Mol Biotechnol (2014) 56:126–135
DOI 10.1007/s12033-013-9688-y

RESEARCH

Molecular Cloning, Characterization, and Expression Pattern of the Ultraspiracle Gene Homolog (*RXR/USP*) from the Hemimetabolous Insect *Periplaneta americana* (Dictyoptera, Blattidae) During Vitellogenesis

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Published online: 20 July 2013

Abstract Ecdysteroid and sequiterpenoids juvenile hormones play a gonadotrophic role in the insect adult female vitellogenesis. The molecular basis of hormone action has been analyzed in great detail in flies and moths, but rarely in primitive insect orders. The primitive hemimetabolous insect *Periplaneta americana* was used, as a model, to isolate and characterize, for the first time, two cDNAs of *RXR/USP*, a component of the heterodimeric ecdysone receptor. These two cDNAs correspond to two isoforms, named *PamRXR-S* (short form) and *PamRXR-L* (long form). Both are identical except for 25 amino acids deletion/insertion located in the loop between helices H1 and H3 of the ligand-binding domain. The two isoforms are differentially expressed in different tissues as revealed by RT-PCR and northern blot analysis. In fat body, brain, ovary, and muscle tissues, the predominant form was *PamRXR-S*, whereas *PamRXR-L* was abundant in ovaries. The *PamRXR* transcript was detected during all stages of vitellogenesis in the fat body with different levels. It was little low during the early vitellogenic period (days 2, 3), then a peak of increase was detected during days 4–6 (day 5) which was followed by another peak of increase at the end of vitellogenesis, day 9. We assumed that *PamRXR* might play a dual role of induction of vitellogenin through JH at early vitellogenesis and suppression through 20E during late vitellogenesis. The present work will pave the way for several other investigations to understand both the ecdysteroid-dependent genetic hierarchy and JH mechanism controlling vitellogenesis in the American cockroach, *P. americana.*

Keywords *RXR* · Vitellogenesis · *Periplaneta americana* · Female fat body · Expression pattern

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Introduction

Most of the vital events occurring in an insect's life, such as development and reproduction, are regulated by steroid hormones. Steroids elicit various physiological responses by modulating gene expression through interaction with their nuclear receptors that serve as ligand-dependent transcription factors [1]. The ecdysteroid hormone, mainly its biologically active form 20-hydroxyecdysone (20E) exerts its effects via direct binding to a heterodimeric nuclear receptor [2]. That functional 20E receptor is a heterodimer of two nuclear proteins, the ecdysone receptor (*EcR*) and the retinoid X receptor (*RXR*)-homolog ultraspiracle (*USP*) [3, 4]. The heterodimer *EcR–RXR/USP* binds to ecdysteroid and in turn, the ecdysteroid/receptor complex binds to sequence specific DNA elements called ecdysteroid response elements (*EcREs*) in target genes and directly induces the expression of a number of primary response genes including a set of transcription factors. Consequently, these genes amplify the effect of 20E by triggering the expression of a large battery of secondary responsive genes [3, 5]. This genetic hierarchy has been studied in detail not only during the molting process and

pupal development of *Drosophila melanogaster* and *Manduca sexta* [3, 6, 7], but also in vitellogenesis of the mosquito, *Aedes aegypti* and oogenesis of the silk worm, *Bombyx mori* [8, 9].

Hormonal regulation of insect vitellogenesis has been studied for many years [10–12]. During insect vitellogenesis, the precursor egg yolk protein vitellogenins (*Vgs*) are synthesized in the fat body, secreted into the hemolymph and taken up by the developing oocytes [13, 14]. 20E and juvenile hormone (JH) play a gonadotrophic role in the adult female and regulate vitellogenesis [15–17]. Insects can be divided into three types based on hormonal regulation of the *Vg* genes transcription. Type I includes insects that use only JH for *Vg* gene expression, such as *Leucophaea maderae* [18]; *Locusta migratoria* [19]; *Blattella germanica* [12, 15, 16]; *Nauphoeata cinerea* [20] and lubber grass hopper [21]. Type II includes insects which require both JH and ecdysone for *Vg* gene expression, such as *A. aegypti*, *Anopheles stephensi*, and *D. melanogaster* [22–24]. Type III includes some lepidopterans that require JH, ecdysteroids, and additional hormones to regulate their reproductive physiology, such as *Choristoneura fumiferna* [25] and *Manduca sexta* [26].

Several *RXR/USP* genes were isolated from many species of most derived insect orders, such as Diptera (*D. melanogaster, Lucilia cuprina*, *Chironomus tentans* and *A. aegypti*) [4, 27–29], Lepidoptera (*M. sexta, C. fumiferana, B. mori*) [7, 30, 31], Coleoptera (*Tenebrio molitor)* [32], and Hymenopterans (*Apis mellifera)* [33]. However, within the primitive hemimetabolous species, only two isoforms of *RXR/USP, RXR-S* (short form) and *RXR-L* (long form), have been completely sequenced in the locust, *L. migratoria* [34, 35], and the German cockroach, *Blatella germanica* [36]. Each isoform is expressed in a tissue- and stage-specific manner and respond differently to insect hormones, indicating different function for each isoform [8, 28, 37]. Indeed, data on the molecular action of 20E during vitellogenesis in hemimetabolous insects are very scarce. In addition, comparative analyses of *RXR/USP* proteins have shown that the *RXR/USP* DNA-binding domain (DBD) is highly conserved among insect's species, whereas the ligand-binding domain (LBD) showed an interesting evolutionary divergence [38–40]. 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 [1, 2, 40]. Such hypothesis was confirmed through the In vitro experiments with the *B. germanica* fat body cells which showed that the expression of the RXR/USP is not affected by 20E or JH III [36, 41]. Previously, we have shown that the expression of *Vg* genes in the American cockroach, *Periplaneta americana*, is synchronized and up regulated by the hemolymph JH titer and suppressed by the ecdysteroid titer during vitellogenesis [42]. However, the detailed molecular mechanisms that regulate vitellogenesis in this model cockroach remained unclear. In the present work, we have cloned and characterized two cDNAs encoding *RXR/USP* homologs, called *Pam RXR-S* and *Pam RXR-L*, for the first time in *P. americana.* In addition to the structural and phylogenetic analysis, we characterized the spatial and temporal expression of both homologs mRNAs during vitellogenesis.

Materials and Methods

Insects and Sample Preparation

Stock cultures of *P. americana* were maintained in the laboratory as described previously [42]. Newly emerged females were collected from the stock colonies, kept separately and used when required. Fat body, midgut, brain, ovary, and muscle tissues were isolated in phosphate-buffered saline (PBS 1×: 2 mM KH_2PO_4, 137 mM NaCl, 10 mM Na_2HPO_4, 2.7 mM KCl, pH 7.4), frozen immediately in liquid nitrogen and stored at −80 °C until required.

RNA Extraction and cDNA Construction

Total RNA was extracted from the above samples using Isogen reagent (Nacalai tesque, Kyoto, Japan) according to the manufacturer's instructions. Poly (A) + RNA was purified from total RNA using mRNA purification kit (Amersham-Pharmacia, Piscataway, NJ, USA). A total of 1.5 μg of mRNA was used to generate ds cDNA using Avian Myelobastosis Virus reverse transcriptase (20 units) and an oligo (dT) primer [a cDNA synthesis primer (10 μM)] with the dNTP mixture (10 mM) from the Marathon cDNA amplification kit (Clontech, Palo Alto, CA, USA). For second strand synthesis, a 20× second strand enzyme cocktail (RNase H, *Escherichia coli* DNA polymerase I and *E. coli* DNA ligase) was used. The ds cDNA, following the creation of blunt ends with T4 DNA polymerase (10 units), was ligated to a Marathon cDNA adaptor (Clontech). This adaptor-ligated ds cDNA library was then used as a template for the cloning of a complete *RXR/USP* cDNA through 5′- and 3′-RACE-PCR.

Cloning of *RXR/USP* cDNA

The two cDNAs prepared from adult female fat body and ovary and degenerate primers based on the conserved DBD and LBD of the *L. migratoria* and *B. germanica RXR* [34, 36] were used to obtain *PamRXR* homolog cDNA fragment. Briefly, the degenerate primers designed were as follows: forward primer 5′-TAYCCNCCNAAYCAYC CN-3′, and reverse primer 5′-RTCCATYTTCATYTC-3′. Amplification conditions employed were heating to 94 °C for 2 min,

followed by 35 cycles of 94 °C for 30 s, 50 °C for 30 s, and 68 °C for 2 min. The amplified fragment using cDNA from the fat body (702 bp) was sub-cloned into pT7Blue vector (Novagen) and sequenced. The remaining fragment of *P. americana RXR* cDNA was obtained by 5′-and 3′ RACE-PCR, using a Marathon cDNA amplification kit (Clontech, Mountain View, CA, USA). 5′-RACE-PCR was performed using a reverse gene-specific primer 1 (named: RXR-R1) and an AP1 (Clontech). The PCR conditions used were 94 °C for 1 min, followed by 35 cycles of 94 °C for 5 s and 59 °C for 3 min. In the second reaction for 5′-RACE-PCR, RXR-R2-specific primer (nucleotide position: 860–876 bp) was also used with AP1. 3′-RACE-PCR was performed using a forward gene-specific primer 1 RXR-F1 (nucleotide position: 504–523 bp) and an AP1 (Clontech). The PCR conditions used were 94 °C for 1 min, followed by 35 cycles of 94 °C for 5 s and 59 °C for 3 min. A second 3′-RACE-PCR was performed with RXR-F2 (nucleotide position: 640–659 bp). The amplified PCR products were cloned into the pT7Blue vector (Novagen, Madison, WI, USA) and sequenced. The overlapping sequences of the above PCR-produced fragments were assembled to obtain the full-length sequences. Sequencing was performed using an ABI prism Big-Dye terminator cycle sequencing ready reaction kit (PE Applied Biosystems, Foster City, CA, USA) and a DNA sequencer (ABI, Prism 3100Genetic Analyzer, PE Applied Biosystems). The sequences were checked for homology with other *USP/RXR* nuclear receptor family members using a BLAST homology search of the DDBJ database. The zinc finger domain and the putative phosphorylation sites were predicted using the Prosite, EXPASY computer program. Sequence comparisons were performed using the CLUSTALW program [38] and the GENETYX Ver 5.1 program (Genetyx Corporation, Tokyo, Japan). Two *RXR* sequences were obtained, a short one *PamRXR-S* from the fat bodies and a long one *PamRXR-L* from the ovaries (GenBank accession number AB751607 and AB781156, respectively).

Structural Comparison and Phylogenetic Inference

PamRXR-S sequence (GenBank accession number: AB751607) was compared with other insect *USP/RXR* sequences and other *RXR* receptor family members in which LBD was completely sequenced using DDBJ (CLUSTALO program). A molecular phylogenetic (neighbor-joining) tree was constructed using MEGA (5.1). Sequences used for the phylogenetic analysis were (accession number in parentheses): *D. melanogaster* (P20153), *L. cuprina* (AAG01569), *Bactrocera dorsalis* (ADM64635), *C. tentans* (AAC03056), *A. aegypti* (AAG24886), *Aedes albopictus* (AAF19033), *Xenos peckii* (AY827155), *C. fumiferana* (AAC31795), *B. mori* (S44490), *M. sexta* (P54779), *Chilo suppressalis* (BAC53670), *Heliothis virescens* (CAD28568), *L. migratoria* (AAF00981), *B. germanica* (AJ854490), *Diploptera punctata* (AEZ64360), *A. mellifera* (AAF73057), *Acromyrmex echinatior* (EGI63885), *Polistes fuscatus* (AY827156), *Gryllus firmus*, (ADL09403), *Mus musculus* (AAB36778), *Saccoglossus kowalevskii* (ADB22634), *Tribolium castaneum* (CAL25729), *Leptinotarsa decemlineata* (BAD99298), *Xenopus laevis* (P51128), *Gallus gallus* (NP990625), *Melipona scutellaris* (AAW02952), *Pediculus humanus corporis* (XP002424949), *Nezara viridula* (ADQ43369), *Leptopilina heterotoma* (AAO18153), *Uca pugilator* (AAC32789), *Daphnia magna* (BAF49028), *Lithobius peregrinus* (CCA61269), *Ixodes scapularis*, (EEC07894), *Danio rerio* (NP571292), *Scaptotrigona depilis* (ABB00308), *Tripedalia cystophora* (AAC80008), *Crangon crangon* (ACO44668), *Amblyomma americanum* (AAC15589), and *Homo sapiens* (CAA36982).

Expression Analysis of *RXR/USP*

To investigate the tissue-specific expression of *PamRXR* during vitellogenesis, pooled total RNA isolated from day 5 to day 7 of adult *P. americana* female fat body, midgut, brain, ovary, and muscle were purified using Isogen reagent (Nacalai tesque, Kyoto, Japan). For the developmental expression profile assay, fat bodies from 2 to 9 days old female adults were used. RNA samples were stored at −80 °C until required. Aliquots of 15 μg of total RNA from the tissues described above was subjected to electrophoresis using 1 % agarose/0.66 M formaldehyde gel in 3-morpholino- propanesulfonic acid (MOPS) buffer. The resolved RNAs were transferred to Hybond N+ membrane (Amersham-Pharmacia) through capillary transfer. The blots were then hybridized with the *PamRXR* fragment (5′end part) probe which was fluorescein-labeled using a Gene Images Random Prime Labeling Module (Amersham-Pharmacia), according to the kit instruction manual. The probe was hybridized based on the protocol from Hybond N+ (Amersham). Briefly, the membranes were blocked for 30 min at 68 °C using ExpressHybTM Hybridization solution (Clontech) and then probed for 1 h at 68 °C, followed by repeated washes in 2× sodium chloride–sodium citrate in water (SSC) at 68 °C and 0.1× SSC–0.1 % SDS at 55 °C. The hybridization blots were visualized and analyzed by BAS 2000 (Fuji Photo Film, Tokyo, Japan). Samples were qualitatively normalized by determining ribosomal RNAs after staining with ethidium bromide.

Developmental RT-PCR Analysis

Two μg DNase-treated total RNA samples of fat bodies from 2 to 9 days old female adults were reverse transcribed using ReverTra Ace (Toyobo Co., Osaka, Japan) and poly dT primer according to the manufacturer's instructions.

Primers set (RT-F and RT-R, Table 1) for *PamRXR* gene was used to amplify RT-PCR products. The PCR amplification cycles were as follows: 35–40 cycles of 94 °C for 30 s, then 60 °C for 30 s, and 72 °C for 1 min with 1 min extension. As a reference, the cDNA was subject to the same PCR conditions with specific primer pair to *Actin* (Actin F, and Actin R).The RT-PCR products were run on 1.5 % agarose gel, which was stained with ethidium bromide, and photographed with the AE-6910 gel imaging system (ATTO, Tokyo, Japan).

Results and Discussions

Cloning and Structural Analysis of *PamRXR*

We identified two cDNAs corresponding to two isoforms of the *PamRXR/USP*, namely, *PamRXR-S* and *PamRXR-L*. cDNAs were cloned by a RT-PCR approach using degenerate primers designed on the bases of the conserved sequences of the DBD and LBD from *L. migratoria* and *B. germanica*. Using a fat body cDNA, a partial clone of 702 bp encoding the *P. americana* (*PamRXR*) was first cloned and analyzed. The sequence of this fragment was very similar to other insect *RXR/USPs*. The full length of *P. americana RXR/USP* was obtained by 3′ and 5′ rapid amplification of cDNA ends PCR (RACE-PCR) (Fig. 1). Two complete sequences of cDNAs from both Fat body (*PamRXR-S*) and ovary (*PamRXR-L*) of 1,468 and 1,543 bp- long, were obtained, which encoded proteins of 416 and 441amino acids with predicted molecular masses of 46.5 and 49.4 kDa, respectively. BLAST database search (www.ddbj.nig.ac.jp/search/blast-e.html) [43] of these two cDNAs indicated that both encoded *P. americana* orthologues of *RXR/USP* (*PamRXR-S*, GenBank accession number: AB751607 and *PamRXR-L*, GenBank accession number: AB781156). The 33 bp-5′ and 187 bp-3′ untranslated regions of the two isoforms are identical, which suggests that they derive from the same gene. Both predicted amino acids sequences are identical except for an insertion/deletion of 25 amino acids in the loop between helix H1 and H3 within the LBD (Fig. 1b).

The deduced amino acid sequence of *PamRXR* contained the five domain structures characteristic for the nuclear receptor superfamily. These domains are a ligand-independent A/B activation domain (amino acids 1–91), a two-zinc-fingered DBD (C domain, 92–167), a hinge region (D domain, 168–183), and LBD (E domain, 184–end) containing the putative ligand-dependent activation domain AF-2 (EFFLMEMLE). The in silico analysis revealed projected phosphorylated residues including two serine at 265, 337 and four threonine at 122, 223, 295, 358 as predicted by ScanProsites SIB Swiss Institute of Bioinformatics server (Fig. 1a). The presence of such a large number of phosphorylated residues in *PamRXR* indicates that it is a highly phosphorylated receptor. These phosphate moieties with a negative charge may play a role in the receptor ligand-binding activity. However, further investigation will be required to emphasis this suggestion. Similar sites for phosphorylation by different protein kinases including casein kinase II (*CK II*) and protein kinase C (*PKC*) sites have been identified in the LBD region of *D. melanogaster RXR/USP* [44]. Also, the phosphorylation of *RXR/USP* is believed to play an important role in gene regulation during adult development and salivary glands of *D. melanogaster* [45–47].

The DBD and LBD of *PamRXR* showed high homology to *RXR/USP* of other arthropods and vertebrates. The highest identity was found in the DBD domain (57–86 %) whereas it was 26–65 % within the LBD domain. Multiple alignments of DBD and LBD were performed for more detailed analysis (Fig. 2). Interestingly, the loop between helices H1 and H3, where locates the insertion/deletion, is highly conserved in Diptera (*D. melanogaster*) and Lepidoptera (*M. sexta*, *B. mori*, and *H. versica*), but quite divergent in length and sequence in other arthropods and invertebrate *RXRs*. This loop is quite similar in *PamRXR-L* and *BgRXR-L* except for insertion of 2 amino acids (RG) in *PamRXR-L* (Fig. 2b). In fact, in Diptera and Lepidoptera

Table1 The name, sequence and position of the primers used in the cloning of *PamRXR* gene and RT-PCR experiments and the reference gene *Actin*

Primer name	Sequence	Position in *PamRXR* (bp)
RXR-F1	TATGGGCATGAAAAGGGAAG	504–523
RXR-F2	GAGTGCAAAACAGAGCACCA	640–659
RXR-R1	CCTGCTCGAAGGAGTAGCAC	778–797
RXR-R2	AAGACCCGTTGCTAAGACGA	860–876
RT-F	TATGGGCATGAAAAGGGAAG	504–523
RTR	TGGTGCTCTGTTTTGCACTC	640–659
Actin F	TGACTGAGCGTGGTTACAGC	330–394
Actin R	CAGGAAGGAAGGTTGGAACA	534–553

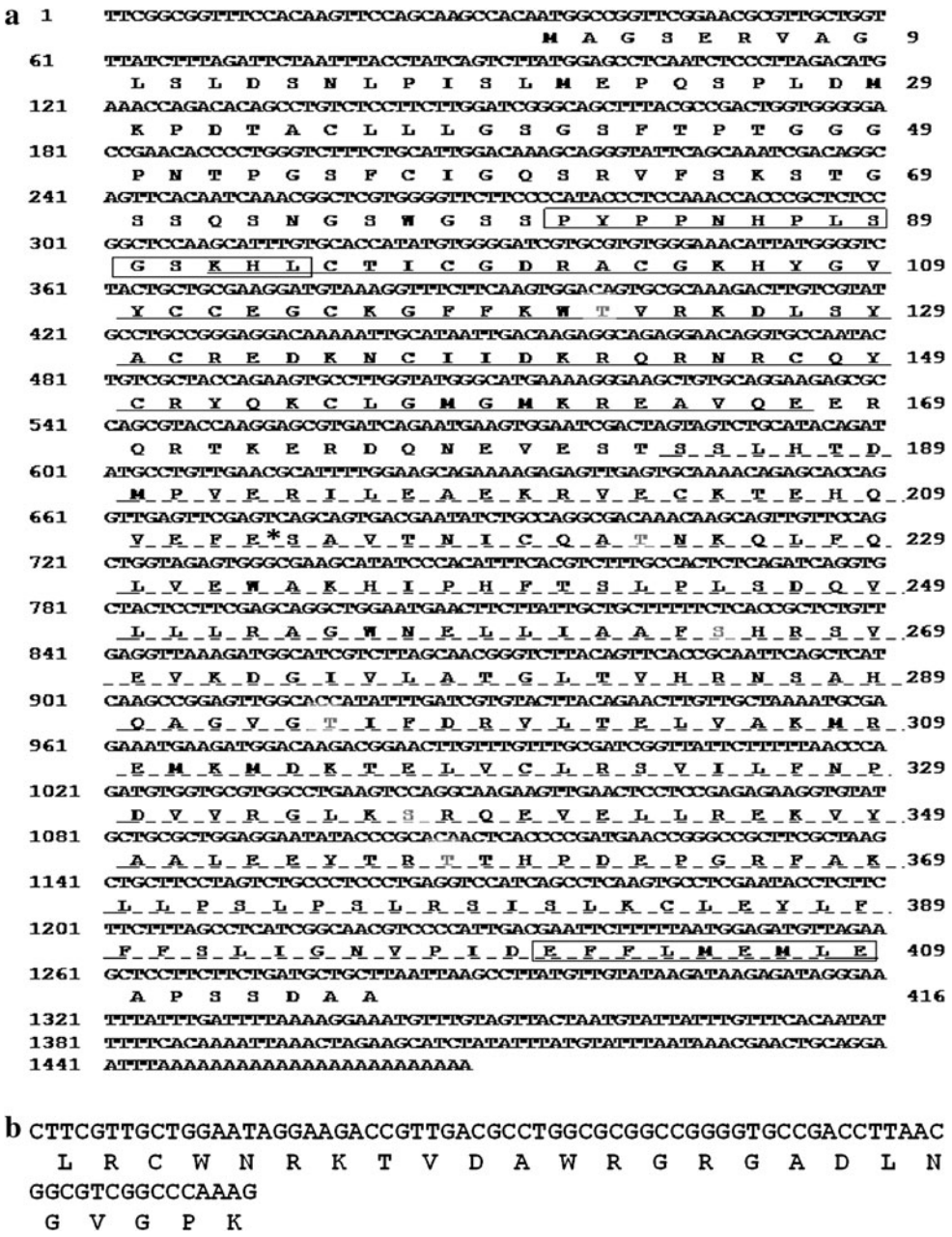

Fig. 1 Nucleotides and deduced amino acid sequences of the *PamRXR- S* and L isoforms of *P. americana.* (Genbank accession number: AB751607, AB781156). **a** *PamRXR-S* sequence was determined by 5′ and 3′ RACE and translated into amino acids. The *left numbers* indicate nucleotide length and the *right numbers* indicate the amino acids. The DNA-binding domain (DBD) is *underlined* and the ligand-binding domain (LBD) is *underlined* with *dashes*. The amino acids motifs conserved in all *RXR/USPs* are *boxed*. Possible serine (S) and threonine (T) phosphorylation sites are in *gray color*. The *asterisk* showed the position of the 25 amino acids insertion forming the long isoform *PamRXR-L.* **b** Nucleotide sequence and amino acid sequence of the insertion fragment characterize the *PamRXR-L* isoform

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 [38, 48]. 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* [38, 48], which suggests that it plays a very important role in the functionality of the receptor. As shown in Fig. 2b, this loop in *Pam RXR-L* is not conserved with holometabolous insects and vertebrate; however, in *PamRXR-S,* it is almost absent. Our results are in complete accordance with those obtained in other primitive insects, *L. migratoria* and *B. germanica* [34–36].

Another two regions within the *PamRXR* LBD are also detected. The first region is located within helix 10 (AKLLLRLPSLR), which believed to be necessary for receptor heterodimerization [49]. The second region is within helix 12, and identified as the ligand-dependent AF-2 activation domain (EFFLMEMLE) (Fig. 1). The latter sequence is well conserved in *L. migratoria, B. germanica* and *T. molitor RXR* proteins, whereas some conservative changes are observed in *A. mellifera* and in other non-insect arthropods [39, 40]. 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

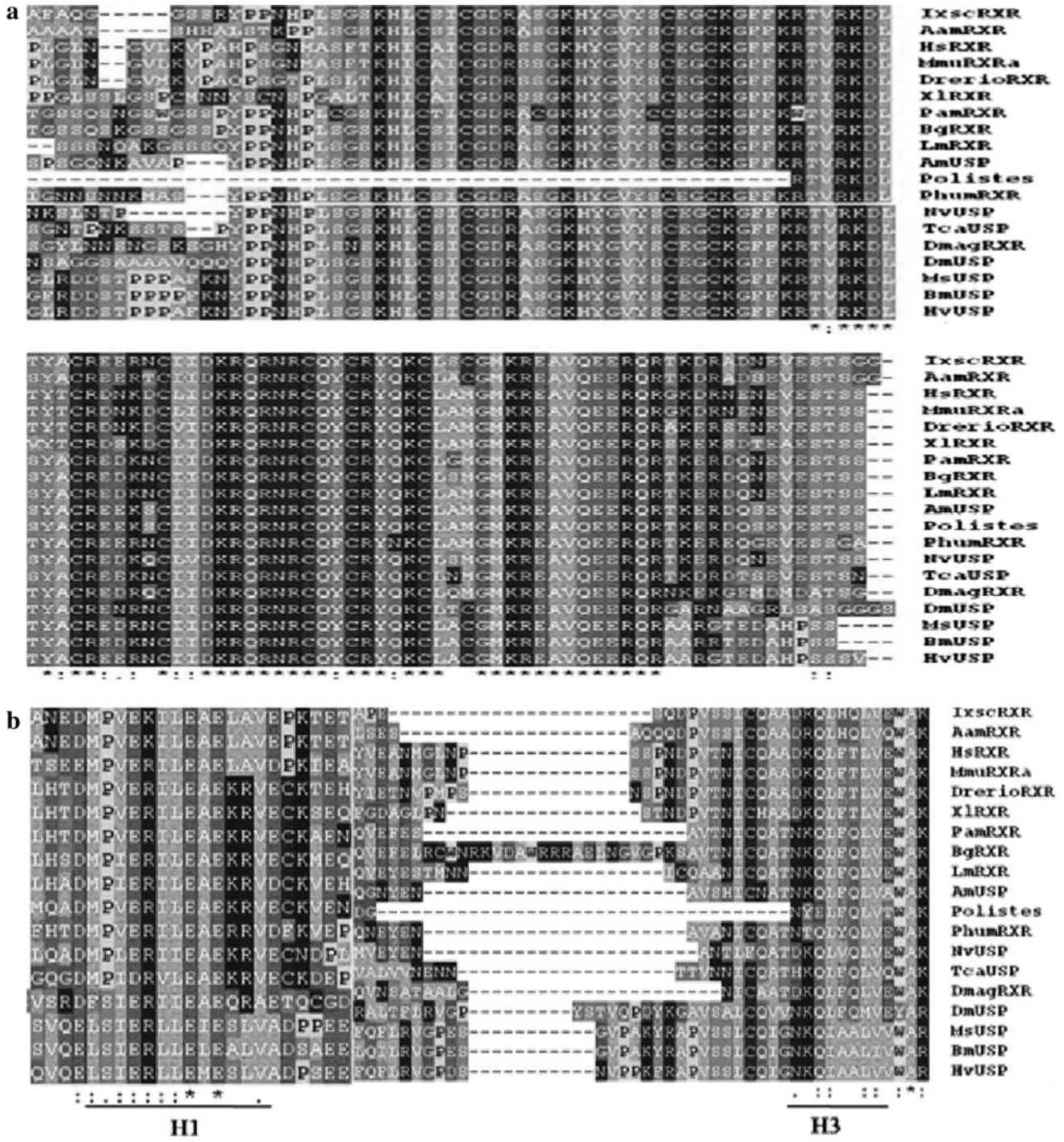

Fig. 2 Comparison of the DBD (*a*) and LBD (*b*) sequence of the *PamRXR* of *P. americana* with other species. **a** The *PamRXR* is aligned with the homologous region of *locusta migratoria* (*LmRXR*), *D. melanogaster* (*DmUSP*), *Bombyx mori* (*BmUSP*), *Manduca sexta* (*MsUSP*), *Heliothis virescens* (*HvUSP*), *Blattela germanica* (*BgRXR*), *Apis mellifera* (*AmRXR*), *Polistes fuscatus RXR* (*Polistes*), *Daphnia magna* (*DmgRXR*), *Ixodes scapularis*, (*IxscRXR*), *Danio rerio* (*DrerioRXR*), *Mus musculus* (*MmuRXRα*), *Tribolium castaneum* (*TcaUSP*), *Amblyomma americanum* (*AamRXR*), *Xenopus laevis* (*XlRXR*), *Pediculus humanus corporis* (*PhumRXR*), *Nezara viridula* (*NvRXR*), and *Homo sapiens* (*HsRXR*). Conserved sequences are shaded. **b** The Region of helices *H1* and *H3* in the LBD are *underlined*

[39, 50], 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.

Phylogenetic Analysis of *PamRXR*

A neighbor-joining tree construction method based on distances of 39 *RXR/USP* protein sequences (27 insects,

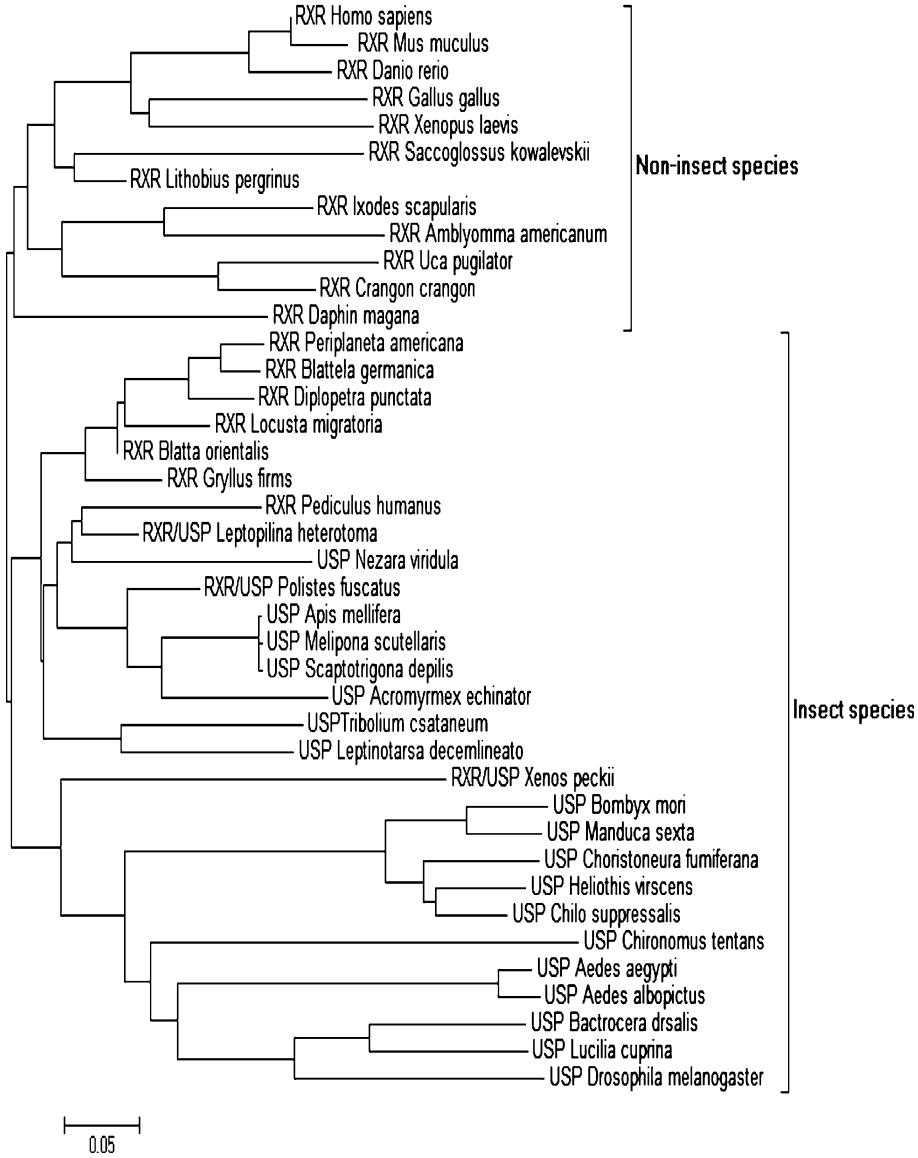

Fig. 3 Phylogenetic tree of 40 *insects* and *non-insects* species *USP/RXR* constructed by neighbor joining method using CLUSTALW program and OMEGA 5.1. The *reference bar* represents the distance (number of amino acid substitution per site). *Branches* lengths are proportional to sequence divergence

and 12 non-insect species) was used to build the phylogenetic tree shown in Fig. 3. The resulted tree was unrooted and consisted of two major branches insect and non-insect *RXR/USP* proteins. In the non-insect branch, vertebrates clustered separately from other arthropods *RXRs*. Although, insects are grouped in the same cluster, Lepidoptera and Diptera formed a monophylogenic branch separated from other holometabolous insect orders such as Coleoptera and Hymenoptera. Also, in the tree Diptera and Lepidoptera had the longest branch lengths, which indicating a rapid rate of divergence of these sequences compared to other arthropods and vertebrates branches' lengths. The *PamRXR* is shown in the same cluster group with the hemimetabolous insects and sister group to the *B. germanica RXR*, *BgRXR* [36, 39, 40].

Tissue-Specific Distribution of *PamRXR*

To determine the tissue-specific expression of *PamRXR*, samples of total RNA extracted from the adult female brain, midgut, muscles, fat body, and ovaries were subjected to RT-PCR with a primer set shown in Table 1. Results (Fig. 4a) show that *PamRXR-S* (short form) was detected in all tissues analyzed, namely fat body, midgut, brain, ovary, and muscle. *PamRXR-L* (long form) was detected obviously in ovary. In fat body, ovary, and muscle samples, the predominant form was *PamRXR-S*, whereas *PamRXR-L* was more abundant in ovary. The existence of *PamRXR* transcript(s) in all tissues tested should not be surprising. Similar and recent molecular studies on different insect species indicated that insect *RXR/USP* gene is

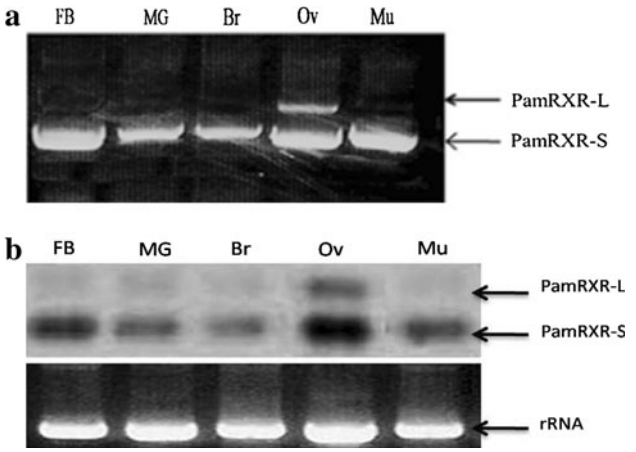

Fig. 4 Tissue-specific expression of *PamRXR* in *P. americana*. **a** Pooled total RNA from 5- to 7-day-old adult female fat body (FB), midgut (MG), brain (Br), ovary (ov), and muscle (Mu) were reverse transcribed to cDNA which used as template to amplify *PamRXR* with a specific primers (RT-F and RT-R) through RT-PCR. **b** Northern blot analyses were performed on total RNA extracted from same above-mentioned tissues. The transcript signals were probed with fluorescein-labeled cDNA fragments of *PamRXR* (see Materials and Methods). *PamRXR-L*: the long isoform; *PamRXR-S*: the short isoform. Ribosomal RNAs (rRNA, *bottom panel*) are shown as internal controls after staining with ethidium bromide. **a**, **b** are representative of two to three replicates

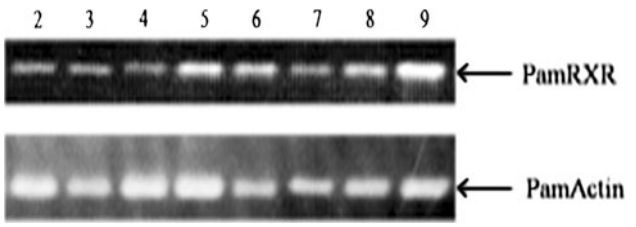

Fig. 5 Developmental expression patterns of *PamRXR-S* in the fat body of adult female *P. americana* during vitellogenic period. The total RNA from 2- to 9-day-old adult female fat bodies were reverse transcribed to cDNA which used as template to amplify *PamRXR-S* with specific primers (RT-F and RT-R). The PCR amplification cycles were as follows : 35–40 cycles of 94 °C for 30 s, then 60 °C for 30 s and 72 °C for 1 min with 1 min extension. As a reference, equal amounts of the cDNA were subject to the same PCR conditions with specific primer pair for *Actin* (Actin F, and Actin R)

expressed in a wide range of tissues including ovary, fat body, midgut, brain, and testis [7, 29, 34–36]. The presence of two *RXR* transcript(s) in different tissues of *P. americana* with different levels suggests the tissue-specific usage of these isoforms. Similarly, in *L. migratoria,* and *B. germanica* two *RXR/USP* isoforms (long and short) have been reported [34, 36]. On the other hand, in *A. aegypti*, two *USP* genes (A and B) have been cloned [37]. The obtained results from RT-PCR have been confirmed by northern blot analyses which revealed similar detectable pattern for *PamRXR* (S and L fragments) (Fig. 4b).

RXR/USP is necessary to mediate ecdysteroid and juvenile hormone functions. Therefore, *PamRXR* gene expression was analyzed in many adult tissues and throughout the vitellogenic period of female *P. americana*.

Developmental Expression Pattern of *PamRXR* in the Adult Female Fat Body During Vitellogenesis

The fat body of female *P. americana* is the main site of vitellogenins (*Vgs*) synthesis [14, 42]. *PamRXR* (S-isoform) transcript level was studied by RT-PCR in the fat body of adult females during the vitellogenic period (Fig. 5). The *PamRXR* transcript was detected during all stages of vitellogenesis with different levels. It was little low during the early vitellogenic period (days 2, 3), then a peak of increase was detected during days 4–6 (day 5) which was followed by another peak of increase at the end of vitellogenesis, day 9 (Fig. 5).

Vg synthesis in the fat body of female *P. americana* was found to be started from day 3 due to the effect of JH III, then secreted into the hemolymph and incorporated into the oocytes [14]. The obtained *PamRXR* early expressed peak is in coincidence with the high titer of JH in the female at the beginning of vitellogenesis [51], which might suggest a role of *RXR* and JH in induction of *Vg* expression at the early vitellogenic period. The second *PamRXR* peak detected on day 9 is also in coincidence with the measured hemolymph ecdysteroid at the end of the vitellogenesis [52], which might suggest another role of *RXR* and ecdysteroid in the suppression of *Vg* expression at the post-vitellogenic period. Combining the present results for *PamRXR* expression level with those previously obtained for *Vg* genes expression levels, we assume that *PamRXR* might play a dual role of induction of vitellogenin through JH regulation at early vitellogenesis and suppression through 20E during late vitellogenesis. However, this assumption will need further investigation to be confirmed. In contrast, in higher insect such as *D. melanogaster* [13, 44] and *A. agypti* [8, 37], the ecdysteroid was found to activate the induction of both *USP* and *Vg*. A rapid and transient up regulation of honey bee, *A. mellifera USP* has been reported in the fat bodies from queens and workers when exposed to JH [33]. The divergence of *RXR* among insects suggests differences in the partner for heterodimeriziation and ligand response [40].

In summary, we have isolated and characterized, for the first time, two isoform for *RXR/USP* gene from *P. americana*. The tissue expression pattern of both *USP/RXR* isoforms indicated a differential regulatory function. Moreover, the obtained results of *PamRXR* developmental expression pattern in the female fat body during vitellogenesis will pave the way for several other investigations to understand both the ecdysteroid-dependent genetic hierarchy and JH mechanism controlling vitellogenesis in the American cockroach, *P. americana.*

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