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Redundant ecdysis regulatory functions of three nuclear receptor HR3 isoforms in the direct-developing insect *Blattella germanica*

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Abstract

In hemimetabolous insects, the molecular basis of the 20-hydroxyecdysone (20E)-triggered genetic hierarchy is practically unknown. In the cockroach *Blattella germanica*, we had previously characterized one isoform of the ecdysone receptor, *BgEcR-A*, and two isoforms of its heterodimeric partner, *BgRXR-S* and *BgRXR-L*. One of the early-late genes of the 20E-triggered genetic hierarchy, is *HR3*. In the present paper, we report the discovery of three isoforms of HR3 in *B. germanica*, that were named BgHR3-A, BgHR3-B_1 and BgHR3-B_2. Expression studies in prothoracic gland, epidermis and fat body indicate that the expression of the three isoforms coincides with the peak of circulating ecdysteroids at each nymphal instar. Experiments *in vitro* with fat body tissue have shown that 20E induces the expression of BgHR3 isoforms, and that incubation with 20E and the protein inhibitor cycloheximide does not inhibit the induction, which indicates that the effect of 20E on *BgHR3* activation is direct. This has been further confirmed by RNAi *in vivo* of BgEcR-A, which has shown that this nuclear receptor is required to fully activate the expression of BgHR3. RNAi has been also used to demonstrate the functions of BgHR3 in ecdysis. Nymphs with silenced *BgHR3* completed the apolysis but were unable to ecdyse (they had duplicated and superimposed the mouth parts, the hypopharinge, the tracheal system and the cuticle layers). This indicates that BgHR3 is directly involved in ecdysis. Finally, RNAi of specific isoforms has showed that they are functionally redundant, at least regarding the ecdysis process.
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Keywords: HR3; Nuclear hormone receptor; *Blattella germanica*; 20-Hydroxyecdysone; RNAi; Ecdysis

1. Introduction

In insects, the ecdysteroidal hormone 20-hydroxyecdysone (20E) controls key developmental processes during embryogenesis, molting, metamorphosis and reproduction. Among these processes, metamorphosis in holometabolous species, particularly in the fruitfly *Drosophila melanogaster*, has been extensively studied. At the molecular level, 20E exerts its regulatory functions upon binding to a heterodimeric receptor formed by two members of the nuclear receptor superfamily, the ecdysone receptor (EcR) and the retinoid-X-receptor (RXR) ortholog ultraspiracle (USP) (Yao et al., 1993). Once activated, the receptor elicits cascades of gene expression that mediate and amplify the ecdysteroidal signal. Most of the early response genes encode transcription factors, like E75, E74 and Broad, which in turn regulate later genes (reviewed by Thummel, 1995; Riddiford et al., 2001; King-Jones and Thummel, 2005). Remarkably, the activity of the early genes at the onset of the pupal stage is further refined, in terms of timing and specificity of activation, by the presence of other 20E-dependent genes, namely *HR3* (or *Hr46*) and βFTZ-F1 (Lam et al., 1997, 1999; Broadus et al., 1999).

The situation is very different in hemimetabolous insects, which do not develop through complete metamorphosis and whose juvenile forms are morphologically similar to the adult, only differing, in general, by color, wing and genitalia details. Indeed, with the exception of few and isolated studies (Erezyilmaz et al., 2006), data concerning the molecular basis of the 20E-triggered genetic

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hierarchy is practically non-existent in hemimetabolous insects. However, if we aim to understand the molecular basis of the evolution towards complete metamorphosis in insects, then characterization of the 20E-induced genetic hierarchy in hemimetabolous species becomes of paramount importance.

In this context, we started isolating the components of the 20E receptor in the hemimetabolous cockroach *Blattella germanica*. Firstly, we cloned one isoform of the ecdysone receptor, *BgEcR-A*, and two isoforms of its heterodimeric partner, *BgRXR-S* and *BgRXR-L*, and we found that they are expressed following a housekeeping-like pattern during the last two nymphal instars (Cruz et al., 2006; Maestro et al., 2005). Furthermore, silencing of BgEcR-A and both isoforms of BgRXR by RNA interference (RNAi) *in vivo*, showed that these nuclear receptors are essential for nymphal development (Cruz et al., 2006; Martín et al., 2006).

One of the genes following the 20E-triggered genetic hierarchy, and which has been functionally characterized in *D. melanogaster*, is *HR3*, again a member of the nuclear receptor superfamily. The corresponding mammalian ortholog of *HR3* is the RAR-related orphan receptor (ROR) (Giguere et al., 1994). In *D. melanogaster*, *HR3* resets the genetic cascade triggered by 20E at level of the early genes, repressing their expression at the prepupal stage, while activating the nuclear receptor βFTZ-F1, which provides competence to respond to 20E in late prepupae (Woodard et al., 1994; Lam et al., 1997; White et al., 1997; Kageyama et al., 1997; Broadus et al., 1999).

Phenotypically, mutation analysis of *HR3* in *D. melanogaster* has revealed that it is required for metamorphosis (Carney et al., 1997), in particular for the correct prepupal–pupal transition and for the differentiation of adult structures (Lam et al., 1999). Orthologs of *D. melanogaster HR3* have been reported in other holometabolous insects, like in the dipteran *Aedes aegypti* (Kapitskaya et al., 2000), and the lepidopterans *Manduca sexta* (Palli et al., 1992), *Galleria mellonella* (Jindra et al., 1994), *Bombyx mori* (Eystathioy et al., 2001), *Choristoneura fumiferana* (Palli et al., 1996, 1997) and *Helicoverpa armigera* (Zhao et al., 2004).

In the present work, we extend the knowledge of the 20E genetic hierarchy in the hemimetabolous *B. germanica* by identifying three HR3 homologs in this cockroach, by studying their regulation by ecdysteroids and by determining their functions in nymphal development using RNAi approaches.

2. Results

2.1. Cloning of HR3 isoforms in Blattella germanica

Cloning of BgHR3 cDNAs was accomplished by a RT-PCR approach using degenerate primers designed on the basis of conserved motifs of the DNA binding domain (DBD) of available sequences of insect HR3 orthologs. Using cDNA from *B. germanica* UM-BGE-1 cells as a template, a 129 bp PCR fragment was obtained, and its sequence was highly similar to insect HR3 sequences. 3′-RACE and 5′-RACE experiments using cDNA from UM-BGE-1 cells, gave three full-length cDNAs of 3.50, 3.79 and 2.36 kb. Database BLAST search with the complete sequences revealed that they encoded *B. germanica* orthologs of HR3. Two HR3 cDNAs encoded identical proteins except for an insertion/deletion of 121 amino acids in the D domain (hinge region), and were named BgHR3-B_1 (accession number: AM259129) and BgHR3-B_2 (accession number: AM259130). The third BgHR3 differed from the other two sequences in the 5′UTR and in most of the A/B domain, and was named BgHR3-A (accession number: AM259128). With the exceptions mentioned, the three BgHR3 are identical not only in terms of amino acids, but also at level of nucleotide sequence, which suggests that they are splice variants of the same gene.

BgHR3-A encodes a 607 amino acid protein with a predicted molecular mass of 68 kDa, whereas *BgHR3-B_1* and *BgHR3-B_2* encode two proteins of 651 and 530 amino acids with predicted molecular masses of 72.9 and 59.2 kDa, respectively. In all sequences, putative start codons are preceded by in-frame stop codons, indicating that these sequences represented full-length open reading frames (ORFs). To verify that the cloned cDNAs contained translatable ORFs, they were expressed in a coupled TNT system under the control of the SP6 promoter. SDS–PAGE and fluorography showed that the molecular sizes of the proteins synthesized *in vitro* closely corresponded to those expected.

In general, nuclear hormone receptor isoforms are based on divergences in their A/B domains. Therefore, we carried out a maximum-likelihood analysis of the A/B domain for all insect HR3 sequences reported to date, except DHR3B from *D. melanogaster*, which has a very short A/B domain. The analysis generated the tree shown in Fig. 1a, which clusters the sequences into three main groups, A, B and C, with notably high bootstrap support values (782 for nodes A and C, and 744 for node B, with respect to 1000 replicates). Classification of *B. germanica* HR3 isoforms into A and B categories was based on that analysis.

Aside from the A/B domain, amino acid sequence comparisons revealed that the three cDNAs include the domain structure characteristic of the members of the nuclear hormone receptor superfamily. The comparison with other HR3/ROR sequences (Fig. 1b), revealed that the most conserved domains are the DBD (92–96% similarity compared to other invertebrates and 77% compared to human RORα), and a 23 amino acids region named carboxy-terminal extension (CTE) of the DBD (91–100% and 73% similarity, respectively). Conversely, the ligand binding domain (LBD) of BgHR3 showed lower similarity with other insect orthologs, 60% compared with dipteran species and 55% with lepidopterans. Similarity was still lower when compared with nematode (27%) and human (35%) sequences. However, the LBD contains the highly

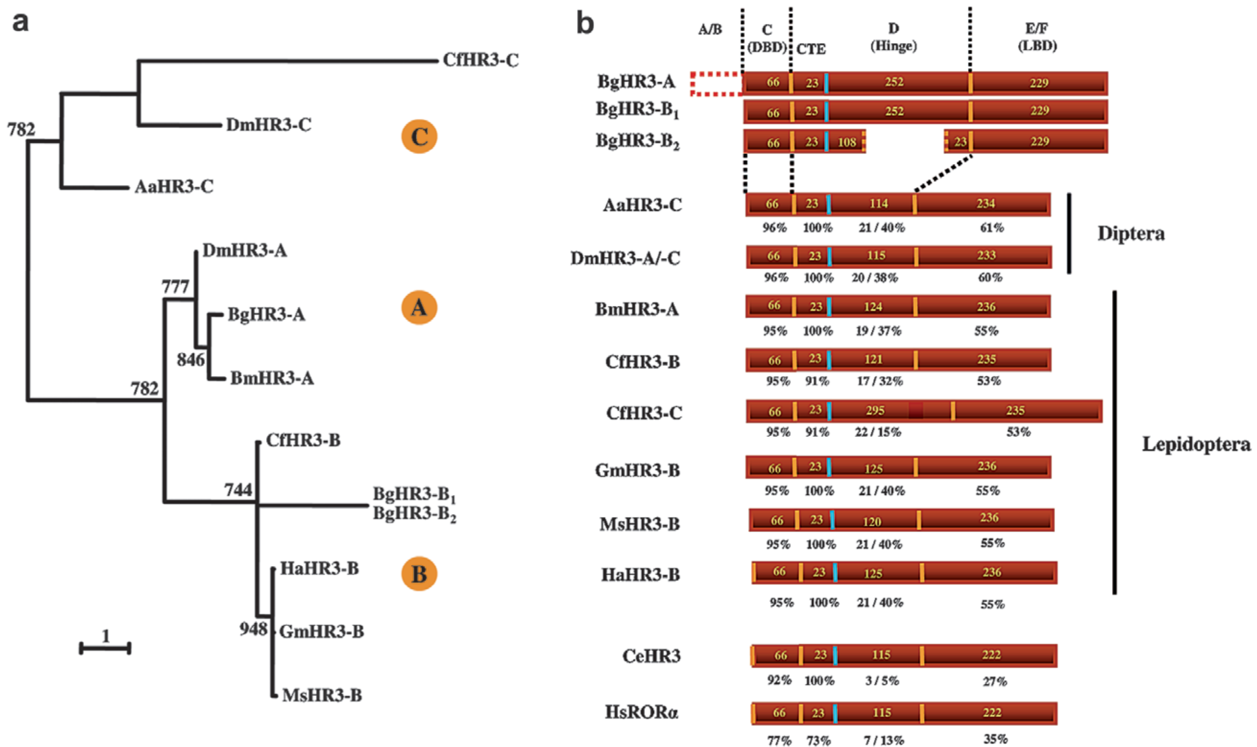

Fig. 1. Sequence comparisons of *Blattella germanica* HR3 isoforms with other HR3/ROR nuclear receptors. (a) Phylogenetic analysis of the A/B domain showing the position of *B. germanica* isoforms with respect to other insect HR3 sequences. The tree was constructed based on the maximum-likelihood method and branch lengths are proportional to sequence divergence. The bar represents 1 difference per site. Bootstrap values higher than 500 (from 1000 replicates) are indicated. Sequences cluster into three groups, A, B and C. For homogeneity, the two-letter code for the species name and the classification A, B and C, according to the results of the analysis, has been used to label all the sequences. (b) Domain comparison of *B. germanica* HR3 isoforms with other HR3/ROR nuclear receptors. Letters above the BgHR3-A indicate functional domains. The D-domain includes the carboxy-terminal extension (CTE). Numbers within each domain indicate the number of amino acids. The percentages of identities between corresponding domains of BgHR3 and the other orthologs are indicated below each domain. In domain D, the first percentage refers to comparison with BgHR3-A and BgHR3-B_1, and the second with BgHR3-B_2. Sequences and species considered are BgHR3-A, BgHR3-B_1 and BgHR3-B_2 from *B. germanica* (present study), DmHR3-A (=DHR3-A) and DmHR3-C (=DHR3-C) from *Drosophila melanogaster*, AaHR3-C (=AHR3) from *Aedes aegypti*, BmHR3-A from *Bombyx mori*, MsHR3-C (=MHR3) from *Manduca sexta*, CfHR3-B (=CHR3-B) and CfHR3-C (=CHR3-C) from *Choristoneura fumiferana*, GmHR3-B (=GHR3) from *Galleria mellonella* and HaHR3-B (=HHR3) from *Helicoverpa armigera*. As non-insect references, the CeHR3 from *Caenorhabditis elegans* (Kostrouch et al., 1995) and HsRORα from *Homo sapiens* (Giguere et al., 1994) are also included in panel b.

conserved AF-2 domain (PALHKELF) that is present in all HR3 orthologs. The most prominent feature of the cockroach sequences was the remarkably long D domain present in BgHR3-A and BgHR3-B_1, composed by 252 amino acids, whereas most of HR3/ROR proteins have between 115 and 125 amino acids.

2.2. Expression of BgHR3 coincides with the peaks of ecdysteroids

Expression patterns of the three *BgHR3* isoforms were obtained in the prothoracic gland, which synthesize ecdysteroids in nymphs, during the fifth and sixth nymphal instars of *B. germanica*, using RT/PCR followed by Southern blot. The mRNA expression pattern of the three isoforms (Fig. 2) coincided with the peaks of circulating ecdysteroids occurring in the second half of each instar, which are associated to molting. Furthermore, we studied the expression patterns of *BgHR3-A*, *BgHR3-B_1* and *BgHR3-B_2* in the epidermis, the tissue that synthesizes the new cuticle during apolysis, and in the fat body, the main metabolic tissue of the insects functionally equivalent to the mammalian liver. The results (Fig. 2) showed that the patterns in the epidermis and the fat body were similar to those observed in the prothoracic gland, although in the epidermis, expression of BgHR3-B_2 seem to cease somewhat earlier than that of BgHR3-B_1.

2.3. 20-Hydroxyecdysone directly induces the expression of BgHR3

The above results, showing a clear parallelism between the levels of circulating ecdysteroids and the expression of *BgHR3* in terms of mRNA, suggest that ecdysteroids induced that expression. To test this hypothesis, we studied the expression of *BgHR3* in abdominal segments with their epidermis and associated fat body from 1-day-old sixth instar female nymphs, that were incubated *in vitro* with 5×10^{-6} or 5×10^{-7} M of 20E (Fig. 3a). As expected, 20E induced the expression of *BgHR3-A*, *BgHR3-B_1* and

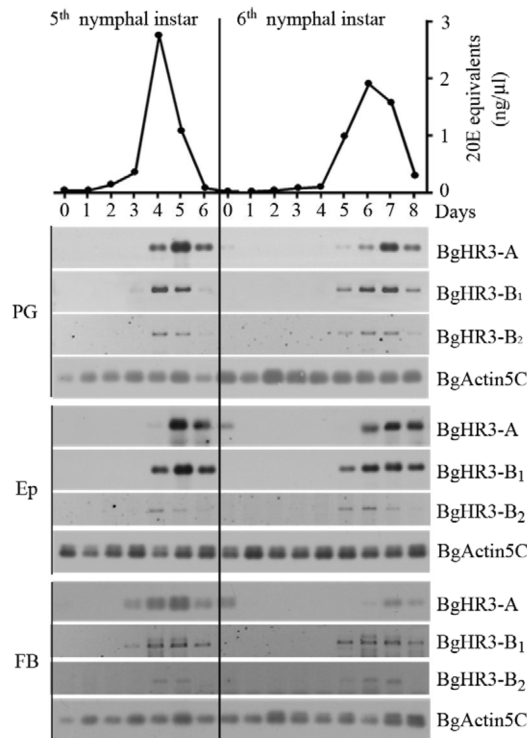

Fig. 2. Hemolymph ecdysteroids and mRNA expression of BgHR3-A, BgHR3-B_1 and BgHR3-B_2 during the last two nymphal instars of *Blattella germanica*. Ecdysteroid levels (upper panel) are from Cruz et al. (2003). mRNA levels were studied in prothoracic gland (PG), epidermis (Ep) and fat body (FB) (lower panels). RNA of the corresponding tissue from staged nymphs was analyzed by RT-PCR/Southern blotting using BgHR3 specific probes for each isoform. BgActin5C levels were used as a reference. The Southern blots are representative of three replicates.

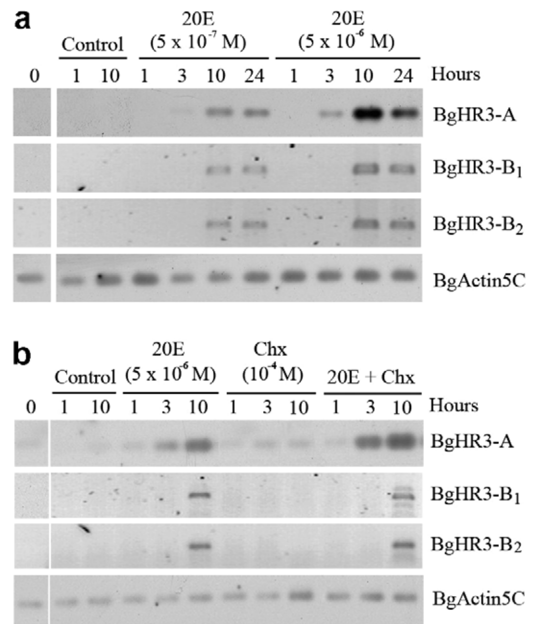

Fig. 3. Induction of BgHR3 expression by 20-hydroxyecdysone (20E) in *Blattella germanica*. (a) Effect of 20E on BgHR3 mRNA expression. Dorsal segments with the corresponding epidermis and associated fat body from sixth instar female nymphs were incubated and treated with 5×10^{-7} M or 5×10^{-6} M 20E for the time indicated. (b) Effect of cycloheximide (Chx) on BgHR3 induction by 20E. The same tissues as above were incubated in the presence of either 5×10^{-6} M 20E; 10^{-4} M of Chx; or both 20E and Chx for the time indicated. Equal amounts of total RNA from the tissues incubated *in vitro* were analyzed by RT-PCR/Southern blotting using BgHR3 specific probes for BgHR3-A, BgHR3-B_1 and BgHR3-B_2. BgActin5C levels were used as a reference. The Southern blots are representative of three replicates.

BgHR3-B_2 in a dose-dependent manner. Incubations without 20E did not result in any detectable expression increase of the three BgHR3 isoforms. To determine whether protein synthesis was required for *BgHR3* activation by 20E, the same kind of tissue was incubated with 10^{-4} M of cycloheximide (Chx), a reversible inhibitor of translation, and with both 20E (5×10^{-6} M) and Chx (Fig. 3b). As above, *BgHR3-A*, *BgHR3-B_1* and *BgHR3-B_2* were induced by 20E, but after 10 h of incubation, they were induced to the same or somewhat higher levels when Chx was added to 20E. Conversely, incubation with Chx alone did not result in any significant induction of *BgHR3-B_1* and *BgHR3-B_2* expression, whereas only a slightly activation of *BgHR3-A* was detected.

To assess whether 20E induces the expression of BgHR3 also *in vivo*, we studied mRNA levels of *BgHR3* isoforms in sixth instar nymphs in which the expression of *BgEcR-A* (one of the components of the functional 20E receptor) had been silenced by RNAi. For that, 1 µg of double stranded RNA (dsRNA) complementary to the A/B domain of BgEcR-A (described in Cruz et al., 2006) was injected in newly ecdysed sixth instar nymphs and mRNA levels of BgEcR-A and the three BgHR3 isoforms in the prothoracic glands (Fig. 4) and in the epidermis with adhered fat body (data not shown) were measured 6–8 days later. Compared with specimens treated with a dsRNA complementary to a non-specific sequence (dsControl), BgEcR-A expression was dramatically reduced in specimens treated with dsBgEcR-A

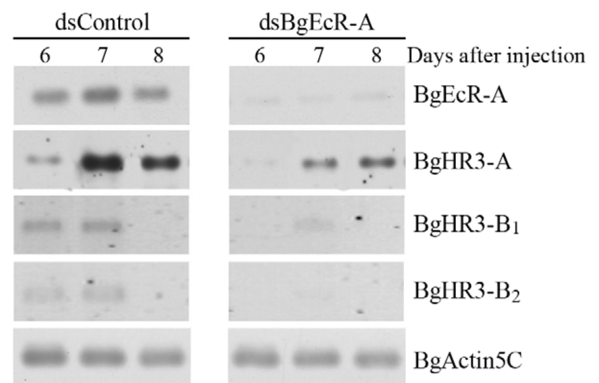

Fig. 4. Effects of BgEcR-A silencing by RNAi on BgHR3 mRNA levels in *Blattella germanica.* A dose of 1 µg of dsRNA targeted to BgEcR-A (dsBgEcR-A) was injected in sixth instar female nymphs, and mRNAs corresponding to BgEcR-A and the three BgHR3 isoforms were measured in the prothoracic gland 6–8 days later, using RT-PCR/Southern blotting and specific probes. Equivalent experiments injecting a non-specific sequence (dsControl) served as negative controls. BgActin5C levels were used as a reference. The Southern blots are representative of six replicates.

(Fig. 4). Remarkably, levels of the three *BgHR3* mRNAs in prothoracic glands were lower in the dsBgEcR-A treated nymphs than in controls (Fig. 4).

2.4. RNAi in nymphs reduces BgHR3 mRNA levels

To analyze the functions of *BgHR3* during nymphal development, we first studied whether it was possible to reduce BgHR3 levels by RNAi *in vivo*. Therefore, we obtained a 411 bp dsRNA targeted to the DBD and D domains (including the CTE region) of BgHR3 (hence, able to silence the three BgHR3 isoforms described herein), that was named as dsBgHR3-1 (Fig. 5a). A dose of 1 μg of dsBgHR3-1 was injected to newly ecdysed sixth instar nymphs, and mRNA levels of *BgHR3-A*, *BgHR3-B*$_1$ and *BgHR3-B*$_2$ were measured 7 days after the treatment, just at the point of maximal level of expression of the three isoforms. In parallel, nymphs were injected with dsControl. Results (Fig. 5b) showed that mRNA levels of the three *BgHR3* isoforms in the epidermis with the adhered fat body as well as in the prothoracic glands decreased substantially in dsBgHR3-treated nymphs when compared to controls. Interestingly, mRNA levels of *BgEcR-A* remained unchanged in dsBgHR3-treated nymphs, thus indicating that RNAi was gene-specific (Fig. 5b).

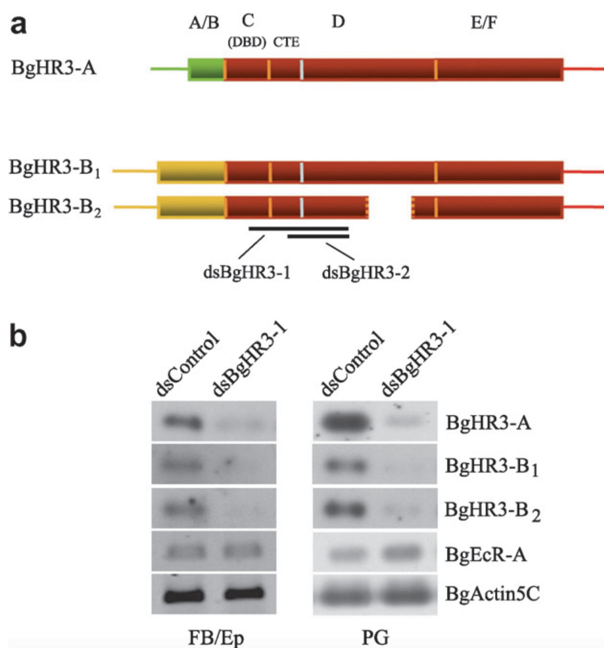

Fig. 5. Silencing of BgHR3-A, BgHR3-B$_1$ and BgHR3-B$_2$ by RNAi *in vivo* in sixth instar female nymphs of *Blattella germanica*. (a) Scheme of the three BgHR3 isoforms showing the regions considered to generate the dsRNAs. (b) Effects of dsBgHR3-1 on mRNA levels of BgHR3-A, BgHR3-B$_1$, BgHR3-B$_2$ and BgEcR-A. A dose of 1 μg of dsBgHR3-1 was injected in sixth instar female nymphs, and mRNA levels were measured 7 days later in the epidermis with adhered fat body and in the prothoracic gland by RT-PCR/Southern blotting using the corresponding specific probes. Equivalent experiments injecting a non-specific sequence (dsControl) served as negative controls. BgActin5C levels were used as a reference. The Southern blots are representative of six replicates.

2.5. RNAi of BgHR3 impairs ecdysis

The efficiency of the RNAi *in vivo* allowed us to analyze the functions of BgHR3 during nymphal development. Sixth instar nymphs that had been treated with 1 μg of dsBgHR3-1 when freshly ecdysed, exhibited normal appearance and behavior throughout the instar. However, at the end of the instar (day 8), 94% of them ($n = 68$) did not molt properly, stopped moving, and 48 h later they arrested development and died. Conversely, all dsControl-treated nymphs ($n = 83$) molted on day 8 (Fig. 6, compare A and B). The arrested nymphs had duplicated and superimposed the ectodermal structures, such as mandibles, parts of the maxilla and the hypopharinge (Figs. 6C–E). The tracheal system was also duplicated with the old trachea included within the lumen of the new ones (Fig. 7, compare A and B). Furthermore, histological sections of the abdominal cuticle of arrested specimens showed the newly formed adult endo- and exocuticle layers below the old nymphal cuticle. Interestingly, the nymphal endocuticle layer had been digested, and only the exocuticle was observed in the arrested specimens (Figs. 7C and D).

To further confirm that these phenotypes were not dependent on the particular dsRNA used, we designed a second dsRNA of 242 bp targeted to the D-domain including the highly conserved CTE region, that was named dsBgHR3-2 (Fig. 5a), and 1 μg of it was injected into the abdomen of freshly ecdysed sixth instar nymphs. As in the case of dsBgHR3-1, 100% of the dsBgHR3-2-treated nymphs ($n = 20$) failed to molt and arrested between days 8 and 10 after the treatment. These specimens exhibited exactly the same phenotype as that obtained with dsBgHR3-1.

In order to characterize whether the requirement of BgHR3 was exclusive of the nymphal-adult transition or it was also necessary for nymphal–nymphal molt, 1 μg of dsBgHR3-1 was injected into the abdomen of freshly ecdysed fifth (penultimate) instar nymphs. Remarkably, 6 days after of the treatment, 89% of the dsBgHR3-1-treated nymphs ($n = 18$) were unable to molt, arrested development and died. As in the experiments with last instar nymphs, they had duplicated the mouth parts, the hypopharinge, the tracheal system, and the cuticular layers.

2.6. The isoforms of BgHR3 have redundant functions in ecdysis

Finally, we aimed to study whether BgHR3-A and -B isoforms had distinct functions during the nymphal development. For this purpose, we prepared dsBgHR3-A (225 bp of the 5′UTR and A/B regions, Fig. 8a), specifically targeted to BgHR3-A, and dsBgHR3-B (323 bp of the 5′UTR and A/B regions, Fig. 8a), targeted to BgHR3-B$_1$ plus B$_2$. Injections of 1 μg of dsBgHR3-A or dsBgHR3-B separately into freshly ecdysed sixth instar nymphs resulted in a clear isoform-specific reduction of the respective transcript in the epidermis and fat body when analysed 6 days after the

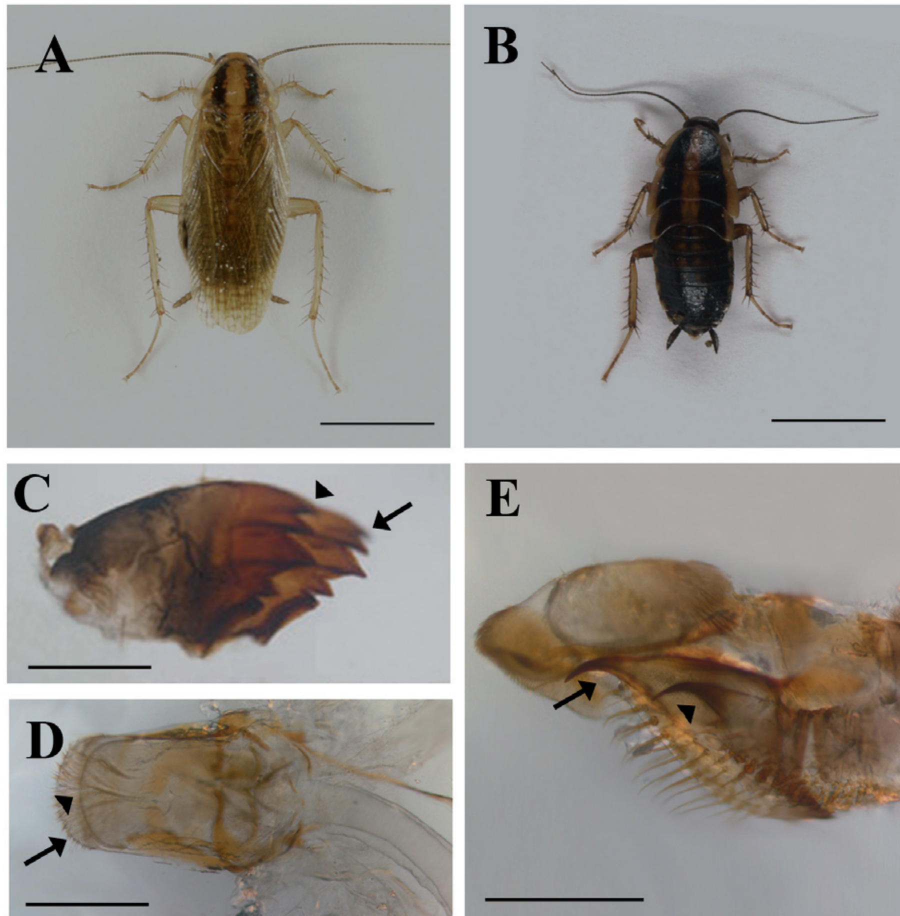

Fig. 6. Effect of RNAi of BgHR3 on ecdysis of *Blattella germanica*. Sixth instar female nymphs were injected with 1 μg of dsBgHR3-1 or with dsControl at day 0 and left until the time of imaginal molt, 8 days later. (A) dsControl specimen 1 day after completing the imaginal molt, showing a normal adult appearance. (B) dsBgHR3-1-treated specimen of the same age, which was not able to ecdyse. (C–E) Specimens that did not ecdyse showed duplicated and superimposed mandibles (C), hypopharinge (D) and lacinia (E). Nymphal structures are indicated with an arrow, and adult ones with a arrowhead. Scale bars: 5 mm in A and B; 500 μm in C, D and E.

treatment (Fig. 8b, lanes 2 and 3). Moreover, when both dsRNAs were injected simultaneously, the levels of the three BgHR3 mRNAs were clearly down regulated (Fig. 8b, lane 4). Then, we studied the phenotypical outcome of the isoform-specific interference. Injection of 1 μg of dsBgHR3-A ($n = 25$) or dsBgHR3-B ($n = 25$) separately into freshly ecdysed sixth instar nymphs did not provoke any effect, and the treated nymphs molted properly into morphologically normal adults 8 days after the treatment. However, when both dsRNAs were injected simultaneously to the same nymph (1 μg + 1 μg, $n = 25$), 65% of the specimens did not molt and arrested at day 8. These specimens showed the same phenotype as that described above for the dsBgHR3-1-treated nymphs (not shown), that is, they had duplicated and superimposed the mouth parts, the hypopharinge, the tracheae and the cuticle layers.

3. Discussion

The present study describes three isoforms of an ortholog of the nuclear receptor HR3 in the hemimetabolous insect *B. germanica*, which were named *BgHR3-A*, *BgHR3-B*$_1$ and *BgHR3-B*$_2$. In this cockroach, we had previously characterized the two components of the functional 20E receptor, one isoform of the ecdysone receptor, *BgEcR-A* (Cruz et al., 2006), and two isoforms of USP/RXR, *BgRXR-S* and *BgRXR-L* (Maestro et al., 2005; Martín et al., 2006). The identification of three isoforms of *HR3* in *B. germanica* demonstrates that the conservation of the 20E-responsive genetic hierarchy in hemimetabolous insects extends beyond the receptor itself. The three BgHR3 isoforms show the typical features of the HR3/ROR subfamily of nuclear receptors, i.e., a highly similar DBD, starting from the seventh amino acid preceding it, and showing the highly conserved 23 amino acids carboxy-terminal extension of the DBD itself. The remaining domains of the protein are poorly conserved when compared to other members of the HR3/ROR subfamily, especially the D domain, which is remarkably long in BgHR3-A and BgHR3-B$_1$ isoforms. Moreover, there is a relative low conservation between the LBD of BgHR3-A, -B$_1$ and -B$_2$ and those of other insects (53–61%), which

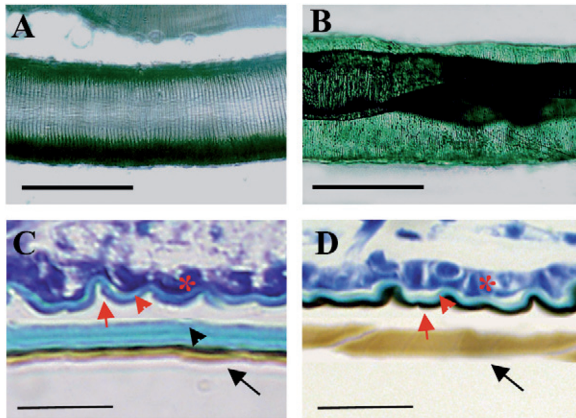

Fig. 7. Effect of RNAi of BgHR3 on the tracheal system and cuticle layers of *Blattella germanica*. Sixth instar female nymphs were injected with 1 μg of dsBgHR3-1 or with dsControl at day 0 and left until the time of imaginal molt, 8 days later. (A) Trachea from a dsControl specimen 1 day after the imaginal molt showing a normal appearance. (B) Double trachea from a dsBgHR3-1-treated specimen that did not ecdyse. The old nymphal trachea is included within the lumen of the new adult one. (C) Cuticle layers of a dsControl specimen on day 8. (D) Cuticle layers of a dsBgHR3-1-treated specimen that did not ecdyse. Epicuticle (arrows), endocuticle (arrowheads) and epidermis (asterisks) are indicated. Black arrows and arrowheads represent nymphal epi- and endocuticle. Red arrows and arrowheads represent adult epi- and endocuticle. Scale bars: 200 μm in A and B; 50 μm in C and D.

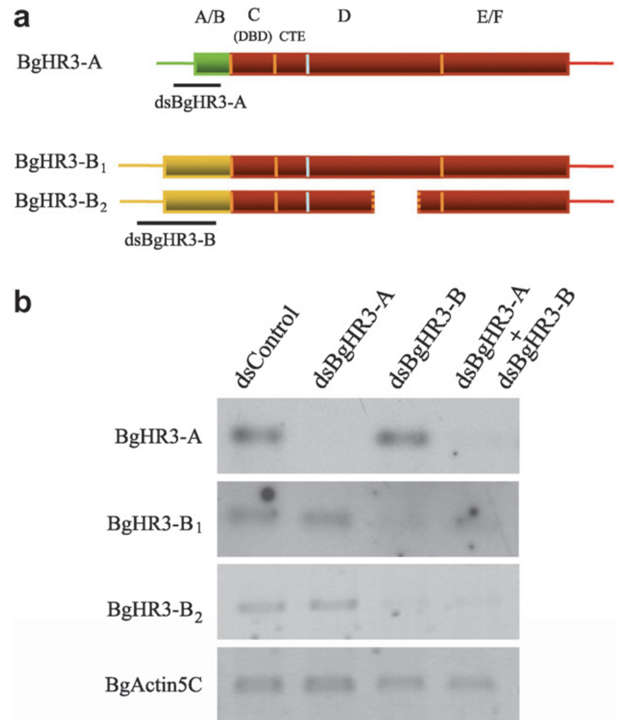

Fig. 8. Effect of RNAi specific for BgHR3-A, BgHR3-B_1 and BgHR3-B_2 on respective transcript levels in sixth instar nymphs of *Blattella germanica*. (a) Scheme of the three BgHR3 isoforms showing the regions considered to generate the isoform-specific dsRNAs. (b) Effects of injection of dsBgHR3-A and dsBgHR3-B separately or simultaneously on the expression of the three BgHR3 isoforms. A dose of 1 μg of dsBgHR3-A or dsBgHR3-B were injected separately or simultaneously in sixth instar female nymphs, and mRNA levels of the three BgHR3 isoforms were measured 6 days later in the epidermis with adhered fat body by RT-PCR/Southern blotting using the corresponding specific probes. Equivalent experiments injecting a non-specific sequence (dsControl) served as negative controls. BgActin5C levels were used as a reference. The Southern blots are representative of six replicates.

does not allow a prediction as to whether the insect HR3 receptors would share the same ligand, if any.

Expression studies in prothoracic gland, epidermis and fat body tissues show that the restricted induction of BgHR3 coincides with the peak of circulating ecdysteroids at each nymphal instar of *B. germanica*. The requirement of ecdysteroids for BgHR3 induction was assessed by the results of experiments incubating fat body tissue *in vitro* with increasing doses of 20E. These results, together with the fact that Chx do not inhibit the induction by 20E, confirm that *BgHR3* is a gene directly activated by 20E. Interestingly, BgHR3 isoforms are induced to the same or even to higher levels when fat body is incubated with 20E and Chx than when incubated with 20E alone. A similar effect has been described in the mosquito *A. aegypti* by Kapitskaya et al. (2000), thus suggesting that in these species there is a 20E-inducible inhibitor protein that was inhibited by Chx. Conversely, Chx decreased the 20E-induced expression of HR3 in *D. melanogaster* (Horner et al., 1995) and in *M. sexta* (Palli et al., 1992), which suggests that Chx inhibited an activator protein in these species.

Furthermore, our experiments of RNAi *in vivo* showed that BgEcR-A, the receptor of 20E, is required to fully activate the expression of BgHR3. Activation of HR3 by ecdysteroids has been also reported in incubated organs of *D. melanogaster* (Horner et al., 1995), in incubated silk glands of *G. mellonella* (Jindra and Riddiford, 1996), in fat bodies of adult *A. aegypti* (Kapitskaya et al., 2000), in larval epidermis of *M. sexta* (Palli et al., 1992) and in ovaries of *B. mori* (Eystathioy et al., 2001). Our previous studies on *B. germanica* had demonstrated that neither *BgEcR-A* nor the two *RXR* isoforms, *BgRXR-S* and *BgRXR-L*, are induced by 20E (Cruz et al., 2006; Maestro et al., 2005, respectively). Therefore, *BgHR3* is the first gene directly induced by 20E that has been isolated in *B. germanica*.

Functional studies on *HR3* have been reported only in *D. melanogaster*, in which *HR3* mutants exhibit defects in the nervous system of the embryo and die during embryonic development (Carney et al., 1997). Furthermore, *HR3* mutants that are rescued to the last larval instar, die during the prepupal and early pupal stages showing defects in the tracheal cuticle, gas bubble translocation and head eversion, which indicates that *HR3* plays crucial roles during the onset of metamorphosis (Lam et al., 1999). A detailed clonal analysis of *HR3* mutants in *D. melanogaster* also revealed functions in bristle, wing and cuticular development in pupae (Lam et al., 1999).

The present paper reports a detailed analysis of *BgHR3* functions in *B. germanica*, a hemimetabolous species with incomplete metamorphosis. RNAi experiments *in vivo*

showed that *BgHR3* is directly involved in the ecdysis process. *BgHR3* mRNAs appear in the epidermis by day 5 of the last nymphal instar and remain present a high levels during the next days, that is, when the new cuticle is secreted (between days 7 and 8). This coincidence suggests that BgHR3 proteins directly or indirectly regulate the expression of cuticle genes. The expression of *HR3* in the epidermis of lepidopteran species, such as *M. sexta* and *H. armigera*, is also correlated with the synthesis of new cuticles (Langelan et al., 2000; Zhao et al., 2004), although no functional studies have been carried out in these species. A direct role of *HR3* in molting has been reported only in *Caenorhabditis elegans*. In this nematode, disruption of *CeHR3* (also known as nhr-23) function leads to incomplete molting (Kostrouchova et al., 1998, 2001). Recently, it has been shown that CeHR3 induces the expression of a number of genes in the hypodermis, and that it is also required for the expression of dpy-7, a collagen protein present in the larval cuticle of the nematode (Frand et al., 2005). Nevertheless, the nymphs of *B. germanica* that arrested development as a consequence of dsBgHR3 treatment were able to synthesize the new cuticle and to digest the old endocuticle. This indicates that *BgHR3* silencing affected particularly the ecdysis process, including exuvia shedding. Ecdysis depends on fast fluctuations of circulating ecdysteroids occurring when the levels decline after the molting peak, which regulate the synthesis and release of peptides governing the behavior associated to ecdysis (for a review, see Zitnan and Adams, 2005). Nymphs with silenced *BgHR3* formed a new cuticle, which indicates that they were able to increase their ecdysteroid levels and undertake the apolysis. However, they were unable to ecdyse, which suggest that BgHR3 is involved in the regulation or sense of the decline in ecdysteroid levels that occur at the end of the nymphal instar rather than directly regulating cuticle genes expression.

Injections with dsRNA that specifically targeted only BgHR3-A or only BgHR3-B1 + B2 had no effect on the development of *B. germanica*. However, coinjection of both dsRNAs prevented ecdysis in 65% of treated animals. This percentage is lower than the 100% penetrance of the same phenotype achieved with dsRNA targeted to common regions of the three BgHR3 isoforms described herein. The different efficiency of RNAi is likely not due to the dsRNA length, since all used fragments were of similar size. These results, then, raise the possibility that there are other BgHR3 isoforms that escaped the RNAi. In any case, the high percentage of arrested animals when dsBgHR3-A and BgHR3-B were injected simultaneously, demonstrates that the three isoforms of BgHR3 reported have redundant functions, at least in the context of the ecdysis process of *B. germanica*. In general, isoforms of HR3 are based in divergences in the A/B domain, and our phylogenetic analysis of this domain suggests that these isoforms were generated in ancestral species before the splitting of the main insect orders. At present, HR3 isoforms occur in greater or lower number depending on the species (based on Northern blot analysis, the number of RNA isoforms detected are: 5 in *H. armigera*, 4 in *C. fumiferana*, 3 in *D. melanogaster* and *A. aegypti*, and 2 in *M. sexta* and *G. mellonella*), which suggests that some of them were lost during evolution. However, isoform loss in a given species would be possible only if their functions were redundant, which is just what we have found in *B. germanica*, as described in the present paper.

4. Experimental procedures

4.1. Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% r.h. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens.

4.2. Cloning of BgHR3 cDNAs

Degenerate primers based on the DBD of insect HR3 sequences were used to obtain a *B. germanica* ortholog cDNA fragment by RT-PCR. PCR amplification was carried out using as a template cDNA generated by reverse transcription from polyA$^+$ RNA obtained from 20E treated-UM-BGE-1 cells (Maestro et al., 2005; Cruz et al., 2006). The primers for BgHR3 amplification were, forward (BgHR3F1): 5′-TGY GARGGNTGYAARGG-3′, reverse (BgHR3R1): 5′-ARRCAYTTYTG NAGNCGRCA-3′. The amplified fragment (129 bp) was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. This was followed by 5′ and 3′ RACE (5′- and 3′-RACE System Version 2.0; Invitrogen) to complete the sequence. For 5′-RACE, reverse primers were BgHR3R2: 5′-TACTGGCATCGGTTCCTGTTGAC-3′ and the nested BgHR3R3: 5′- TGCGTGAGCACTGGTAGTTGAC-3′. For 3′-RACE, forward primer was BgHR3F2: 5′-TCCCAGAGCTCCGTCGTCAACTA-3′. All PCR products were subcloned into the pSTBlue-1 vector (Novagen) and sequenced in both directions. Following this approach, we isolated three different BgHR3 isoforms.

4.3. cDNA transcription and translation in vitro

The entire BgHR3 ORF for each of the three isoforms was amplified by PCR using Accutaq polymerase (Sigma). The three PCR fragments of the expected size were subcloned into pSTBlue-1 (Novagen) and sequenced. cDNAs were transcribed and translated using the TNT coupled reticulocyte lysate system (Promega), according to the manufacturer's instructions.

4.4. Sequence comparisons and phylogenetic analysis

In order to classify the HR3 orthologs of *B. germanica*, a phylogenetic analysis was carried out on the A/B domain of the sequences. HR3 sequences used in the analyses were obtained from GenBank, and were the following: DHR3C and DHR3A from. *D. melanogaster* (NP_788301 and NP_788303, respectively), AHR3 from *A. aegypti* (AAF36970), CHR3B and CHR3C and from *C. fumiferana* (U375228 and U63929, respectively), BHR3A from *B. mori* (AF073927), MHR3 from *M. sexta* (X74566), HHR3 from *H. armigera* (AF337637), GHR3 from *G. mellonella* (U02621). The protein sequences of the A/B domain were aligned with those obtained from *B. germanica* using clustalX. The resulting alignment was analyzed by the PHYML program (Guindon and Gascuel, 2003) based on the maximum-likelihood principle with the amino acid substitution model. Four substitution rate categories with a gamma shape parameter of 1.444 were used. The data was bootstrapped for 1000 replicates using PHYML.

4.5. RT-PCR/Southern blot analyses

RT-PCR followed by Southern blotting with specific probes was used to establish the expression patterns of the three BgHR3 transcripts. Total RNA was extracted from different tissues using the GenElute™ Mammalian Total RNA kit (Sigma). An amount between 0.3 and 1 μg of each RNA preparation was used for cDNA synthesis, as previously described (Maestro et al., 2005). For mRNA detection, cDNA samples were subjected to PCR amplification (with a number of cycles within the linear range of amplification, ranging from 18 to 28 depending on the tissue and physiological stage) at 94 °C for 30 s, 64 °C for 30 s and 72 °C for 45 s (Maestro et al., 2005; Cruz et al., 2006). Primers for BgHR3-A were: forward (BgHR3F3), 5′-GGCGCACCAAAAGTGGATTAATTGA-3′ and reverse (BgHR3R4), 5′-CGCTGATGTCGTACGGCATCT-3′; for BgHR3-B_1 were: forward (BgHR3F4), 5′-TTGCTGGCGCCCGACAAA AGATC-3′ and reverse (BgHR3R5), 5′-CAAGAAGACCCAGAGAAC TAGCTT-3′; and BgHR3-B_2 were: forward, BgHR3F4 and reverse (BgHR3R6), 5′-GATAACTGCGCCGGATCTGTTGTAA -3′. Primers for BgEcR-A were: (EcRF1), 5′-TACTCCGGAGGTAGCGTCATCAT-3′; and reverse (EcRR4), 5′-GACGGTGAAGACAACCAGTCATC-3′ (accession number of BgEcR-A: AM039690). As a reference, the same cDNAs were subjected to RT-PCR with a primer pair specific of *B. germanica* actin5C: forward, 5′-TCGTTCGTGACATCAAGGAGAAGCT-3′ and reverse, 5′-TGTCGGCAATTCCAGGGTACATGGT-3′. cDNA probes for Southern blot analyses were generated by PCR with the same primer pairs using plasmid DNAs containing the corresponding cDNA clones as templates. The probes were labeled with fluorescein using the Gene Images random prime-labeling module (Amersham Biosciences). RT-PCR followed by Southern blotting of total RNA without reverse transcription did not result in amplification, thus indicating that there was no genomic contamination.

4.6. Incubation of epidermis with associated fat body in vitro

Dorsal segments of the abdominal region, with the epidermis and associated fat body tissue, were dissected from 1-day-old sixth instar female nymphs and incubated in 1 ml of Grace's medium, with L-glutamine and without insect hemolymph (Sigma) at 30 °C in the dark. For hormonal treatments, 20E (Sigma) was dissolved in water with 10% ethanol. To inhibit protein synthesis, 10^{-4} M Chx (Sigma) was added to the incubation medium. This concentration reversibly inhibits protein synthesis ca. 100% (Comas et al., 2001). The incubation medium was changed every 5 h to avoid contamination. At indicated time intervals, total RNA was isolated from the incubated tissues and subjected to RT-PCR amplification followed by Southern blotting.

4.7. Synthesis of dsRNAs and injection

To produce the dsBgHR3-1, designed to interfere all BgHR3 transcripts, a 411 bp DNA encoding a protein sequence specific of the common DBD and D domain, from nucleotide 685 to 1096 according to the sequence of BgHR3-B_1, was amplified by PCR and cloned into pSTblue-1 vector (Novagen). dsBgHR3-2, a second dsRNA that would knockdown all possible isoforms was similarly prepared, by selecting a 242 bp DNA sequence of the D region including the CTE (from nucleotide 854 to 1096 of BgHR3-B_1). To specifically silence BgHR3-A, a 225 bp DNA fragment encompassing its 5′UTR and A/B regions (from nucleotide 55 to 280 of BgHR3-A sequence) was selected (dsBgHR3-A). Finally, to interfere BgHR3-B_1 plus BgHR3-B_2, a 323 bp DNA fragment encompassing their 5′UTR and A/B regions (from nucleotide 246 to 569 of the BgHR3-B_1 sequence), was used (dsBgHR3-Bcom). To prepare the dsRNA targeted to BgEcR-A (dsBgEcR-A), a 700 bp fragment encompassing the A/B region of BgEcR-A was used, and as a control, we used a non-coding sequence from the pSTBlue-1 vector (dsControl), as described by Cruz et al. (2006).

The respective RNAs were generated by transcription *in vitro* using either SP6 or T7 RNA polymerases from the corresponding plasmids, and resuspended in water. To prepare the dsRNAs, equimolar amounts of sense and antisense RNAs were mixed, heated at 90 °C for 5 min, cooled down slowly to room temperature and stored at −20 °C until use. The obtained dsRNAs were suspended in diethyl pyrocarbonate-treated water and dissolved in Ringer saline with a final concentration of 1μg/μl. Formation of dsRNAs was confirmed by running 1 μl of these reactions in a 1% agarose gel. One microliter of the solution (1 μg/μl) was injected into the abdomen of newly emerged sixth instar nymph females. In case of coinjection of two dsRNAs, 1 μl of each solution was applied in a single injection of 2 μl.

4.8. Microscopy and histological examination

All dissections of nymphal tissues were carried out in Ringer's saline. Mouth parts and tracheae were directly immersed in glycerol 50% and observed under the microscope. To examine the cuticle layers, a portion of abdominal ventral cuticle was fixed in 4% paraformaldehyde, dehydrated and paraffin embedded. Cuticle sections (6 μm) were stained with toluidine blue. All samples were observed on a Zeiss Axiophot microscope.

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