The BmE75 Nuclear Receptors Function as Dominant Repressors of the Nuclear Receptor BmHR3A*

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The orphan nuclear receptors BmE75 and BmHR3 are induced by 20-hydroxyecdysone in the ovary of the silk moth Bombyx mori at the beginning of pupation and show stage-specific expression in ovarian follicles during pharate adult development. To analyze the function of these receptors, we have developed a transactivation assay based on the transcriptional stimulation of a retinoic acid receptor-related receptor response element (RORE)-linked promoter-reporter construct. Co-transfection of a Bombyx cell line with a BmHR3A expression construct results in constitutive activation of the reporter, whereas expression of BmE75 has no measurable effects on reporter expression. However, when the BmE75 receptors are co-introduced with BmHR3A into the cells, the BmHR3A-mediated transactivation is repressed. Repression of BmHR3A by BmE75 occurs by two distinct mechanisms. Increasing doses of BmE75 efficiently displace BmHR3A bound to the RORE target site in gel retardation assays, indicating that both receptors compete for common DNA target sites. However, analysis of the function of deletion mutants of BmE75 in the transactivation assay indicates that repression can also occur in the absence of the DNA-binding domain and that the C-terminal F domain is sufficient for repression. In gel retardation assays, the two receptor types form a ternary complex on a single RORE, suggesting that repression is also mediated by protein interactions on the DNA target site. Yeast two-hybrid assays show that BmHR3A interacts with BmE75 and that this interaction is dependent on the C terminus of BmHR3A and the F domain of BmE75. Because the C terminus of BmHR3A contains a strong activation domain, we predict that BmE75 blocks activation by BmHR3A through competition for co-activator binding sites located at the C terminus of BmHR3A. Our data also indicate that the transcriptional activities of BmHR3A and BmE75 are integrated in such a way that activation of RORE-linked target genes depends on the relative expression levels of the two receptor types.

The orphan nuclear receptors E75 and HR3 were originally isolated because of their ubiquitous activation as early-response genes by 20-hydroxyecdysone (20E) in target tissues of insects (1). The mRNA of E75 is typically induced within 30 min of hormone treatment, and the induction also occurs in the presence of protein synthesis inhibitors, suggesting a direct control of E75 gene transcription by the hormone-bound ecdysone receptor complex (2–4). The accumulation of HR3 mRNA, on the other hand, occurs more slowly (starting 2–3 h after hormone exposure) and requires synthesis of additional factors for maximal expression (5–7). Ecdysone-induced E75 and HR3 proteins are subsequently thought to function as transcription factors and play essential roles in the implementation of the 20E-induced gene expression cascade (8).

The ovary of the silk moth Bombyx mori is a target of 20E during early pupation (9, 10). Administration of 20E to developmentally arrested abdomens causes abrupt induction of BmE75 and BmHR3, the silk moth homologs of the E75 and HR3 nuclear receptors, with kinetics similar to those observed in target tissues of other insects (11, 12). Interestingly, however, different isoforms for both types of receptors, characterized by unique N-terminal regions, exist in the silk moth ovary and differ in their hormone induction patterns. Among the BmE75 isoforms, BmE75A and BmE75C mRNAs are induced within 30 min by the hormone, but the decline of the BmE75C mRNA occurs much faster than that for the mRNA of BmE75A (12). Similarly, among the BmHR3 isoforms, BmHR3B and BmHR3C mRNAs are induced after 2–3 h, in contrast with the mRNA of the BmHR3A isoform, which accumulates in ovarian tissue 2 days after hormone administration (11).

In addition to the hormonal induction in the immature ovary at the beginning of pupation, the expression of HR3 and E75 isoforms in the silk moth ovary is also regulated in a hormone-independent fashion during pharate adult development and can be correlated with specific stages of oogenesis. Thus, the expression of BmHR3A coincides with vitellogenesis (11), whereas BmE75C is only expressed during a brief period at the transition from vitellogenesis to chorogenesis (12). BmE75A, on the other hand, has a more ubiquitous expression pattern and is present in both previtellogenic and vitellogenic follicles. The expression at specific stages of oogenesis suggests a role for BmE75 and BmHR3 in the implementation of the developmental program of follicle differentiation in the ovary.

The E75 and HR3 nuclear receptors were previously shown to bind as monomers to similar extended nuclear hormone receptor DNA half-sites consisting of the core motif AGGTCA preceded by an AT-rich extension (6, 11, 13–15). Such extended half-sites are known as retinoic acid receptor-related receptor response elements (RORES) because they were origin-
nally identified as the binding sites for the ROR receptors, the mammalian homologs of the insect HR3 receptors (16). The ability of E75 and HR3 to bind to similar DNA target sites, combined with the simultaneous expression of both receptors in tissues that are challenged by hormone, raises questions regarding whether the receptors will compete for common target sites in the DNA and the effects this competition will have on the transcriptional activation of target genes.

In this study, we tested the capacity of several Bombyx E75 and HR3 receptors for binding to a consensus RORE in gel retardation assays and activation of a minimal promoter-reporter construct harboring four copies of a RORE motif in its upstream region in transient expression assays using B. mori tissue culture cells. BmHR3A was found to bind efficiently to and HR3 receptors for binding to a transcriptional activation of target genes.

Regarding whether the receptors will compete for common target sites in mammalian homologs of the insect HR3 receptors (16). The receptor expression vector and additional pBS/SK plasmid pEA.e75a and pEA.e75c were obtained after cloning a 2.4-kb fragment encompassing the complete ORF of BmE75A and a 2.6-kb NotI fragment encompassing the complete ORF of BmE75C, respectively, into the NotI site of the polylinker of pJ3315 (12).

Yeast Two-hybrid Library Screening—Gal4 activation domain/cDNA fusion (prey) plasmid libraries were screened for proteins that interact with BmE75C using the MATCHMAKER Two Hybrid System (Clontech). A Gal4 DBD/BmE75C protein hybrid (bait) plasmid was constructed by cloning the 2.6-kb BmE75C ORF and BmHR3A ORF into a yeast expression vector, the BamHI/HindIII restriction fragment region into the BamHI/NotI site of the pGBT9 plasmid (Clontech) in-frame with Gal4-DBD. Two “prey” plasmid libraries were constructed by directional cloning of cDNAs prepared from follicular epithelial cells of B. mori follicles into the pGAD424 plasmid (Clontech). One library contained cDNAs (insert lengths ≤ 0.5 kb) prepared from previtellogenic follicles whereas the other library contained cDNAs prepared from chorionic follicles (insert lengths ≥ 1 kb). The two libraries, each consisting of 1.6 × 10^6 and 1 × 10^5 primary clones, respectively, were mixed before screening. An initial screening of 10^7 transformants performed in the absence of 3-aminotriazole (Sigma) yielded a large number of colonies (≈20,000). The cells of these colonies were pooled and used for a second screening in the presence of 20 μM 3-aminotriazole. From the second screening, 31 true positive clones were isolated that produced significant levels of β-galactosidase only in the presence of the bait plasmid.

Yeast Two-hybrid Assays for β-Galactosidase—Fresh overnight cultures of Saccharomyces cerevisiae H717c strain (Clontech) harboring BmE75A and BmHR3A and prey plasmid pGBD9, or their homologs, were grown to mid-log phase in SD medium with 50 μg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside (Sigma; 160 μg/ml) and 1 mM 3-aminotriazole. From the second screening, 31 true positive clones were isolated that produced significant levels of β-galactosidase only in the presence of the bait plasmid.

Materials and Methods

In Vitro Transcription/Translation and Gel Retardation Assays—Recombinant derivatives of the Bluescript plasmid (pBS/SK′; Stratagene) containing the complete open reading frames (ORFs) of BmHR3A, BmE75A, and BmE75C (11, 12) were linearized with SoI or BamHI and transcribed in vitro with T3 RNA polymerase (Amersham Biosciences). The transcribed RNA was translated in a rabbit reticulocyte lysate (Promega) using the manufacturer’s protocol. Gel mobility shift assays were carried out as described previously (17). The double-stranded oligonucleotide probe ROREdr, which was derived from the Drosophila FTZ-F1 promoter and used in the bandshift assays, has been described previously (11). Competition assays were carried out in the presence of a 10-fold molar excess of unlabeled oligonucleotides or probes (Sigma). Samples were incubated at 30 °C for 5 min and cell pellets were resuspended in 200 μl of zymolase buffer (50 mM Tris-HCl, pH 7.5, 10 mM MgCl2, and 1 mM NAD) containing 10 μg/ml 5-bromodeoxyuridine (Sigma). 5000 U of zymolase buffer (100 mM sodium phosphate buffer, pH 7, 10 mM KCl, and 1 mM MgSO4) containing 40 mM β-mercaptoethanol. O-Nitrophenyl β-D-galactoside (Sigma; 160 μl of a 4 mg/ml solution) was added, and samples were incubated at room temperature. After 90 min, the reactions were quenched by the addition of 600 μl of 1 N Na2CO3—O N-mercaptoethanol.

Cell Transfections, CAT Assays, and Dot Blot Hybridizations—All procedures for transfection, cell harvest, protein extraction, CAT assays, and dot blot hybridizations were as described previously (18). For each condition, one million cells were transfected with 0.5 μg of each reporter construct in the presence or absence of 0.5 μg of each nuclear receptor expression vector and additional pBS/SK′ (to a total amount of 1.5 μg) in 0.5 ml of basal IPL–41 medium (Invitrogen) containing 15 μl of Lipofectin (Invitrogen). Quantification of cat gene activity was carried out by PhosphorImager analysis (Amersham Biosciences). The probe used in dot blot hybridizations was a 0.9-kb BamHI fragment containing the CAT ORF.

The cat gene reporter construct pBm/ROREdoro.cat consisted of the basal actin promoter harboring four copies of the ROREdoro element in its upstream region (11). The expression plasmids for BmHR3A, BmE75A, and BmE75C (as well as their deletion mutants; see below), i.e. pEA.hr3a, pEA.e75a, and pEA.e75c, respectively, were based on plasmid p13315 (19), which contains a viral enhancer sequence inserted upstream of the actin promoter in pBmA (18). The construction of plasmid pEA.hr3a has been described previously (11). Plasmids pEA.e75a and pEA.e75c were obtained after cloning a 2.4-kb NotI fragment encompassing the complete ORF of BmE75A and a 2.6-kb NotI fragment encompassing the complete ORF of BmE75C, respectively, into the NotI site of the polylinker of pJ3315 (12). Yeast Two-hybrid Library Screening—Gal4 activation domain/cDNA fusion (prey) plasmid libraries were screened for proteins that interact with BmE75C using the MATCHMAKER Two Hybrid System (Clontech). A Gal4 DBD/BmE75C protein hybrid (bait) plasmid was constructed by cloning the 2.6-kb BmE75C ORF and BmHR3A ORF into a yeast expression vector, the BamHI/HindIII restriction fragment region into the BamHI/NotI site of the pGBT9 plasmid (Clontech) in-frame with Gal4-DBD. Two “prey” plasmid libraries were constructed by directional cloning of cDNAs prepared from follicular epithelial cells of B. mori follicles into the pGAD424 plasmid (Clontech). One library contained cDNAs (insert lengths ≤ 0.5 kb) prepared from previtellogenic follicles whereas the other library contained cDNAs prepared from chorionic follicles (insert lengths ≥ 1 kb). The two libraries, each consisting of 1.6 × 10^6 and 1 × 10^5 primary clones, respectively, were mixed before screening. An initial screening of 10^7 transformants performed in the absence of 3-aminotriazole (Sigma) yielded a large number of colonies (≈20,000). The cells of these colonies were pooled and used for a second screening in the presence of 20 μM 3-aminotriazole. From the second screening, 31 true positive clones were isolated that produced significant levels of β-galactosidase only in the presence of the bait plasmid.
CGG (at 1080–1086). The PCR fragments were digested with NotI and MluI and used to replace a 0.8-kb NotI/MluI fragment encompassing the N terminus of BmE75C.

To obtain the expression plasmid for the F domain of BmE75, the cDNA clone of BmE75 in PBS/ SK * was digested with Hinfl (nt 1558, in the first codon of the F domain) and Xhol (polylinker of PBS/ SK * ) to release the sequences upstream of the F domain of BmE75. A double-stranded oligonucleotide that contains a Xhol 5' overhang, a NotI site, the translation start of BmE75C (the latter two in italic), the last codon of the ligand-binding domain, and the first base of the F domain with the sequence 5'-TCGAGCCGGCGCGACTGCGTCG-3' ('CGCGGGG- GCTGATACCGCACG-5') was subsequently ligated upstream of the F domain between the Hinfl1 and Xhol sites.

To obtain C-terminal deletion mutants of BmE75, a 0.2-kb PCR fragment was generated by combining forward primer CGGCCTGCCTGGCAAGAAC (nt 1351–1368 in BmE75C) with reverse primer AAGCG- GCCGCCGCAACAGGATATCCG-3.

To examine whether BmHR3A is capable of recognizing RORE DNA target sites, bandshift experiments were carried out using BmHR3A produced by in vitro transcription/translation. The HRE selected for the gel retardation assay was derived from the Drosophila FTZ-F1 promoter (the “A” element; Ref. 13) and corresponded to a perfect match to the consensus vertebrate RORE (6). As shown in Fig. 1A, BmHR3A binds FTZ-F1 RORE in a specific manner.

To investigate whether BmHR3A is capable of activating RORE-linked reporter genes, transient expression experiments were carried out. In these experiments, a BmHR3A expression vector (pEa.Hr3A) was transfected into silkmoth tissue culture cells (Bm5 cells) together with a reporter construct containing four copies of the FTZ-F1 RORE linked to a basal promoter (pBmAb/R ORE). As demonstrated previously (11), BmHR3A activates the RORE-linked promoter by −75-fold (76.1 ± 1.3, n = 9; Fig. 1C).

Most nuclear receptors contain two distinct regions that are involved in the activation of transcription: a ligand-independent activation function 1 (AF-1) residing in the N terminus, and a ligand-dependent activation function 2 (AF-2) residing in the ligand-binding domain (20). Whereas different subregions in the ligand-binding domain contribute to AF-2, the integrity of the ligand-binding domain (helix 12 in the conserved structure of the ligand-binding domain of nuclear receptors) (21) was shown to be indispensable for ligand-dependent activation. A similar amphipathic α-helix, characterized by the conserved motif ΦXΕΦXΦ (Φ being a hydrophobic amino acid and X being a nonconserved amino acid) (20), is also present at the C terminus of the ligand-binding domain of BmHR3A (LYKELF; aa residues 474–479 in Ref. 11).
To determine whether similar activation functions exist in BmHR3A, mutant BmHR3A receptors encompassing a deletion of the isoform-specific region at the N terminus and/or a deletion of the 22 C-terminal amino acids that included the short F domain and the LYKELF motif at the end of the ligand-binding domain (BmHR3AΔN, BmHR3AΔC and BmHR3AΔAN/C; Fig. 1B) were tested in the transactivation assay for their capacity to stimulate transcription from the RORE-coupled promoter construct. Whereas the N terminus deletion mutant was capable of stimulating transcription of the reporter construct as efficiently as full-length BmHR3A (72.6 ± 1.1, n = 3), only very minor stimulation of transcription was observed for the C-terminal deletion mutants (stimulation by 3.1 ± 0.8 (n = 3) for BmHR3AΔC and 2.9 ± 1.1 (n = 3) for BmHR3AΔAN/C). These results suggest that the activation functions of BmHR3A reside in its C terminus (AF-2) and that its N terminus is not involved in transcriptional activation in any significant manner.

The Nuclear Receptors BmHR3A and BmE75 Compete for DNA Binding and Form a Ternary Complex on a Single RORE Motif in Gel Retardation Assay—To deduce whether the BmE75 nuclear receptors interact as avidly as BmHR3A with the consensus RORE, we performed bandshift assays using the FTZ-F1 RORE element as probe together with in vitro-transcribed/translated BmE75A and BmE75C. The results shown in Fig. 2A demonstrate that BmE75A and BmE75C specifically bind the RORE sequence. The amount of shifted complex in these experiments was less than that seen for BmHR3A, presumably because of the lower amount of protein produced by the in vitro transcription/translation mixtures (Fig. 2A, lanes 2–7; compare with Fig. 2, lane 8 and Fig. 1A, lanes 2–4).

To investigate whether binding of BmE75 to the RORE can interfere with the binding of BmHR3A, a small amount of BmHR3A (1 μl of translation mixture) was incubated with a limiting concentration of RORE probe and increasing concentrations of BmE75C protein (1–10 μl of translation mixture). As shown in Fig. 2B, the complex corresponding to BmHR3A was gradually displaced by the BmE75 complex at increasing doses, indicating that both proteins can compete with each other for binding the RORE motif (Fig. 2B). However, weak supershifted activity could also be observed when high doses of BmE75 were added to BmHR3A (arrow in Fig. 2B), suggesting the formation of ternary complexes. When higher amounts of BmHR3A translation mixtures (5 μl) were used in the gel retardation assay together with high amounts of BmE75 (5 μl), a new complex of slower mobility was indeed clearly observed, indicating the presence of a BmHR3A/BmE75(A or C) ternary complex on the target DNA (Fig. 2A, lanes 9 and 10). Because the bandshifts were carried out with a probe containing a single RORE, the latter results indicate that BmHR3A and BmE75 interact with each other.

Repression of BmHR3A-dependent Transactivation by BmE75—When BmE75 expression constructs were transfected into the Bm5 cells together with the RORE-linked promoter-reporter construct, reporter gene activity was not measurably affected (data not shown). Because BmE75 was shown to interact with the RORE in bandshift assays (Fig. 2), we assume that BmE75 is recruited to the RORE elements present upstream of the promoter but may not be capable of influencing transcription because it lacks the activation and/or repression functions normally present in other nuclear receptors.

To investigate the possibility that BmE75 affects the function of BmHR3A, BmE75 and BmHR3A expression constructs were co-transfected into the Bm5 cells together with the RORE promoter-driven reporter construct. The results of the co-transfection experiments shown in Fig. 3B demonstrate that all isolated BmE75 isoforms significantly inhibit the BmHR3A-mediated, RORE-dependent activation of the reporter gene. Interestingly, differences were detected among the BmE75 iso-
forms regarding their capacity to inhibit BmHR3A-mediated gene activation. Thus, BmE75C functions as a 2–3-fold stronger repressor than BmE75A (12.8 ± 1.9-fold repression (n = 7) by the C isoform compared with 5.2 ± 0.7-fold (n = 10) for the A isoform; Fig. 3B).

To investigate which domains of BmE75 are responsible for repression, deletion mutants of BmE75 were co-expressed with BmHR3A in the tissue culture cells. Our results show that BmE75 receptors with their N termini deleted still function as potent repressors (13.2 ± 1.9-fold reduction (n = 5) in BmHR3A function for BmE75AN; Fig. 3B). Importantly, further deletion of the DNA-binding domain did not affect the repression activity (12.2 ± 1.1-fold repression (n = 3) by BmE75AN/DBD; Fig. 3B), indicating that DNA binding is not required for BmE75 to repress activation by BmHR3A.

By contrast, C-terminal deletions compromise the capacity of BmE75 to silence BmHR3A. Although repression is still significant, it is diminished to only 1.8 ± 0.2-fold (n = 3) and 2.0 ± 0.2-fold (n = 4) in the F domain deletion mutants of BmE75A and BmE75AN, respectively. Deletion of the F domain has a less significant effect for BmE75C, although the capacity to repress transactivation is reduced by ~2-fold (to 5.5 ± 1.5-fold repression (n = 3)) in the F domain deletion mutant compared with full-length BmE75C (Fig. 3B).

In view of our finding that the F domain contributes significantly to the BmE75-mediated repression of BmHR3A, we investigated the possibility that the F domain alone is sufficient for effecting the silencing of the BmHR3A function. As shown in Fig. 3B, potent repression of BmHR3-mediated gene activation was observed upon co-transfection of the cells with the F domain of BmE75. Repression by the F domain (9.7 ± 0.6 (n = 4)) was comparable with that of the full-length BmE75 receptors. By contrast, no repression was observed when two other constructs of BmE75, consisting of either the DBD/hinge or hinge/LBD regions, were tested in the transactivation assay (Fig. 3B). Finally, the repression activity of the F domain was specific to BmHR3A-mediated gene activation because overexpression of the F domain did not influence the activity of the Bombyx actin promoter, which is not regulated by BmHR3A (data not shown).

In conclusion, the experiments with the BmE75 deletion mutants show that the F domain of BmE75 contains most of the determinants for repression of the transcriptional activity of BmHR3A and is sufficient for function. However, deletion of the F domain from the full-length receptors does not completely abolish repression, indicating that partial repression of BmHR3A function is also accomplished by competition for DNA binding sites. In this respect, it should be noted that in the case of BmE75C, which exhibits stronger DNA binding than BmE75A (Fig. 2A), deletion of the F domain results in a lesser reduction of its repression activity relative to BmE75A (Fig. 3). Interaction between BmHR3A and BmE75 Nuclear Receptors in Yeast Two-hybrid Assays—During an initial screening of a yeast two-hybrid expression library of follicular cell cDNA using full-length BmE75C as bait, two classes of clones encoding interacting proteins were obtained. The first class, represented by ~90% of the positive clones, contained clones encoding the nuclear receptor BmHR3. The second class, represented by the remaining 10% of positives, consisted of clones encoding a putative adaptor protein containing multiple SH3 domains. Interestingly, two isoforms of BmHR3 preys differing from each other in their hinge regions were isolated in the yeast two-hybrid screen. The first one corresponded to the previously characterized A isoform (11), whereas the second one contained a hinge region that is similar to that described for the C isoform of Choristoneura fumiferana HR3 receptor (83% aa identity; Ref. 15).

To map the interaction domain of BmE75, full-length BmE75 isoforms and a series of deletion mutants were tested for interaction with BmHR3A in the yeast two-hybrid assays. As shown in Fig. 4B, all isoforms interact with full-length BmHR3A in these assays, but the interaction is much stronger for the A isoform than for the C isoform. Deletion of the isoform-specific N terminus of BmE75 did not result in a decrease in the interaction between the two receptors (Fig. 4B). By contrast, deletion of the F domain (bait BmE75ΔN/F and BmE75ΔN/LBD/F; Fig. 4A) strongly compromised the interaction with BmHR3A (Fig. 4B). Most importantly, the F domain alone was capable of interacting strongly with BmHR3A (Fig. 4B).

Curiously, whereas the F domain alone showed strong interaction with BmHR3A, the same domain in combination with the ligand-binding domain (bait BmE75ΔN/DBD/H) did not show much activity when combined with BmHR3A (Fig. 4B). This may be due to steric hindrance exerted by the ligand-binding domain of BmE75.

Finally, to map the interaction domain of BmHR3, full-length BmHR3A, the C-terminal region of BmHR3 that is common to the A and C isoforms (from the C-terminal part of the hinge region to the end of the F domain), or BmHR3A with a short deletion at the C terminus were tested for interaction with the BmE75ΔN mutant or the F domain alone in the yeast two-hybrid system (Fig. 5, B and C). These assays clearly showed that the BmE75-interacting domain resides in the C-terminal half of BmHR3, starting from the 20 C-terminal aa of BmHR3A (Fig. 5, B and C).
the hinge region that is common to all BmHR3 isoforms (Fig. 5, A and B). Most importantly, a smaller deletion encompassing the 22 C-terminal aa of BmHR3A including the amphipathic α-helical activation domain (prey BmHR3A ΔN) resulted in the abolishment of the interaction of BmHR3 with BmE75 (Fig. 5, B and C).

In conclusion, the results of the yeast two-hybrid interaction assays suggest a new function for the F domain of BmE75 nuclear receptors, the mediation of the interaction with the C-terminal activation region of BmHR3.

**DISCUSSION**

Previous studies in *Drosophila* have shown that the interaction between the nuclear receptors HR3 and E75 plays an
activation of $\beta$-FTZ-F1 (13, 22–24). However, activation of $\beta$-FTZ-F1 by DHR3 is prevented by overexpression of E75B, an isoform of E75 that lacks a functional DNA-binding domain (22). The inhibition of DHR3 by E75B is therefore thought to function as a timing mechanism for the induction of $\beta$-FTZ-F1, which is dependent on the persistence of DHR3 and the disappearance of E75B. With regard to the mechanism by which E75B represses DHR3, it was found that in bandshift assays the two receptors form a ternary complex on ROREs that exist in the $\beta$-FTZ-F1 promoter, suggesting that repression is carried out through DHR3-E75B interactions on this promoter (22).

Our results on the interactions between BmE75 and BmHR3 in yeast and in Bombyx tissue culture cells confirm and expand the findings in transgenic flies. Using silk moth homologs of HR3 and E75 and an artificial promoter-reporter construct, we have shown that BmHR3A is a potent transrepressor of the DHR motif-containing promoters that becomes repressed upon simultaneous expression of BmE75. Repression of the BmHR3-activating function is mediated by the two BmE75 isoforms tested (A and C; Fig. 3). Because the B isoform of E75 differs from the other isoforms by a unique N terminus that replaces the first half of the DNA-binding domain (2, 25, 26), our findings indicate that the unique N-terminal sequences of E75B are not necessary for repression. Based on the structural features of the E75B isoform of B. mori, which has also been described recently (27), we predict that this isoform will also function as an efficient repressor of BmHR3-mediated transactivation.

With regard to the mechanism by which BmE75 represses BmHR3A, we have shown that overexpression of the F domain alone is sufficient to achieve efficient repression of BmHR3A (Fig. 3). Thus, despite the fact that BmE75 is capable of binding to the RORE element, DNA binding by BmE75 is not required for repression. The fact that BmE75A and BmE75C form ternary complexes with BmHR3A on a single RORE in bandshift assays (Fig. 2A) also indicates that repression can be mediated through protein interactions. However, the fact that BmE75 is capable of competing efficiently with BmHR3A for binding to the RORE motif in bandshift assays (Fig. 2A) and the observation that BmE75 mutants with an intact DNA-binding domain but with the F domain deleted still mediate considerable repression of HR3-mediated activation (Fig. 3) indicate that competition for common DNA binding sites also contributes to repression.

The yeast two-hybrid assays have shown that the F domain of BmE75 interacts with the C terminus of BmHR3A (Fig. 4). Interestingly, deletion of the 22 C-terminal aa of BmHR3A results in the abolishment of the interaction with the F domain of BmE75 (Fig. 4). The BmHR3A mutant lacking the same C-terminal aa is also defective in transactivation of the RORE-linked reporter construct in tissue culture cells (Fig. 3), indicating that binding by the F domain of BmE75 occurs at the transactivation domain of BmHR3A. The deletion in BmHR3A that abolishes transactivation and interaction with BmE75 covers the short F domain and 12 aa at the C terminus of the ligand-binding domain and encompasses an amphipathic $\alpha$-helix that is conserved among nuclear receptors (20). The integrity of this $\alpha$-helix has been shown to be a requirement for transactivation and interaction between the ligand-binding domain and intermediary transcriptional factors or co-activators (21, 28, 29). We therefore propose that in analogy with other (mammalian) nuclear receptors, the amphipathic $\alpha$-helix at the C terminus of BmHR3A is involved in the stimulation of transcription through interactions with co-activators and that the binding of the F domain of BmE75 prevents the recruitment of the co-activators that are needed for stimulation of transcriptional activity.

A similar mechanism of transcriptional repression through blocking of binding sites for co-activator proteins has also been reported for the nuclear receptor SHP (small heterodimerization partner) (30). Despite the fact that SHP consists only of a ligand-binding domain, it effectively represses the estrogen receptor through binding to the C-terminal transactivation $\alpha$-helix of estrogen receptor and competing out co-activator binding to the estrogen-bound ligand-binding domain of the estrogen receptor. Interestingly, the binding of SHP for the co-activator binding site occurs via LXXLL-related motifs. The LXXLL motifs that exist in nuclear receptor co-activators are the primary determinants for high-affinity binding to the ligand-binding domain of the receptors (29, 31–33). Furthermore, intermediary transcriptional factors that mediate repression of nuclear receptors (co-repressors) associate with the unliganded receptors through similar motifs (34). Although an exact copy of the LXXLL motif was not found in BmE75, a similar motif, LXXVL, exists in a region in the middle of the F domain of BmE75 that is evolutionarily conserved (25). In BmE75, this motif is flanked by proline residues that may allow it to fold as an independent domain, a proposed prerequisite for efficient recognition of the coactivator-binding site (33). Mutation of this motif through site-directed mutagenesis will determine whether it is directly involved in the interaction with BmHR3A and in the repression of its transcriptional activation.

The mammalian homologs of BmHR3 and BmE75 are the ROR/retinoid Z receptor and the Rev-Erb/Rev-Erb-related receptor, respectively (2, 16). The antagonism between the two types of receptors seems to be conserved between insects and mammals because ROR acts as a constitutive activator whose major activation function resides at its C terminus, similar to HR3 (35, 36), whereas Rev-Erb functions as a repressor (37, 38). When co-expressed, Rev-Erb represses transcriptional activation by ROR (35). However, Rev-Erb lacks the F domain, and inhibition of ROR seems to be achieved mainly through competition for common DNA-binding sites. On certain response elements, on the other hand, Rev-Erb functions as a constitutive repressor by the active recruitment of co-repressor proteins to the ligand-binding domain (38, 39). However, no such “active” repression function was observed for BmE75 on the reporter constructs used in our experiments.

During the early phases of the ecdysone response in the Bombyx ovary, the nuclear receptors BmE75A and BmE75C become induced immediately upon hormone addition, whereas the accumulation of BmHR3 occurs with a delay of 2 h (11, 12). Similarly, the decline in expression occurs faster for BmE75 than for BmHR3. Because BmE75A and BmE75C act as efficient repressors of BmHR3, it can be predicted that, as was reported for Drosophila E75B (22), the BmHR3 function is effectively blocked during the early phases of the 20E-induced cascade, whereas it is relieved during the later phases of the response. A similar situation also exists during vitellogenesis in the Bombyx ovary, which is initiated 2 days after the first exposure to 20E (11). During vitellogenesis, the levels of BmHR3A mRNA increase steadily as follicle development progresses and reach their maximum levels at stages 13/18 (11). The A isoform of BmE75 is also expressed during vitellogenesis, but its expression actually declines during the stages when BmHR3A expression is highest (12). Interestingly, the period with the highest ratio in expression levels of BmHR3A
relative to BmE75A coincides with the induction of the mRNA for BmFTZ-F1 (40), indicating that the mechanism for activation of BmFTZ-F1 during ovarian development in Bombyx occurs in a fashion similar to that of β-FTZ-F1 during metamorphosis in Drosophila.

During vitellogenesis, the accumulation of BmHR3A mRNA also coincides with the expression of the gene encoding the follicle cell-specific yolk protein ESP, whereas it is reciprocal to the accumulation of the mRNA of the chorion gene regulator BmGATAβ (11). Based on these observations, we propose that BmHR3A could act, in concert with cell and promoter-specific cofactors, as both an activator of the ESP gene and a repressor of the BmGATAβ gene. BmE75A, which is co-expressed with BmHR3A during vitellogenesis, is therefore predicted to be recruited more efficiently to the BmGATAβ promoter than to the ESP promoter. Preliminary experiments indeed suggest that repression of the BmGATAβ promoter occurs in Bm5 cells upon overexpression of BmHR3A and BmE75A.3 A role for repression through HR3 receptors is not unprecedented because it has been demonstrated that during Drosophila metamorphosis, DHRR3 receptors down-regulate the expression of the early response genes E74, E75, and Broad-Complex, presumably through a mechanism that involves direct interaction with the edysone receptor (13, 22).

Finally, it is also clear that BmE75 receptors play functional roles during Bombyx oogenesis that are independent of BmHR3. The C isoform of BmE75 becomes markedly up-regulated at the end of vitellogenesis and the beginning of chorionogenesis (12), when no BmHR3 protein can be detected in the follicular cells (11). Interestingly, in yeast two-hybrid screens, a putative adaptor protein containing multiple SH3 domains was isolated that interacts with the proline-rich N terminus of BmE75C.3 The expression pattern of the putative adaptor protein during oogenesis also overlaps with that of BmE75C (40). Thus, the data indicate a role for BmE75C as a key player in a transduction cascade that is independent of 20E and BmHR3 and governs the transition from vitellogenesis to chorionogenesis.

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