RESEARCH ARTICLE

Nuclear Receptor BgFTZ-F1 Regulates Molting and the Timing of Ecdysteroid Production During Nymphal Development in the Hemimetabolous Insect *Blattella germanica*

Josefa Cruz,[†] Claudia Nieva, Daniel Mané-Padrós,[†] David Martín,[*] and Xavier Bellés[*]

Postembryonic development of holometabolous and hemimetabolous insects occurs through successive molts triggered by 20-hydroxyecdysone (20E). The molecular action of 20E has been extensively studied in holometabolous insects, but data on hemimetabolous are scarce. We have demonstrated that during the nymphal development of the hemimetabolous insect *Blattella germanica*, 20E binds to the heterodimeric receptor formed by the nuclear receptors BgEcR-A and BgRXR activating a cascade of gene expression, including the nuclear receptors BgE75 and BgHR3. Herein, we report the characterization of BgFTZ-F1, another nuclear hormone receptor involved in 20E action. BgFTZ-F1 is activated at the end of each instar, and RNAi has demonstrated that BgHR3 is needed for BgFTZ-F1 activation, and that BgFTZ-F1 has critical functions of during the last nymphal instar. Nymphs with silenced BgFTZ-F1 cannot ecdyse, arrest development, and show structures of ectodermal origin duplicated. BgFTZ-F1 also controls the timing of the ecdysteroid molting pulse. *Developmental Dynamics 237:3179–3191, 2008.* © 2008 Wiley-Liss, Inc.

Key words: FTZ-F1; Nuclear hormone receptor; *Blattella germanica*; 20-hydroxyecdysone; RNAi

INTRODUCTION

Ecdysteroids play a key role in postembryonic development in insects. In holometabolous species, such as *Drosophila melanogaster*, a periodic pulse of 20-hydroxyecdysone (20E, the biologically active form of ecdysteroids) trigger larval molts, whereas a pulse of 20E at the end of the last larval instar signals the onset of pupariation. Moreover, 20E induces the destruction of larval tissues and the formation of the adult body plan during metamorphosis (Thummel, 1995). 20E exerts its effects upon binding to the ecdysone receptor (EcR), a member of the nuclear receptor superfamily, which heterodimerizes with another nuclear receptor, the RXR-ortholog ultraspiracle (USP; Yao et al., 1992). This heterodimer elicits cascades of gene expression that mediate and amplify the ecdysteroidal signal (reviewed in Thummel, 1995; Riddiford et al., 2000; King-Jones and Thummel, 2005).

In more basal hemimetabolous, direct-developing insects data on the mechanisms of 20E action are practically nonexistent, with few exceptions (e.g., Erezyilmaz et al., 2006). The postembryonic development of hemimetabolous species shows significant differences with respect to holometabolous, especially because growth and maturation occur simultaneously throughout successively nymphal stages until the imaginal molt, without an intermediate pupal stage. During the last years, and with the aim of finding regularities as well as differences in ecdysteroid action between both types of insect development, we have been characterizing the 20E-triggered genetic hierarchy of transcription factors in the German cockroach *Blattella germanica*, as model of hemimetabolous

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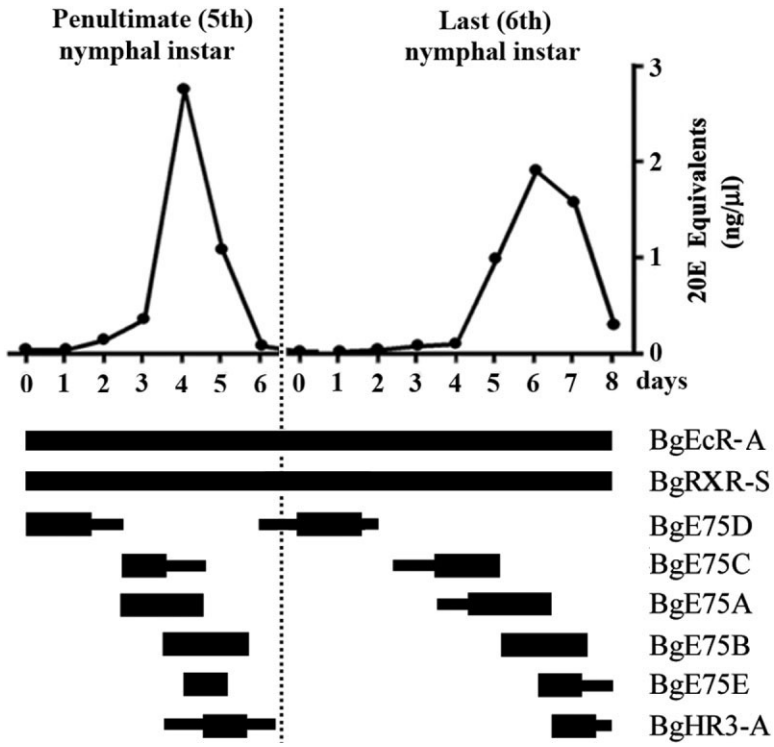

Fig. 1. Schematic representation of circulating ecdysteroid levels and mRNA expression of several nuclear receptors during the last two nymphal instars of *Blattella germanica*. The hormone titer is redrawn from Cruz et al. (2003). The diagrams of mRNA expression, where the width of the box represents approximate levels of expression, are based on Cruz et al. (2006), Cruz et al. (2007), Maestro et al. (2005), and Mané-Padrós et al. (2008).

species. We have identified the functional 20E heterodimeric receptor, namely BgEcR-A, and two isoforms of RXR/USP, BgRXR-S, and BgRXR-L (Maestro et al., 2005; Cruz et al., 2006), as well as five isoforms of the early transcription factor E75 (Mané-Padrós et al., 2008) and three isoforms of the early-late HR3 (Cruz et al., 2007). In *B. germanica*, whereas BgEcR-A and BgRXR are expressed in a housekeeping-like pattern during the last two nymphal instars, BgE75 and BgHR3 isoforms are activated and repressed sequentially in response to the periodic pulse of circulating ecdysteroids (Fig. 1). Moreover, RNA interference (RNAi) in vivo of BgEcR-A, BgRXR, and BgHR3 during nymphal development, results in significant impairment of molting to the next stage. Remarkably, all knockdown nymphs that arrested development showed duplication of all structures of ectodermic origin, such as mouthparts, hypopharinge, tracheal system and cuticle layers (Cruz et al., 2006; Martín et al., 2006; Cruz et al., 2007). We have also shown that BgE75 is required for the rise of circulating ecdysteroids at the end of the last nymphal instar. Consequently, BgE75 knockdown nymphs do not molt into adults and live for up to 90 days remaining as nymphs (Mané-Padrós et al., 2008).

In *D. melanogaster*, the four nuclear receptors that we have characterized in *B. germanica* up to now, EcR, RXR, E75, and HR3, constitute a functional axis that controls the expression of the nuclear hormone receptor FTZ-F1 in a very precise way (White et al., 1997; Lam et al., 1999). Therefore, the obvious next step to further study the role of 20E in the development of *B. germanica* has been the characterization of the ortholog of FTZ-F1 in this species. In *D. melanogaster*, the *ftz-f1* gene generates two isoforms differing only in their N-terminal part. The αFTZ-F1 isoform, from maternal origin, is only present during early embryonic development, where it regulates early segmentation through the interaction with the homeotic gene *ftz* (Guichet et al., 1997; Yu et al., 1997). The other isoform, βFTZ-F1, is detected during late embryogenesis and throughout the postembryonic development (Ueda et al., 1990; Sullivan and Thummel, 2003). In all stages, β*Ftz-F1* is expressed as the ecdysteroid titer declines, and controls each developmental transition (Yamada et al., 2000). Furthermore, the presence of βFTZ-F1 during the prepupal stage plays a crucial role as a competence factor for stage-specific responses to a second 20E pulse that triggers the onset of pupal development. Thus, β*Ftz-F1* mutants show defects in adult head eversion, leg elongation and salivary gland degeneration at the prepupal-pupal transition (Broadus et al., 1999). The role of FTZ-F1 in molting has also been demonstrated in the coleopteran *Tribolium castaneum* (Tan and Palli, 2008), whereas its role as a competence factor for 20E action has been reported during the previtellogenic-vitellogenic transition in the dipteran *Aedes aegypti* (Li et al., 2000; Zhu et al., 2003). FTZ-F1 orthologs have also been identified in other holometabolous insects, namely the lepidopterans *Manduca sexta* (Weller et al., 2001) and *Bombyx mori* (Sun et al., 1994), and the hymenopteran *Apis mellifera* (Velarde et al., 2006).

In the present study, we describe the cloning and functional characterization of the homolog of FTZ-F1 in *B. germanica*, named BgFTZ-F1. We report a detailed analysis of its developmental expression throughout the last two nymphal stages, and we show that BgFTZ-F1 expression is dependent on the presence of BgHR3. Finally, the functions of BgFTZ-F1 during nymphal development have been also studied, using an RNAi in vivo approach.

RESULTS

Cloning and Characterization of BgFTZ-F1 cDNA

Using a reverse transcriptase-polymerase chain reaction (RT-PCR) approach followed by 5′ and 3′ rapid amplification of cDNA ends (RACE), we isolated a 2.3-kb sequence encoding a polypeptide of a predicted molecular mass of 65.5 kDa. The putative start codon was preceded by an in-frame stop codon, and the final stop codon

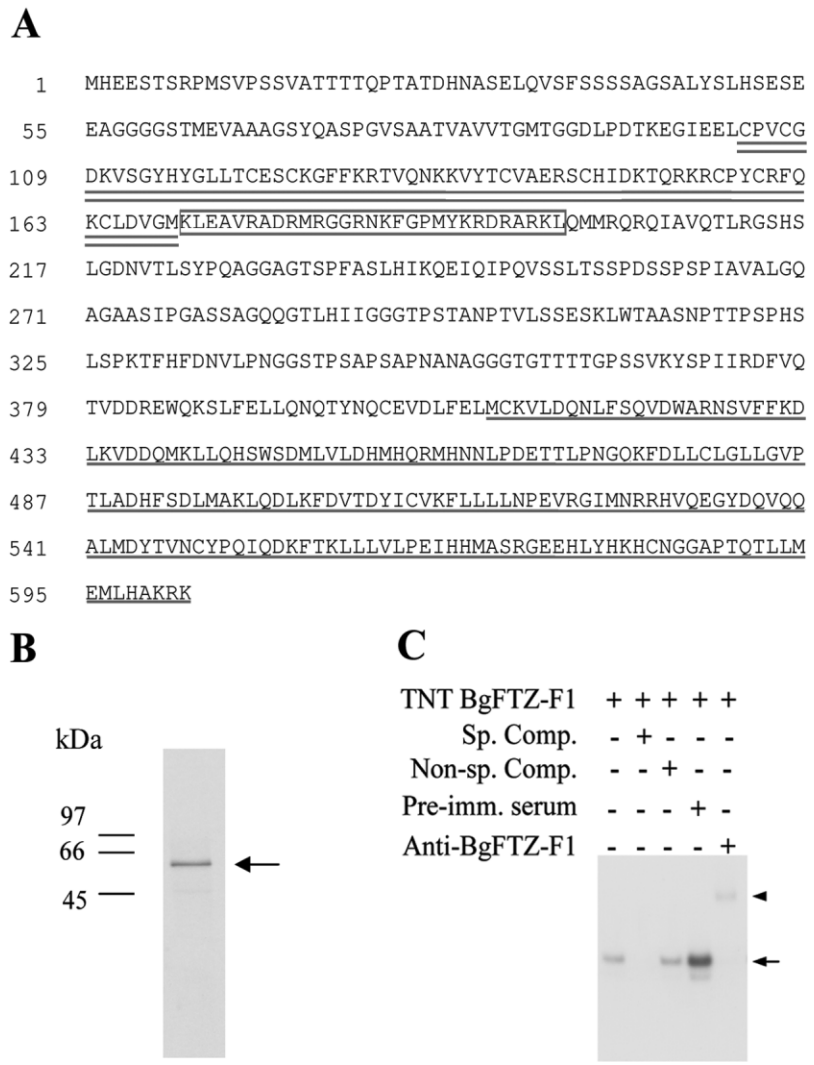

Fig. 2. **A:** Amino acid sequence of the *Blattella germanica* FTZ-F1 (BgFTZ-F1). The DNA binding domain is double underlined and the ligand binding domain is underlined. The FTZ-F1 box is boxed. **B:** Translational analysis in vitro. The cDNA clone encoding BgFTZ-F1 was translated by a coupled transcription-translation system in vitro and labeled with [^{35}S]-methionine. The translation product was resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis using a 10% gel followed by autoradiography. Molecular mass standards are shown on the left. **C:** Electrophoretic mobility shift assay (EMSA) analysis of the BgFTZ-F1 protein translated in vitro. The protein sample was incubated with ^{32}P-labeled FTZ-F1 response element (F1RE). The specificity of the interaction was tested by the addition of 50-fold molar excess of the unlabeled F1RE (Sp. Comp.), or a nonspecific DNA (Non-sp. Comp.). In addition, anti-BgFTZ-F1 antibody (Anti-BgFTZ-F1) and pre-immune serum (Pre-imm. serum) were added to the reactions.

was followed by a poly (A) sequence, indicating that it was a full-length open reading frame (ORF), and it was named BgFTZ-F1 (GenBank accession no. FM163377). The protein presented the domain organization characteristic of a nuclear hormone receptor: a ligand-independent A/B activation domain (amino acids 1-103), followed by the DNA-binding domain (DBD; C domain, 104-168) with an adjacent stretch of 29 highly conserved amino acids known as the FTZ-F1 box, a hinge region (D domain, 169-407), and a ligand-binding domain (LBD; E domain, 408-601), containing the putative ligand-dependent activation motif, AF-2 (LLMEML; Fig. 2A). The comparison of BgFTZ-F1 with FTZ-F1 proteins from other insects, crustaceans, nematodes, and the two closest human homologs, steroidogenic factor 1 (SF-1) and liver receptor homolog 1 (LRH-1), showed that the DBD and FTZ-F1 box are the highest conserved domains, with an overall 78–100% amino acid identity in the FTZ-F1 box and 81–98% in the DBD (Table 1). High similarity among insect FTZ-F1 proteins was also observed in the LBD, being more pronounced with coleopteran and hymenopteran proteins (86 and 82% amino acid identity, respectively) than with those of lepidopteran and dipterans (58 and 79%). Finally, the A/B and hinge domains showed very weak similarities (Table 1).

The cloned BgFTZ-F1 cDNA contained a translatable ORF, since its expression in a coupled TNT system followed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography, showed that the protein synthesized in vitro closely corresponded to that expected, that is, 64 kDa (Fig. 2B). To elucidate whether the BgFTZ-F1 protein recognized the DNA-binding site characteristic for the FTZ-F1 family, the BgFTZ-F1 protein translated in vitro was tested in electrophoretic mobility shift assay (EMSA), using a 9 base pairs (bp) binding sequence identified previously in the upstream regulatory region of the *D. melanogaster ftz* gene (F1RE; Ohno et al., 1994), as a probe. A binding complex was detected with BgFTZ-F1 and the F1RE (Fig. 2C). The specificity of the complex was confirmed by competition with an excess amount of the specific unlabeled F1RE and also by supershifting the complex by incubation with an antibody that specifically recognizes the A/B domain of BgFTZ-F1 (Fig. 2C).

Expression of BgFTZ-F1 During the Two Last Nymphal Instars

To study the expression of BgFTZ-F1 during the two last nymphal instars, we carried out semiquantitative RT-PCR on RNA isolated from prothoracic glands (responsible of ecdysteroid synthesis), the epidermis (responsible of cuticle synthesis), and the fat body (the main metabolic organ), using a primer pair located within the A/B domain of the receptor (Fig. 3). In all tissues, BgFTZ-F1 mRNA was present during the first days of each instar, when circulating ecdysteroids were very low. Then, in parallel with the increase of ecdysteroid titer, BgFTZ-F1 mRNA levels

declined until being undetectable by the time of the ecdysteroid peak. Finally, when ecdysteroid levels declined during the last 2 days of each instar, BgFTZ-F1 mRNA levels increased again, showing a maximum just before molting (Fig. 3). The same patterns were obtained when the PCR was conducted with a second primer pair located within the LBD (data not shown).

To test whether there was a correlation between mRNA and protein levels, we examined by EMSA analysis the pattern of BgFTZ-F1 protein in fat body nuclear extracts during the last nymphal instar. As a previous step, we characterized the occurrence of BgFTZ-F1 protein in nuclear extracts from fat bodies from 1-day-old sixth instar nymphs, by incubating the extract with the F1RE (Fig. 4A). We detected a retarded DNA-protein complex (Fig. 4A), showing similar mobility to that of the complex formed by F1RE and the BgFTZ-F1 protein translated in vitro (data not shown). The complex was removed by competition with excess amount of the specific unlabeled F1RE or by incubation with the anti-BgFTZ-F1 (Fig. 4A), thus indicating that this complex corresponds to BgFTZ-F1 bound to F1RE. Then, we tested the binding activity of BgFTZ-F1 in nuclear extracts from staged last nymphal instar fat bodies. Interestingly, the binding activity parallels that of the mRNA pattern. BgFTZ-F1 binding was high during the first days of the instar, declining from day 2 to disappear at day 5. At the end of the instar, BgFTZ-F1 appeared again very clearly (Fig. 4B).

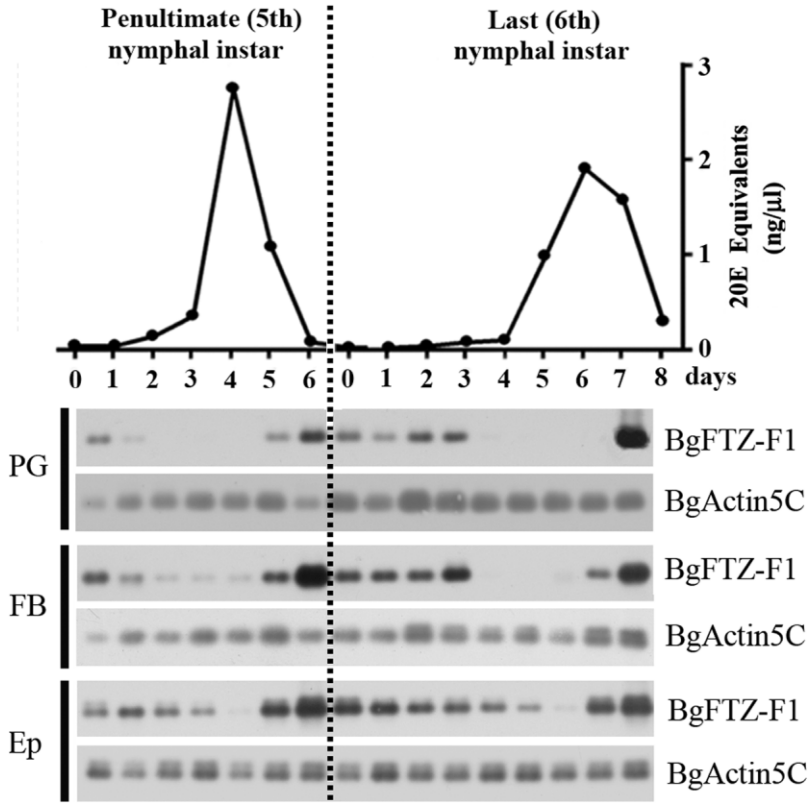

Fig. 3. Expression pattern of BgFTZ-F1 mRNA during the last two nymphal instars of *Blattella germanica*. Ecdysteroid levels in terms of 20E equivalents (upper panel) are from Cruz et al. (2003). mRNA levels were analyzed in the prothoracic gland (PG), fat body (FB) and epidermis (Ep) from staged nymphs by reverse transcriptase-polymerase chain reaction (RT-PCR) /Southern blotting, using BgFTZ-F1 specific probe (lower panels). BgActin5C levels were used as a reference. The Southern blots shown are representative of three replicates.

Effects of 20E on BgFTZ-F1 Expression

To determine whether the negative correlation between BgFTZ-F1 mRNA levels and the increase of circulating ecdysteroids observed at the mid-nymphal instar reflected possible inhibitory effects of 20E, we carried out in vitro experiments.

We studied BgFTZ-F1 mRNA levels in abdominal segments with associated epidermis and fat body from 1-day-old sixth instar female nymphs (which present detectable levels of BgFTZ-F1 mRNA) incubated in vitro for 1 to 24 hr with either 20E (5×10^{-6} M), the protein synthesis inhibitor cycloheximide (Chx; 10^{-4} M), or both 20E and Chx. This approach in vitro had been previously used to demonstrate the 20E-dependency of BgE75 and BgHR3 expression in *B. germanica* (Cruz et al., 2007; Mané-Padrós et al., 2008). Results (Fig. 5A) showed that incubation in hormone-free medium up to 24 hr did not affect the expression of BgFTZ-F1, whereas in the presence of 20E, BgFTZ-F1 mRNA levels moderately decreased at 24 hr of incubation. Interestingly, when the tissues were incubated in the presence of Chx or Chx plus 20E, BgFTZ-F1 mRNA levels notably increased.

Given that the appearance of BgFTZ-F1 mRNA coincides with the decline of the ecdysteroid pulse (Fig. 3), we hypothesized that BgFTZ-F1 expression might depend not only on the presence but also on the subsequent absence of 20E. To test this hypothesis, we incubated abdominal segments with epidermis and fat body tissues in 20E-containing medium for a 24-hr period, after which with transferred the tissues to a hormone free-medium where they were incubated for an additional 16-hr period. Under these conditions, BgFTZ-F1 transcript levels remained unchanged throughout the incubation (data not shown).

Despite the above results, it is worth noting that in all tissues analyzed in vivo, the expression of BgFTZ-F1 at the end of each nymphal instar was preceded by the expression of the nuclear receptor BgHR3 (Fig. 1; Cruz et al., 2007). Given that DHR3 regulates the expression of β*FTZ-F1* at the prepupal stage of *D. melanogaster* (White et al., 1997; Lam et al., 1999), we sought to determine whether BgHR3 was required for BgFTZ-F1 activation in *B. germanica*. In this line, we studied the BgFTZ-F1 mRNA levels in prothoracic glands and in abdominal segments with epi-

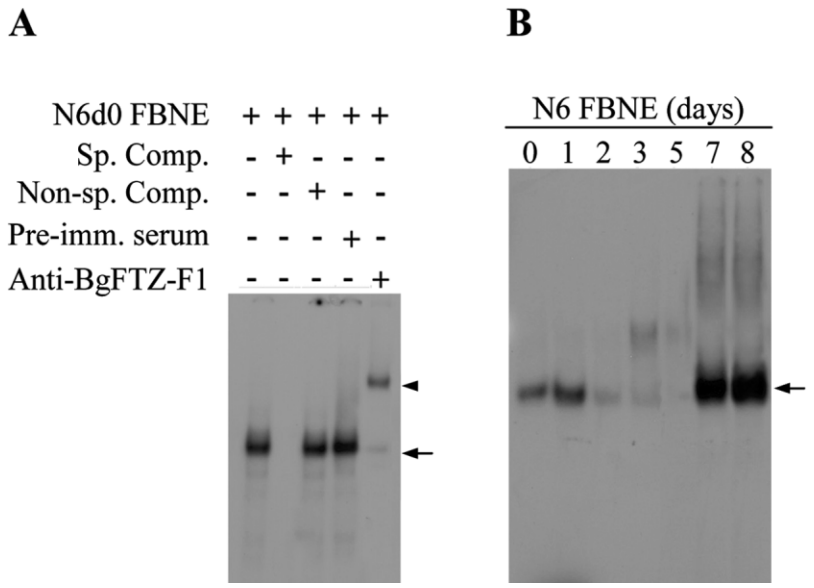

Fig. 4. BgFTZ-F1 protein in the fat body of sixth instar nymphs of *Blattella germanica*. **A:** Identification of functional BgFTZ-F1 protein in fat body nuclear extracts (FBNE) of freshly emerged sixth instar nymphs (N6d0FBNE). The protein samples were incubated with ^{32}P-labeled F1RE. The specificity of the interaction was examined by the addition of 50-fold molar excess of unlabeled F1RE (Sp. Comp.), or a nonspecific DNA (Non-sp. Comp.). In addition, BgFTZ-F1 antibody (Anti-BgFTZ-F1) and preimmune serum (Pre-imm. serum) were added to the reactions. **B:** Developmental profile of functional BgFTZ-F1 protein present in FBNE from sixth instar nymphs of different days incubated with labeled F1RE. One fat body equivalent was used in each binding reaction.

dermis and fat body tissues (Fig. 5B), and in the corpora allata (data not shown) of sixth instar nymphs in which the expression of *BgHR3* had been interefered by RNAi. Results showed that the absence of BgHR3 impaired the activation of *BgFTZ-F1* at the end of the instar in all tissues analyzed, whereas mRNA levels of the two components of the ecdysone receptor, BgEcR-A and BgRXR, remained unchanged (Fig. 5B).

RNAi reduces BgFTZ-F1 mRNA levels

To study the function of BgFTZ-F1 during nymphal development, we lowered its expression by RNAi. A 502-bp dsRNA fragment encompassing the A/B and DBD domains (dsBgFTZ-F1, Fig. 6A; Table 2) was injected in the abdomen of freshly ecdysed last-instar female nymphs. BgFTZ-F1 mRNA levels were determined in the prothoracic gland and in abdominal segments with epidermis and fat body tissues 8 days later. Specimens injected with dsControl were used as negative controls.

Results showed that BgFTZ-F1 mRNA levels clearly decreased in the dsBgFTZ-F1-treated insects compared with controls (Fig. 6B). Similarly, the BgFTZ-F1 protein levels were drastically reduced in dsBgFTZ-F1-treated nymphs (Fig. 6C). The same reduction in BgFTZ-F1 expression was obtained when a second 601-bp dsRNA encompassing the hinge and LBD domains (dsBgFTZ-F1-2; Fig. 6A; Table 2) was used (data not shown).

BgFTZ-F1 Knockdowns Exhibit Molting Defects

Once demonstrated the effectiveness of RNAi on mRNA and protein levels, we investigated the BgFTZ-F1 functions by injecting dsBgFTZ-F1 or dsControl to freshly ecdysed sixth instar nymphs. Specimens treated with dsBgFTZ-F1 (n = 72) showed normal behavior until the end of the instar. However, 46% of them did not molt into adults, arrested development and died 24 hr later (Fig. 7C). Furthermore, 29% started to ecdyse but failed to shed the exuvia completely (Fig. 7B). Finally, 25% ecdysed properly into adults, although showing deficiencies in the extension of the fore- and hindwings, as well as malformations in the leg bristles (Fig. 8). Conversely, 100% of the dsControl-treated nymphs (n = 83) molted into adults normally (Fig. 7A). Similar phenotypical proportions were obtained when dsBgFTZ-F1-2 was injected (51% of arrested nymphs; 23% of nymphs showing incomplete ecdysis; 26% of successful molt but adults showing wing and bristles defects).

The arrested specimens presented all structures of ectodermal origin duplicated. In the cephalic region, two pairs of mandibles (Fig. 7D) and two laciniae within the maxilla (Fig. 7E) were well apparent. They also showed a double superimposed tracheal system, with the old tracheae visible in the lumen of the new one (Fig. 7F). Moreover, histological sections of the abdomen of these nymphs showed the occurrence of the adult endocuticle and exocuticle layers below the nymphal exocuticle (Fig. 7G).

When dsBgFTZ-F1 was injected to 5-day-old sixth instar nymphs (thus allowing the presence of BgFTZ-F1 during the first days of the instar), 95% of specimens arrested development at the nymphal–adult transition. As in the previous experiments, these knockdown nymphs showed duplication of ectoderm-derived structures (data not shown). This indicates that the problems to complete the molting process were due to the reduction of BgFTZ-F1 levels at the end of the nymphal instar, after the ecdysteroid peak.

BgFTZ-F1 Controls the Timing of Ecdysteroid Production

In *B. germanica*, the last nymphal stage lasts for 8 days. The synthesis of the adult cuticle starts on day 6, and the adult cuticle is totally formed below the nymphal one just before ecdysis at day 8, although untanned. Interestingly, the deposition of the adult cuticle in dsBgFTZ-F1-treated last instar nymphs took place 24–36 hr before the controls. Upon dissection of the abdomen just before arresting development at day 8, the adult cuticle of dsBgFTZ-F1-treated nymphs was already tanned and sclerotized with well formed adult bristles (Fig. 7H). Given that the timing of cuticle synthesis and its tanning depends of the

pulse of circulating ecdysteroids, we compared the titer of ecdysteroids in dsControl and dsBgFTZ-F1-treated nymphs between days 2 and 6, when ecdysteroids reach the highest levels in the haemolymph (Cruz et al., 2003). Remarkably, we observed a normal pulse of ecdysteroids in dsBgFTZ-F1-treated nymphs, although starting 24 hr earlier than in dsControl nymphs (Fig. 9), in good correlation with the advancement of the cuticle synthesis and tanning processes.

Finally, we wondered whether this advancement in the ecdysteroid pulse was accompanied by changes in the expression of 20E-regulated genes previously characterized in *B. germanica*. To answer this question, we isolated RNA from prothoracic glands from both dsControl and dsFTZ-F1-treated nymphs between days 2 and 6 of the last nymphal instar, and mRNA levels of BgE75A, BgE75B, BgE75C, BgE75E, and BgHR3-A were examined by RT-PCR. Results (Fig. 10) showed that the 20E-regulated genes were expressed at levels similar to those of controls, but the timing of their expressions was advanced 24 hr, in agreement with the advancement of the ecdysteroid peak observed in the dsBgFTZ-F1-treated nymphs.

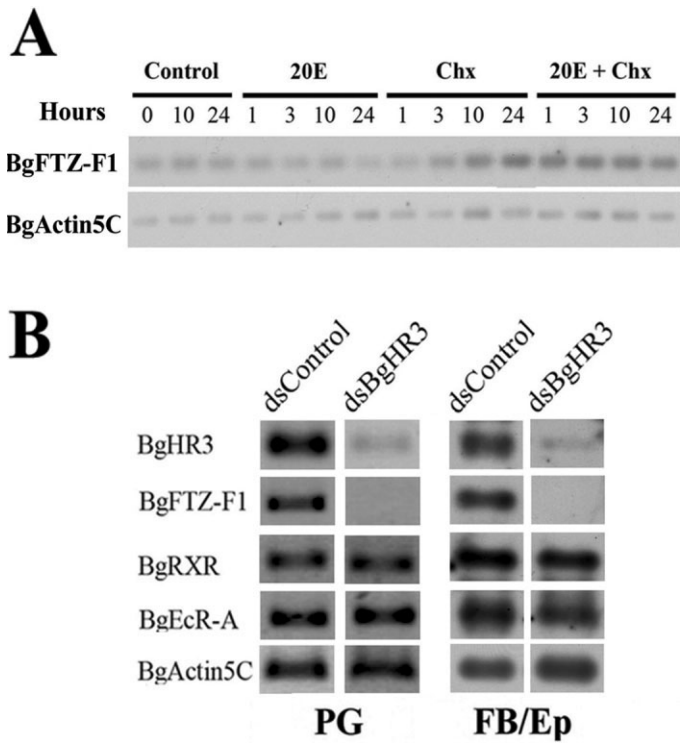

Fig. 5. Regulation of BgFTZ-F1 expression in *Blattella germanica*. **A:** Effect of 20E on BgFTZ-F1 mRNA levels in abdominal segments with epidermis and fat body tissues from 1-day-old sixth instar female nymphs. Tergites were incubated in vitro in the presence of either 5×10^{-6} M 20E; 10^{-4} M of cycloheximide (Chx); or both 20E and Chx for the time indicated. Equal amounts of total RNA from the tissues incubated in vitro were analyzed by reverse transcriptase-polymerase chain reaction (RT-PCR)/Southern blotting. BgActin5C levels were used as a reference. The Southern blots shown are representative of three replicates. **B:** Effect of BgHR3 RNAi on BgFTZ-F1 mRNA levels. One microgram of dsRNA targeting BgHR3 (dsBgHR3) was injected in newly emerged sixth instar female nymphs and mRNAs corresponding to BgHR3, BgEcR-A, BgRXR and BgFTZ-F1 were studied in the prothoracic gland (PG) and in abdominal segments with epidermis and fat body tissues (FB/Ep) 8 days later, using RT-PCR/Southern blotting. Equivalent experiments injecting a nonspecific sequence (dsControl) served as negative controls. BgActin5C levels were used as a reference. The Southern blots shown are representative of six replicates.

DISCUSSION

Conversely to the development of holometabolous, hemimetabolous insects do not have an intermediate metamorphic stage, the pupae. In these insects, such as the cockroach *B. germanica*, postembryonic development occurs throughout several nymphal instars before emerging as adults, and the nymphal–nymphal molts and the final nymphal–adult transition are triggered by pulses of ecdysteroids. Using RNAi in vivo in *B. germanica*, we have demonstrated that these ecdysteroid pulses tightly control the induction of a genetic cascade of nuclear hormone receptors that ultimately controls the correct developmental progression of the insect (Fig. 1). For example, during the last nymphal instar of *B. germanica*, BgE75 inhibits the premature degeneration of the prothoracic gland, thus allowing the production of the ecdysteroid peak at the end of the instar (Mané-Padrós et al., 2008). Furthermore, the heterodimeric receptor of 20E, BgEcR-A and BgRXR, as well as three isoforms of BgHR3, are required for successfully completing the molting process. Nymphs with these receptors interfered completed the apolysis but were unable to ecdyse (Cruz et al., 2006, 2007; Martín et al., 2006).

In the present study we have further characterized the 20E-dependent genetic cascade in *B. germanica* by isolating and characterizing another member of the nuclear receptor superfamily, namely BgFTZ-F1. It shows the characteristic modular structure of a nuclear receptor, with highly conserved DBD and LBD. Moreover, the DBD is followed by the "FTZ-F1 box" that distinguishes this family of nuclear receptors. In the case of BgFTZ-F1, the box is 100% conserved compared with those of other insect homologs, with the exception of that of *B. mori* (Table 1). The FTZ-F1 box extends the DNA binding site of the protein and hence increases the binding specificity (Ueda et al., 1992), and also contains putative nuclear localization signals (Li et al., 1999). Given the high conservation of the DBD and the FTZ-F1 box in BgFTZ-F1, it was not surprising that the cockroach nuclear receptor bound to the recognition element PyCAAGGPyCPu, as happens with other insect FTZ-F1 homologs (Li et al., 2000; Ueda et al., 1992).

Expression and Regulation of BgFTZ-F1

According to our observations, the induction of BgFTZ-F1 during nymphal development of *B. germanica* always follows the ecdysteroid pulse that oc-

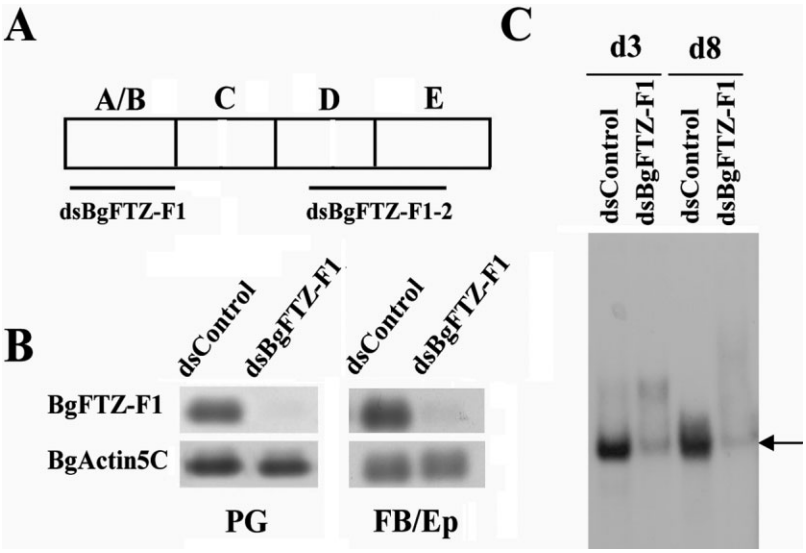

Fig. 6. RNAi in vivo of BgFTZ-F1 in sixth instar female nymphs of *Blattella germanica*. **A:** Scheme of BgFTZ-F1 domain organization showing the regions used to generate the two dsRNAs: dsBgFTZ-F1 and dsBgFTZ-F1-2. **B:** Effects of dsBgFTZ-F1 on mRNA levels of BgFTZ-F1. A dose of 1 μg of dsBgFTZ-F1 was injected in newly emerged sixth-instar female nymphs, and mRNA levels were measured 8 days later in the protoracic gland (PG) and in the abdominal segments with epidermis and fat body tissues (FB/Ep) by reverse transcriptase-polymerase chain reaction/Southern blotting. Equivalent experiments injecting a nonspecific sequence (dsControl) served as negative controls. BgActin5C levels were used as a reference. The Southern blots shown are representative of at least ten replicates. **C:** Electrophoretic mobility shift assay (EMSA) analysis of nuclear extracts of fat bodies from dsBgFTZ-F1- and dsControl-treated nymphs 3 and 8 days (d3 and d8, respectively) after the treatment. Equal amount of protein extracts were incubated with labeled F1RE as a probe.

curs at the end of each instar. This timing of induction has been observed during all instars in the holometabolous insects investigated so far, namely *D. melanogaster* (Yamada et al., 2000; Sullivan and Thummel, 2003), *M. sexta* (Weller et al., 2001), *B. mori* (Sun et al., 1994) and *A. aegypti* (Li et al., 2000). RNAi of the 20E-dependent nuclear receptor BgHR3 demonstrated that the highly precise timing of induction of BgFTZ-F1 at the end of each instar is promoted by BgHR3, which is expressed slightly before BgFTZ-F1 (see Fig. 1; Cruz et al., 2007). The HR3-dependent induction of FTZ-F1 has been also demonstrated in *D. melanogaster*, where β*FTZ-F1* expression is clearly reduced during the prepupal–pupal transition in a DHR3 mutant (Lam et al., 1999). Furthermore, DHR3 binds to three sites downstream of the transcription initiation site of the *ftz-f1* gene and mutations of these sites reduce the levels of expression of the gene in transgenic flies (Kageyama et al., 1997; Lam et al., 1997). Because BgHR3 is a direct target of 20E (Cruz et al., 2007), the results presented here confirm the occurrence of a genetic axis formed by the nuclear receptors BgEcR-BgRXR-BgHR3 and BgFTZ-F1 in *B. germanica*, which is therefore conserved in holometabolous insects.

Finally, our experiments in vitro incubating abdominal segments with epidermis and fat body tissues, have shown that 20E has a modest inhibitory effect on *BgFTZ-F1* expression, and that addition of Chx into the medium increases BgFTZ-F1 mRNA levels. The later result suggests that the expression of *BgFTZ-F1* is repressed by one ore more unstable repressors, maybe BgFTZ-F1 itself. Similar in vitro responses have been observed with previtellogenic fat bodies from the mosquito *A. aegypti* (Li et al., 2000).

BgFTZ-F1 Controls Molting

Using RNAi, we have demonstrated that the occurrence of BgFTZ-F1 at the end of each instar is required to complete the molting process. This function is conserved among holometabolous insects, as βFTZ-F1 regulates the transitions between larval instars in *D. melanogaster* (Yamada et al., 2000) and *T. castaneum* (Tan and Palli, 2008). Moreover, and as occurs in *B. germanica*, arrested larvae of βFTZ-F1 mutants of *D. melanogaster* showed duplication of ectodermal structures, such as two pairs of mouthhooks, anterior and posterior spiracles, double tubular tracheae, and double cuticle (Yamada et al., 2000). The dsBgFTZ-F1-treated nymphs arrested molting with the new adult cuticle well formed and the old nymphal endocuticle digested, which indicates that BgFTZ-F1 is mostly required during the last step of molting, probably by regulating the expression of genes controlling the ecdysis. In this sense, it is worth noting that in *D. melanogaster*, RNAi of β*FTZ-F1* in Inka cells, which produce pre-ecdysis and ecdysis triggering hormones, blocks the release of these hormones, resulting in ecdysis defects (Zitnan et al., 2007). Furthermore, ecdysis impairment observed in dsBgFTZ-F1-treated nymphs of *B. germanica* is indistinguishable from that showed by dsBgHR3-treated specimens (Cruz et al., 2007). This coincidence, in addition to the fact that BgHR3 is responsible for the activation of *BgFTZ-F1* at the end of the instar, indicates that the effects of BgHR3 during this stage of development are mainly channeled through BgFTZ-F1.

Remarkably, the functions of HR3 and FTZ-F1 are conserved not only in hemimetabolous and hemimetabolous insects, but also in the clade Ecdysozoa, because in the nematode *Caenorhabditis elegans*, the nuclear hormone receptors nhr-23 and nhr-25, the homologs of insect HR3 and FTZ-F1, respectively, also govern molting. As observed in insects, expression of nhr-23 and nhr-25 mRNAs oscillates with each molting cycle (Gissendanner et al., 2004) and RNAi of both receptors at the intermolt results in a defective molt, with associated problems of cuticle shedding (Ashaina et al., 2000; Gissendanner and Sluder, 2000). However, whereas HR3 controls the expression of FTZ-F1 in insects, *nhr-25* expression seems not dependent of nhr-23 (Krostrouchova et al., 2001).

TABLE 1. Percent Amino Acid Identity Among FTZ-F1 Homologs

Species	A/B	DBD	FTZ-F1 box	Hinge	LBD
Tribolium castaneum	19	98	100	53	86
Apis mellifera	6	98	100	48	82
Drosophila melanogaster	7	98	100	33	59
Aedes aegypti	4	96	100	44	79
Anopheles gambiae	8	96	100	46	78
Manduca sexta	13	96	100	43	66
Bombyx mori	15	94	93	42	58
Metapenaeus ensis	11	95	100	27	49
Homo sapiens (SF-1)[a]	1	86	92	13	35
Homo sapiens (LRH-1)[a]	9	86	96	12	45
Caenorhabditis elegans	2	81	78	12	21
Schistosoma mansoni	7	63	44	10	17

[a]The human nuclear receptors showing the highest percentage amino acid identity within the DNA-binding domain (DBD) and ligand-binding domain (LBD) with the insect FTZ-F1. SF1, steroidogenic factor 1; LRH-1, liver receptor homolog 1.

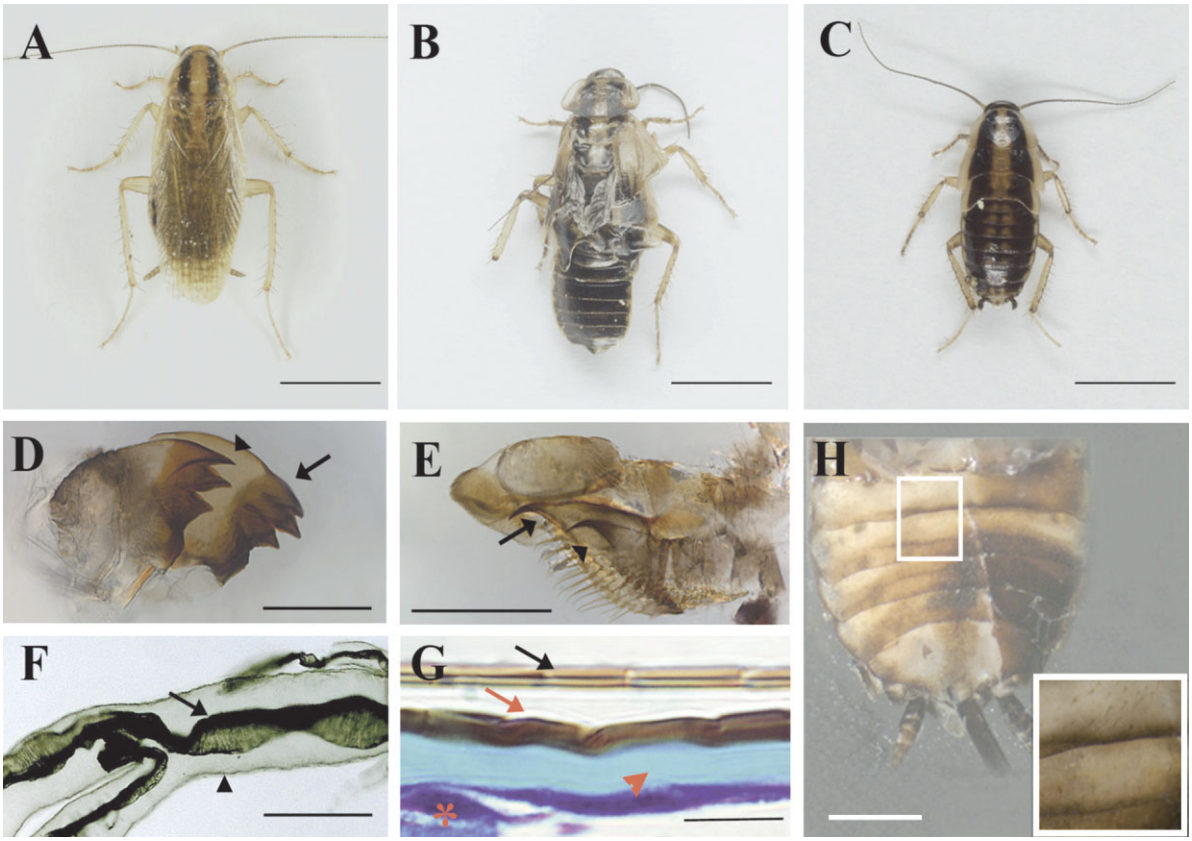

Fig. 7. Effect of RNAi-mediated knockdown of BgFTZ-F1 on the imaginal molt of *Blattella germanica*. Sixth-instar female nymphs were injected with 1 μg of dsBgFTZ-F1 or with dsControl at day 0 and left until the imaginal molt, 8 days later. **A:** dsControl specimen 1 day after the imaginal molt, showing a normal winged adult shape. **B,C:** dsBgFTZ-F1-treated specimens at the same time point arrest development showing incomplete shedding of the nymphal cuticle (B) or completely arresting development without molting (C). **D–F:** The arrested specimens show duplication of cuticular structures (nymphal structures indicated with arrows, and adult structures with arrowheads), like double mandibles (D), double laciniae (E), and two superimposed trachea (F). **G:** Cuticle layers of an arrested specimen showing the nymphal epicuticle (black arrow) above the adult endocuticle (red arrowhead) and epicuticle (red arrow) as well as the epidermis (asterisks). **H:** Abdomen of an 8-day-old arrested dsBgFTZ-F1 treated nymph. The left half of the nymphal cuticle has been removed to show the occurrence of the new adult cuticle already tanned and sclerotized. The insert shows a detail of the adult cuticle with well formed adult bristles. Scale bars = 5 mm in A–C, 500 μm in D,E, 200 μm in F, 50 μm in G, 1.7 mm in H.

Developmental Dynamics

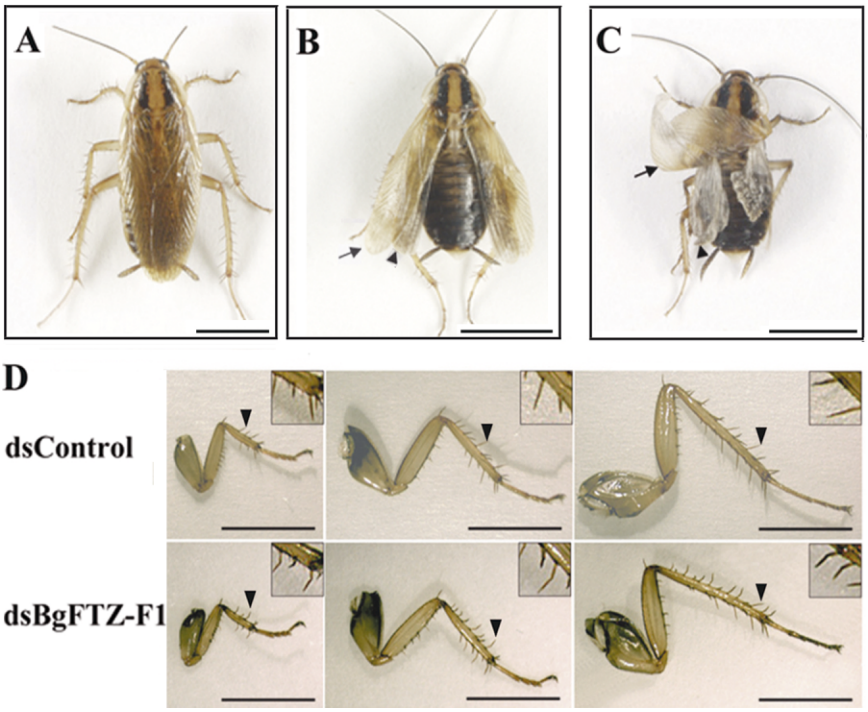

Fig. 8. Effect of RNAi-mediated knockdown of BgFTZ-F1 on adult *Blattella germanica*. **A:** dsControl specimen 1 day after the imaginal molt, showing a normal winged adult shape. **B,C:** dsBgFTZ-F1-treated nymph 1 day after the imaginal molt showing defects of wing extension in both the forewings (arrow) and hindwings (arrowhead). Wing extension phenotypes vary from moderately (B) to severe (C) in both wing pairs. **D:** BgFTZ-F1 knockdown specimens display bristle defects in the three pair of legs (arrowheads in upper panels), compared with well formed leg bristles of dsControl-treated specimens (arrowheads in lower panels). Inserts show in more detail the bristles of each leg represented. Scale bars = 5 mm in A–C, 2.5 mm in D.

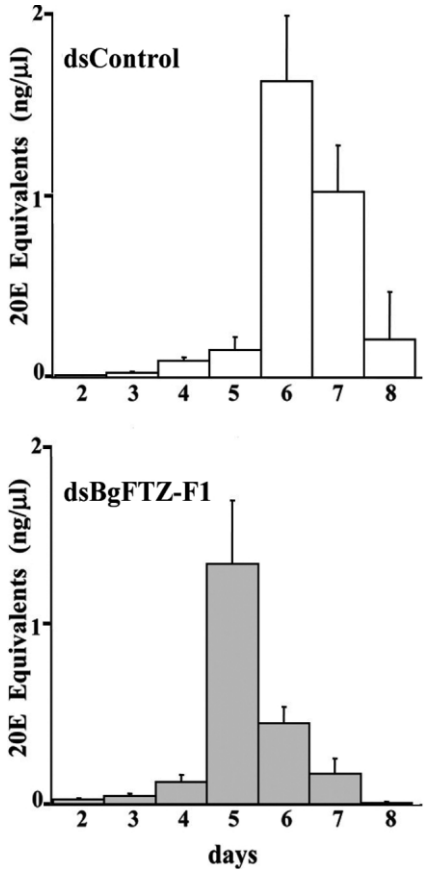

Fig. 9. Effect of RNAi-mediated knockdown of BgFTZ-F1 on ecdysteroid levels in *Blattella germanica*. Hemolymph from dsBgFTZ-F1 and dsControl-treated nymphs was collected between days 2 and 8, and ecdysteroid levels were determined by enzyme-linked immunosorbent assay. Results are expressed as nanograms of 20E equivalents. Vertical bars indicate the SEM (n = 8–18).

BgFTZ-F1 Controls the Timing of Ecdysteroid Production

In *B. germanica*, BgFTZ-F1 is expressed at significant levels, in terms of mRNA and protein, during the first days of nymphal instars (Figs. 3, 4). The fact that nymphs with interfered BgFTZ-F1 display a normal ecdysteroid pulse, although advanced 24–36 hr with respect to controls, suggests that the main function of BgFTZ-F1 at the intermolt is to control the timing of the ecdysteroid peak. This represents a significant novel function of this nuclear receptor, which has not been described in *D. melanogaster*. In a previous report, we had demonstrated that BgEcR-A is necessary for the normal rise of circulating ecdysteroids at day 5 of the last nymphal instar of *B. germanica* (Cruz et al., 2006). Taken together, the results suggest that during the nymphal development of *B. germanica*, BgFTZ-F1 acts antagonistically with BgEcR-A, with BgEcR-A inducing and BgFTZ-F1 repressing the onset of the ecdysteroid pulse. In holometabolous insects, the signal for the initiation of massive ecdysteroid production during immature stages by the prothoracic glands is the prothoracicotropic hormone (PTTH) produced in the brain (see Gilbert, 2004; McBrayer et al., 2007). Although the existence of a PTTH controlling developmental timing in hemimetabolous insects cannot be ruled out, no *PTTH* gene has been identified in insects outside Diptera and Lepidoptera. Interestingly, it has been shown recently that PTTH production is not essential for molting in *D. melanogaster*, and that loss of PTTH during larval development results in remarkably delayed larval development between molts (McBrayer et al., 2007), resembling the molting timing of an hemimetabolous insect.

Although the timing of ecdysteroid production is clearly altered in BgFTZ-F1 knockdowns, the pulse of ecdysteroids was otherwise as in controls. In *D. melanogaster*, βFTZ-F1 regulates the expression of genes involved in ecdysteroid biosynthesis in the ring gland during larval development, and using clonal analysis, levels of the steroidogenic enzymes phantom and disembodied were reduced in FTZ-F1 mutant ring glands (Parvy et al., 2005). However, the authors did not report direct measurements of ecdysteroid levels in the mutant animals, making difficult to establish a direct relation between FTZ-F1 and the ecdysteroid titer. In this sense, it has been shown that low levels of phantom and disembodied do not impair the production of significant amounts of ecdysteroids and the formation of the pupae (McBrayer et al., 2007). Recently, it has also been shown that reduced levels of SUMO (a

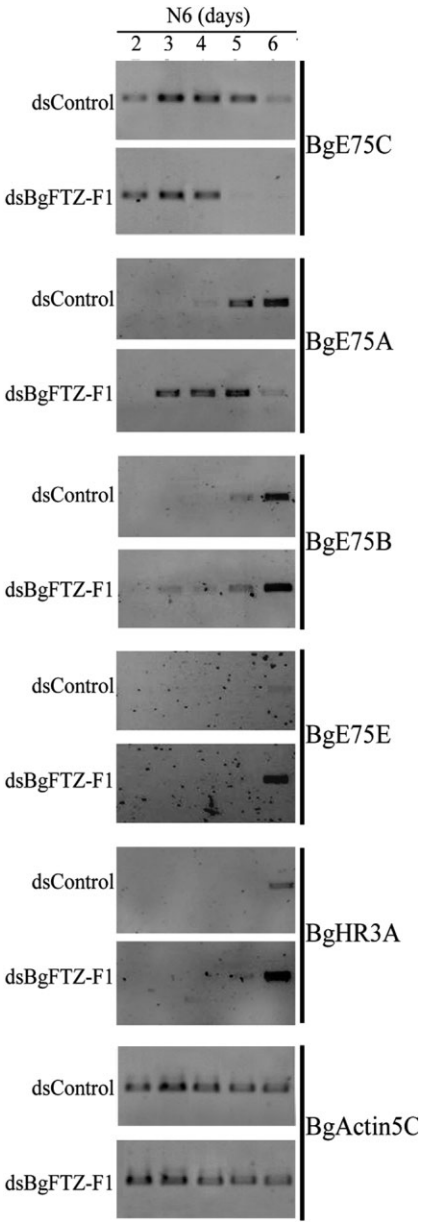

Fig. 10. Premature expression of 20E-regulated genes in dsBgFTZ-F1-treated sixth-instar nymphs of *Blattella germanica*. A dose of 1 μg of dsBgFTZ-F1 or dsControl was injected in newly emerged sixth instar female nymphs, and mRNA levels of several 20-E dependent genes were analyzed in the prothoracic gland of 2- to 6-day-old nymphs by reverse transcriptase-polymerase chain reaction/Southern blotting using specific probes. BgActin5C levels were used as a reference. The Southern blots shown are representative of three replicates.

small ubiquitin-related modifier of target proteins) in the ring gland cells of *D. melanogaster* resulted in lower levels of βFTZ-F1 and disembodied proteins in this gland (Talamillo et al., 2008). Remarkably, these animals were ecdysteroid-deficient and arrested development after an extended third larval instar, although the deficiency in ecdysteroid synthesis was related to an impaired cholesterol uptake in the prothoracic glands rather than to the reduced levels of ecdysteroid enzymes. Work directed to clone and characterize key ecdysteroidogenic enzymes in *B. germanica* to establish regulatory relationship between BgFTZ-F1 and the synthesis of ecdysteroids is currently in progress in our laboratory.

FTZ-F1 has been defined as a transcriptional competence factor of 20E action in dipterans. In β*FTZ-F1* mutants of *D. melanogaster*, the expression of the 20E-dependent genes *Br-C*, *E74A*, *E75A*, and *E93* is clearly attenuated during the prepupal–pupal transition (Broadus et al., 1999). Furthermore, βFTZ-F1 plays the role of competence factor in adult fat bodies during the previtellogenic-vitellogenic transition of *A. aegypti*. In βFTZ-F1 knockdown adult mosquitoes, activation of several early genes, like *EcR-B*, *E74B*, and *E75A*, as well as the *vitellogenin* gene, a direct target of 20E (Martín et al., 2001b), was clearly reduced in vivo at the onset of the vitellogenic cycle (Zhu et al., 2003). Conversely, we have not observed a transcriptional competence activity of BgFTZ-F1 in relation to 20E signaling during the nymphal development of *B. germanica*. In the absence of BgFTZ-F1, the induction of the early and early–late genes tested (*BgE75A*, *BgE75B*, *BgE75C*, *BgE75E*, and *BgHR3A*), although advanced in time, was not otherwise affected (Fig. 10). Conversely, the timing of activation and repression of 20E-dependent genes characterized in *B. germanica* is almost identical during nymphal–nymphal and nymphal–adult transitions. This suggests that there are no stage-specific responses to 20E in hemimetabolous and, therefore, that the role of βFTZ-F1 as a competence factor for 20E action must have been an innovation of more modified, holometabolous insects.

In summary, we have further characterized the 20E-triggered genetic response during development of hemimetabolous insects by cloning and functionally characterizing the nuclear hormone receptor BgFTZ-F1. Data presented here, combined with previous results obtained in our laboratory, show that the genetic module formed by BgEcR-A, BgRXR, and BgHR3 converge, at the end of a nymphal stage, on the expression of BgFTZ-F1, which in turn controls the last steps of molting. Importantly, the genetic axis, as well as its function regarding molting, is highly conserved in holometabolous insects. Conversely, the role of BgFTZ-F1 in controlling the onset of production of ecdysteroids at mid-nymphal stages has only been reported in our insect model. These results suggest that different nuclear receptors of the 20E-triggered hierarchy could act as positive as well as negative regulators of the dynamics of ecdysteroid production during nymphal development and hence on developmental timing in hemimetabolous insects. Work directed to characterize this regulatory functions of nuclear receptors is currently in progress in our laboratory.

EXPERIMENTAL PROCEDURES

Insects

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

Cloning of BgFTZ-F1 cDNA

The *B. germanica* FTZ-F1 homologue was obtained by PCR using a cDNA template from 20E-treated UM-BGE-1 cells, as described by Maestro et al. (2005). Degenerate primers were designed on the basis of conserved DBD sequences of insect homologues, as follows: forward, 5′-TGYGARTC-NTGYAARGG-3′; reverse, 5′-AR-RCAYTTYTGRAANCGRCA-3′. The amplified fragment (134 bp) was subcloned into the pSTBlue-1 vector (Novagen) and sequenced. This was followed by 5′- and 3′-rapid amplification of cDNA ends (5′- and 3′-RACE System Version 2.0; Invitrogen) to complete the sequence. For 5′-RACE, the reverse primer was 5′-CGACGCATGTGTA-GACCTTCTT-3′, and for 3′-RACE, the forward primer was 5′-TTCTTCAA-GAGGACAGTGCAA-3′. All PCR prod-

TABLE 2. Features of the dsRNAs Designed to Specifically Interfere BgFTZ-F1 and BgHR3 of *Blattella germanica*

dsRNA	DNA fragment	Region targeted
dsBgFTZ-F1	502 bp	A/B and DBD domains (nucleotide 138 to 639 in BgFTZ-F1; accession no. FM163377)
dsBgFTZ-F1-2	601 bp	Hinge and LBD domains (nucleotide 1482 to 2081 in BgFTZ-F1; accession no. FM163377)
dsBgHR3	411 bp	DBD and hinge domains (nucleotide 400 to 808 in BgHR3A; accession no. AM259128)

ucts were subcloned into the pSTBlue-1 vector (Novagen) and sequenced.

Translation In Vitro

The entire BgFTZ-F1 ORF was amplified by PCR using Accutaq polymerase (Sigma) and the primers: forward (FTZ-F1-F3), 5′-TGTCCTACAGACCTACATTTATACT-3′; and reverse (FTZ-F1-R3), 5′-GGACTTTGTCAACAATTCATTTCAGT-3′, which spans the stop codon. A fragment of the expected size (2366 bp) was subcloned into pSTBlue-1 (Novagen) and sequenced. The BgFTZ-F1 cDNA was transcribed and translated using the TNT coupled reticulocyte lysate system (Promega), according to the manufacturer's instructions.

BgFTZ-F1 Antibody Production

The cDNA fragment encompassing the A/B domain of BgFTZ-F1 was subcloned into the pET14b vector (Novagen) to produce a histidine-tag fusion protein. Using the cloned BgFTZ-F1 full-length cDNA as template, the fragment was amplified by PCR with primers: forward (FTZ-F1-F4), 5′-CTCGAGCATGAGGAGTCAACCAGTAGG-3′; and reverse (FTZ-F1-R4), 5′-CTCGAGCTCTTCGATGCCCTCCTTCGT-3′. *Escherichia coli* cells (BL21) were transformed with the resultant plasmid and the synthesis of the recombinant protein was induced by adding 1 mM IPTG, and then purified using Ni chelation resins according to the manufacturer's instructions (Quiagen). Polyclonal antibodies were raised against the bacterially expressed fusion protein in New Zealand White rabbits. For validation of the antibody, a Western blot was carried out using in vitro produced BgFTZ-F1 protein. A single band of the expected size (65.5 kDa) was obtained.

Fat Body Nuclear Extracts and EMSA

Preparations of nuclear extracts from *B. germanica* fat bodies were carried out according to the method described by Miura et al. (1999) and Martín et al. (2001a). Twenty fat bodies were used for each time-point extraction. Binding reactions were carried out in a 20 μl volume containing 1 fat body equivalent of nuclear extracts or 1 μl of the TNT sample, 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM$MgCl_2$, 0.5 mM DTT, 0.5 mM EDTA, 4% (v/v) glycerol, 1 μg poly(dI.dC), 1 μg of a single-stranded DNA (5′-TAATACGACTCACTATA-3′), and the indicated amount of competitor DNA or antibody when appropriate. After 15 min incubating at 4°C, 0.05 pmol of ^{32}P-labeled DNA probe was added, and the incubation was continued for another 45 min at the same temperature. The reaction was resolved on 5% nondenaturing polyacrilamide gel run at 4°C and at a constant voltage of 150 V in 0.5 × TBE. The gel was then dried and autoradiographed. Oligonucleotides (only sense strands are shown) used to generate the DNA probe for EMSA were: (F1RE) 5′-GCAGCACCGTCTCAAGGTCGCCGAGTAGGAGAACC-3′.

RT-PCR/Southern Blot Analyses

Given the extremely low expression levels of transcription factors, a semiquantitative RT-PCR/Southern blot approach was used (Maestro et al., 2005; Mané-Padrós et al., 2008). Total RNA was extracted from different tissues using the GenElute Mammalian Total RNA kit (Sigma). A sample of 0.3–1 μg of each RNA preparation was used for cDNA synthesis, as previously described (Maestro et al., 2005). For BgFTZ-F1 mRNA detection, cDNA samples were subjected to PCR amplification with several cycles within the linear range of amplification at 94°C for 30 sec, 64°C for 30 sec, and 72°C for 45 sec. Primers used for BgFTZ-F1 detection were as follows: forward (BgFTZ-F1-F5), 5′-TATAGTCTACACAGCGAATCAGA-3′ and reverse (FTZ-F1-R5), 5′-GTACATCGGGCCGAATTTGTTTCT-3′; primers used for the amplification of the different nuclear receptors were as described in Cruz et al. (2006, 2007), Maestro et al. (2005), and Mané-Padrós et al. (2008). As a reference, the actin5C transcript of *B. germanica* was amplified by PCR using the same cDNA template samples (Maestro et al., 2005). Southern blot probes were generated by PCR with the same primer pairs, using plasmid DNAs containing the corresponding cDNA clones as a template. 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, indicating that there was no genomic contamination.

RNA Interference

RNAi in vivo in sixth instar nymphs of *B. germanica* was performed as described by Martín et al. (2006) and Cruz et al. (2007). The regions selected to generate templates by means of PCR for transcription of the dsRNAs are described in Table 2. A volume of 1 μl of each dsRNA solution (1

μg/μl) was injected into the abdomen of newly emerged sixth instar female nymphs.

Microscopy

All dissections were carried out in Ringer's saline. Mouthparts and tracheae were directly immersed in 50% glycerol and examined microscopically. To study cuticle layers, a portion of abdominal ventral cuticle was fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Cuticle sections (6 μm) were stained with toluidine blue. All samples were examined with a Zeiss Axiophot microscope.

Quantification of Ecdysteroids

Hemolymph ecdysteroids were quantified by enzyme-linked immunosorbent assay following the procedure described by Porcheron et al. (1989), and adapted to *B. germanica* by Pascual et al. (1994) and Romañá et al. (1995). The ecdysteroid antiserum has the same affinity for ecdysone and 20E (Porcheron et al., 1989), but because the standard curve was obtained with the latter compound, results are expressed as 20E equivalents.

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