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Differential expression of two RXR/ultraspiracle isoforms during the life cycle of the hemimetabolous insect *Blattella germanica* (Dictyoptera, Blattellidae)

Oscar Maestro, Josefa Cruz, Núria Pascual, David Martín *, Xavier Bellés *

Department of Physiology and Molecular Biodiversity, Institut de Biologia Molecular de Barcelona (CSIC), Jordi Girona 18, 08034 Barcelona, Spain

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Abstract

In insects, the molecular basis of ecdysteroid action has been analysed in great detail in flies and moths, but rarely in primitive orders. Using the primitive hemimetabolous insect *Blattella germanica*, the German cockroach, as a model, we isolated two cDNAs of RXR/USP, a component of the heterodimeric ecdysone receptor. These two cDNAs correspond to two isoforms, named BgRXR-S (short form) and BgRXR-L (long form). Both are identical except for a 23-amino acid deletion/insertion located in the loop between helices H1 and H3 of the ligand-binding domain. Pattern expression studies show that the two isoforms are differentially expressed throughout the life cycle of *B. germanica*. During embryogenesis, BgRXR-L occurs in early embryos, whereas BgRXR-S is highly expressed in middle and late embryogenesis. In the penultimate and last larval instars, BgRXR-S mRNA is the predominant form in the fat body and in the prothoracic gland. In the adult female, BgRXR-S mRNA predominates in the fat body, whereas BgRXR-L mRNA predominates in the ovary. Experiments performed with fat body and embryo cells incubated in vitro showed that the expression of BgRXR-S and BgRXR-L is not affected by 20-hydroxyecdysone or by juvenile hormone III.

Keywords: Ultraspiracle; Ecdysone receptor; *Blattella germanica*; Nuclear receptor; RXR; Juvenile hormone

1. Introduction

Most of the critical events occurring in an insect's life are controlled by ecdysteroid hormones, mainly by the form 20-hydroxyecdysone (20E). The molecular basis of 20E action has been clearly established in the fruit fly, *Drosophila melanogaster*, in relation to moulting and metamorphosis (reviewed in Thummel, 1995, 1996; Kozlova and Thummel, 2000), and in critical morphogenetic events of embryogenesis, such as germ band retraction and head involution (Kozlova and Thummel, 2003). The functional receptor for 20E is a heterodimer of two proteins belonging to the nuclear receptor gene family, the ecdysone receptor (EcR) and the retinoid X receptor (RXR)-homologue ultraspiracle (USP) (Yao et al., 1993). The heterodimer EcR-RXR/USP binds to sequence-specific DNA elements in target genes and directly induces the expression of a number of primary response genes, including a set of transcription factors, E75, E74 and Broad-complex among others. In turn, these genes amplify the effect of 20E by triggering the expression of a large battery of secondary-responsive genes (reviewed in Thummel, 1995, 1996; Riddiford et al., 2001).

This genetic hierarchy has been characterized in detail, not only with regard to the moulting process and pupal development of *D. melanogaster* and *Manduca sexta* (Sullivan and Thummel, 2003; Kozlova and Thummel, 2003), but also in vitellogenesis of mosquitoes and in oogenesis of silk moths and mosquitoes (reviewed in Raikhel et al., 2002; Swevers and Iatrou, 2003). The pleiotropic functions of 20E

Abbreviations: EcR, ecdysone receptor; DBD, DNA-binding domain; 20E, 20-hydroxyecdysone; JH III, juvenile hormone III; LBD, ligand-binding domain; RXR, retinoid X receptor; USP, ultraspiracle

* Corresponding authors. Tel.: +34 934006124; fax: +34 932045904.
E-mail addresses: dmcagr@cid.csic.es (D. Martín), xbragr@cid.csic.es (X. Bellés).

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are performed by several EcR and RXR/USP isoforms expressed with different developmental and tissue specificities (reviewed in Riddiford et al., 2001). Nevertheless, most of the EcR and RXR/USP sequences described so far were isolated from species belonging to the most derived insect orders, such as Diptera and Lepidoptera. In addition to these orders, within the holometabolous insects, RXR/USP has been reported in the coleopteran *Tenebrio molitor* (Nicolaï et al., 2000) and in the hymenopteran *Apis mellifera* (Barchuk et al., 2004). Within the primitive hemimetabolous species, only two isoforms of RXR/USP have been completely sequenced in the locust, *Locusta migratoria* (Hayward et al., 1999, 2003). Indeed, data on the molecular action of 20E in hemimetabolous insects are very scarce, which hinders the analysis of functional relationships between evolutionarily distant species. In addition, recent comparative analyses have shown that EcR and RXR/USP from Diptera and Lepidoptera have co-evolved during the course of holometabolous insect diversification, possibly leading to a functional divergence of the receptor (Bonneton et al., 2003). Hence, results on the mode of action of 20E obtained from insects such as *D. melanogaster* or *M. sexta* might not be extended to primitive hemimetabolous insects.

As a first step towards the clarification of the role of 20E in embryonic and post-embryonic processes (moulting and reproduction) in hemimetabolous species, we have cloned and characterized two cDNAs encoding RXR/USP homologues, called BgRXR-S and BgRXR-L, in the German cockroach, *Blattella germanica*. In addition to the structural and phylogenetic analysis, we characterized the spatial and temporal expression of both mRNAs during embryogenesis, moulting and reproduction, and studied the effect of 20E and juvenile hormone III (JH III) on their expression.

2. Experimental procedures

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared in the dark at 30 ± 1 °C and 60–70% r.h. Freshly ecdysed fifth or sixth instar larvae or adult females were selected and used at appropriate ages. All dissections and tissue sampling were carried out on carbon dioxide-anaesthetized specimens.

2.2. Cloning of RXR/USP cDNAs

Total RNA was isolated with the GenElute™ Mammalian Total RNA kit (Sigma) and cDNAs were obtained as described (Cruz et al., 2003). Degenerate primers based on the sequences of the DNA-binding domain (DBD) of RXR/USP insect homologues were used for PCR to obtain a *B. germanica* homologue cDNA fragment. PCR amplification was performed using, as a template, cDNA generated by reverse transcription from polyA$^+$ RNA obtained from the UM-BGE-1 cell line derived from *B. germanica* embryos (see below). The primers for BgRXR amplification were as follows: forward (RXR-dF1), 5′-TGYGARGGNTGYAARGG-3′; and reverse (RXR-dR1), 5′-ARRCAYTTYTGRTANCGRCA-3′. Amplification was carried out for five cycles at 94 °C for 30 s, 52 °C for 1 min, 72 °C for 1 min, and for 35 cycles of 94 °C for 30 s, 55 °C for 1 min and 72 °C for 1 min. The amplified fragment (129 bp) was sub-cloned into the pSTBlue-1 vector (Novagen) and sequenced. To complete the BgRXR cDNA sequences, 5′- and 3′-rapid amplifications of cDNA ends (RACE) were applied to polyA$^+$-RNA extracted from UM-BGE-1 cells and ovaries using a 5′- and 3′-RACE system Version 2.0 (Invitrogen), according to the manufacturer's instructions. For 5′-RACE, reverse primers were as follows: RXR-R1 (position 514–494; Fig. 1), 5′-CTCCCGGCAGGCGTACGACAA-3′, and nested RXR-R2 (position 487–467; Fig. 1), 5′-GCGCACTGTCCTCTTGAAGAA-3′. For 3′-RACE, forward primers were as follows: RXR-F1 (position 70–95; Fig. 1), 5′-GGTGCTAATTAGTTACTGGGTGATTA-3′; and nested forward RXR-F2 (position 1124–1147; Fig. 1), 5′-AGCCAGGAAGTGGAACTTCTTCGA-3′. PCR products were sub-cloned into the pSTBlue-1 vector (Novagen) and sequenced from both the directions. Two RXR/USP sequences were obtained, a short one called BgRXR-S and a long one called BgRXR-L.

2.3. Translation in vitro

To obtain the entire BgRXR-S and -L open reading frames (ORFs), a new primer pair combination was used, the forward primer RXR-F1 and a new reverse one (RXR-R3): 5′-AGCGAATGAATTAAGCATCAGAGGA-3′ (position 1367–1343; Fig. 1A), which contains the stop codon in the C-terminal region. Template cDNA was generated from total RNA of UM-BGE-1 cells for BgRXR-S amplification, and from ovarian tissues for BgRXR-L amplification. cDNAs were PCR-amplified with Accutaq polymerase (Sigma) with 40 cycles of 30 s at 94 °C, of 30 s at 62 °C and of 2 min at 68 °C. Two fragments of the expected sizes, 1298 and 1367 bp, were sub-cloned into pSTBlue-1. These cDNAs were transcribed and translated with the TNT coupled reticulocyte lysate system (Promega, according to the manufacturer's instructions).

2.4. Cloning of BgActin5C cDNA

First, a fragment of *actin* 5*C* gene of *B. germanica* was amplified using degenerate primers designed on the basis of conserved sequences from *M. sexta* and *D. melanogaster.* Then, using specific primers by RT-PCR, a partial 308 bp cDNA clone was obtained. These primers were as follows: forward (AcF1), 5′-TCGTGACATCAAGGAGAAGCT-3′, and reverse (AcR1), 5′-TGTCGGCAATTCCAGGGTACA-TGGTGGT-3′. These primers were combined with cDNA synthesized from total RNA from UM-BGE-1 cells and

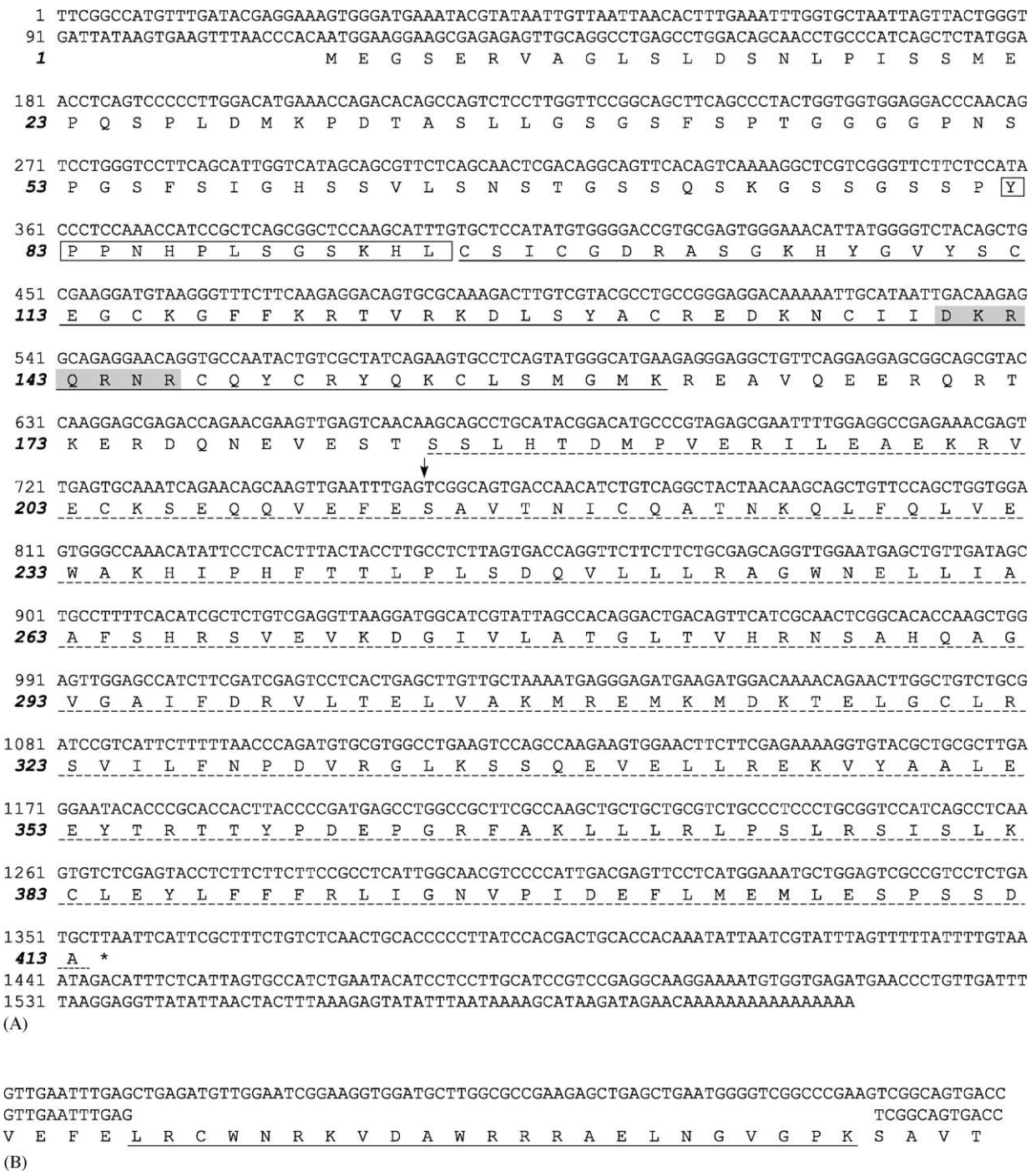

Fig. 1. Nucleotide and deduced amino acid sequence of the BgRXR isoforms of *B. germanica*. (A) BgRXR-S amino acid sequence is shown below the nucleotide sequence. The DNA-binding domain (DBD) is underlined and the ligand-binding domain (LBD) is underlined with dashes. The 12 amino acids motif conserved in all RXR/USPs located upstream from the DBD is boxed. The putative nuclear localization signal is shaded. The location of the insertion present in the longer BgRXR-L isoform is indicated by an arrow. (B) Nucleotide and amino acid sequence of the insertion specific to the BgRXR-L isoform (underlined).

used for PCR amplification under the conditions: 94 °C for 30 s, 68 °C for 30 s and 72 °C for 60 s, for 40 cycles. The final product was sub-cloned into the pSTBlue-1 vector (Novagen) and sequenced (GenBank accession number AJ862721). The deduced amino acid sequence showed high identity with the *M. sexta* (86% identity) and *D. melanogaster* (85% identity) equivalent portion of the actin genes.

2.5. Phylogenetic analyses

We used the arthropodan species in which the ligand-binding domain (LBD) of RXR/USP was completely sequenced. In addition to that of *B. germanica* described herein, the sequences were: *D. melanogaster* (GenBank accession number: P20153), *Lucilia cuprina* (AAG01569), *Chironomus tentans* (AAC03056), *Aedes aegypti* (AAG24886), *Aedes albopictus* (AAF19033), *Choristoneura fumiferana* (AAC31795), *Bombyx mori* (S44490), *M. sexta* (P54779), *Chilo suppressalis* (BAC53670), *Heliothis virescens* (CAD28568), *Xenos pecki* (AY827155), *L. migratoria* (AAF00981), *A. mellifera* (AAF73057), *Polistes fuscatus* (AY827156), *T. molitor* (CAB75361), *Uca pugilator* (AAC32789) and *Amblyomma americanum* (RXR1: AAC15588; RXR2: AAC15589). As a reference, the following vertebrate sequences were used: *Homo sapiens* (RXRα: CAA36982; RXRβ: AAA60293; RXRγ: AAA80681) and *Xenopus laevis* (RXRα: P51128; RXRβ: S73269; RXRγ: P51129). The tree was rooted using the cnidarian *Tripedalia cystophora* sequence (AAC80008) as outgroup. Protein sequences were aligned using ClustalX (Thompson et al., 1997). Poorly aligned positions and divergent regions were eliminated using Gblocks 0.91b (Castresana, 2000) following block parameters by default or using the option "with half" in the parameter "allowed gap positions", which resulted in 153 and 181 final positions, respectively. The obtained alignments were analysed by the PHYML program (Guindon and Gascuel, 2003) based on the maximum-likelihood principle with the model of amino acid substitution reported by Jones et al. (1992). Four substitution rate categories optimizing the gamma shape parameter were used. The data sets were bootstrapped for 100 replicates using PHYML.

2.6. Developmental RT-PCR analyses

Temporal profiles of transcript abundance for the two BgRXR isoforms were carried out using RT-PCR followed by Southern blotting with specific probes. Procedures for RNA extraction and cDNA synthesis were as previously described (Cruz et al., 2003). PCR amplification of the two BgRXR isoforms was carried out simultaneously in a single reaction containing the following primers: forward (RXRF3, position 527–549; Fig. 1), 5′-ATAATTGACAAGAGGCAGAGGAA-3′; and reverse (RXRR4, position 858–836; Fig. 1), 5′-TGGTCACTAAGAGGCAAGGTAGT-3′. Thermal cycling conditions were as follows: 34–42 cycles at 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min. The weight of the amplified fragments was in agreement with the expected lengths: 346 bp for BgRXR-S and 415 bp for BgRXR-L. To discard the possibility of preferential amplification, we performed PCRs using as template equal amounts of plasmids containing full-length BgRXR-S and BgRXR-L at increasing non-saturating number of cycles; results (not shown) indicated that amplification kinetics was identical in both the isoforms. As a control of all expression patterns, the same cDNAs were subjected to RT-PCR/Southern blotting with a primer pair specific to *B. germanica* actin5C (AcF1 and AcR1, see above). Amplification of actin5C gave the expected 308 bp band, although in a number of cases (especially in embryos) another slightly heavier, unspecific band appeared. cDNA probes for Southern blot analyses were generated by PCR with the same primer pairs, using plasmid DNAs containing the corresponding cDNA clones as a template. The probes were labelled with fluorescein by the Gene Images random prime-labelling 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.

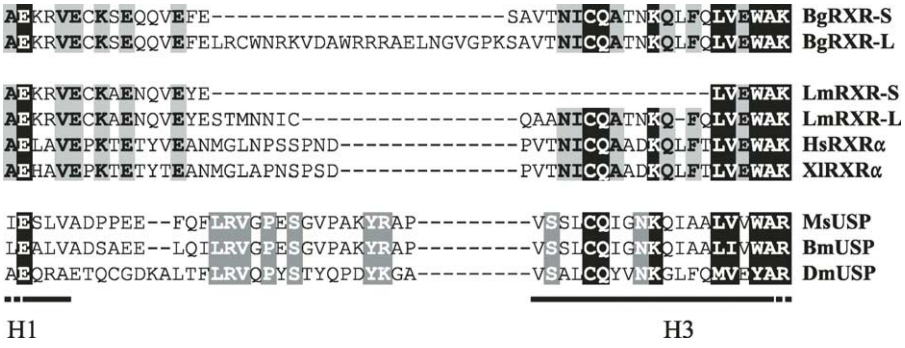

Fig. 2. Comparison of the LBD sequences of the BgRXR-S and BgRXR-L isoforms of *B. germanica* with other species. The region from helices H1 to H3 including the loop between the two helices is aligned with the homologous region of the two isoforms from *Locusta migratoria* (LmRXR-S and LmRXR-L), *Homo sapiens* (HsRXRα) and *Xenopus laevis* (XlRXRα), as well as with *Manduca sexta* USP (MsUSP), *Bombyx mori* (BmUSP) and *Drosophila melanogaster* (DmUSP). Conserved sequences are shown in boxes. Regions corresponding to helices H1 and H3 (with reference to *H. sapiens*) are underlined.

2.7. Culture of UM-BGE-1 cells in vitro

UM-BGE-1 is a 20E-responsive cell line derived from 4- to 5-day-old embryos of *B. germanica* (Kurtti and Brooks, 1977). UM-BGE-1 cells, kindly provided by T. Kurtti, were maintained at 25 °C in Leibovitz-15 medium (Sigma) supplemented as recommended in Munderloh and Kurtti (1989). For hormonal treatments, 10^6 cells ml^{-1} were seeded into 24-well cell-culture clusters (Costar). JH III (Sigma) was dissolved in acetone and 20E (Sigma) in water with 10% ethanol, and added to the media in a total volume of 10 μl. The same volume of solvent was added as control.

2.8. Quantification of ecdysteroids in embryos

Staged oothecae were stored in methanol at −20 °C for ecdysteroid extraction. Oothecae were homogenized, rinsed with 100 μl of methanol, sonicated and centrifuged at 10,000 × *g* for 10 min. The supernatant was stored and the pellet was re-extracted twice in the same way. The supernatants were pooled and ecdysteroids were quantified by ELISA following the procedure previously described in Porcheron et al. (1989) and subsequently adapted to *B. germanica* by Pascual et al. (1992) and Romañá et al. (1995). 20E (Sigma) and 20E–acetylcholinesterase (Cayman) were used as standard and enzymatic tracer, respectively. The antiserum (AS 4919, supplied by P. Porcheron) was used at a dilution of 1/50,000.

3. Results

3.1. Cloning and characterisation of RXR/USP

BgRXR cDNAs in *B. germanica* were cloned by a RT-PCR approach using degenerate primers designed on the basis of conserved sequences of the DBD of a number of RXR/USP sequences from different insect species. Using cDNA obtained from the UM-BGE-1 cells as a template, a 129 bp PCR fragment was obtained. The sequence of this fragment was very similar to other insect RXR/USPs. To obtain full-length cDNAs, 3′-RACE and 5′-RACE methods were used, with cDNA obtained from the UM-BGE-1 cells and ovarian tissue. Two complete sequences, 1589 and 1658 bp-long, were obtained, which encoded proteins of 413 and 436 amino acids with predicted molecular masses of 46.0 and 48.6 kDa, respectively (Fig. 1A). BLAST database search of these two cDNAs indicated that both encoded *B. germanica* orthologues of RXR/USP. They were called BgRXR-S (short form, GenBank accession number AJ854489) and BgRXR-L (long form, GenBank accession number AJ854490). Both proteins are identical except for an insertion/deletion of 23-amino acids in the loop between helix H1 and H3 within the LBD (Fig. 1B). The putative start codon is preceded by an in-frame stop codon, indicating that these sequences represent full-length ORFs. The 5′ and 3′ UTR sequences of the two isoforms are identical, which suggests that they derive from the same gene. 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 proteins synthesised in vitro corresponded closely to their expected molecular size of 46.0 and 48.6 kDa. Amino acid sequence comparisons revealed that these cDNAs included the five-domain structure characteristic of all members of the nuclear hormone receptor superfamily, i.e. a ligand-independent A/B activation domain (amino acids 1–94), a two-zinc-fingered DBD (C domain, 95–160), a hinge region (D domain, 161–183) and an LBD (E domain, 184–end) containing the putative ligand-dependent activation domain AF-2 (FLMEMLE). Interestingly, the loop between helices H1 and H3, where locates the insertion/deletion, is highly conserved in Diptera and Lepidoptera, but quite divergent in length and sequence in other arthropods and in vertebrate RXRs (Fig. 2).

3.2. Sequence comparison and phylogenetic analysis

Maximum-likelihood analysis using the LBD region of RXR/USP sequences gave the tree shown in Fig. 3. Using the sequence of the cnidarian *T. cystophora* RXR as outgroup, the topology of the resulting tree closely follows the current phylogeny of these species. Vertebrate clustered in a branch separated from arthropod RXRs. In addition, all insects grouped in the same cluster, although the internal topology of it is incongruent with current phylogenies, especially because coleopterans and hymenopterans become separated from other holometabolous insects (dipterans, lepidopterans and strepsipterans). A remarkable feature of the tree was the great difference in length of the branches. Diptera and Lepidoptera had the longest lengths, clearly indicating a much more rapid rate of divergence of these sequences with respect to other arthropod and vertebrate sequences. Vertebrate branches were shorter, indicating the great conservation of these sequences, whereas the branches of the arthropods other than Diptera and Lepidoptera had intermediate lengths.

3.3. Tissue distribution of BgRXR

To determine the tissue distribution of the BgRXR-L and BgRXR-S mRNAs, samples of total RNA from various tissues were subjected to RT-PCR with a primer pair that discriminates between both isoforms. Results (Fig. 4) show that BgRXR-S was detected in all tissues analysed, namely fat body, ovary, brain, midgut, prothoracic gland, as well as in embryos and UM-BGE-1 cells. BgRXR-L was detected in fat bodies, ovaries, embryos and prothoracic glands. In fat body and prothoracic gland samples, the predominant isoform was BgRXR-S, whereas BgRXR-L was more abundant in ovaries.

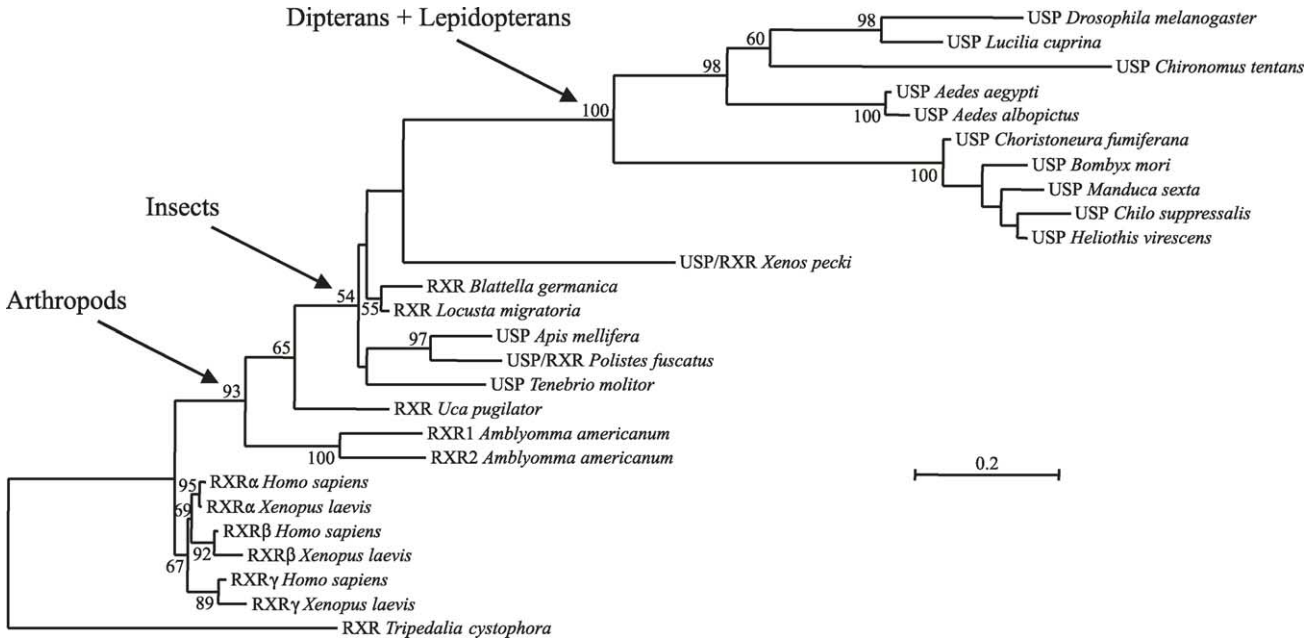

Fig. 3. Phylogenetic position of the BgRXR-LBD of *B. germanica*. The tree was constructed following the maximum-likelihood method using the LBD region of RXR/USP sequences. Poorly aligned regions were eliminated using Gblocks with the option "with half" in the parameter "allowed gap position", resulting in 181 final positions. Branch lengths are proportional to sequence divergence. The bar represents 0.2 substitutions per site. Only bootstrap values ≥50% are shown. The sequence from the cnidarian *Tripedalia cystophora* was used as outgroup. The analysis using the complete LBD sequence, or using Gblocks by default, gave trees with practically identical topologies.

3.4. Developmental pattern of BgRXR mRNAs during embryogenesis

As a first step towards clarifying the mechanism of action of 20E during embryo development, we determined the titre of ecdysteroids in egg batches contained in staged oothecae of *B. germanica*. Ecdysteroid contents were generally low, but two clear-cut ecdysteroid peaks were observed (Fig. 5). The first one occurred between days 5 and 7, at approximately 30–40% of embryonic development, reaching the highest mean level of 6 ng of 20E equivalents per ootheca on day 6. The second peak occurred between days 12 and 14 (at 65–90% of embryogenesis), reaching a mean value of 34 ng of 20E equivalents per ootheca on day 13.

Of the two BgRXR mRNAs (Fig. 5), only BgRXR-L expression was detected in very early embryos (days 0–1). The fact that this transcript was also present in unfertilized, unviable eggs (see below) clearly points to a maternal origin. After day 1, BgRXR-L mRNA levels decreased dramatically and became almost undetectable on day 3. They peaked on day 6 and then vanished. In contrast, BgRXR-S mRNA levels

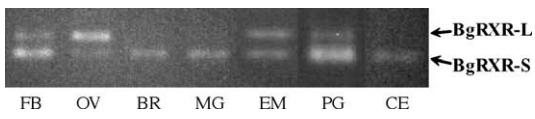

Fig. 4. Tissue distribution of BgRXR-S and BgRXR-L transcripts in *B. germanica*. RT-PCR amplification of BgRXR mRNAs was carried out from fat bodies (FB), ovaries (OV), brains (BR) and midguts (MG) of a 3-day-old adult female, from prothoracic glands (PG) of a 6-day-old sixth instar larva, and from 4-day-old embryo (EM) and UM-BGE-1 cells (CE) with a specific primer combination that discriminates between the two isoforms, as described in Section 2.

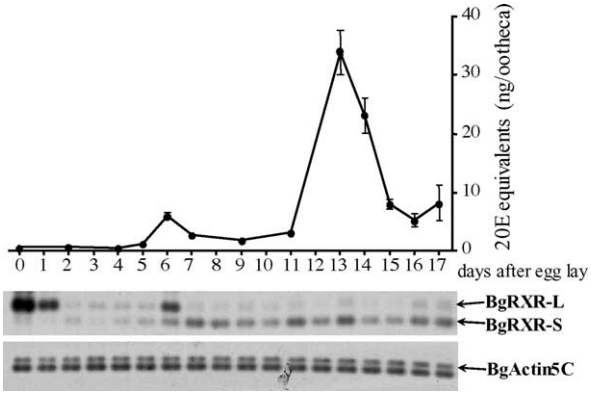

Fig. 5. Expression patterns of BgRXR-S and BgRXR-L mRNAs, and total ecdysteroid contents during embryonic development of *B. germanica*. Ecdysteroids are expressed as ng of 20E equivalents per ootheca, which contains an average of 44 embryos. Vertical bars indicate the S.E.M. ($n = 4$–10). For transcript analysis, equal amounts of total RNA from staged embryos were analysed by RT-PCR using a specific primer combination, as described in Section 2, followed by Southern blotting with a specific probe. The Southern blots are representative of two to three replicates. BgActin5C levels were used as a reference. In this case, amplification of actin5C gave the expected 308 bp band plus another slightly heavier, unspecific band.

were first detected at low levels on day 2, steadily increased up to day 7, and then were maintained until the end of embryogenesis (Fig. 5).

3.5. Developmental pattern of BgRXR mRNAs during moulting

We analysed the expression pattern of BgRXR-S and BgRXR-L mRNAs in the fat body of penultimate (fifth) and last (sixth) larval instars. Little attention has been paid to fat body in relation to the molecular action of 20E. However, fat body may be a suitable research subject in this sense, given that, as the epidermis, it undergoes metamorphic changes associated with moulting and 20E action (Kunkel, 1981; our unpublished results). The patterns of BgRXR mRNAs in the fat body during these periods are shown in Fig. 6, compared with the ecdysteroid titres reproduced from Cruz et al. (2003). BgRXR-S mRNA was the predominant form, occurring throughout both instars. In fifth instar mRNA levels were quite constant, whereas in sixth instar they were high at the beginning, decreased in mid instar, and slightly increased towards the end of the instar. This profile contrasts with that of ecdysteroids, which show a cyclic pattern peaking 3 days before each moult (Fig. 6; data from Cruz et al., 2003). We also studied the expression pattern of both BgRXR isoforms in staged prothoracic glands from females in sixth larval instar. The prothoracic gland is responsible for the synthesis and secretion of ecdysteroids and so is clearly involved in the moulting process. As in the case of fat body, BgRXR-S was predominant; its levels were high until day 2, then decreased and remained relatively constant until the end of the instar (Fig. 7).

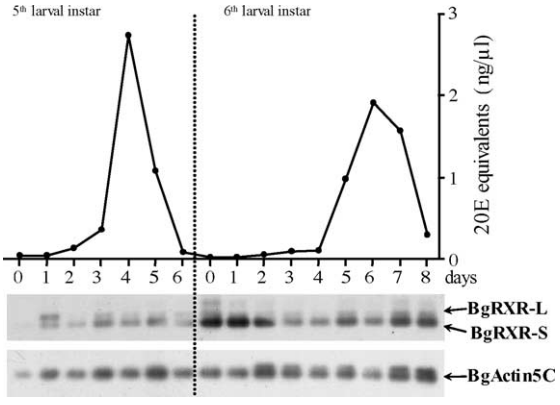

Fig. 6. Expression patterns of BgRXR-S and BgRXR-L mRNAs in the fat body of fifth and sixth larval instars of *B. germanica*. mRNA values are compared with the haemolymph ecdysteroids profile reported by Cruz et al. (2003). Equal amounts of total RNA from staged larvae fat bodies were analysed by RT-PCR using a specific primer combination, as described in Section 2, followed by Southern blotting with a specific probe. Southern blots are representative of two to three replicates. BgActin5C levels were used as a reference.

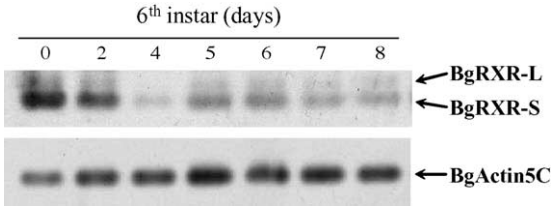

Fig. 7. Expression patterns of BgRXR-S and BgRXR-L mRNAs in the prothoracic gland of sixth larval instar of *B. germanica*. Total RNA from three staged larval prothoracic glands were analysed by RT-PCR using a specific primer combination, as described in Section 2, followed by Southern blotting with a specific probe. The Southern blots are representative of two to three replicates. BgActin5C levels were used as a reference.

3.6. Developmental pattern of BgRXR mRNAs in the adult

BgRXR mRNA levels were studied in the fat body and the ovary of adult females during the first gonadotrophic cycle. In the fat body, BgRXR-S mRNA was the most abundant transcript, and its level remained practically unchanged throughout the cycle (Fig. 8A). The patterns of BgRXR mRNAs contrast with those of circulating ecdysteroids reported by Pascual et al. (1992), which increase steadily along the cycle (Fig. 8A). In the ovary, BgRXR-L mRNA was clearly predominant. The levels of this transcript were high during the first 3 days of adult life, and then declined slowly over the course of oogenesis (Fig. 8B). This pattern is almost the opposite of that of ovarian ecdysteroids reported by Pascual et al. (1992), which steadily increased from day 2 to 7 (Fig. 8B).

3.7. 20E and JH III effects on BgRXR expression in UM-BGE-1 cells

We studied the effects of 20E, at concentrations from 10^{-8} to 10^{-5} M, on BgRXR transcription in UM-BGE-1 cells. After 4 h incubation, increasing amounts of 20E had no effect on BgRXR mRNA levels (Fig. 9A). To confirm and extend these results, we determined the temporal profile of BgRXR-S expression in UM-BGE-1 cells cultured with a high concentration of 20E (5×10^{-6} M) for different periods of time. Results showed that the transcripts also remained unchanged under these conditions (Fig. 9B). Our results (manuscript in preparation) indicating that 20E (at 10^{-6} to 10^{-7} M) increase mRNA levels of E75 and HR3, early genes of the 20E cascade, in UM-BGE-1 cells, serve as positive controls of these experiments. Negative results were also obtained with fat bodies from females in sixth larval instar, incubated in the same dose- and time-dependent conditions (data not shown). We also tested the response of BgRXR mRNAs to JH III exposure. As in 20E assays, we carried out a time-course experiment by incubating UM-BGE-1 cells up to 24 h in the presence of 10^{-6} M of JH III. However, JH III did not exert any appreciable effect on transcript levels (Fig. 9C). Similar results were obtained with fat bodies from sixth instar larvae in the presence of the same JH III concentrations, or when JH

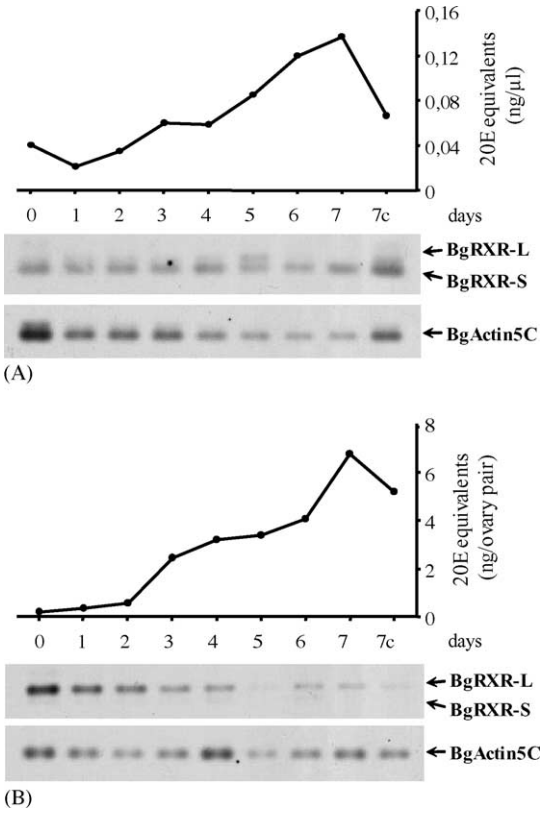

Fig. 8. Expression patterns of BgRXR-S and BgRXR-L mRNAs in the adult female *B. germanica* during the first gonadotrophic cycle. BgRXR-S and BgRXR-L mRNAs were measured in the fat body (A) and in the ovary (B). Equal amounts of total RNA from staged fat bodies and ovaries were analysed by RT-PCR using a specific primer combination, as described in Section 2, followed by Southern blotting with a specific probe. The Southern blots are representative of two to three replicates. BgActin5C levels were used as a reference. In both panels 7c corresponds to females with chorionated oocytes. Data on haemolymph and ovarian ecdysteroids are from Romañá et al. (1995) and Pascual et al. (1992), respectively.

III was topically applied at doses from 0.01 to 1 μg on adult females (data not shown).

4. Discussion

4.1. Structure of BgRXR from B. germanica

We identified two cDNAs corresponding to two isoforms of the *B. germanica* RXR/USP, namely BgRXR-S and BgRXR-L. The sequences of the predicted proteins only differ by a 23-amino acid insertion/deletion in the loop connecting helices H1 and H3 within the LBD. In invertebrates, RXR/USP isoforms differing in insertions/deletions of this type have been previously reported in the locust *L. migratoria* (Hayward et al., 1999, 2003), and in the crab *U. pugilator* (Durica et al., 2002). However, this type of isoforms has not been reported in species of more derived insect orders, such as Diptera and Lepidoptera, whose isoforms are usually produced by differential splicing of the N-terminal region of the genes (Kapitskaya et al., 1996; Jindra et al., 1997; Vogtli et al., 1999). In fact, in Diptera and Lepidoptera the loop connecting helices H1 and H3 is very well conserved and is responsible for contacting with the helix H12 of the LBD, thus locking the receptor in an inactive position (Billas et al., 2001; Clayton et al., 2001). Moreover, this loop contains conserved residues that interact with the phospholipid ligand that was co-crystallized with the LBD of *D. melanogaster* and *H. virescens* (Billas et al., 2001; Clayton et al., 2001), which suggests that it plays a very important role in the functionality of the receptor. Interestingly, this loop is almost absent (BgRXR-S) or not conserved (BgRXR-L) in *B. germanica*, as happens in other primitive insects (Fig. 2).

Two other regions within the LBD are also of interest. The first is located within helix 10 (AKLLLRLPSLR) and, as demonstrated in other species, is necessary for receptor heterodimerization (Lee et al., 1998). The second region is within helix 12, and is known as the ligand-dependent AF-2 activation domain (FLMEMLE). This sequence is well conserved in *L. migratoria* and *T. molitor* RXR proteins, whereas some conservative changes are observed in *A. mellifera* and in other non-insect arthropods (see Bonneton

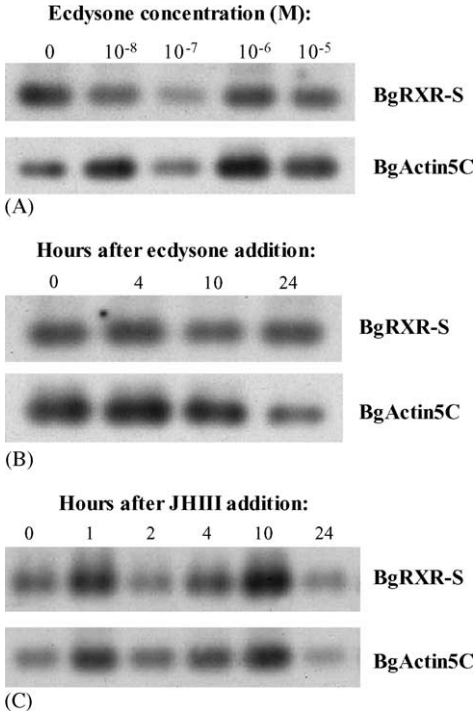

Fig. 9. Effect of 20E and JH III on BgRXR mRNA levels. (A) Dose-response effects of 20E. UM-BGE-1 cells were cultured and treated with different concentrations of 20E for 4 h. (B) Time-course of BgRXR transcription in UM-BGE-1 cells cultured with 5×10^{-6} M 20E for the periods of time shown. (C) Time-course of BgRXR transcription in UM-BGE-1 cells cultured with 10^{-6} M JH III for the periods of time shown. In all the cases, equal amounts of total RNA were-extracted after the incubation and analysed by RT-PCR using a specific primer combination, as described in Section 2, followed by Southern blotting with a specific probe. BgActin5C levels in the same samples were used as a reference. The Southern blots are representative of two to three replicates.

et al., 2003). In contrast, this domain is quite divergent in dipteran and lepidopteran species. Since this region is necessary for interaction with co-regulator proteins having the LXXLL domain (Glass and Rosenfeld, 2000), divergence of Diptera and Lepidoptera RXR/USP suggests that these proteins may not be able to act as active ligand-dependent homodimers in these insect orders.

The LBD differences of Diptera and Lepidoptera from other arthropod and vertebrate RXRs are reflected in the phylogenetic tree shown in Fig. 3. In previously reported phylogenetic analyses, the dipterans + lepidopterans appeared as the sister-group of all other arthropods + vertebrates (Guo et al., 1998; Hayward et al., 1999; Bonneton et al., 2003). This topology points to the great divergence of these two highly modified insect orders, but does not reflect the current animal phylogeny. Our RXR/USP phylogenetic analysis shows for the first time all arthropods grouped in the same clade and with high support (93% bootstrap), and in this context, the divergence of RXR/USP from Diptera and Lepidoptera is reflected by the length of their branches. This remarkable length is not observed in any other insect, including two hymenopteran species, *A. mellifera* and *P. fuscatus*. Current insect phylogenies place Hymenoptera as the sister-group of the lineage leading to Diptera + Lepidoptera, which suggests that the strong divergence of RXR/USP only affected this lineage (see Bonneton et al., 2003). In our analysis, the strepsipteran *X. pecki* appears as the sister-group of lepidopterans + dipterans, but with low support (40% bootstrap). The position of Strepsiptera, however, remains enigmatic, whatever the phylogenetic analysis used (see Hayward et al., 2005).

4.2. Expression during embryogenesis

Unlike *D. melanogaster* embryogenesis, which has only a major mid-embryonic ecdysteroid pulse around 25–50% of embryonic development (Maroy et al., 1988), during *B. germanica* embryogenesis there are two clear-cut pulses of ecdysteroids, one at 30–40% embryogenesis and one at 65–90%. The occurrence of several ecdysteroid bursts during embryogenesis has been previously detected in other hemimetabolous insects, such as the locust *L. migratoria* (Hoffmann et al., 1980), and the cockroaches *Nauphoeta cinerea* (Lanzrein et al., 1985) and *Blaberus craniifer* (Bullière et al., 1979). The bursts correlate with embryo dorsal closure or processes of cuticle deposition (Hoffmann et al., 1980). In *D. melanogaster*, the embryonic pulse of ecdysteroids is required not only for cuticle deposition, but also for germ band retraction and for head involution (Chavez et al., 2000; Kozlova and Thummel, 2003), two major morphogenetic events required to shape the larval body plan.

Interestingly, the expression of the two BgRXR isoforms throughout embryogenesis is remarkably different. BgRXR-L is the only isoform detected at the earliest stages, strongly suggesting that it is of maternal origin and that it is related to processes occurring early in embryogenesis, namely blastoderm formation, germ band extension, serosa deposition and dorsal closure. Maternal contribution to embryonic RXR/USP mRNAs has also been described in *D. melanogaster* (Kozlova and Thummel, 2003; Sullivan and Thummel, 2003) and in *C. fumiferana* (Perera et al., 1998). Conversely, BgRXR-S is expressed after dorsal closure, at day 6, i.e. after the first ecdysteroid peak, and is probably involved in processes relating to organogenesis.

4.3. Expression in larvae and adults

The requirement for RXR/USP during larval development was established in *D. melanogaster*, in which USP mutants show multiple defects in ecdysteroid-dependent processes at the larval–prepupal transition (Hall and Thummel, 1998). Moreover, variations of RXR/USP mRNA levels have been described in the epidermis of *T. molitor* and *M. sexta* larvae (Nicolaï et al., 2000; Jindra et al., 1997). In *B. germanica*, however, BgRXR mRNA levels showed little variation in the fat body and the prothoracic gland (Figs. 6–8). The expression patterns observed do not correlate with ecdysteroid levels, which suggest that these hormones do not direct the transcription of RXR in *B. germanica*, at least in the periods studied. Similar expression patterns have also been reported in larvae of *D. melanogaster* (Sullivan and Thummel, 2003) and *C. fumiferana* (Perera et al., 1998).

At the adult stage the prothoracic gland degenerates, and circulating ecdysteroids come mainly from the ovary (Pascual et al., 1992; Romañá et al., 1995). The function of ovarian ecdysteroids is poorly understood, although their inductive function in choriogenesis has been described in *B. germanica* (Bellés et al., 1993). Consistent with this, BgRXR-L mRNA was detected in the ovaries of *B. germanica* (Fig. 6). mRNA levels remained high during the first 4 days of adult life, and then declined slowly during oogenesis, a pattern that coincides with those reported for *L. migratoria* (Hayward et al., 2003), *A. aegypti* (Wang et al., 2000) and *B. mori* (Swevers and Iatrou, 2003). The need for an ecdysteroid-dependent genetic hierarchy for oogenesis has been demonstrated in Diptera and Lepidoptera. In *D. melanogaster*, oogenesis was defective in EcR mutant females, which showed abnormal egg chambers and loss of vitellogenic egg stages (Carney and Bender, 2000). In addition, studies on ovarian expression of EcR and RXR/USP in the mosquito *A. aegypti* have also confirmed that the transition from previtellogenesis to vitellogenesis is regulated by factors derived from the ecdysteroid-triggered cascade (Wang et al., 2000). Finally, in the lepidopteran *B. mori*, ovarian development is also induced by ecdysteroids through such a regulatory cascade (see Swevers and Iatrou, 2003).

4.4. Hormone regulation

Experiments in vitro with embryo cells and larval fat body tissue showed that 20E does not modify BgRXR mRNA levels. These results are reminiscent of those described in

B. mori, in which 20E had no effect on BmUSP mRNA in wing discs incubated in vitro (Matsuoka and Fujiwara, 2000). However, in *M. sexta* and *A. aegypti*, 20E has an isoform-specific differential regulatory function. Thus, in the epidermis of *M. sexta*, 20E rapidly increases the abundance of MsUSP-2, whereas MsUSP-1 mRNA is down regulated by high levels of the same hormone in the presence of JH (Jindra et al., 1997; Hiruma et al., 1999). In *A. aegypti*, 20E upregulates AaUSP-B transcription, and its presence is necessary to maintain a high level of AaUSP-B expression, whereas it inhibits AaUSP-A transcription (Wang et al., 2000).

Nor did we observe in our experiments any effect of JH III on mRNA levels of BgRXR-S and BgRXR-L. In recent years, results obtained in *D. melanogaster* have shown that DmUSP might act as a receptor for JH (Jones and Sharp, 1997), and that it can activate gene transcription in an EcR-independent manner on binding to JH (Xu et al., 2002). This led to the hypothesis that RXR could be inducible by JH. However, the only antecedent in this sense has been reported in the honeybee *A. mellifera*, where a rapid and transient up-regulation of AmUSP has been observed in fat bodies from queens and workers when exposed to JH (Barchuk et al., 2004).

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