequent development would fail due to lack of fertilization. Another possible explanation is that ectopic expression of *BR-C* can disrupt some other Bric-a-brac/Tramtrack/Broad Complex (BTB)-containing protein that can dimerize with the *BR-C* and thus modulate its function.

Heatshock alone causes eggshell defects. The data of the control experiments with the heatshocked wild-type OrR flies and w^{1118}, the host line for the transgenic flies, is presented in Table 2. We observed in the few abnormal eggs wide-branched dorsal appendages of approximately normal length. It is quite clear that the results of misexpressing *BR-C* in oogenesis significantly affects the eggshell.

Other zinc-finger isoforms are expressed in oogenesis: Although only Z1 expression was clearly observed by *in situ* hybridization we observed defects in chorion formation and morphology by overexpressing all four zinc-finger isoforms available. It became essential, therefore, to establish if this was due to some degree of functional redundancy between the isoforms with respect to eggshell development or if the other zinc fingers are, in fact, expressed at lower levels in oogenesis. To check this we used RT-PCR using primers for Z1, Z2, Z3, and Z4 and the core DNA binding domain. The organization of the zinc-finger isoforms in relation to the *BR-C* is shown in Figure 1. The primers used are illustrated in Figure 1B and should generate products of 974 bp, 780 bp, 728 bp (Z1); 320 bp (Z2); 784 bp (Z3); and 1082 bp (Z4), respectively, based on published data (Hodgetts *et al.* 1995; Bayer *et al.* 1996b). The results clearly show that all four zinc fingers are expressed in oogenesis (Figure 6). The identity of the PCR-generated products was confirmed with Southern blot analysis.

It seems likely therefore that, as in metamorphosis, all zinc-finger isoforms are expressed and function to regulate downstream gene expression. However, only Z1 is expressed at a high-enough level to detect the spatial distribution of the RNA and protein in oogenesis.

Ectopic *BR-C* expression during mid-oogenesis affects endoreplication and chorion gene amplification: Ectopic *BR-C* expression appears to induce premature production of the chorion. Figure 5, C and D, shows that the chorion is already present in stage-11 egg chamber. This could isolate the oocyte from the nurse cells and physically prevent dumping of the nurse cell components into the oocyte. This could result from an altered pattern of transcription and translation of the chorion genes, or from abnormalities in amplification of the chorion genes, or both. We investigated, therefore, whether the alterations in *BR-C* expression affected the timing or pattern of chorion gene amplification. Since the chorion is synthesized by most follicle cells, this function could be related to the earlier expression of the *BR-C*. To monitor amplification we investigated the incorporation of BrdU in the follicle cell nuclei of wild-

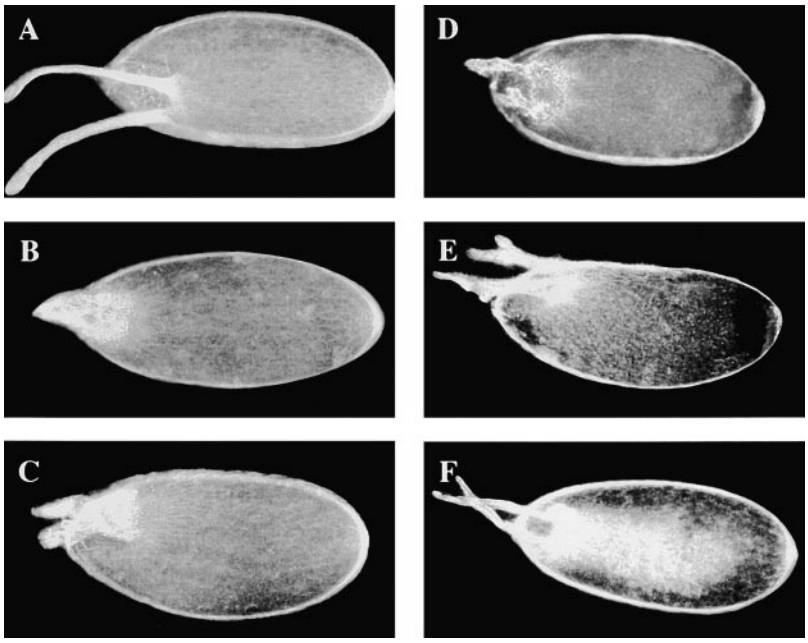

Figure 4.—Ectopic expression of different *BR-C* zinc-finger isoforms during oogenesis. A–F, dark-field microscopy. (A) A wild-type egg after heatshock treatment. (B and C) Two distinctive *hsp70/Z1* (527-5 and 708-1) transgenic lines exhibit a similar phenotype. Dorsal appendage materials are present in the dorsalmost region, resulting in fused, thickened, and shortened dorsal appendages. (D–F) A similar, but less severe, eggshell phenotype in *hsp70*/Z1 flies; see explanation in the text. Fused, thickened, and irregularly shaped appendages are also found in eggshells produced by the Z2, Z3, and Z4 transgenic lines.

type ovaries and in ovaries misexpressing various isoforms of the *BR-C*.

In wild-type ovaries, after eight mitotic cell divisions, the endoreplication phase of the follicle cell development of oogenesis begins (stage 6), and is completed by stage 10B; during this process the entire nucleus is labeled by BrdU. The endoreplication is asynchronous in wild-type and w^{1118} (the host strain used to produce the transgenic lines) ovaries and occurs in both nurse cells and follicle cells (Figure 7, A and C). We observed a continuous endoreplication in the nurse-cell-associated follicle cells at stage 10B (Figure 7C). This is followed by the chorion gene amplification phase when 4 spots of incorporation are seen per nucleus in the follicle cells overlying the oocyte (Figure 7, C, G, and K). These 4 spots represent amplification of the two clusters of chorion genes (Figure 7, G and K). Two are always larger, presumably due to the higher level of amplification of the cluster on chromosome 3 compared to the X-chromosome cluster (Orr-Weaver and Spradling 1986; Delidakis and Kafatos 1989). This amplification was first observed at the border between the oocyte and nurse cells and it soon spread to the rest of the follicle cells. When the *BR-C* isoforms are misexpressed, there is prolonged and synchronized endoreplication until late stage 10B, followed by specific amplification of genes in each nucleus (Figure 7, D, F, H, and L). These results are observed from 3.5 to 4.5 hr after heatshock.

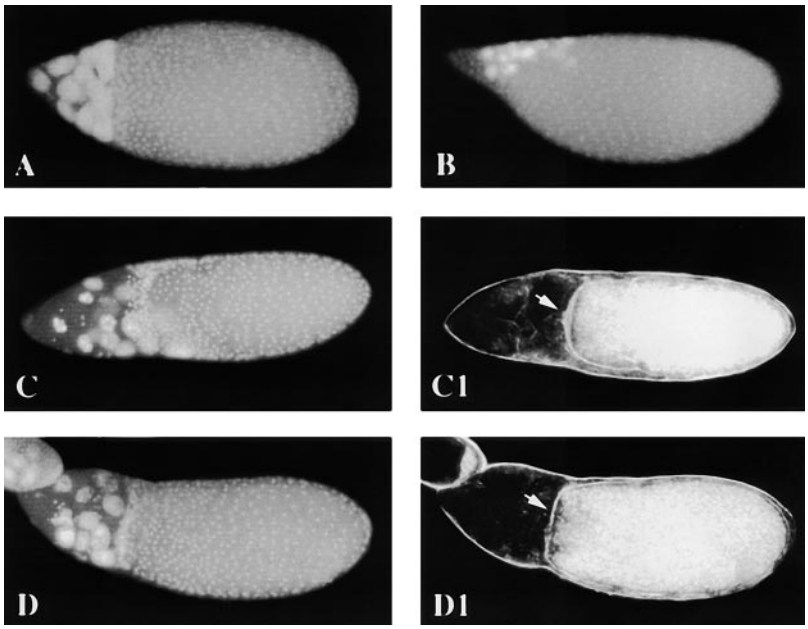

Figure 5.—Ectopic *BR-C* expression affects the onset of chorion synthesis. A–D, Hoechst staining; C1–D1, dark-field microscopy. (A) A wild-type egg chamber at late-stage 11. The oocyte is larger than the nurse cell complex due to the onset of nurse cell dumping into the oocyte. (B) Wild-type egg chamber, late stage 12. Dumping is complete. C–C1 (late stage 11) and D–D1 (late stage 12) show that transgenic Z1–Z4 also causes inappropriate chorion synthesis (arrows), and this blocks dumping of the nurse cell cytoplasm into the oocyte during stage 11–12 of oogenesis.

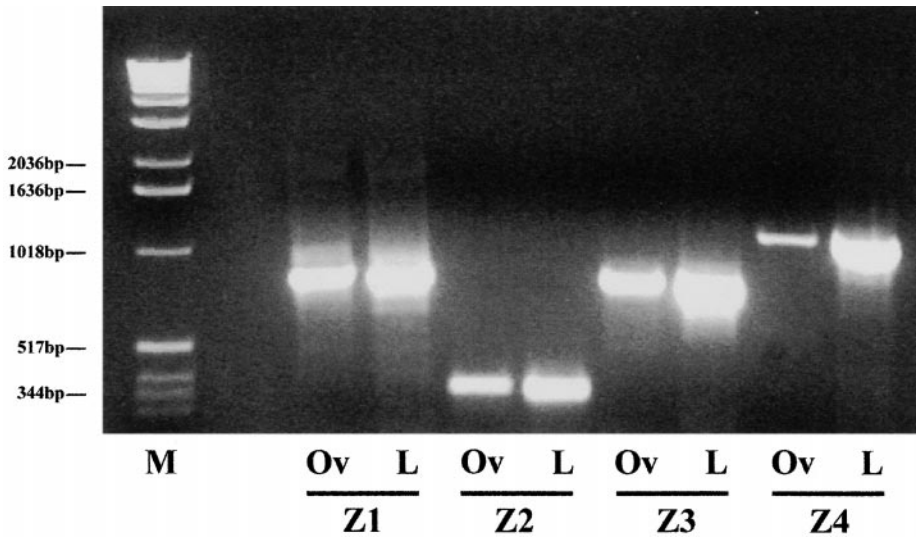

Figure 6.—PCR analysis of the *BR-C* transcripts in *Drosophila melanogaster* (wild-type, Oregon R) ovaries. Total RNA from ovaries was reverse transcribed and the subsequent cDNA PCR amplified using the primer pairs shown with arrows below the exon map (Figure 1B). All four PCR reactions were carried out with a common primer located at the 3′ end of the core domain of *BR-C*. The other primers are located just within, or immediately 3′ to, the respective Z1–Z4 domain. M, marker–1-kb ladder (Gibco BRL); Ov, ovaries; L, larvae (whole organism) used as a control. The primer sets generate the following products from transcribed larvae RNA: 974 bp, 780 bp, 728bp (Z1); 320bp (Z2); 784bp (Z3); 1082 bp (Z4). The PCR does not generate the 728-bp Z1 product from transcribed ovarian RNA, indicating the lack of this transcript during oogenesis.

We also observe extra spots of incorporated BrdU in the nuclei (Figure 7, H and L). There were three possible explanations for this: either the heatshock could be responsible, the homologues of the chromosomes could have separated due to a defect in the cell cycle, or there could be amplification of DNA at additional sites in the genome. The host flies used to produce the transgenic lines, w^{1118}, were heatshocked and still showed 4 spots per nucleus, so heatshock itself was not responsible for the results. We counted the number of spots per nucleus and found 6 or 12 spots in ~80% of the nuclei. Occasionally we observed up to 28 spots. If the cell cycle was affected, and the homologues had separated, we would expect to see many more than 28 spots per nucleus due to the polyploidy of the follicle cells. If the amplification sites varied we could not predict the numbers, and indeed it may well be variable. This suggests that there are other sites in the genome induced to replicate by *BR-C* overexpression. We conclude that the endoreplication of DNA and the amplification of the chorion genes depends upon the *BR-C* encoded proteins or an unknown protein that is encoded by one of the downstream targets of the *BR-C*. This observation is consistent with the report that a mutation in the *BR-C* locus causes premature arrest of chorion gene amplification (Huang and Orr 1992).

Ectopic *BR-C* expression in relation to chorion gene expression: The chorion is produced by the columnar follicle cells to provide a shell around the egg. Later in oogenesis, two groups of cells migrate anteriorly to produce the chorionic appendages and very large amounts of chorion material. Ectopic expression of the Z1 isoforms leads to chorionic appendage deposition by extra cells lying at the anterior of the egg filling in the middorsal gap observed in wild-type eggs. Often the follicle cells fail to migrate anteriorly over the remaining nurse cells at stage 11 and they are present, therefore, at a more posterior position. The pattern of *chorion* gene expression was compared in wild-type ovaries and in those expressing the Z1 isoform ectopically. In the wild type, we observe a high concentration of chorion transcripts in all follicle cells at stage 9, prior to their translation; they then become inactive and transcripts are again seen in stages 11–14 (Figure 8, A, C, E, and G). In the ovaries with ectopic *BR-C* gene expression, examined 3.5–4.5 hr after the heatshock, the same high concentration of chorion transcripts is observed in anterior follicle cells, even though their location in relation to the nurse cells is more posterior. This suggests the expression pattern is not dependent on the *BR-C* along the anterior-posterior axis (Figure 8, B, D, F, and H). Moreover, posterior follicle cells do not produce substantially more chorion material even though they express Z1 protein after heatshock. The fact that more dorsal cells produce appendage material and express the chorion genes means that *BR-C* expression in the most dorsal anterior cells does induce additional chorion production. Thus we observed two different effects. Initially the ectopic expression of *BR-C* prevents the migration of the dorsal follicle cells in an anterior direction. Then the midline cells that normally express chorion protein at that stage start depositing chorion material in the wrong location. This results in the production of aberrant dorsal appendages. It is possible that the *BR-C* activates downstream genes which, in turn, activate the chorion genes. In anterior cells high levels of *BR-C* expression "win" over *trans*-acting repressors, but in posterior cells they do not. The *in situ* hybridization results and the observed characteristic phenotypes following ectopic *BR-C* expression are both consistent with this observation. Alternatively, *BR-C* could be essential for the cell migrations to position the follicle cells and endoreplication

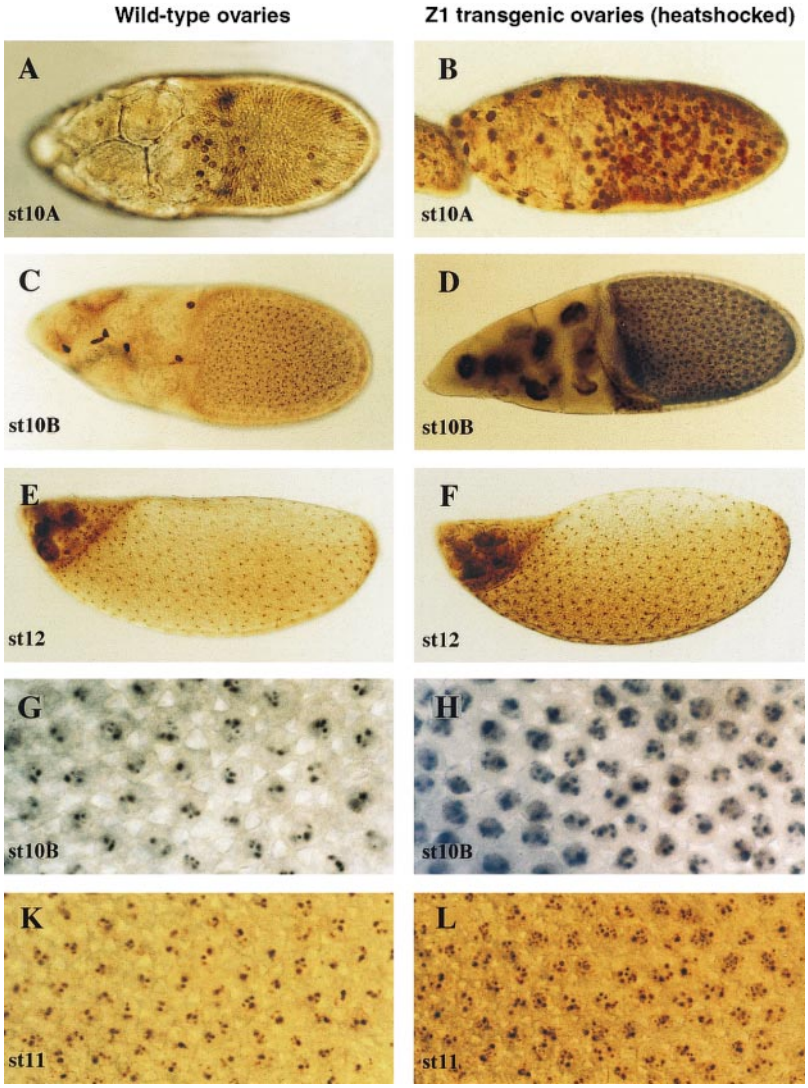

Figure 7.—Chorion gene amplification. BrdU incorporation associated with endoreplication and amplification. (A) BrdU incorporation in the nuclei of wild-type Drosophila (Oregon R) ovaries and w^{1118}, the host strain used for the production of the *BR-C* transgenic lines at stage 10A. The endocycles are not synchronized. Thus, just some of the nuclei are positive. (Calvi *et al.* 1998). (B) Overexpression of a *BR-C* (Z1) transgene in ovaries at stage 10A. Most of the nuclei show synchronized endoreplication. (C) Normal synchronized amplification in wild type and w^{1118} at stage 10B. By that time the endoreplication associated with the main body follicle cells is completed. (D) BrdU incorporation in the nuclei of heatshocked *BR-C* (Z1) transgenic ovaries, stage 10B. Strong BrdU incorporation is present in the nurse cells. (G) Higher magnification of wild-type and w^{1118} nuclei at stage 10B to show the four spots of amplification normally occurring in wild-type ovaries. (H) BrdU incorporation in the nuclei of *BR-C* (Z1) expressing ovaries 3 hr after the heatshock, stage 10B. Some of the nuclei contain extra spots of replication. The amplification pattern overlaps with the labeling due to continued endoreplication. (E–K) The nuclei of heatshocked w^{1118} ovaries still show four spots of amplification. (F–L) The nuclei of *BR-C* (Z1) transgenic ovaries 4.5 hr after heatshock exhibit a multispotted pattern.

of the chorion genes but not in regulating chorion gene expression.

DISCUSSION

The *BR-C* complementing groups and zinc-finger isoforms: It has been shown by genetic analysis that rbp^+ function is required for dorsal appendage formation, and it was observed that Z1 is the sole zinc-finger isoform expressed at high levels in the appendage-producing cells. These observations are compatible with the report that Z1 provides rbp^+ function (Bayer *et al.* 1997). It was also found that rbp^+ function is partially provided by Z4, but is not provided by Z2 and Z3 (Bayer *et al.* 1997). However, heatshock-induced expression of all four zinc-finger isoforms (Z1, Z2, Z3, and Z4) leads to a similar phenotype of extra dorsal appendage material production in the dorsal gap, indicating that the different transcripts may substitute for each other functionally in dorsal appendage formation.

The homozygous viable mutant $br^1$was the first mutant identified in the *BR-C* locus. It exhibits a broad wing phenotype and fails to complement other mutations that are categorized in the *br* complementation group. However, the complementation analysis presented in this article suggests that the br^1 mutations also partially remove *rbp* function. Therefore, it is in fact a *2Bab* allele. It is known the *2Bab* mutations cause reduction of both Z1 and Z2 expression. Thus in the br^1/br^{A47} and br^1/npr^6 females, both Z1 and Z2 are reduced. The reduction of Z1 levels results in the reduction of dorsal appendages, while no effect is produced by the reduction of Z2 levels. This is why no defects were observed in eggs produced by br^1/br^5 and br^1/br^6 females.

The mutant phenotypes clearly show the need for the *BR-C* in chorionic appendage formation. PCR experiments have shown that all zinc-finger isoforms are in fact expressed in oogenesis, but as yet we have no evidence that they perform different functions. Neither do we know the spatial and temporal distribution of Z2–Z4, which are not present at sufficiently high levels for detection by *in situ* hybridization. Overexpression

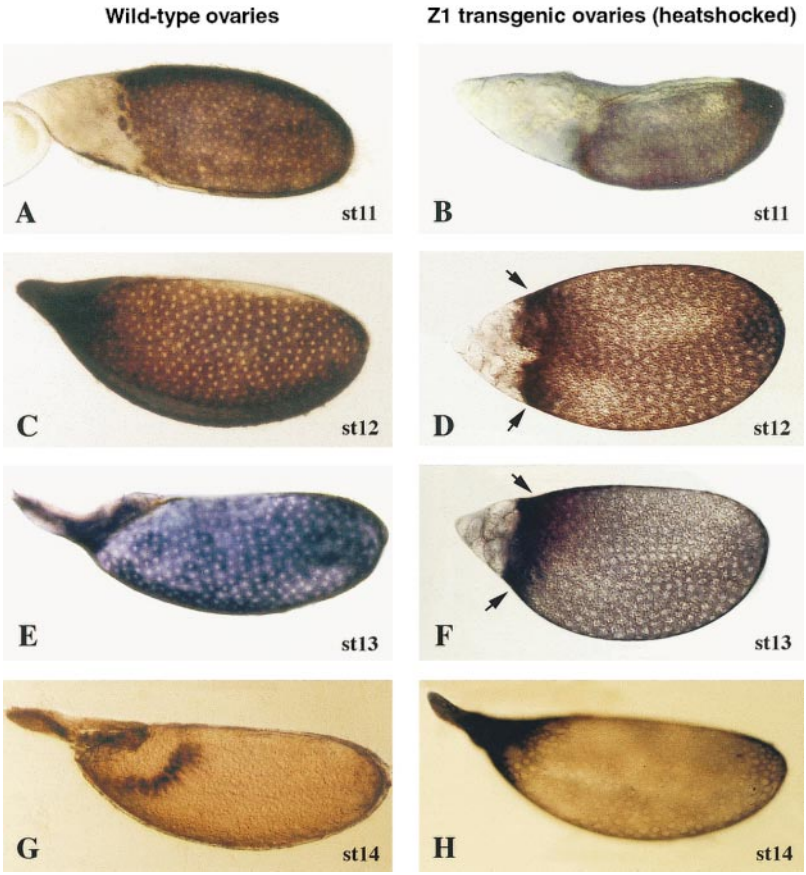

Figure 8.—*In situ* hybridization to chorion RNA in ovaries. (A, C, E, G) *In situ* hybridization to wild-type (Oregon R) Drosophila ovaries with a DIG-labeled probe hybridizing to chorion mRNA. The chorion probe, 7C8, was generated from cDNA encoding the c38 chorion protein, which maps to the X chromosome. Similar results were obtained using the alternative 7B7 probe (cDNA encoding c15 protein), which maps to a site on the third chromosome. For more information about the probes see Spradling *et al.* (1980). (B) *In situ* hybridization to heatshocked Drosophila *BR-C* (Z1) transgenic ovaries with 7C8 probe, stage 11. At that stage an increase in the amount of chorion mRNA is observed at the border between the nurse cells and the oocyte. The misexpression of the *BR-C* leads to abnormal migration of the anterior follicle cells. (D) *In situ* pattern of chorion mRNA expression in heatshocked *BR-C* (Z1) transgenic ovaries, stage 12. A gap (arrows) is formed at the anterior pole as a result of failed migration of the follicle cells. (F) Strong expression of the chorion gene clusters in heatshocked *BR-C* (Z1) transgenic ovaries at stage 13. The variations in the egg chamber shape result from the heatshock-induced misexpression of *BR-C*. (H) Strong continuous expression of chorion mRNA in heatshocked *BR-C* (Z1) transgenic ovaries, stage 14. The anterior gap is not present because the nurse cells have completed dumping and the follicle cells have migrated correctly by the time of heatshock.

studies using transgenic flies carrying heatshock-controlled Z1 and Z4 isoforms lead a failure of proper migration of the follicle cells that will secrete the appendages, premature chorion deposition, and abnormal appendage formation.

Relationship between the *BR-C* and chorion production: We have shown here that the *BR-C* is important for controlled DNA replication in oogenesis. Overexpression does not affect the timing of the onset of endoreplication and amplification, but endoreplication is prolonged beyond that observed in wild-type ovaries and it leads to additional replication sites in the genome. These additional sites presumably share sequence similarities with the *cis*-acting sites regulating chorion gene amplification. This suggests that the *BR-C* is a key regulator of endoreplication and chorion gene amplification. The early *BR-C* expression pattern is in all the follicle cells and it is presumably at this stage that it is involved in this function. The expression of the *BR-C* is first observed in wild-type flies at stage 6 and it is also at stage 6 that the endoreplication cycles begin. Since we did not observe premature endoreplication with *BR-C* overexpression, presumably other components essential for endoreplication are absent until stage 6 of oogenesis. The active role of the *BR-C* in endoreplication is also apparent from the fact that we observed prolonged incorporation of BrdU in the nurse cell nuclei when the *BR-C* is overexpressed. This presumably results in expression of the *BR-C* in the nurse cells, where it is normally not expressed. This shows that the proteins encoded by the *BR-C* can function to prolong replication of DNA even in cells where it is not normally used to control this process. It also suggests that an alternative regulator for DNA replication to the *BR-C* is used in the nurse cells. In normal development, the later *BR-C* expression, which is maintained in the dorsal-anterior cells making the appendages, is probably needed for cell migration, chorion deposition, and other follicle cell differentiation events.

It is possible that the initial activation of the *BR-C* in all follicle cells is regulated by the ecdysone/USP heterodimer (Yao *et al.* 1992; Horner *et al.* 1995) as is observed in metamorphosis. There is also some evidence that in intermoult puffs a second heterodimer with DHR38 (a nuclear receptor related to NGF1-B from mammals) can compete for the same binding sites (Crispi *et al.* 1998), so it is also possible that other DNA binding proteins, besides the ecdysone receptor, will be involved in *BR-C* expression. This would suggest a role for ecdysone during these stages of oogenesis. Later, the *BR-C* could be repressed in specific follicle cells by competitive and cooperative interactions with other gene products initiated by the *grk* and *dpp* signaling pathways. There is a significant amount of evidence that ecdysone and juvenile hormone [which binds to ultra spiracle proteins (USP)] are important for the progress

of oogenesis (Wilson 1982; Bownes 1989, 1994) and we have recently shown that there is a control point in oogenesis that regulates whether egg chambers will proceed with development or undergo apoptosis, which is regulated by the balance of juvenile hormone and ecdysone (Soller *et al.* 1999). However, there is little evidence as to precisely what role these hormones have in regulating oocyte development and egg chamber differentiation. We have shown that the ecdysone receptor is present in the follicle cells at the time *BR-C* is activated (D. Mauchline, W.-M. Deng and M. Bownes, unpublished results) by antibody staining.

Once activated, as we have shown, the *BR-C* gene is involved in endoreplication, the selective amplification of the chorion genes, and in the subsequent morphogenesis of the chorionic appendages. Calvi *et al.* (1998) have recently shown that the selective amplification of the chorion genes is closely linked with the cell cycle and the cycles of endoreplication that occur in the follicle cells earlier. Somehow the chorion genes escape the rereplication controls that influence other parts of the genome. Our BrdU labeling experiments confirm their results on the timing of endoreplication and chorion amplification and the close association between endoreplication and selective amplification. Using overexpression of the *BR-C* we see not only the two extra sites they mentioned that may represent another chorion gene amplified for a function in later oogenesis, but also a number of additional sites. These may be sites with sequence similarity to the *cis*-acting sites regulating amplification. Calvi *et al.* (1998) propose that there are amplification complexes located at chorion genes. Whether the *BR-C* encoded proteins are associated with these complexes or regulate the synthesis of one or more of their components remains to be elucidated. We have confirmed the link between endoreplication and chorion amplification and shown that it involves the *BR-C.* This may therefore provide a crucial link between hormones and the control of the cell cycle, and hence of differentiation, of the egg chamber during oogenesis.

In summary, our working model would be that the *BR-C* is activated by ecdysone in all follicle cells at stage 6 of oogenesis where its key function is the control of endoreplication, and then selective amplification. Later, when it is turned off in all but the anterior-dorsal follicle cells that will secrete the appendages it has a second set of functions and is involved in the migration of cells and morphogenesis of the chorionic appendages. Recently this link between ecdysone, the *BR-C,* and morphogenesis has also been described for the progression of the furrow in the developing eye imaginal disc of Drosophila (Brennan *et al.* 1998).

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