ISOLATION OF RETINOIC X RECEPTOR (RXR)-LIKE RECEPTOR SEQUENCES FROM *AMBLYOMMA AMERICANUM*

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CHAPTER I

INTRODUCTION

Background and Significance

Ticks are obligate blood-sucking arthropods that affect human and animal health. They exceed all other disease vectors in the variety of pathogens they transmit. The pathogens include viruses, rickettsiae, spirochetes, bacteria, fungi, and protozoa (Hoskins 1991). Babesiosis, Encephalitis, Rocky Mountain Spotted Fever, and Lyme disease are prominent examples of tick-borne diseases (Hoskins 1991). In addition, tick feeding alone may have deleterious effects such as local irritation, blood loss, and cutaneous wounds with possible secondary infection and paralysis. There are also a number of pharmacologically active agents in tick saliva that are secreted into the host during feeding and affect the host's physiological responses (Hoskins 1991).

There are two major families of ticks: argasid ticks and ixodid ticks. Both families have four stages in their life cycle: the embryo, the larva, the nymph and the adult. Each stage (except the embryo) requires a bloodmeal to molt to the next stage. Ixodid ticks have a single nymphal stage while argasid ticks may have multiple nymphal stages (Sonnenshine 1991). Pathogens may be acquired in various blood-feeding stages and can be passed trans-stadially (within the development stages) or sometimes trans-ovarially (from female to progeny). Many ixodid ticks are slow feeders and the long attachment to

hosts increases the chance of pathogen transmission (Hoskins 1991). Ixodid females feed for 7-10 days in which time their weight increases greatly and the ratio of fed to unfed body weight may exceed 100. After mating, a large portion of bloodmeal imbibed by females is converted into eggs. Ixodid females are capable of producing thousands of eggs (Sonnenshine 1991).

Ecdysteroid Hormones in Insects and Ticks

Steroid hormones are a major class of hormones that play regulatory roles in development, differentiation, and in physiological responses in both vertebrates and invertebrates (Voet and Voet 1990). In insects, ecdysteroids are the principal steroid hormone (Koolman 1990). However, most insects are unable to synthesize cholesterol, the precursor of ecdysteroids *de novo* and must acquire it from the diet (Nijhout 1994). The main sources of ecdysone in insects are the prothoracic (or corresponding glands) in immature stages (Rees 1985) and the ovary in adults (Koolman 1990). The production of ecdysone in prothoracic glands is triggered by prothoracicotropic hormone (PTTH) that is secreted by neurosecretory cells and released from the corpora cardiaca. Ecdysone is secreted into the blood and hydroxylated into its active form 20-OH ecdysone in target tissues.

In insects, the role of ecdysone has been best characterized in the molting process. In *Drosophila melanogaster*, ecdysone coordinates tissue-specific morphogenetic changes as a temporal signal. Each stage of development (embryo, three larval instars, prepupa and pupa) is triggered by a pulse of ecdysone (Richards 1981). During the first and second instars, ecdysone triggers molting of the larval cuticle. A high titer ecdysone pulse at the end of third instar triggers metamorphosis. Twelve hours later, another ecdysone pulse triggers the prepupal-pupal transition. This is followed by a

prolonged high titer of ecdysone during pupal development, which affects virtually all tissues during metamorphosis.

Ecdysteroids are not only present in immature stages, but also in adult females. In some insects, ovarian ecdysteroids may regulate vitellogenin synthesis in the fat body (Nijhout 1994). Ecdysteroids may also control cycles of cuticulogenesis in embryos. In some insect species, there is a clear correlation between peaks of free ecdysteroids and the formation of the embryonic membrane (Rees and Issac 1984).

Relative to insects, the central nervous system of ticks is condensed and lacks the classical neuroendocrine and endocrine structures: the corpora cardiaca, corpora allata and prothoracic glands (Sonnenshine 1991). Analogous sites that are responsible for the synthesis, metabolism, storage or release, and control of the ecdysone have not been identified in ticks (Diehl et al. 1986).

Investigation of steroid synthesis in the ixodid tick *Dermacentor andersoni* suggests that ticks lack the enzymes required to close the isoprenoid ring to produce cholesterol (Maroun and Kamal 1976). Thus, ticks also appear to depend on the diet for their cholesterol requirement. Although little is known about the biosynthetic pathway leading to the production of ecdysone from cholesterol in ticks, ecdysone and 20-hyroxyecdysone can be synthesized from ^{14}C-labeled cholesterol *in vivo* (Sonenshine et al. 1985). A number of structures have been suggested as sites for the production of ecdysteroids including lateral organs (Binnington 1981) and fat body (Schriefer et al. 1987). Recently, Zhu et al. (1993) have shown that the epidermis is the source of ecdysone production in the argasid tick *Ornithodoros parkeri*. Incubation of the whole integument *in vitro* results in the production of ecdysone but not 20-hydroxyecdysone or other ecdysteroids.

However, when the integument and the fat body are incubated together, a large amount of labeled 20-hydroxyecdysone and a small amount of ecdysone are produced, suggesting that conversion of ecdysone to 20-hydroxyecdysone occurs in the fat body. Thus, the epidermis may serve as both a source and target of ecdysteroids in *O. parkeri*. Since both the prothoracic glands and epidermis are of ectodermal origin, this may represent a more primitive form of hormone regulation in argasid ticks. However, analogous information about the synthesis, storage or metabolism of ecdysone does not exist for ixodid ticks.

In ticks, ecdysteroids may control a number of developmental transitions including molting, salivary gland degeneration, oogenesis, reproduction, embryogenesis, and diapause (Sonnenshine 1991). During the molting cycle, fluctuating titers of ecdysteroids accompany physiological and morphological changes in larvae and nymphs. In the larvae of *Ornithodoros moubata* (Diehl et al. 1986) and nymphs of *Amblyomma hebraeum* (Diehl et al. 1982), rising ecdysteroid titers parallel apolysis. Deposition of the epicuticle occurs at the ecdysteroid peak. During the declining phase of ecdysteroid titers, the procuticle is synthesized.

In adult females, the salivary glands lose most of their fluid secretory capacity following mating and repletion and the acini undergo autolysis and degeneration (Sonnenshine 1991). Kaufman et al. (1986) have indicated that salivary gland degeneration is triggered by very high concentrations of ecdysone and 20-OH ecdysone. In 1994, Kaufman's group (Mao and Kaufman 1994) demonstrated that salivary gland extracts from partially fed *A. hebraeum* females display saturable and reversible Ponasterone A binding, confirming that the salivary glands express an ecdysone receptor during the later stages of feeding. More recently, our group has demonstrated that EcR

mRNA is present in both unfed salivary glands and throughout feeding and repletion (see Chapter 4). Therefore, it is likely that EcR receptors are expressed throughout feeding and may regulate both differentiative and degenerative events in the salivary glands.

Ecdysteroids may also influence oogenesis and embryogenesis in ticks. Ingestion of high doses of ecdysteroids can suppress oogenesis (Connat and Dotson 1988). Administration of 20-OH ecdysone inhibits egg production (Mansingh and Rawlins 1977) and decreases female fecundity in ixodid ticks (Khalil et al. 1984). Ingested ecdysteroids are metabolized to apolar conjugates in adult *Boophilus microplus* females (Wigglesworth et al. 1985) and in *O. moubata* females (Connat et al. 1986). These apolar conjugates have been identified as ecdysone 22-long-chain fatty acid esters (Crosby et al. 1986). The maternal ecdysteroid esters in *B. microplus* seem to function as a storage form of hormone which are hydrolyzed to release free hormones for the initiation of embryonic cuticles (Wigglesworth et al. 1985). While apolar conjugates of ecdysteroids are found in freshly laid eggs of *O. moubata,* Connat et al. (1986) have been unable to show that they are metabolized to biologically active ecdysteroids during embyrogenesis. Dotson et al. (1993) have suggested that in *O. moubata* embryos, the endogenous ecdysteroids peak is synthesized *de novo* and then is inactivated by conjugation to form apolar products. Based on these results, they propose that the apolar pathway in the female *O. moubata* is a detoxification mechanism for ingested ecdysteroids that prevents vitellogenesis. However, they did not investigate the possibility that the radiolabel may have been removed to produce unlabeled hormones during metabolism of the apolar conjugates. Therefore the role of the apolar conjugates in this species remains speculative.

Exogenously applied ecdysone has been shown to terminate diapause (Wright 1969) and has also been shown to produce effects in tick cell lines (Kurtti and Munderloh 1983). Twenty-hydroxyecdysone has been shown to exhibit dose dependent effects on the growth, attachment, and morphology of an embryonic *Rhipicephalus appendiculatus* cell line (RAE25) (Kurtti and Munderloh 1983). All these studies suggest that ecdysteroids may act in ticks in an analogous fashion to insects.

Steroid Hormone Receptors

Steroid hormones are important in regulating development, differentiation, and homeostasis of both vertebrates and invertebrates. Steroid hormones share a common mode of action. These nonpolar molecules pass through the plasma membranes of their target cells by passive diffusion. They either bind to specific receptors in the cytosol and then are translocated into the nucleus, or they bind to receptors in the nucleus directly (Voet and Voet 1990). Hormone-receptor complexes are composed of protein dimers that bind to hormone response elements (HREs) in target genes to either activate or repress their transcription (Voet and Voet 1990). Regulation of target genes may also require that steroid receptors interact with other tissue-specific and signal-induced transcriptional factors. Thus, the number and type of steroid receptors, as well as the presence or absence of additional transcriptional factors may determine the response of a cell to its hormonal signal (Schcile and Evans 1991).

Steroid hormone receptors belong to a superfamily of ligand-dependent transcriptional factors that include receptors for steroid, vitamin D, thyroid hormones, and retinoic acid (Evans 1988). In addition, several orphan receptors have been identified for which there is no known ligand

(Seagraves 1991). Most nuclear receptors share a highly conserved structure, suggesting that they may all have evolved from a common ancestral gene prior to the divergence of invertebrates and vertebrates (Laudet et al. 1992). The structural organization of nuclear receptors consists of six functional domains (Figure 1). The amino-terminal domain A/B is involved in transcriptional activation and varies in length and amino acid composition. The central DNA-binding domain (C) consisting of 66-68 amino acids, is the most conserved domain among steroid receptors. The hormone-binding domain E binds the hormone and participates in dimerization, nuclear translocation and hormone-dependent transcriptional activation. Regions of domain E that are involved in hormone binding are highly conserved among receptors with the same binding specificity but lack similarity among different receptor types. Domain D provides a hinge between the DNA-binding domain and the hormone-binding domain. The function of the carboxy terminal domain F is unknown (Evans 1988).

Out of the 66-68 amino acids located in the highly conserved DNA binding domains of steroid hormone receptors, 20 are invariant and fold into two "zinc finger" motifs, each of which is coordinated with a zinc atom and four cysteine residues. Residues located at the base of the first zinc finger, referred to as the proximal box (P box), provide specificity for recognition of hormone response elements (HRE) in target genes. Residues located in the distal box (D box) in the second zinc finger form a dimerization interface between hormone receptor monomers.

Recent studies have shown that some nuclear receptor genes produce variant receptor proteins that can negatively modulate the function of their wild type receptors in a dominant negative manner. Bigler et al. (1992) reported that the erbA/thyroid hormone receptor in embryonic avian

erythroid cells is expressed as a nested set of several proteins. The smaller receptor forms, which are generated by alternative translational initiations at internal AUGs in the full length receptor mRNA, contain the hormone binding domain but lack the N-terminal DNA-binding domain. These truncated proteins specifically suppress both transcriptional repression and hormone-dependent transcriptional activation by the full length receptor.

Dominant negative receptors may also be generated by alternative mRNA splicing. In rats, alternative splicing of a thyroid hormone receptor transcript generates a non-hormone binding protein, which inhibits the thyroid hormone-dependent inductive effects of the wild type receptors on gene expression (Koenig et al. 1989) in tissues where both isoforms are expressed. In a similar fashion, a truncated retinoic acid receptor can cause retinoic acid (RA) resistance in RAC65 cells, which are defective in both $RAR\beta_2$ induction and RA-induced differentiation. Mutational studies indicate that mutants that contain a dimerization subdomain, but are defective in either DNA binding, RA binding or/and transactivation are dominant negative. These mutants may exert their effects by forming inactive heterodimers with RXRs, which mediate high affinity binding of RARs to retinoic acid response elements (Shen et al. 1993). Several mechanisms have been suggested for the dominant negative effects. The dominant negative receptors may compete with the functional receptor for binding to the hormone response element or may bind to the functional receptor to form inactive heterodimers, or may compete for accessory factors required for receptor function (Koenig et al. 1989).

Most members of nuclear receptor superfamily bind their hormone response elements (HREs) as dimers (Evans 1988). The glucocorticoid receptor (GR) and estrogen receptor (ER) bind as homodimers to their HREs

(Kumar and Chambon 1988; Tsai et al. 1988). Other receptors, including retinoic acid receptors (RAR), thyroid hormone receptor (TR), peroxisome proliferator-activated receptor (PPAR), and vitamin D receptor (VDR) pair with retinoid X receptors (RXRs), additional members of the superfamily, to form heterodimers (Kliewer et al. 1992). However, recently a number of orphan receptors have been found to bind as monomers (Wilson et al. 1993).

In vertebrates, retinoids have profound effects on growth, differentiation, and homeostasis. At least two classes of receptors are involved in retinoid acid (RA) signalling. The two classes are retinoid acid receptors (RAR α, β, and γ) (Zelent et al. 1989) and retinoid X receptors (RXR α, β, and γ) (Leid et al. 1992). RARs show strong affinity for both the *all-trans* and 9-*cis* isoforms of RA. The three isoforms of RARs, which are generated by different promoter usage and/or alternative splicing, share a high degree of structure conservation. However, they exihibit specific temporal and spatial expression patterns during development (Zelent et al. 1989). RXRs show marked specificity for 9-*cis* RA and bind as homodimers to their HREs (Mangelsdorf et al. 1992). The three subtypes of RXRs are encoded by separate genes and each gene produces multiple isoforms (Mangelsdorf et al. 1992). All three RXR genes show conservation in their DNA-binding domains and hormone-binding domains but differ greatly in their amino- terminal domains, which may confer different transactivation functions.

RXRs also act as common partners with other receptors including RAR, TR, PPAR, and VDR as described above and stimulate their binding to cognate HREs (Kliewer et al. 1992). The DNA-binding domains of RXRs and their insect homologue, *ultraspiracle* (USP) differ from other receptors in that they contain a third helix, in addition to the two zinc fingers (Lee et al. 1993). The eight amino acids comprising the third helix are conserved among the

isoforms of the RXR proteins in human, mouse, and *Xenopus laevis*, as well as in USPs (Oro et al. 1990). This additional helix may be required for high-affinity binding with DNA and/or serve as a dimerization interface (Lee et al. 1993).

Hormone Response Elements

Typically, HREs for nuclear receptors are composed of two copies of a six nucleotide motif, termed half-sites (Evans 1988). The binding specificity of HREs to receptors is determined by both the half-site sequence and the number of nucleotides separating the two half-sites. The HREs for GR and ER are oriented as inverted repeats and are separated by 3 bps (Martinez et al. 1987). The HREs for RAR, TR, PPAR, and VDR are oriented as direct or inverted repeats and are separated by spacers of defined length (Umesono et al. 1991). The HREs for RXRs are composed of direct repeats with a single nucleotide spacer (Heery et al. 1994). HREs for a number of orphan receptors are configured as a single half-site preceded by a short AT-rich sequence (Wilson et al. 1993).

Recently, several ecdysone-response elements (EcREs) have been isolated and characterized from *Drosophila melanogaster* (Antoniewski et al. 1993). The consensus sequence is: PuG(G/T)T(C/G)A(N)TG(C/A)(C/A)(C/t)Py, an imperfect palindromic structure consisting of two pentamer half-sites separated by a single nucleotide. The integrity of both the half-sites and the 1 bp spacing are critical for binding the EcR. The sequence flexibility of this consensus sequence implies a large degree of variation of EcREs that may confer different EcR-binding affinities in response to changing ecdysone titres during development.

Receptors Associated with Ecdysone Responses

Like other steroid hormones, ecdysone acts on target genes via intracellular receptors. The first direct demonstration of gene regulation by ecdysone arose from studies of the puffing patterns of the salivary gland of the midge *Chironomus tentans* (Cleaver and Karlson 1960). Injection of ecdysone prematurely induced puffs that would normally occur later in development. Cleaver also demonstrated that the rapid induction of some puffs was independent of protein synthesis, while the induction of others was delayed and required protein synthesis (Cleaver 1964). He was the first to suggest that "early acting genes are involved in those processes leading to sequential activation of puffs that appear late" (Cleaver 1964).

Ashburner confirmed these observations in *D. melanogaster*, and as suggested by Cleaver, demonstrated that late puffs were under the control of early puffs (Ashburner et al. 1974). In 1974, Ashburner et al. proposed a formal model in which ecdysone, when associated with its receptor, activates early genes but represses late genes. As the products of the early genes accumulate over time, the ecdysone receptor complex is displaced from both early and late genes, resulting in the repression of the early genes and the activation of the late genes.

The isolation of early genes has confirmed the predictions of the Ashburner model and led to the identification of the ecdysone receptor (EcR) gene in *D. melanogaster* (Koelle et al. 1991). The EcR gene in *D. melanogaster* is a member of the steroid hormone superfamily. Recently, additional cDNAs encoding EcRs from two other dipterans, *C. tentans* (Imhof et al. 1993) and *Aedes aegypti* EcRs (Cho et al. 1995) have been cloned. The DNA-binding domain and the hormone-binding domain of *C. tentans* EcR share 95% and

75% amino acid identity respectively with the *D. melanogaster* EcR. The *A. aegypti* EcR share 97% and 87% identity with the DNA- and hormone-binding domains of the *Drosophila* EcR. cDNAs encoding two lepidopteran EcRs have been cloned from the tobacco hornworm *Manduca sexta* EcR (Fujiwara et al. 1995) and the silkmoth *Bombyx mori* EcR (Swevers et al. 1995) and also share a high percentage of identity in DNA- and hormone-binding domains with other EcRs (Table 1). Our laboratory has also cloned a partial EcR cDNA from *A. americanum,* which shares approximately 78% and 61% identity in the DNA and hormone binding domains respectively, with the *Drosophila* EcRs.

The EcR gene in *D. melanogaster* encodes three functional isoforms (A, B1, and B2) that have common DNA- and hormone-binding domains but different N-terminal regions including the trans-acting domains(Talbot et al. 1993). EcR B1 and B2 transcripts are produced from the same promoter but are alternately spliced. The promoter for EcRA is upstream of that for two EcRB transcripts and the EcRA transcription unit overlaps the EcR-B1 and B2 transcription unit. At the onset of metamorphosis, EcRA and B1 isoforms exhibit different tissue expression patterns. The EcRA isoform predominates in the imaginal discs, which will form adult structures, and in the larval prothoracic glands responsible for ecdysone biosynthesis. Conversely, the EcRB1 isoform predominates in larval tissues destined to degenerate which include the fat body, muscle, foregut, midgut, salivary glands and epidermis. The EcRB1 isoform also predominates in the imaginal histoblasts and midgut cells that will replace the larval epidermis.

Perhaps the most surprising results have been that EcR, when expressed in heterologous cells, is unable to bind DNA or hormone and is incapable of transcriptionally activating a reporter gene containing an

ecdysone response element (Koelle et al. 1991; Yao et al. 1992). Rather, EcR requires the product of another *Drosophila* steroid hormone receptor gene, *ultraspiracle* (USP), or its vertebrate counterpart, RXR, for DNA binding and transactivation (Thomas et al. 1993). Therefore, the functional ecdysone receptor is the heterodimer of both EcR and USP receptors (Yao et al. 1993).

USP is the insect homologue of vertebrate RXRs (α, β, γ) and shares 86% amino acid identity in the DNA-binding domain and 49% amino acid identity in the hormone-binding domain with RXRα(Oro et al. 1990). USP genes have been isolated from *D. melanogaster* (Henrich et al. 1990; Oro et al. 1990) and *B. mori* (Tzertzinis et al. 1994). The two genes share 96% identity in DNA-binding domains and 56% identity in hormone -binding domains respectively. Like RXRs, USP can form heterodimers with vertebrate receptors including receptors for retinoic acid, T3, vitamin D, and peroxisome proliferator activators (Yao et al. 1992). Therefore, it appears that nuclear receptor heterodimer formation is a conserved mechanism used in both vertebrates and invertebrates. The finding that the functional EcR is a heterodimer raises the possibility that EcR activity can be controlled in multiple ways, including presence of ligands, control of alternate isoforms, and regulation or replacement of its partners.

In *D. melanogaster*, USP is required in multiple tissues and at multiple times during the life cycle. It is expressed at high levels in adult females and at low levels throughout development. The USP protein is involved in a number of ecdysone-regulated responses including embryogenesis, larval development, pupation, and female reproduction (Oro et al. 1992). Recently, USP has been found to partner with at least one other *D. melanogaster* hormone receptor, the insect homologue of rat nerve growth factor induced protein B (Sutherland et al. 1995), raising the possibility that there may be

competition among receptor proteins for pairing with USP. These findings indicate that USP may be an integral part of many hormone responses, in addition to those which are ecdysone-induced.

Nuclear receptors plays important roles in ecdysone responses during feeding, development, and reproductive processes in arthropods. Thus, characterization of these receptors and their relationship to the tick life cycle might provide a better understanding of ecdysone responses in ticks.

The purpose of my project is to clone a RXR/USP-like receptor cDNA from the ixodid tick *Amblyomma americanum* and to determine the number, size, and stage- and tissue- specific distribution of RXR/USP-like transcripts in this species. Characterization of RXR/USP-like receptors will provide new insights into the molecular mechanism of ecdysone action in ticks and facilitate the development of new strategies for effective tick control.

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CHAPTER II

ISOLATION OF RXR-LIKE SEQUENCES UTILIZING REVERSE TRANSCRIPTION- POLYMERASE CHAIN REACTION

Introduction

Ecdysone acts on target genes via intracellular receptors. The functional ecdysone receptor in insects is a heterodimer of EcR and USP proteins (Thomas et al. 1993; Yao et al. 1992; Yao et al. 1993). Both proteins are required for DNA-binding, hormone-binding and transactivation of target genes (Thomas et al. 1993; Yao et al. 1993). Because ecdysone mediates analogous processes in ticks and insects (Diehl et al. 1986), we hypothesized that ticks should possess EcR and USP homologs. Consistent with this prediction, our laboratory has recently isolated a partial EcR cDNA from *A. americanum* that shows approximately 78% and 61% amino acid identity in the DNA- and hormone-binding domains respectively, with the insect EcR proteins (unpublished results). The purpose of the experiments described in the following chapter was to isolate a USP-like cDNA from *A. americanum.*

The Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR) combines technologies for the synthesis and amplification of complementary DNAs (cDNAs) (Veres et al. 1987). In RT-PCR, mRNA is converted to cDNA using reverse transcriptase and the resulting cDNA is subsequently amplified by PCR. The first strand cDNA synthesis can be primed with random hexamers, oligo (dT), or a gene-specific primer. Following synthesis of the

cDNA, and degradation of the RNA template, two gene-specific primers are used to amplify the target DNA using *Taq* DNA polymerase.

Steroid hormone receptors share a high degree of similarity in their DNA-binding domains. The proximal box (P box) region, which is a critical determinant of half-site recognition specificity, is conserved among subfamilies (Forman and Samuels 1990). All USP, RXR, RAR receptors possess identical P-box regions (CEGCKGF). In addition, another downstream region (CQYCRYQ) within the second zinc finger of the DNA-binding domain is also conserved in this subfamily (Figure 2). Therefore, we designed two degenerate DNA primers corresponding to CEGCKGF and CQYCRYQ sequences to amplify a 42 amino acid region of the DNA-binding domain. The predicted 126 base pair (bp) cDNA fragment, encoding part of the DNA-binding domains of RXR-like receptors, was subsequently amplified from the RNA of molting *A. americanum* larvae and nymphs, and a tick cell line RAE25 (Kurtti and Munderloh 1983) by RT-PCR.

Materials and Methods

Animals and cell lines

Lone star ticks, *Amblyomma americanum*, were reared on rabbits (larvae) or sheep (nymphs) according to the methods of Patrick and Hair (1976). Heterogerous populations of larvae and molting nymphs were collected and frozen at -70°C.

RNA from an ecdysone responsive tick cell line RAE 25 (Kurtti and Munderloh 1983) isolated from *Rhipicephalus appendiculatus* was a gift from Drs. Ulriche Munderloh and Tim Kurrti, University of Minnesota.

Isolation of RNA

RNA was prepared from molting *A. americanum* larvae and nymphs using a modification of the hot phenol/chloroform extraction method (Joett 1986). One gram of ticks were weighed, ground in liquid nitrogen to a fine powder using a mortar and pestal, and placed into 10 ml phenol saturated with 0.2 M sodium acetate (pH 5.0) at 65°C in a conical tube. The tissue was disrupted with a high speed homogenizer for 1 minute at 20 seconds intervals. Seven and one half ml of 0.2 M sodium acetate (pH 5.0) and 1 ml of 20% SDS were added. The tube was incubated at 65°C for 5 minutes and then vortexed and cooled to room temperature. Ten ml of chloroform was then added. After vortexing, the mixture was centrifuged for 5 minutes in a table top centrifuge to separate the phases. The lower organic phase was withdrawn by aspiration through a RNase-free Pasteur tube and discarded, leaving the interphase and the upper phase behind. Five ml of phenol saturated with 0.2 M sodium acetate (pH 5.0) and 5 ml of chloroform was added, votexed, and centrifuged as before. The aqueous phase was then extracted with 10 ml of chloroform 2-3 times until the interface was clear. The upper aqueous phase was withdrawn to a fresh tube and RNA was precipitated with 2.5 volumes of absolute ethanol at -20°C overnight. RNA was recovered by centrifugation for 20 minutes at 4°C and resuspended in DEPC (diethy pyrocarbonate)-treated distilled water. The RNA concentration was determined spectrophotometrically at 260 nm and 280 nm (Davis et al., 1986). RNA was precipitated with 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of absolute ethanol and stored at -70°C.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Reverse Transcriptase-Polymerase Chain Reactions (RT-PCR) were performed using the SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technology) from the RNA of *A. americanum* molting larvae and nymphs according to manufacturer's instructions. Random hexamers were used to prime synthesis of the first strand cDNA. One μg of total RNA and 50 ng of random hexamers were mixed and incubated at 70°C for 10 minutes to disrupt any secondary structure in the RNA. Reverse transcription was performed with 200 u SuperScript II Reverse Transcriptase. The reaction was incubated in 20 mM Tris·HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 500 μM dNTP mix, 5 mM DTT at 42°C for 50 minutes. Following first strand cDNA synthesis, the RNA template was destroyed with RNase H at 37°C for 20 minutes. The resulting cDNA was amplified by PCR using one of the forward degenerate primers and a reverse degenerate primer shown in Figure 3. Because the identity of the last 3' nucleotide is critical for elongation, two different forward primers that differed in the last nucleotide were used. Ten percent of the first strand reaction was used for PCR amplification. The PCR reaction was set up on ice in 20 mM Tris·HCl (pH 8.4), 50 mM KCl, 1.5 mM or 2.5 mM $MgCl_2$, 200 μM dNTP mix, 0.1 μM of each primer, and 5 units Taq DNA polymerase. After overlaying 2 drops of mineral oil over the reaction, the reaction was performed in an Ericomp Singleblock Easycycler PCR apparatus. The amplification program was: 5 minutes at 94°C followed by 3 cycles of 95°C for 1 minute, 42°C for 2 minutes (low annealing temperature) and 72°C for 2 minutes followed by 25 cycles of 94°C for 1 minute, 52°C for 2 minutes, 72°C for 3 minutes and then the reaction was held at 30°C. To increase the yield of the PCR products, 10% of

the primary PCR products were re-amplified using the same primers and PCR program.

Subcloning and hybridizations

Following PCR amplification, 15% of the primary and secondary PCR products were analyzed on a 2% agarose gel stained with ethidium bromide. The gel was denatured in 0.5 M NaOH and 1.5 M NaCl and neutralized in 1 M ammonium acetate. DNA was transferred to the nitrocellulose membrane by blotting the gel with 1 M ammonium acetate and fixed by UV-crosslinking (Smith and Summers 1980). The blot was prehybridized several hours at 42°C and hybridized 16-24 hours at 42°C at low stringency (5X Denhardts, 5X SSPE, 0.1% SDS, and 100 μg/ml ssDNA). A 352 bp *Pst* I-*Bam*H I fragment of the *Drosophila* USP cDNA, pZ7-1, spanning the DNA-binding domain (Henrich et al. 1990) was ^{32}P-labeled by random priming (Feinberg and Vogelstein, 1983) and used as a probe. Fifty ng of the probe (in 7.1 μl distilled water) was denatured at 95°C for 5 minutes and incubated with 11.4 μl LS solution (HEPES/DTM/OL in a ratio of 25/25/7; HEPES= 1M HEPES pH 6.6; DTM= 100 μM dATP, 100 μM dGTP, 100 μM dTTP in 250 mM Tris pH 8.0, 25 mM $MgCl_2$, and 50 mM Beta mercaptoethanol; OL= 1mM Tris pH 7.5, 1mM EDTA pH 8.0, and 90 OD units/ml of oligonucleotide hexamers), 1 μl 10mg/ml BSA, 5 μl 3000 Ci/mmol (α-^{32}P)dCTP, and 0.5 μl of Klenow (5 units/μl) at room temperature for three hours to overnight. The probe was purified from unincorporated (α-^{32}P)dCTP by spinning a Sephadex G-50 column (Sambrook et al. 1989). The probe was added to the hybridization solution at a concentration of ~1X10^6-5X10^6 counts/ml. After hybridization , the filter was washed twice in 5XSSPE and 0.1% SDS at 52°C for 30 minutes and exposed to Kodak X-ray film at -70°C overnight.

The remainder of the PCR products were resolved on a 8% acrylamide gel. The band(s) of interest was excised and eluted with elution buffer (0.5 mM ammonium acetate, 1 mM EDTA, pH 8) according to standard procedures (Chrambach et al., 1971). Purified DNA fragments were cloned into the pCR™II vector according to manufacturer's (Invitrogen, San Diego, CA) instructions using the TA cloning kit. The kit takes advantage of the nontemplate-dependent activity of the thermostable Taq polymerase used in PCR amplification that adds a single deoxyadenosine to the 3' ends of duplex molecules. PCR products with 3' A-overhangs are inserted into a vector with 3' T-overhangs at the insertion site. The purified PCR product was ligated with 50 ng of pCR™II vector with T4 DNA ligase at 14-15°C overnight. One μl of the ligation reaction were used to transform 50 μl One Shot competent cells mixed with 1 μl of 0.5 M β-mercaptoethanol. The mixture were incubated on ice for 30 minutes and then heat-shocked in a 42°C water bath for 30 seconds. Transformed cells were then agitated with 450 μl SOC medium at 37°C for 1 hour and spread on LB agar plates containing 50 μg/ml ampicillin and 25 μl 40 mg/ml X-gal. After incubation at 37°C overnight, colonies containing recombinant plasmids appeared as white or light blue, due to the disruption of the *lac*Z open reading frame and were picked for plasmid isolation.

Plasmid DNA was purified by the alkaline lysis miniprep method (Sambrook et al. 1989). Cells were incubated in Luria Broth (LB) medium containing 100 μg/ml ampicillin at 37°C overnight and centrifuged at 12,000g for 30 seconds. The pellet was resuspended in solution I (50 mM glucose, 25 mM Tris·HCl, pH 8.0, and 10 mM EDTA, pH 8.0), denatured in solution II (0.2 N NaOH, 1% SDS) and neutralized in solution III (3 M potassium and 5 M acetate). After centrifugation, the supernant was extracted with equal volume

of phenol:choloroform and precipitated with 2 volume of 95% ethanol. Purified plasmids were digested with *Eco*R I to remove inserts. DNA was electrophoresed on a 2% agarose gel stained with ethidium bromide and hybridized to the 352 bp *Pst* I-*Bam*H I fragment of the *Drosophila* pZ7-1 USP cDNA (Henrich et al. 1990) as described above. Positive clones were identified by comparing the intensity of the hybridization signals with a *Drosophila* USP control cDNA digested with *Pst* I and *Bam*H I.

DNA Sequencing

Plasmid DNAs from positive clones were purified with the Wizard Miniprep DNA purification System (Promega) according to manufacturer's instructions. Purified double-stranded DNA templates were sequenced by the dideoxynucleotide chain termination method (Sanger et al. 1977) using a Sequenase 2.0 kit (United States Biochemical) according to manufacturer's instructions. ^{35}S-dATP was used to radiolabel DNA. Four to six μg of DNA was denatured with 0.2 N NaOH and 0.2 mM EDTA, annealed to 100 ng of the oligonucleotide primer, and precipitated with 0.1 volume of 3 M NaOAc (pH 5.2) and 2.5 volumes of absolute ethanol. After centrifugation, washing and drying, the DNA templates and the primers were resuspended in 6.5 μl of distilled water. 1.5 μl of sequenase buffer (200 mM Tris·HCl, pH7.5, 100 mM $MgCl_2$, 250 mM NaCl), 1μl 0.1M Dithiothreitol, 2 μl 1: 9 diluted Labeling mix (7.5 μM dGTP, 7.5 μM dCTP, 7.5 μM dTTP), 1 μl ^{35}S-dATP, and 2 μl 1:7 diluted Sequenase Version 2.0 T7 DNA polymerase (13 units/μl) were added for DNA elongation. The mixture was incubated at room temperature for 4 minutes. One fourth of the elongation reaction was transferred to each termination tube containing ddA, ddC, ddG, ddT and incubated at 37°C for 6 minutes. The

reaction was stopped by adding 4 µl stop solution (95% Formamide, 20 mM EDTA, 0.05 % Bromophenol Blue, 0.05% Xylene Cyanol FF). The sample was heated to 95°C for 3 minutes before loading a 6% denaturing polyacrylamide sequencing gel. Following electrophoresis, the gel was dried and exposed to Kodak X-ray film at room temperature overnight. DNA sequences were visualized by autoradiography. Sequences of the PCR clones were analyzed using Macvector (Kodak, Rochester, NY) sequence analysis software.

Northern Blot Analysis

Total RNA from heterogeneous populations of *A. americanum* embryos, molting larvae and nymphs was isolated as described above. Total RNA was extracted and mRNA was isolated from total RNA using oligo (dT) cellulose spin columns according to manufacturers' instructions (5' prime-3' prime, Inc.). Approximately, 2 mg of total RNA was dissolved in 2 ml of 0.1% diethylpyrocarbonate (DEPC)-treated distilled water and heated at 65°C for 5 minutes. The sample was then mixed with 0.5 ml of loading buffer (2.5 M NaCl, 100mM Tris-Cl, 5 mM EDTA, pH 7.5) and applied to the oligo (dT) cellulose spin column and centrifuged. The column was washed with 0.5 M NaCl buffer (0.5 M NaCl, 20 mM Tris-Cl, 1 mM EDTA, pH 7.5) and 0.1M NaCl buffer (0.1 M NaCl, 20 mM Tris-Cl, 1 mM EDTA, pH 7.5). poly $(A)^+$ RNA was eluted with elution buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.5). 10 µg of mRNA was electrophoresed on a 1% denaturing formaldehyde gel (Sambrook et al. 1989). RNA was partially hydrolyzed by 0.05 N NaOH and transferred to nitrocellulose (Nitro Me, Micron Separation Inc.) by capillary elution using 20XSSC for 6-18 hours. RNA was fixed to filters by UV irradiation (Stratalinker). Anti-sense RNA probes were prepared using an *in*

vitro transcription System (Promega, Madison, WI) according to manufacturers' instructions. Linearized DNA templates were *in vitro* transcribed with T7 or SP6 RNA polymerase and radiolabeled with [α-^{32}P] CTP. Following transcription, the DNA templates were removed with RNase-free DNase. The probe was then purified with phenol/chloroform and purified by spinning a RNase-free Sephadex G-50 column. The filter was prehybridized in 50% formamide, 0.75 M NaCl, 0.15 M Tris-HCl (pH 8.0), 10 mM EDTA, 0.2 M sodium phosphate (pH 6.5), 1X Denhardts, and 0.1% SDS for 1-3 hours at 50°C and hybridized to the probe in a fresh solution for 16 hours at 50°C. After hybridization, the filter was washed 3 times in 5XSSC for 5 minutes at room temperature and then washed in 2XSSC for 15 minutes at room temperature. The filter was exposed to Kodak X-ray film at -70°C for 24-72 hours.

Results and Discussion

Isolation of RXR-like cDNAs from A. americanum using the Reverse Transcriptase Polymerase Chain Reaction

Because production of specific PCR products is dependent on a variety of factors, we varied several reaction parameters to produce a specific product. Because annealing of the 3' terminal base is critical for elongation of the template, we used two forward primers that were degenerate but whose terminal base was either a T or a C (see Figure 3). Magnesium concentration can also influence a variety of reactions parameters including primer annealing, strand dissociation temperatures, and product specificity (Innis and Gelfand 1990). Therefore, we performed duplicate PCR reactions using either 1.5 mM or 2.5 mM $MgCl_2$. In addition, we also performed secondary PCR reactions using 10% of the primary reaction product to ensure that we

would get sufficient amplification that the products would be visible in agarose gels following ethidium bromide staining.

Following RT-PCR, a band of 126 bp was predicted based on the forty-two amino acid interval between the forward and reverse PCR primers. When RT-PCR products were resolved by agarose gel electrophoresis, no bands were seen following primary amplification whereas the secondary amplifications resulted in several bands (Figure 4A). A prominent 126 bp band of the expected size was seen in samples amplified with the forward primer 1 and the reverse primer from the RNA of both molting larvae and nymphs. A much fainter band of approximately 126 bp was also detected in some samples amplified with the forward primer 2 and the reverse primer (see below).

When secondary amplifications were resolved on an 8% acrylamide gel (Figure 5), a 126 bp product was seen in samples amplfied with both 1.5 mM and 2.5 mM $MgCl_2$. However, while amplification in 1.5 mM $MgCl_2$ resulted in the production of a more robust 126 bp product, there was also production of more non-specific products. In addition, when the forward primer 2 was used, very faint 126 bp band was also observed in the samples amplified from both the larval and nymphal RNAs using 1.5 mM $MgCl_2$. A faint 126 bp band was detected in the nymphal sample only when 2.5 mM $MgCl_2$ was used.

Following electrophoresis, the agarose gel was blotted and hybridized at low stringency to a *Pst* I-*Bam*H I fragment of the *Drosophila* USP cDNA spanning the DNA-binding domain (Henrich et al. 1990) to determine if the 126 bp product contained identity to the *Drosophila* USP DNA binding domain. Following an overnight exposure at -70°C, a single hybridizing band of approximately 126 bp was detected in lanes corresponding to amplifications using forward primer 1 and the reverse primer (Figure 4B). Although the

fainter band seen in amplifications using the forward primer 2 and the reverse primer might have been detected following a longer exposure, we chose to analyze the more abundant product. However, we don't rule out the possibility that products amplified with the forward primer 2 and the reverse primer might correspond to some larger transcripts derived from alternative RNA splicing or correspond to some transcript intermediates. To determine if the hybridizing fragment contained significant DNA sequence identity to RXR or USP DNA binding domains, a fraction of the secondary PCR reactions were purified from an 8% acrylamide gel and cloned into the PCRII vector for DNA sequence analysis.

To determine the size of the DNA inserts, DNA from individual clones was isolated by alkaline lysis (Sambrook et al. 1989), digested with *Eco*RI and resolved by electrophoresis on a 2% agarose gel. The DNA was then transferred to nitrocellulose and hybridized to the 352 bp*Pst* I-*Bam*H I fragment of the *Drosophila* USP cDNA (Henrich et al. 1990). Positive signals were verified by comparing the intensity of the hybridization signals with a *Drosophila* USP control cDNA digested with *Pst* I and *Bam*H I (data not shown).

In total, approximately 20 clones that were positive by hybridization were sequenced by the dideoxy chain termination method (Sanger et al. 1977). Analysis of the DNA sequences of individual clones showed that all the PCR clones contained predicted open reading frames of the correct length (42 amino acids) and the sequences of the termini were consistent with that of the primers. Conceptual translation of the clones showed a high degree of identity to the DNA-binding domains of RXR and USP receptors. However, comparison of the individual clones showed that they contained an unusually high number of sequence transitions (one purine or pyrimidine

replaced by another) and transversions (a purine replaced by a pyrimidine or *vice versa*). In addition, the majority of differences was clustered, indicating that the changes in the sequences might reflect multiple products of identical size.

Based on the DNA and putative amino acid sequence identity between individual clones, they were placed into six classes that are likely to reflect distinct genes. An example of the alignment of the DNA and predicted amino acid sequence from one clone, pCRTUSP17 to clones in the other classes is shown in Figure 6A and B. When the DNA sequences were compared, most substitutions were clustered between nucleotide positions 40 and 100 (Figure 6A). Furthermore, the base changes which would result in amino acid substitutions were found in regions of the DNA sequence that would encode the second zinc finger, near or in the distal box (D box) region (Figure 6B). The D box region has been proposed to form a dimerization interface between the two protein monomers (Lee et al. 1993).

When the putative translational products from each class were compared to USP, RXR and RAR sequences (Figure 7), there was conservation in the number and placement of cysteine residues critical for zinc-finger formation. The proximal (P) box region and the distal (D) box region (Umesono and Evans 1989) are delineated by two pairs of arrows in Figure 7. When amino acids from the D box regions of the *A. americanum* DNA binding domain were compared to either vertebrate RXR and RAR, or to insect USP proteins, four of the six classes of RT-PCR products were placed in either the USP, RAR, or RXR subfamilies (Figure 7). pCRTUSP17 (class I) was most related to the USP subfamily while pCRTRXR4 and pCRTRXR29 sequences (class II and III) were most related to the RXR subfamily. pCRTXRXR26 (class IV) was most related to the RAR subfamily. Products in

the remaining two classes (pCRQ4RXR and pCRTRXR11) contained novel D box amino acids.

The percentage of DNA sequence identity of the *A. americanum* RXR/USP DNA-bindng domain to vertebrate RXR genes and the *Drosophila* USP gene is shown in Figure 8A. TRXR4 (class II) and TRXR29 (class III) and TRXR11(class VI) showed a higher degree of DNA identity to vertebrate RXR sequences than to the insect USP sequences while TUSP17 (class I), QRXR4 (class V), and TRXR26 (class IV) showed a higher degree of sequence identity to the insect USP sequences.

The percentage of putative amino acid sequence similarity (amino acids with similar polarity of their side chains) and identity (identical amino acids) of the six classes to vertebrate RXR receptors and the *Drosophila* USP receptor is shown in Figure 8B. Clones from all classes showed the same degree of similarity to RXRα and *Drosophila* USP. The *Drosophila* USP protein is most closely related to RXRα (Oro et al. 1990). However, when amino acid sequence identity was compared, TRXR4, TRXR29, and TRXR11 showed higher identity to RXRα than to USP. TUSP17 showed higher identity to USP. QRXR4 and TRXR26 had approximately the same degree of identity to RXRα and USP, with slightly higher identities to RXRβ and RXRγ. Together, these results suggest that *A. americanum* may possess a family of RXR receptor genes similar to that found in vertebrates. This result is in striking contrast to insects, in which a single RXR gene (USP) has been isolated. In addition, our results suggests that this family contains some members whose DNA-binding domains are more closely related to vertebrate RXR and RAR genes as well as at least one member that is more closely related to the insect RXR gene, USP.

Isolation of a USP-like product from RAE25 cell line

We also performed RT-PCR using RNA derived from an ecdysone-responsive tick cell line RAE25. RAE25 cells are derived from *Rhipicephalus appendiculatus* embryos and exhibit dose dependent effects on attachment, growth, and morphology in response to 20-hydroxyecdysone (Kurtti and Munderloh 1983). Therefore, we predicted that these cells should express EcR and USP mRNAs. Following amplification with both gene-specific forward primer 1 and 2, and the reverse primer, a faint band of 126 bp was visible after secondary PCR only when forward primer 1 was used (data not shown). The 126 fragment was isolated, subcloned and sequenced. An alignment of the predicted amino acid sequence of one clone, RAUSP36 to *Drosophila* USP and TUSP17 from *A. americanum* is shown in Figure 9. As predicted, the amino acid sequence was most similar to USP.

Are RXR/USP RT-PCR Products Encoded by A. americanum?

Because the PCR products were amplified from the RNA of molting larvae and nymphs, which previously had taken a bloodmeal from vertebrates, there was a possibility that some or all products could result from amplification of small RNA fragments present in the bloodmeal. To rule out this possibility, we synthesized antisense RNA probes from RXR4, RXR11, USP17, and RXR26 and performed Northern blot analysis of mRNA isolated from embryos and molting larvae and nymphs. After a 24-72 hour exposure, TRXR11 (Figure 10, left panel), TRXR26 and TUSP 17 (data not shown) detected a prominent transcript of ~2.7 kb that is most abundant in molting larvae and nymphs. This transcript is very close in size to that detected in *Drosophila* mRNA with USP probes (Henrich et al. 1990; Oro et al. 1990).

Larger transcripts were detected in all stages with RXR11, 26, and TUSP17, in addition to a faint 4.4 Kb transcript in the embryonic mRNA. In contrast, When TRXR4 was used as a probe (Figure 10, left panel), it detected a strong doublet of ~2.5-2.7 Kb in embryonic RNA, in addition to faint larger transcripts (molting larvae and molting nymph were not tested).

These data show that the PCR products hybridize to transcripts in embryos, a stage which does not have the potential to contain host blood. In addition, they show the presence of abundant transcripts in larvae and nymphs which are molting, and in which there has already extensive digestion of the bloodmeal. Therefore, these results are consistent with the presence of multiple RXR-like transcripts in ticks. Furthermore, the detection of at least two size classes of transcripts in different stages with the same probe, and the detection of an abundant doublet of approximately 2.5-2.7 kb embryonic RNA only with the TRXR4 probe, suggests that RXR genes in ticks are expressed in tissue- and/or temporal-specific patterns and that there may be alternative processing of RNAs.

We had previously performed Western blot analysis of crude extracts of *A. americanum* embryos, and molting larvae and nymphs, using a polyclonal antibody directed against the amino terminus of the *Drosophila* USP protein (Yao et al. 1992). As shown in Figure 10, right panel, we detected a single cross-reactive band of approximately 55 KDa on immunoblots of molting larvae, suggesting that ticks express a cross-reactive protein. When protein is overloaded (data not shown), we can also detect faint bands in nymphs, suggesting that the protein is expressed at this stage as well. Therefore, our earlier Western blot data are consistent with the expression of a USP-like protein in *A. americanum.*

RXR receptors have been identified in mammals (human and mouse), chickens, and frogs. RXR genes encode three isoforms: RXR α, β, and γ (Mangelsdorf et al. 1992). These proteins are closely related to each other both in their DNA-binding and hormone-binding domains, but differ greatly in their amino-terminal domains. Therefore, they may bind common target sequences and ligands but confer distinct trans-activation functions via their amino termini. The insect homolog of the RXRs, USP has been cloned in the fruitfly *Drosophila melanogaster* (Oro, et al., 1990), the silkmoth *Bombyx mori* (Tzertzinis et al., 1994), and the midge *Chironomous tentans* (Henrich, et al., personal communication). Comparison of the insect USP sequences indicates that they are more closely related to one another than to the vertebrate RXRs. Therefore, Tzertzinis et al. (1994) suggests that the three isoforms of RXRs in vertebrates may derive from ancestral genes that arose after the separation of the vertebrate and the arthropod lineages but before the separation of the amphibian and the amniote lineages. Further, they suggest that the mammalian RXR β may represent a diversifying but primitive gene, which was possibly in place when the arthropod and the vertebrate lineages separated.

Evolutionarily, ticks belong to the subphylum chelicerata, while insects belong to a separate subphylum, mandibulata. Therefore, these two branches of Arthropoda are quite distant. DNA sequence analysis of the DNA-binding domains of *A. americanum* RXR-like receptors suggests that ticks may possess multiple RXR-like receptors and the DNA-binding domains of some of these receptors are more related to those of vertebrate RXRs. These data invite speculation that the duplication of the RXR gene family may have occured prior to the divergence of arthropods and vertebrates. Alternately, duplication may have occured following their divergence, but the close

association of ticks with their vertebrate hosts may have selected for convergent evolution of the RXR gene family in ticks. However, the isolation of complete cDNAs corresponding to each of the six classes of *A. americanum* RXR cDNAs is needed to explore the evolutionary significance of these findings.

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CHAPTER III

ISOLATION OF *A. AMERICANUM* RXR CDNAS

Introduction

The diversity of responses to a single hormone can be generated by the production of multiple hormone receptor isoforms which interact differently with tissue-specific transcription factors or which compete with other receptor isoforms for hormone-binding, DNA-binding or transactivation of target genes (Henrich and Brown 1995). In addition, some hormone receptors can pair with multiple partners.

In *Drosophila melanogaster,* the functional ecdysone receptor (EcR) is the heterodimer of the EcR and USP receptors (Yao et al. 1993). The EcR gene encodes three isoforms (A, B1, B2) that contain different trans-activating domains. The three isoforms are generated by alternate splicing of the EcR gene and exhibit tissue-specific expression patterns that are associated with different metamorphic responses to ecdysone (Talbot et al. 1993). Mutiple EcR transcripts have also been identified in *Chironomus tentans* embryos and larvae (Imhof et al. 1993) and previtellogenic *Aedes aegypti* adult females (Cho et al. 1995). In contrast, the *usp* gene in *Drosophila,* produces a single transcript throughout most of development (Henrich et al. 1994). There is evidence for multiple USP isoforms in both *Bombyx mori* (Tzertzinis et al. 1994) and *A. aegypti* vitellogenic ovaries (Henrich and Brown 1995).

The mammalian counterparts of USP, the RXRs, are encoded by three closely related but distinct genes (RXRα, β, and γ) (Mangelsdorf et al. 1992). The three RXR genes exhibit both unique and overlapping patterns of expression in different tissues. To increase the complexity, both RXRβ andRXRγ produce two alternately spliced transcripts (Mangelsdorf et al. 1992) that are expressed in a tissue specific pattern. Results presented in Chapter II suggest that the *A. americanum* genome also encodes multiple RXR-like genes that produce multiple transcripts and are expressed in temporal- and/or tissue-specific patterns. The purpose of the experiments described in the following chapter was to isolate the corresponding RXR-like cDNAs from *A. americanum* .

Materials and Methods

cDNA libraries

A. americanum embryonic and larval cDNA libraries were constructed in our laboratory using the Uni-ZAP XR vector (STRATAGENE). The embryonic library was constructed from poly-A^+ mRNA prepared from a heterogeneous population of embyros (day 1-day 14). The larval cDNA library was constructed from a heterogeneous population of molting larvae. First strand cDNA synthesis was primed with a hybrid oligo (dT) linker-primer containing an *Xho* I site. StrataScript RNase H- reverse transcriptase and 5-methyl dCTP were used in the reverse transcription reaction. Following first strand cDNA synthesis, the RNA template was removed by RNaseH and second strand cDNA synthesis was performed with DNA polymerase I. The double-stranded cDNA was ligated to *EcoR* I adaptors. Following *Xho* I digestion, the cDNAs were size-fractionated and ligated to the Uni-ZAP XR vector arms containing compatible *Eco*RI and *Xho*I restriction sites. The *EcoR* I site at the 5' end and *Xho* I site in the 3' linker-

primer allows the synthesized cDNA to be cloned into the Uni-ZAP XR vector in a sense orientation (*EcoR* I-*Xho* I) with respect to the *lacZ* promoter. The lambda library was packaged in the Gigapack II gold packaging extract and plated on the *E. coli* cell line XL1-Blue MRF'.

Library Screening

The library was screened according to the DNA screening protocol in the cDNA Synthesis Kit (STRATAGENE, Menasha, WI). The library was titered and 50,000pfu were plated on individual 150 mm NZY plates with 600 µl of host cells XL1-Blue MRF' (OD_{600} of 0.5/ plate) in 6.5 ml of 0.7% NZY top agar/plate. Twenty plates were used to screen a total of 10^6 plaques. The plates were incubated at 37°C for ~8 hours and chilled for at least 1 hour before the DNA was transferred onto nylon membranes (MagnaGraph, Micron Separation Inc.). Duplicate lifts of each plate were made. The membranes were denatured in 1.5 M NaCl and 0.5 M NaOH for 5 minutes, neutralized in 1.5 M NaCl and 0.5 M Tris-HCl (pH 8.0) for 7 minutes, and rinsed in 2X SSC for 2 minutes. Then the DNA was fixed to the membrane by UV-crossliking (UV stratalinker).

A class I RXR-like cDNA amplified by RT-PCR, TRXR8 (126 bp) was radiolabeled using the PCR Radioactive Labeling method (Mertz and Rashtchian, 1993). The amplification primers were the degenerate oligonucleotide forward primers 1 and reverse primer described in Figure 4, which were initially used to amplify 126 bp RT-PCR products. PCR-radiolabeling reactions (20 µl) contained 100 pg plasmid DNA, 5 µM each of three nucleotide (dATP, dGTP, and dTTP), 2.5 mM $MgCl_2$, 250 nM of each forward and reverse primer, and 2.5 units of *Taq* DNA polymerase in PCR buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl). 5 µl of [α-^{32}P]dCTP (3000 Ci/mmol, 3.3 µM) were added for a final concentration of 0.825 µM. The PCR program was: 95°C for 5 minutes followed by 30 cycles of

94°C for 30 seconds, 50°C for 75 seconds, and 72°C for 90 seconds followed by 72°C for 5 minute and the reaction was held at 30°C for 2 minutes. The probe was purified from unincorporated (α-^{32}P) dCTP by Sephadex G-50 spin-column chromatography(Sambrook et al. 1989). The 352 bp *Pst*I-*Bam*HI fragment from the pZ7-1 plasmid containing the *D. melanogaster* USP cDNA (Henrich et al. 1990) was ^{32}P-labeled by random priming (Feinberg and Volgestein 1983).

The membranes were prehybridized for several hours and fresh hybridization buffer and probe (~1X 10^7 counts/membrane) were added to the filters. The filters were incubated for an additional 16-24 hours. Two low stringency conditions were used for the screens: In condition A, the filters were prehybridized and hybridized in 5X Denhardts, 5X SSPE, 0.1% SDS, and 100 µg/ml sheared salmon sperm DNA at 42°C and washed in 5X SSPE and 0.1% SDS at 52°C for 30 minutes twice (S. Elledge, personal communication). In condition B, the filters were prehyridized and hybridized in 30% formamide, 1X Denhardts, 5X SSPE, 0.1% SDS, and 100 µg/ml sheared salmon sperm DNA at 38°C and washed in 2XSSC and 0.1% SDS at room temperature for 30 minutes, and then at 50°C for another 30 minutes (Giguere et al. 1987). High stringency conditions were as follows: filters were prehyridized and hybridized in 2X Denhardts, 3X SSC, 0.5% SDS, and 50 µg/ml sheared salmon sperm DNA at 62°C and washed in 2XSSC and 0.1% SDS at 62°C for 30 minutes, in 1XSSC and 0.1% SDS at 62°C for 30 minutes, and in 0.2XSSC and 0.1% SDS at 62°C for 30 minutes.

Following autoradiography, films with positive signals were aligned to the original plates, and cores were taken using the broad end of a pasteur pipette. Plaques were eluted in 500 µl of SM (0.1M NaCl, 10 mM $MgSO_4 \cdot 7H_2O$, 50 mM Tris-HCl, pH 7.5, and 0.01% gelatin), titered, and replated on 88 mm plates at dilutions of 1 X 10^{-4} and 1 X 10^{-5}. Additional rounds of screening were performed until a single hybridizing plaque could be isolated. Single plaques

were isolated with the narrow end of a pasteur pipette and stored in 500 μl of SM buffer for *in vivo* excision.

In vivo excision

The Uni-ZAP XR vector allows efficient *in vivo* excision and recircularization of cloned insert within the lambda vector to form a phagemid containing the cloned insert. Approximately 250 μl of phage stock (>1X10^5 phage particles) containing a single plaque was incubated with 200 μl of XL1-Blue MRF' cells at an OD_{600} of 1.0 and 1 μl of the ExAssist helper phage (>1X10^6 pfu/μl) at 37°C for 15 minutes. Three ml of LB broth was added and the mixture was incubated with shaking at 37°C for 2-2$^1/_2$ hours to allow *in vivo* excision of the phagemids. The reaction was then heated at 70°C for 15 minutes to kill the bacterial cells and centrifuged to remove debris. One μl of the phage supernatent was incubated with 200 μl of freshly grown SOLR cells (to prevent the growth of the helper phage) at 37°C for 15 minutes. The mixture was then plated on LB-ampicillin agar plates (50 μl/ml) and incubated at 37°C overnight. Single colonies were picked and plasmid DNA was purified by the alkaline lysis miniprep method (Sambrook et al. 1989).

Restriction Mapping and hybridization analysis

Plasmid DNAs from positive clones were initially digested with *EcoR*I and XhoI to remove inserts and the DNA was electrophoresed on a 1% agarose gel and stained with ethidium bromide. DNA from selected clones were purified using a Qiagen midiprep kit according to manufacturers' instructions. Restriction enzyme sites in purified cDNAs were mapped with the following restriction enzymes which also contain sites in the multiple cloning sites of the

pBluescript SK (-) phagemid: *EcoR* I, *Xho* I, *Sac* I, *BamH* I, *Pst* I, and *Kpn* I. DNA was digested with the restriction enzymes either singly or in pairwise combinations in 1X universal restriction enzyme buffer (URB -330 mM Tris acetate, pH 7.9, 600 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT, 100 μg/μl BSA, and 400 mM spermidine) at 37°C for 2 hours. DNAs were electrophoresed in 1% agarose containing 0.5 μg/ml ethidum bromide using a 1 kb ladder (Gibco BRL) as a molecular weight standard. Agarose gels were photographed using an MP4 camera system (Polaroid) and bidirectionally transferred to nitrocellulose (Smith and Summers 1980) for Southern blot analysis.

DNA fragments were radiolabeled by random priming (Feinberg and Volgestein, 1983) directly in low melting point gel. DNA fragments were resolved on 0.8% low melting point gel and excised. Three ml of dH_2O/gm of gel slice was added and the samples were boiled for 7 minutes. Twenty μl of diluted gel was incubated with 22.8 μl LS, 2.0 μl BSA (10 mg/ml), 5.0 μl (α-^{32}P)dCTP (3000 Ci/mmol), and 1 μl Klenow (5 u) for at least 3 hours. The probes were purified from unincorporated (α-^{32}P)dCTP by Sephadex G-50 spin-column chromatography (Sambrook et al. 1989).

Southern blot analysis

Genomic DNA was isolated from *A. americanum* embryos according to Levis et al. (1982). Genomic DNA (10 μg) was digested with *EcoR*I, *Hind*III, or double digested with *EcoR*I and *Hind* III. The DNA was resolved on a 0.7% agarose gel. The gel was denatured in 0.5 M NaOH and 1.5 M NaCl, neutralized in 1M Tris (pH 7.4), 1.5 M NaCl and transferred to the nitrocellulose membrane in 10X SSC according to Southern (1975). DNA was fixed to the membrane by UV-crosslinking. The blots were prehybridized several hours at 62°C, hybridized

16-24 hours at 62°C at high stringency (2X Denhardts, 3X SSC, 0.5% SDS, and 50 μg/ml sheared salmon sperm DNA) to radiolabeled cDNAs of group 1 (RXRE6), 2 (RXRE1), 3 (RXRE2), and 4 (RXRE5) that were linearized by digestion with *Sac* I and labelled with (α-^{32}P)dCTP by random priming (Feinberg and Volgestein, 1983). The blot was washed in 2X SSC and 0.1% SDS at 62°C for 30 minutes, in 1X SSC and 0.1% SDS at 62°C for another 30 minutes. The blot was exposed for 2 days at -70°C and developed.

DNA Sequence Analysis

RXRE6 DNA was double digested with *Bam*HI and *XhoI* to purify fragments of approximately 300 bp and 2500 bp. In cases where deletions could not be obtained, the following DNA fragments were also purified from RXRE6 (Figure 11). The large *BamH* I-*Xho* I fragment of RXRE6 (2500 bp) was subcloned into pGEM-7Zf(+) vector. Subequently, this subclone was digested with *Sph* I and *Hind* III separately to create subclones from the internal 760 bp*Sph* I fragment and 1110 bp *Hind* III fragment. The vector DNA was then religated to create *Sph* I-*Bam*HI and *Hind* III-*Bam*HI subclones. The two *Pst* I fragments (~1300 bp and ~200 bp), an *Xba* I-*Sal* I fragment (~1950 bp), and a *Sal* I fragment were isolated from the original RXRE6 cDNA and were subcloned into the pBluescript SK(-) plasmid.

RXRE1, 2 and 5 cDNAs were digested with *Pst*I to purify the following fragments: RXRE1 (1500 bp, 400 bp, and 150 bp), RXRE2 (1900 bp, 400 bp, and 150 bp), and RXRE5 (2000 bp, 400 bp). An additional fragment of ~450 bp from RXRE5 was subcloned by re-ligating the *PstI* digested vector. DNAs were either electrophoresed in 1% agarose and fragments purified by electroelution (Sambrook et al. 1989) or were resolved on 0.8% low melting point gels, excised and purified from low-melting point gel by phenol/chloroform extraction.

Purified DNA (25-50 ng) was then ligated to 50 ng of vector DNA (pGEM 7Zf, pGEM 5Zf, or pBluescript SK$^-$) digested with the appropriate enzymes. Ligation reactions were performed using 1 u of T4 DNA ligase and 1 µl 5 mM ATP in lX ligase buffer (50 mM Tris-HCl, pH 7.6, 10 mM $MgCl_2$, 1 mM ATP, 1 mM DTT, 5% polythylene glycol-8000) at 14-15°C overnight. One or two µl of the ligation reactions were used to transform DH5α cells by the method of Hanahan (1983). Transformations were plated on LB broth plates containing 50 mg/ml ampicillin and X-gal with IPTG. White colonies were picked for plasmid isolation and DNA was purified by alkaline lysis (Sambrook et al. 1989), digested with appropriate restriction enzymes, and analysed by agarose gel electrophoresis to verify the identity of the cloned products. Appropriate subclones were purified with the Wizard Minipreps DNA purification System (Promega) for DNA sequencing.

The Erase-a-Base system (Promega) was used for the construction of subclones containing progressive unidirectional deletions of the RXRE6 and RXRE5 DNAs. The starting DNA was purified by acid-phenol extraction (Zasloff et al. 1978) to separate supercoiled DNA from nicked and linearized DNA. RXRE6 DNA was digested with *Bam* HI and *Bst*X1 to generate a 5' Exonuclease III (Exo III) sensitive 5' overhang and an Exo III resistant 3' overhang. Likewise, the larger *Pst*I fragment from RXRE5 was digested with *Not*I and *Sph*I or *Sal*I and *Bst*XI to create deletions in 5' to 3' and 3' to 5' orientations, respectively. Exo III digestions were performed at 32°C. Aliquots of the reaction were removed at 30 second intervals to tubes containing S1 Nuclease (to remove the remaining single-stranded tails). Klenow DNA polymerase was added create flush (blunt) ends and the DNAs were re-ligated for 1 hour at room temperature. Ten µl (1/10 of the reaction) was added to competent JM109 cells prepared according to Hanahan (1983) and incubated on LB-ampicillin plates overnight at 37°C. Several

colonies were picked from each time point and plasmid DNA was purified using the alkaline lysis miniprep method (Sambrook et al. 1989). Undigested plasmid DNA was electrophoresed with a supercoiled DNA ladder (Gibco BRL) and a nested set of deletion clones were selected. Plasmid DNA was purified with the Wizard Minipreps DNA purification System (Promega) for sequencing.

Double-stranded DNA templates were sequenced by the dideoxynucleotide chain termination method using the Sequenase 2.0 kit (United States Biochemical) according to manufacturer's instructions. DNA sequence was analysed using MacVector 4.5 (Kodak, Rochester, NJ) software. Data base searches were conducted using the National Center for Biotechnology Information Basic Local Alignment Tool (BLAST) search program (Altschul et al. 1990). Multiple sequence alignments were performed using the Clustal multiple-alignment program (Higgins and Sharp 1989). Synthetic oligonucleotides (17-20 bases) were synthesized and purified by the Oklahoma State University Recombinant DNA/Protein Resource Facility.

Results and Discussion

Isolation of A. americanum RXR cDNAs

Previously, we isolated twenty 126 bp RXR-like cDNAs with RT-PCR. Although the sequences were similar, they contained multiple transversions and transitions, suggesting they might encode distinct products. The clones were placed into six classes based on the similarities of their DNA and putative amino acid sequences. Northern blot analysis with four of the six classes showed that all cDNAs detected a faint 4.4 kb transcript in embryonic RNA while class I, represented by TRXR4, detected an abundant transcript(s) of 2.7-28 kb in

embyros. Therefore, a representative clone from class I (TRXR8) was chosen to perform a low stringency screen of an *A. americanum* embryonic cDNA library constructed in our laboratory. Following the primary screen, 20 positive plaques were recovered based on signals present on duplicate lifts. However, none of these plaques were positive in a secondary screen. When the filters were re-screened at high stringency with a 5.5 kb EcR cDNA, 30 positive clones were recovered following three successive rounds of screening (M.J. Palmer, unpublished results). Therefore, we decided to perform an additional screen of the library using an alternate low stringency protocol (condition B, see materials and methods) and a mixed probe.

A newly plated aliquot of the *A. americanum* embryonic cDNA library was screened with a PCR-radiolabeled TRXR8 probe that was mixed with a radiolabeled *Pst* I-*Bam*H I fragment (352 bp) of the *Drosophila* USP cDNA spanning the DNA-binding domain (Henrich et al. 1990). Twenty positive plaques were recovered from the primary screen. In the secondary screen, only one of twenty plaque lifts was positive when filters were hybridized with the mixed probe. The resulting cDNA (RXRE1) was purified in a tertiary screen and subcloned by *in vivo* excision. The RXRE1 DNA was digested with *EcoR* I and *Xho* I, electrophoresed on a 1% agarose gel, blotted bi-directionally (Smith and Summers 1980) and hybridized to either the TRXR8 or a *Pst* I-*Bam*H I fragments of the *Drosophila* USP cDNA at low stringency (condition B). Appoximately 100 ng of restriction digested TRXR8 and pZ7-1 (USP) (Henrich et al., 1990) were used as controls for hybridization. The RXRE1 insert hybridized strongly to each probe, suggesting that it contained sequences with identity to RXR-like DNA binding domains (data not shown).

The ~2.3 Kb insert of RXRE1 was purified, radiolabeled directly in low-melting point agaorose and used to rescreen the library at high stringency.

Twenty-two positive plaques were isolated after three rounds of replating and hybridization. Clones were placed into two groups based on their giving either strong or weak hybridization signals. Altogether, 9 strong clones and 13 weak clones were isolated. Further analysis of weak clones indicated that they were false positives and did not contain signficant sequence identity to RXRE1.

RXRE1 was also used to screen an *A. americanum* cDNA library constructed from molting larvae but no positive clones were recovered. After a rescreen of the same library with an actin cDNA (M.J.P., unpublished results), we found that the *Eco*RI site in the majority of cDNAs had been corrupted, suggesting that the *Eco*RI linkers were damaged prior to library construction. Therefore, it is likely that this library is incomplete, since many cDNAs may not have had an *Eco*RI end suitable for ligation.

Restriction mapping and hybridization analysis

A combination of restriction enzyme digestion, hybridization, and DNA sequence analysis were used to place the remaining nine strongly hybridizing clones into four groups (Figure 12). All 9 clones were digested with *EcoR* I and *Xho*I to remove inserts. RXRE6, RXRE7, RXRE8, and RXRE9 had identically sized inserts of ~2.8 Kb and were placed into Group 1. RXRE1 was ~2.3 Kb in size and was placed into Group 2. RXRE2, RXRE3, RXRE4 contained identically sized inserts of ~2.4 Kb and were placed into Group 3. RXRE5 was ~2.6 Kb in size and was placed into Group 4.

The cDNA clones were mapped using several restriction enzymes which also contained sites in the multiple cloning site of the pBluescript SK (-) phagemid. The enzymes included *EcoR* I, *Xho*I, *Sac* I, *BamH* I, *Pst* I, and *Kpn* I. cDNAs from each group were digested either singly or in pairwise combination, and digests from each group were compared to one another. All groups

contained large *PstI* fragments of differing size, but all four groups contained one or more smaller *PstI* fragments of identical size. In addition, Groups 1 and 2 contained an identical *Eco*RI-*Bam*HI fragment of approximately 300 bp.

The ~300 bp*Eco*RI-*Bam*HI fragment from RXRE6 (group 1), a ~1900 bp *EcoR* I-*Pst* I fragment and a ~400 bp *Pst* I-*Pst* I fragment from RXRE2 (group 3) were isolated, radiolabled and hybridized to Southern blots of restriction digests from all four groups (data not shown). The 1900 bp *EcoR* I-*Pst* I fragment of group 3 hybridized to the large *Pst* I fragments from group 2 and 4, but not group 1. The ~300 bp *Pst* I fragment from group 3 also hybridized strongly to the 300 bp *Pst* I fragments in groups 2 and group 4 but not group 1. However, the 300 bp *Eco*RI-*Bam*HI fragment from RXRE6 hybridized to a similarly sized fragment from group 2 that was absent in group 3 and group 4.

The 5' and 3' end of the representative clones from each of the 4 groups were also sequenced using T3 and T7 primers located in the multiple cloning site of pBS sk-. The 5' sequences of groups 1 and Group 2 were identical and contained an open reading frame corresponding the DNA-binding domain TUSP-17 (class IV RXR receptor cDNA isolated by RT-PCR). Group 3 and Group 4 each had unique 5' sequences. All cDNAs had poly-A+ tracts at their 3' termini.

A schematic representation of the 4 RXR-like cDNAs isolated from the embryonic cDNA library is show in Figure 12. Group 1 and 2 share a common 5' end that encodes part of the DNA-binding domain with sequence that is identical to the TUSP17 RT-PCR product. Although groups 2, 3, and 4 share a large region of identity, groups 3 and 4 lack sequences encoding the DNA-binding domain found in groups 1 and 2.

Unfortunately, group 1 and group 2 cDNAs intitate in the middle of the DNA-binding domain and lack sequences encoding the amino terminal transactivation (A/B) domain. We have also recovered several EcR cDNAs that

initiate at approximately the same place in the DNA binding domain (unpublished results). Because we have experienced difficulty sequencing this region of EcR, it seems likely that this region of the DNA binding domain contains secondary structure that terminates reverse transcription. This may explain the failure of the 126 bp TRXR8 probe to detect RXR-like cDNAs in the cDNA library screen. Since these probes span the region of the DNA binding domain that may have a strong secondary structure, it is likely that RXR-like cDNAs in the embryonic cDNA library would be truncated and would not contain these sequences. The *Drosophila* USP probe used in the second cDNA library screen was larger and therefore, would also detect regions of the DNA binding domain spanning the second and third helices that are present in both RXRE1 and RXRE6 cDNAs. Attempts to recover longer EcR and RXR cDNAs by 5' RACE (Rapid amplification of cDNA ends) (Life Technologies) have thus far, been unsuccessful.

Southern blot analysis

Because the restriction map of RXRE6 differed from RXRE1, 2 and 5, we performed Southern blot analysis of embryonic genomic DNA to determine if hybridization patterns of the four groups were similar. Total DNA from each group was radiolabeled and used to probe a Southern blot of *A. americanum* embryonic genomic DNA that had been digested with *EcoR* I, *Hind* III, or double digested with *EcoR* I and *Hind* III. Blots were hybridized at high stringency and exposed to film at -70^{0}C (data not shown). The hybridizations patterns of RXRE1, RXRE2, and RXRE5 were very similar. RXRE6 contained several bands that were common to RXRE1, 2, and 5, but it also lacked bands present in RXRE1, 2 and 5 and it contained several unique hybridizing bands (see Table 2).

Together with data that show that RXRE1 and RXRE6 contain identical 5' regions and RXRE1, 2, and 5 contain large regions of identity , the Southern blot data suggest that the four cDNAs are most likely derived from a common region of DNA , but that the differences in the restriction patterns are due to alternative RNA processing of transcripts.

DNA Sequence analysis

Representative clones from group 1 (RXRE6) and group 4 (RXRE5) were sequenced entirely on both strands. Because clones in groups 2 and 3 appear to contain several restriction fragments common to group 4, several fragments from groups 2 and 3 were subcloned and selected sequencing was performed to verify that the sequences are identical to RXRE5 (X. Guo, unpublished results). In addition, several oligonucleotide primers were synthesized from selected regions to obtain sequence across restriction sites and to verify that internal sequences in RXRE1 and RXRE2 were identical to either RXRE5 or RXRE6.

The strategy for sequencing RXRE6 is depicted in Figure 11. The *EcoR* I-*BamH* I fragment (~300 bp) of RXRE6 was subcloned into pBluescript SK(-) plasmid and sequenced on both strands. In total, twelve nested deletion clones corresponding to the sense strand of the 2500 bp *BamH* I-*Xho* I fragment of RXRE6 were sequenced. The sequences of individual clones were aligned using MacVector 4.5 and a contig of the entire sequence of the 2500 bp fragment was generated. Where gaps in the sequences existed, nested oligonucleotides were designed as primers to obtain the DNA sequence of the gaps between deletion clones and to join sequence from the *BamH* I-*Xho* I fragment to the 5' *Eco*RI-*Bam*HI containing the DNA binding domain sequence .

Several attempts were made to produce deletion clones for sequencing the anti-sense strand of the *BamH* I-*Xho* I fragment of RXRE6. Ultimately, several

overlapping restriction fragments from the *BamH* I-*Xho* I fragment were subcloned (see Materials and Methods and Figure 11), and the anti-sense (3' to 5') orientation of each clone was sequenced using a combination of primers in the multiple cloning sites and oligonucleotides designed from internal sequences.

The complete DNA and amino acid sequence of RXRE6 is shown in Figure 13. The total sequence of RXRE6 cDNA is 2812 bp long and contains an open reading frame of 906 bp (303 amino acids) that intiates in the DNA binding domain. The remaining 1906 bp corresponds to 3' untranslated region and terminates in a poly-A+ tract. Interestingly, only one polyadenlyation concensus sequence was found in the 3' untranslated region at position 2376. No concensus polyadenylation site precedes the poly-A+ tract in the RXRE6 cDNA sequence. When the conceptual translation of RXRE6 was aligned to RXR, RAR, and USP receptor proteins with MacVector 4.5, RXRE6 was found to contain a high degree of identity to amino acids in the the DNA-binding domain and the C-terminal hormone binding domain. When the putative amino acid sequence of RXRE6 was used to search the non-redundant protein data-base with the BLAST program (Altschul et al. 1990), the sequence showed the highest identity to RXRα from human, mouse, and frog. The next highest scores were those of vertebrate RXRβ and RXRγ. In fact, the USP sequence ranked twenty-second in scoring.

To obtain an optimal alignment, RXRE6 was aligned to RXR and USP receptors using the Clustal program (Higgins and Sharp 1989). As shown in Figure 14, RXRE6 most closely resembles vertebrate RXR receptors. RXRE6 shares the greatest degree of identity (87% and 72% in the DNA- and hormone-binding domain respectively) with the RXR-α receptor. While RXRE6 shows approximately the same degree of identity to USP in the DNA-binding domain, the percentage of identity (50%) to the hormone binding domain of USP is significantly lower. Amino acids located in the third helix of the DNA-binding

domain, a feature unique to the vertebrate RXR and insect USP receptors, are also conserved in RXRE6. However, amino acids in the D-box region which forms a dimer interface between hormone receptor monomers, are more related to amino acids in the insect USP receptors than to the vertebrate RXR receptors.

The RXRE5 clone (group 4) was sequenced completely on both strands by creating a nested set of deletions (X. Guo, unpublished results). However, a search for open reading frames indicated that the clone did not contain any long open reading frames. When the RXRE5 DNA sequence was aligned to RXRE6, RXRE1, and RXRE2, a small region spanning nucleotides 295-490 aligned to a region of RXRE6 sequence that corresponds to a region of the hormone binding domain. When the sequence from RXRE5 was translated in the same frame, a small open reading frame from nucleotide 94 to 512 was found. However. the putative amino acid sequence preceding position nucleotide position 295 did not align to any RXR receptor protein, suggesting that the region preceding position 295 may be derived from a different exon. In addition, the sequence immediately following nucleotide 512 terminated at the stop codon, TGA. A BlastX search was performed in which the DNA sequence of RXRE5 was translated in all six open reading frames and used to search the non-redundant protein data-base (Altschul et al. 1990). When translated in phase 3, the amino acid sequence of a open reading frame from nucleotide 28 to 321 aligned to varying degrees with the transactivation domains of RXR receptors. When translated in phase 1, the amino acid sequence of a open reading frame from nucleotide 312 to 510 was found to have a high degree of identity to a portion of the hormone-binding domains of RXR receptors. This suggests that the RXRE5 transcript contains portion of exons encoding both the transactivation and the hormone-binding domains while lacking exons encoding the DNA-binding domain and part of the hormone-binding domain. This structure most likely results from alternative

splicing and we are currently checking this region of the DNA sequences to determine if splicing of the transactivation exon to the hormone-binding domain exon produces a frameshift that results in termination of the open reading frame. Therefore, RXRE5 may encode a dominant-negative receptor that competes with functional receptor proteins.

Limited DNA sequence analysis of RXRE1 and RXRE2 (Q. Xu and X. Guo, unpublished results) show that both contain a substantial length of sequence that is colinear with RXRE5. When RXRE1 and RXRE2 are aligned, they too show identity to RXRE5 from position 295 (RXRE1) and 370 (RXRE2) and remain colinear with RXRE5. Therefore, the open reading frames of RXRE1 and RXRE2 mostly terminate at the same position as RXRE5. While the 3' sequences of RXRE1 and RXRE2 also align with RXRE5, suggesting they are colinear, there is still a gap of ~370 bp which remains to be sequenced. Therefore, it is possible that some differences may exist between RXRE1, 2, and 5.

These data suggest that at least 3 mRNA isoforms are generated by alternative mRNA processing. RXRE6 most likely corresponds to a full length protein with DNA- and hormone- binding domains. RXRE1 probably encodes a receptor that is truncated in the middle of the hormone-binding domain. RXRE5, since it lacks any long open reading frames, may not be translated, or may produce a very small protein. Since RXRE2 is colinear with RXRE5, presumably it represents a similar cDNA structure.

Therefore, we conclude that RXRE6 encodes a functional RXR-like receptor in *A. americanum.* with a high degree of identity to RXRα. Because sequences in the DNA binding domain are identical to TUSP17, these cDNAs most likely correspond to a class IV RT-PCR product. Unfortunately, RXRE6 is a partial cDNA and lacks sequences encoding the N-terminal A/B domain. Thus far, attempts to extend the cDNA using a 5' RACE have been unsucessful. We

plan to construct additional cDNA libraries using random hexamers to arbitrarily prime cDNA synthesis in combination with inhibitors of secondary structure to recover cDNAs with more 5' sequences. The remaining 3 groups of cDNAs contain regions of overlap, suggesting they are produced from the same gene. Because RXRE1 and RXRE6 are identical for 662 bp starting from 5' end, it is likely that all four groups of cDNAs orginate from the same gene and are produced by alternative splicing. This situation is analogous to that seen in vertebrate hormone receptor genes, where mRNA produced by alternative RNA splicing can produce truncated products which may compete with functional receptor proteins in a dominant negative manner.

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CHAPTER IV

ANALYSIS OF ECR AND RXRE6 mRNA EXPRESSION

Introduction

Ecdysteroids play important roles in embryogenesis, molting, and reproduction in ticks (Sonnenshine 1991). In addition, they may also play important roles in regulating histolysis and remodeling of tissues involved in feeding (i.e. gut and salivary glands). At least one RXR-like receptor in insects, USP has been shown to partner with EcR, and is essential for DNA and hormone binding, and activation of target genes.

We previously performed preliminary Northern blot analysis of poly-A+ RNA from heterogeneous populations of *A. americanum* embryos, molting larvae, and molting nymphs using 126 bp RT-PCR products corresponding to the the DNA-binding domain of RXR-like cDNAs in *A. americanum* as probes. As described in chapter II, a prominent transcript of ~2.5-2.7 Kb was detected in molting larvae and nymphs with all probes tested. Larger transcripts that were much fainter were also detected in all stages with TRXR11, TRXR26, and TUSP17. In contrast, the TRXR4 probe detected abundant transcripts of ~2.5-2.7Kb in embryos, in addition to faint larger transcripts (molting larval and nymphal RNA were not tested). This suggests that *A. americanum* possesses multiple RXR-like genes, which are expressed in temporal- and/or tissue- specific patterns, similar to the multi-gene family of vertebrate RXR genes (Mangelsdorf et al. 1992). If so,

A. americanum RXR receptors could modulate EcR function by expressing alternate RXR genes and/or alternate RXR isoforms.

We previously isolated 4 partial RXR-like cDNAs from an *A. americanum* embryonic cDNA library (Figure 11). A combination of restriction enzyme, hybridization, and DNA sequence analysis suggests they are encoded by a single gene and are produced by alternative RNA splicing. Two cDNAs (RXRE6 and RXRE1) contain identical 5' sequences encoding part of the DNA-binding domain and hormone-binding domain but contain distinct 3' sequences. RXRE2 and RXRE5 lack sequences encoding the DNA-binding domain, but contain some sequence that is colinear with RXRE1 and RXRE6. DNA sequence analysis suggests that RXRE1, 2, and 5 encode truncated receptor isoforms while RXRE6 encodes a functional receptor protein. DNA sequence analysis also confirms that RXRE6 corresponds to the RT-PCR TUSP17 product. Preliminary Northern blot analysis showed that TUSP17 detects a transcript of 2.7 Kb in molting larvae and nymphs and an additional larger 4.4 Kb transcript in embryos.

The size of the 4 partial cDNAs ranges from 2.3 Kb (RXRE1) to 2.8 Kb (RXRE6). Since all four cDNAs lack the sequences encoding the N-terminal A/B region but are at least 2.3 Kb in size, it is likely that they correspond to the larger, less abundant transcripts seen in embryonic RNA. DNA sequence analysis indicated that RXRE6 encodes a functional RXR-like protein with high identity to RXRα in the the DNA- and hormone- binding domain (Figure 14).

Northern blot analysis, in which mRNA is fractionized by size, is used to determine the number, size and relative abundance of transcripts (Alwine et al. 1977). However, due to the level of sensitivity, it can sometimes fail to detect transcripts of low abundance. RNase protection (Melton et al. 1984) and RT-PCR (Veres et al. 1987) assays are more sensitive and can detect lower abundance of transcripts. In addition, they can also differentiate between transcripts which

contain a high degree of identity. When the sample quantity is low or the target mRNA is rare, RT-PCR is the most sensitive assay to measure changes in gene expression. However, to obtain quantitive measurement of RNA levels in different samples, samples should be assayed within the exponential phase of the PCR reaction, before the plateau phase when the amplification efficiency decreases (Gause and Adamovicz 1994). In addition, an internal control that is coamplified with the target RNA is also required to monitor the degradation of RNA and sample-to-sample variations in RT and PCR reactions. An ideal RNA internal standard should be expressed constantly across the samples. Typically, several housekeeping genes such as β-actin, hypoxanthine-guanine phosphoribosyl (HPRT), and glyceraldehyde-3-phosphate dehydrogenase have been used as endogenous standards to detect the relative diffence between individual samples (Finkelman et al. 1991). Variations in samples due to different RNA preparations can also be controlled by normalizing the amount of target products to that of housekeeping gene products. However, even housekeeping genes are not completely invariant in their expression level in some situations. Therefore, a competitive RT-PCR technique has been developed to measure the absolute values of target RNA and to control for differences in amplification efficiency (Uberia et al. 1991).

In competitive RT-PCR, a known amount of control RNA template is coamplified with the target RNA using the same primers. The control RNA can be prepared by *in vitro* transcription of a synthetic gene that differs from the target sequence by the addition of an unrelated DNA sequence or deletion of some portion of the gene. Because primers anneal to identical sequences, the target RNA and control RNA are amplified with the same efficiency. However, products of different size are produced to discriminate between amplification of target and control RNAs.

Experiments in this chapter were designed to determine the number and size of RXRE1 and RXRE6 transcripts and to develop a RT-PCR assay to simultaneously analyse EcR and RXR expression in tick salivary glands.

Materials and Methods

Animals and tissues

Lone star ticks, *Amblyomma americanum*, were reared on rabbits (larvae) or sheep (nymphs and adults) according to the methods of Patrick and Hair (1976). Heterogerous populations of larvae and molting nymphs were collected and frozen at -70°C.

Adult female *A. americanum*, were collected throughout feeding and repletion (unfed, 20-50 mg, 50-100 mg, 100-250 mg, 250-500 mg, >500 mg, replete) and grouped according to weight. Salivary glands from ticks of different weights were dissected at room temperature in cold (4°C) 20 mM TS/Mops (PH 7.0) as described by Needham and Sauer (1979) and frozen in liquid nitrogen. The glands from several ticks were pooled and stored at -70°C.

Extraction of RNA

Total RNA was prepared from *A. americanum* embryos, molting larvae, and molting nymphs using a modification of the hot phenol/chloroform extraction method (Joett 1986). Poly A (+) RNA was isolated from total RNA using oligo (dT) cellulose spin columns according to manufacturers' instructions (5' prime-3' prime, Inc.).

RNA from female salivary glands was extracted as described by Huet et al. (1993), which was originally designed for rapid isolation of total RNA from single salivary glands for RT-PCR. Salivary glands were pooled in 200 µl

extraction buffer (3 M LiCl, 6 M urea, 10m M sodium acetate, pH 5.0, 0.2 mg/ml heparin, and 0.1% SDS) and votexed vigorously. The tubes were left on ice for 1 hour and an equal volume of 4 M LiCl, 8 M urea was added. After vortexing, the tubes were centrifuged for 15 minutes at 4°C. The pellet was resuspended in 250 µl of 0.1 M sodium acetate (pH 5.0) and 0.1% SDS and the tubes were incubated for 5 minutes at room temperature with shaking. Phenol (250 µl) and chloroform (250 µl) were added and the tubes were incubated for 20 minutes at room temperature with shaking. After centrifugation for 5 minutes at room temperature, the supernant was extracted with an equal volume of chloroform. The supernant was then adjusted to 0.2 M sodium acetate (pH 5.0) and precipitated with 2 volumes of ethanol for 1 hour at -80°C with two agitations at 20 minute intervals. The RNA was recovered by centrifugation at 4°C and resuspended in 50 µl of DEPC-treated distilled water. After determination of the concentration of RNA, dilutions were made and stored at -70°C.

Northern blot analysis

Ten µgs of mRNA from embryos, molting larvae, and molting nymphs were electrophoresed on a 1% denaturing formaldehyde gel (Sambrook et al. 1989). RNA was partially hydrolyzed by 0.05 N NaOH and transferred to nitrocellulose filters (Nitro Me, Micron Separation Inc.) by capillary elution with 20X SSC for 6-18 hours. RNA was fixed to filters by UV irradiation (Stratalinker). Anti-sense RNA probes were prepared using an *in vitro* transcription System (Promega) according to manufacturers' instructions. RXRE1 and RXRE6 DNAs were linearized by *Xba* I digestion. Linearized DNA templates were *in vitro* transcribed with T7 RNA polymerase and radiolabeled with [α-^{32}P] CTP to produce anti-sense RNA probes. Following transcription, the DNA templates were removed with RNase-free DNase. The probe was then purified with

phenol/chloroform and purified on a RNase-free Sephadex G-50 spin-column. The filter was prehybridized in 50% formamide, 0.75 M NaCl, 0.15 M Tris-HCl (pH 8.0), 10 mM EDTA, 0.2 M sodium phosphate (pH 6.5), 1X Denhardts, 10% Dextran sulfate, and 0.1% SDS for 1-3 hours at 55°C and hybridized to the probe in a fresh solution for 16 hours at 55°C. After hybridization, the filter was washed three times to remove any nonspecifically bound probes. The first wash was in 0.75 M NaCl, 0.15 M Tris·HCl (pH8.0), 10 mM EDTA, 25 mM $NaPO_4$, 0.1% SDS at 65°C for 1 hour. The second wash was in 0.15 M NaCl, 0.03 M Tris·HCl (pH8.0), 2 mM EDTA, 25 mM $NaPO_4$, 1X Denhardts at 65°C for 1 hour. The third wash was in 0.05 M NaCl, 0.005 M Tris·HCl (pH8.0), 0.4 mM EDTA, 0.1% SDS at 65°C for 40 minutes. The filter was subsequently treated with RNase A. The filter was rinsed three times in 5X SSC for 5 minutes at room temperature and treated with 1 μg/ml RNase in 2X SSC for 15 minutes at room temperature. The filter was then rinsed in 1X SSC, 0.1% SDS at 50°C for 30 minutes and exposed to Kodak X-ray film at -70°C for five days with two intensifying screens.

RT-PCR assay

For amplification of *A. americanum* EcR, RXR, or actin mRNA, gene specific primers were designed based on the sequences of *A. americanum* EcR cDNA, RXRE6, and actin cDNAs (Figure 15). The primers were 19-21 bp in length and had GC content of 50% . Ideally, primer sets should span an large intron (>1 Kb) so that the contaminating genomic DNA will not be coamplified. Studies have shown that most nuclear receptors have introns between the DNA- and hormone-binding domain (Laudet et al. 1992). Since the genomic structure of tick genes is unknown, the primers were selected to amplify a region between the DNA- and hormone-binding domain for amplification of EcR and RXR-like mRNA. Amplifications without the addition of reverse transcriptase were also

performed to assay amplification of genomic DNA or unprocessed RNA. Products of ~530 bp, ~660 bp, and ~580 bp were predicted for amplification of *A. americanum* EcR, RXRE6, and actin mRNA respectively. 10 ng of *A. americanum* EcR cDNA, RXR-like cDNA (RXRE6), and actin cDNA were used as positive controls for PCR amplification in initial experiments.

The RT-PCR assay was performed using embryonic total RNA and the total RNA from salivary glands from ticks of different weights (unfed, 20-50 mg, 50-100 mg, 100-250 mg, 250-500 mg, >500 mg, and replete). Two protocols were used for the assay as described below:

RT-PCR kit— SuperScript Preamplification System for First Strand cDNA Synthesis (Life Technology) was initially used to determine if the assay would work. Random primers or gene-specific reverse primers were first tested for reverse transcription of RNA templates. Total RNA was reverse transcribed with 200 units SuperScript II Reverse Transcriptase. The reaction was incubated in 20 mM Tris·HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 500 μM dNTP mix, 5mM DTT at 42°C for 50 minutes. Following first strand cDNA synthesis, the RNA template was destroyed with RNase H at 37°C for 20 minutes. The resulting cDNA was amplified by PCR using the specific forward and reverse primers. The PCR reaction was set up on ice in 20mM Tris·HCl (pH 8.4), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM dNTP mix, 0.1 μM of each primer, and 5 units Taq DNA polymerase. After overlaying 2 drops of mineral oil over the reaction, the reaction was performed in an Ericomp Singleblock Easycycler PCR apparatus. The amplification program was: 3 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes followed by 72°C for 5 minutes and then the reaction was held at 30°C.

A combined reverse transcription-PCR reaction (Huet et al. 1993) was set up on ice in 25 mM Tris·HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 100 μg/ml

BSA, 100 mM TMAC (tetramethylammonium chloride), 50 μM dNTP mix and 12.5 pmol/primer plus the RNA template in a total volume of 49 μl. After overlaying of 100 μl mineral oil, both the reverse transcription and the PCR amplification were performed in an Ericomp Singleblock Easycycler PCR apparatus. The reaction was denatured for 3 minutes at 94°C and followed by a programmed step (10 minutes) to 55°C. 1 μl of a mix of AMV RT (2.5 units, Pharmacia) and Taq polymerase (1.25 units, Promega) was added and the tube was left for 20 minutes at 55°C. The subsequent amplification program was: 3 minutes at 94°C followed by 30 cycles of 94°C for 1 minute, 55°C for 2 minutes, and 72°C for 3 minutes followed by 72°C for 5 minutes and then the reaction was held at 30°C.

After amplification, 20-30 μl of the reactions were resolved on a 1.5% agarose gel. To increase the sensitivity of detection, the PCR products were blotted onto nitrocellulose membranes and hybridized to ^{32}P-labeled internal oligonucleotides. Oligonucleotides were end-labeled (Sambrook et al. 1989) as follows: 100 ng of the olignucleotide was incubated with 5 μl of 10X T4 kinase buffer, 15 μl of (γ-32P)ATP (10 mCi/ml) and 1μl of T4 kinase (10 u/μl) at 37^{0}C for 45 minutes. The reaction was then heated at 68-70°C for 10 minutes. The probe was purified by spinning through a P6 Bio-Spin Chromatography Column (Bio-Rad) following manufacturer's instructions. The blot was prehybridized for 1 hour at 42°C and hybridized for 4 hours at 42°C in 6X SSC, 1 mM EDTA, 0.5% SDS, 100 μg/ml ssDNA, 1X Denhardts, and 10 mM Na_3PO_4. After hybridization, the blot was washed in 2X SSC and 0.1% SDS at room temperature for 30 minutes and at 50°C for another 30 minutes. The blot was exposed 2 days at -70°C and developed.

Results and Discussion

Northern blot analysis

The action of ecdysteroids in ticks is likely to be mediated by EcR and one or more RXR-like receptors. We previously isolated four partial RXR-like cDNAs from an *A. americanum* embryonic cDNA library. RXRE6 and RXRE1 share a common 5' sequences encoding part of the DNA-binding domain that is identical to the TUSP17 sequences. However, while RXRE6 contains a large open reading frame consistent with a functional receptor, RXRE1 contains distinct 3' sequences whose open reading frame is truncated in the hormone-binding domain. RXRE2 and RXRE5 share a large region of identity to RXRE1 but lack sequences encoding the DNA-binding domain. These results suggest that the four partial RXR-like cDNAs are derived from the same gene by alternative processing. Therefore, we performed Northern blot analysis using RXRE1 and RXRE6 anti-sense RNA probes to examine the number, size, and relative abumdance of RXR-like transcripts in *A. americanum* embryos, molting larvae and nymphs. After a 5 day exposure, RXRE6 detected a faint 3.3 Kb transcript in all stages (data not shown). RXRE1 did not detect any bands. The results suggest that RXRE6 and RXRE1 may be expressed in a very low level or in temporal- or tissue- specific patterns which limit their detection using this method.

RT-PCR assay

At the onset of feeding, salivary glands undergo extensive differentiation and degenerate following repletion in a single feeding cycle. In immature stages, salivary gland tissues are presumably remodeled following repletion. Ecdysone and 20-OH ecdysone stimulate salivary gland degeneration in adult females after

mating and repletion (Kaufman 1986). Recently Kaufman's group (Mao and Kaufman 1994) demonstrated that the salivary glands express an ecdysone receptor during the later stages of feeding. Early stages of feeding were not tested.

Because EcR is likely to mediate both differentiation, degeneration and remodeling of salivary glands, the salivary glands provide a good model for assessing EcR expression. Since it is likely that one or more RXR-like receptors may be dimerization partners of EcR in ticks, both the EcR and the RXR-like receptors might be expressed in the salivary glands throughout feeding and repletion in adult females.

The goal of the following experiments was to develop an assay for the simultaneous detection of EcR and RXR mRNA isoforms in staged salivary glands. Initially we chose to utilize an endogenously expressed RNA as an internal control for sample to sample variation in amplification. We chose to clone an actin cDNA since actins are housekeeping genes that are ubiquitously expressed. An actin mRNA of approximately 660 bp was first amplified from the RNA of molting larvae using degenerate oligonucleotide primers corresponding to conserved amino acids in cytoplasmic actins (M.J. Palmer, unpublished results).

We first used the RT-PCR kit to amplify EcR and actin mRNA (endogenous standard) from serial dilutions of embryonic RNA (1 ng, 10 ng, 100 ng, and 1 μg) using random primers to prime reverse transcription. Gene-specific primers were used for PCR. After electrophoresis in a 1% agarose gel, amplified products were observed for EcR and actin (data not shown). We amplified a single band of ~530 bp for EcR mRNA and a band of ~580 bp for actin mRNA. Next, we used EcR- and actin- specific primers (the same reverse primers for PCR amplification) instead of random primers for reverse

transcription to amplify 10 ng of embryonic RNA. Again, we amplified a band of ~530 bp for EcR mRNA and a band of ~580 bp for actin mRNA.

The Gibco BRL RT-PCR kit was then used in combination with the gene-specific primers to amplify EcR and actin mRNAs from total RNA of salivary glands from ticks of different weights (unfed, 20-50 mg, 50-100 mg, 100-250 mg, 250-500 mg, >500 mg, and replete). After amplification, the products were resolved on a 1.5% agarose gel. A ~530 bp EcR product (Figure 16) and a ~580 bp actin product were observed in salivary glands of all stages. We hoped that actin expression would be invariant in all salivary gland stages. However, we found that actin expression also varies throughout feeding and repletion (data not shown). Both EcR and actin mRNAs were expressed at a low level in unfed ticks and increased in salivary glands from ticks weighing 20-50 mg, 50-100 mg, 100-250 mg, 250-500 mg. Both EcR and actin levels decreased in ticks weighing >500 mg and in replete ticks.

To compare the relative levels of amplified products and to assure that amplified products were specific, gels were blotted and hybridized to internal EcR and actin specific oligonucleotides. Following exposure at -70°C, the hybridization signals were compared between stages (Figure 16). Results were similar to those observed from EtBr stained gels, with one exception. Using an actin-specific oligonucleotide, we detected additional faint bands in the actin amplifications (data not shown), suggesting that multiple actin products were being amplifed. Both vertebrates and arthropods contain multiple cytoplasmic and muscle- specific actin genes that are highly conserved (Mounier et al. 1992). Therefore, actin is not a suitable internal standard for this assay. We will develop competitive internal standards for each EcR and RXR isoforms. Unfortunately, these experiments are beyond the scope of this thesis.

A combined reverse transcription-PCR reaction, similar to that described by Huet, et al. (1993), would allow simultaneous processing of a larger number of samples. Therefore, this protocol was used to amplify EcR and actin mRNA from embryonic total RNA and RNA of tick salivary glands as described above. In the combined RT-PCR assay, both the reverse transcription and the polymerase chain reaction were performed in a single tube in the thermocycler. Reverse transcription is performed at an elevated temperature of 55°C using avian myeloblastosis virus (AMV) reverse transcriptase which contains both the RTase and the RNase H activities. Following reverse transcription, the cDNA is subsequently amplified for 30 cycles. Although we were able to amplify control cDNAs (confirming the reagents were working) and a faint ~580 bp band for actin mRNA, we were unable to amplify EcR mRNA from up to 100 ng of embryonic RNA. When the reverse transcription conditions were modified from 55°C for 20 minutes to 42°C for 30 minutes, a ~580 bp band for actin mRNA and a faint ~530 bp band for EcR mRNA were amplified from 100 ng of embryonic RNA (data not shown). Using the same modified conditions, we amplified a ~580 bp band for actin from 100 ng of RNA from tick salivary glands in several stages (unfed, 20-50 mg, 50-100 mg, 100-250 mg, and 250-500 mg). However, EcR mRNA was not amplified from any stage. We next attempted to optimize the amplification conditions by changing the concentration of $MgCl_2$ from 1.5 mM to 2.5 mM. We also tried substituting SuperScript II RTase for AMV RTase. However, using these modifications, we were still unable to amplify either actin or EcR reproducibly.

Given the time limitations, we opted instead to use the Gibco-BRL pre-amplification system to study the RXRE6 mRNA kinetics in the female salivary glands throughout feeding and repletion. Since RXRE6 has a long open reading

frame and RXRE1, 2, and 5 do not, it is likely that RXRE6 encodes a functional protein.

We designed specific primers spanning DNA- and hormone-binding domains of RXRE6 (see Figure 15) to determine if RXRE6 mRNA is present in salivary glands and could be a potential partner for EcR. To assure the RXRE6 primers were specific, we verified a specific product (~660 bp) amplified with RXRE6 cDNA. Using the same primers, a product of ~660 bp was amplified from 10 ng of the embryonic poly-A+ RNA using random hexamers for reverse transcription after secondary PCR. However, when 10 ng of total salivary gland RNA and total RNA from embryos, larvae and nymphs were used and the RNA templates were reverse transcribed with the gene-specific primers, no product was detected, even after secondary PCR. We subsequently increased the amount of RNA template to 100 ng and performed the reverse transcription using random hexamers. No bands were detected after primary RT-PCR, but following secondary PCR, a product of ~660 bp was amplified from RNA of all stages of salivary glands and from total RNA of embryos (positive control reaction). We also performed a negative control reaction that did not include the reverse transcriptase. No specific band was amplified from this reaction (Figure 17). To assure that the amplified products are RXRE6-specific, we will hybridize the products to internal RXRE6-specific oligonucleotides.

We have demonstrated that both EcR RXRE6 mRNA is expressed in unfed salivary glands and throughout feeding and repletion. Thus, it is likely that EcR receptors are expressed throughout feeding and may regulate differentiative and degenerative events. The fact that we can only amplify a RXRE6 specific product after secondary PCR suggests that RXRE6 is expressed at a much lower level than EcR in salivary glands. Therefore, one or more of the remaining RXR cDNAs amplified from *A. americanum* may be potential partners for EcR in salivary

glands. Because different RXR-like receptors may be expressed in a temporal and/or tissue specific patterns, it is possible that RXRE6 is expressed only in a subset of salivary gland cells. Future work will focus on developing competitive exogenous standards for the RT-PCR assay and on developing and optimizing a combined PCR reaction to assay EcR and RXRE6 transcripts in other tissues. .

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APPENDIX: TABLES AND FIGURES

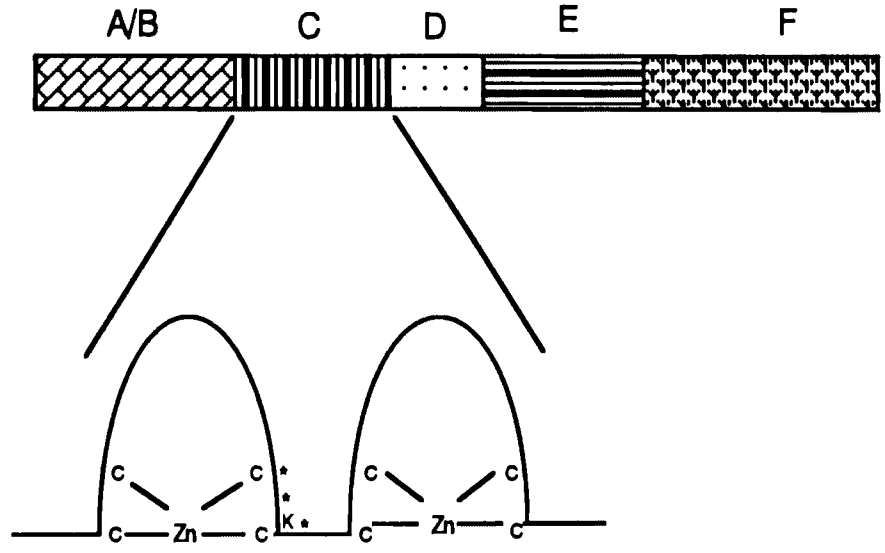


Figure 1. Structure organization of nuclear hormone receptors. The receptor superfamily can be divided into six domains. The amino- terminal domain A/B is involved in the transcriptional activation. The DNA-binding domain (C) comprises two zinc-fingers, each of which is coordinated with a zinc atom and four cysteine residues. The hormone-binding domain (E) binds the hormone and participates in dimerization, nuclear translocation, and hormone-dependent transcriptional activation. The D domain provides a hinge between the DNA- and hormone- binding domain. The function of the F domain is unknown.

SPECIES	% IDENTITY	
	DBD	HBD
DmEcR	100	100
CtEcR	95	75
AaEcR	97	87
MsEcR	95	70
BmEcR	95	70
AmEcR	78	61

Table 1. Percentage of identity of the predicted amino acid sequences of insect and *A. americanum* ecdysone receptors to the *D. melanogaster* EcR. DmEcR, *Drosophila melanogaster* EcR; CtEcR, *Chironomus tentans* EcR; AaEcR, *Aedes aegypti* EcR; MsEcR, *Manduca sexta* EcR; BmEcR, *Bombyx mori* EcR; AmEcR, *Amblyomma americanum* EcR. DBD, DNA-binding domain; HBD, hormone-binding domain.

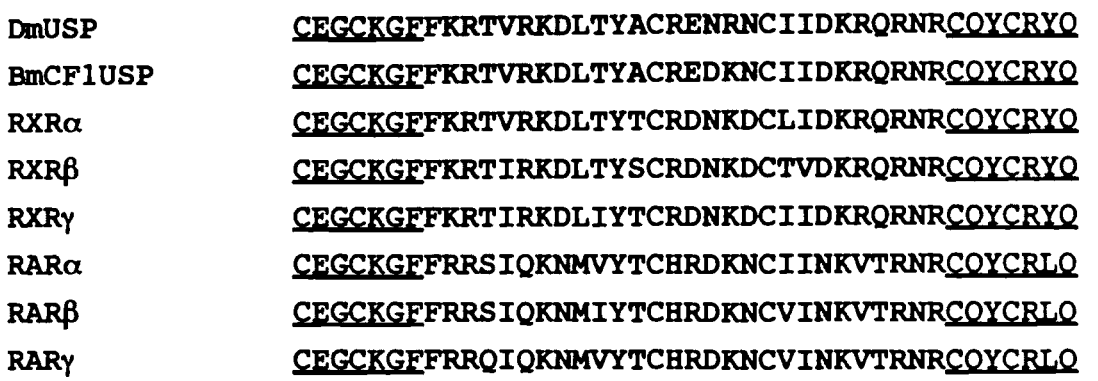


Figure 2. Amino acid sequences of the DNA-binding domains of USP, RXR , and RAR receptors. 42 of the 66 amino acids comprising the DNA-binding domains of USP, RXR, and RAR receptors are shown. Amino acids used to design degenerate oligonucleotides are underlined. Dm = *Drosophila melanogaster,* Bm = *Bombyx mori,* RXR = murine retinoic X receptor, RAR = murine retinoic acid receptor.

PRIMERS	AA SEQUENCE	DNA SEQUENCE
forward 1	CEGCKGF	TGYGARGGITGYAARGGITTC
forward 2	CEGCKGF	TGYGARGGITGYAARGGITTT
reverse	CQYCRYQ	YTGRTAICIRCARTAYTGRCA

Figure 3. Degenerate primers for amplification of the DNA-binding domain of tick RXR-like receptors. Y= C or T, R= A or G, I=inosine.

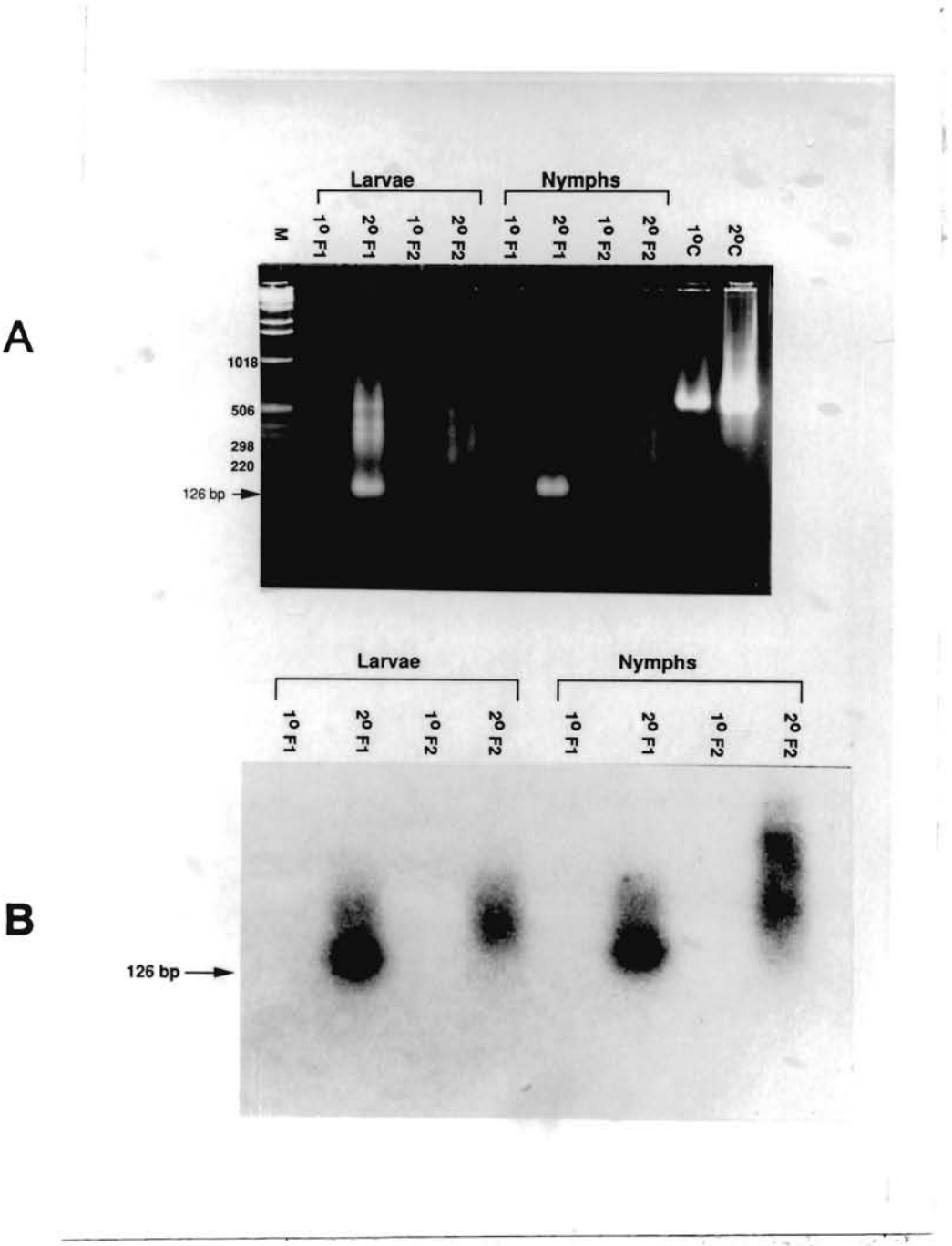


Figure 4. Amplification of RXR-like cDNAs using RT-PCR. RT-PCR products were separated on a 2% agaroese gel stained with ethidium bromide. Panel A shows primary (1°) and secondary (2°) amplification reactions of *A. americanum* larval and nymphal RNAs using two different forward degenerate oligonucleotide primers (F1 or F2) in combination with a degenerate reverse primer. Panel B shows the results of hybridization of the amplified products to a radiolabeled probe from the DNA-binding domain of the *D. melanogaster* USP gene.

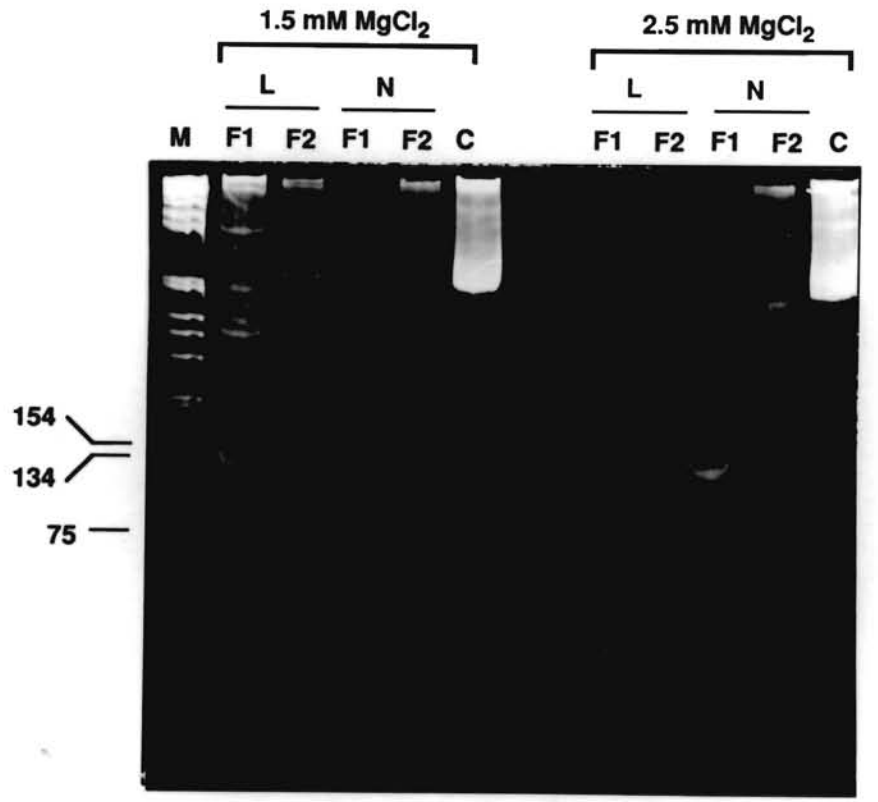
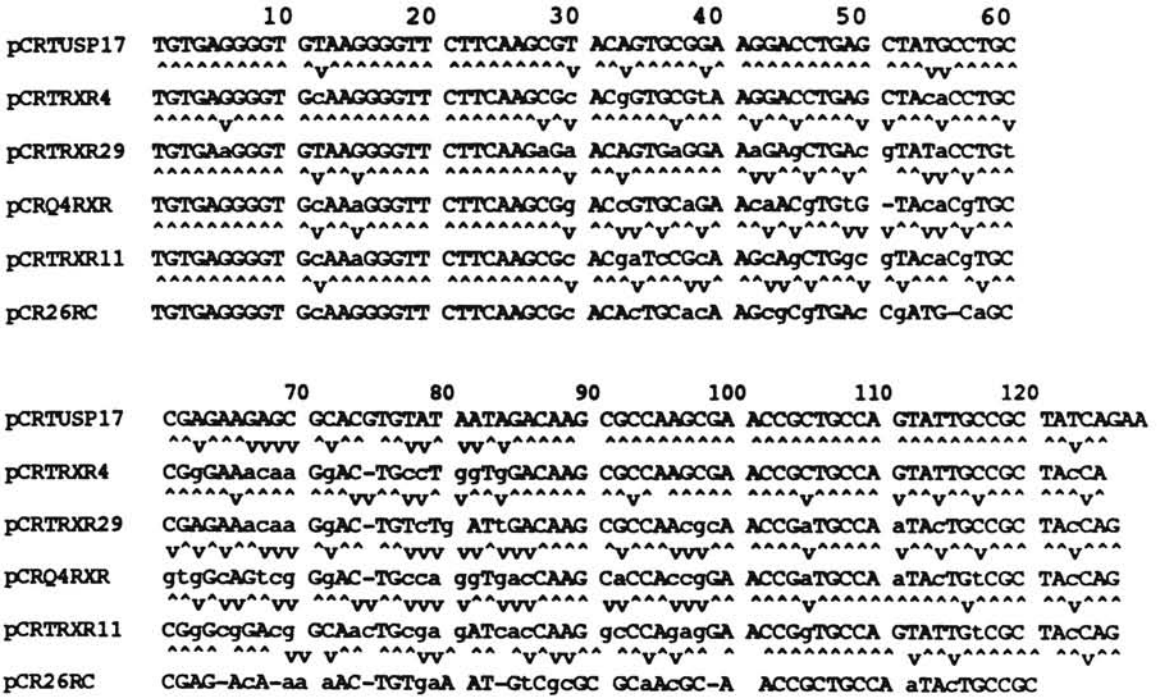


Figure 5. Secondary RT-PCR amplifications of RXR-like cDNAs from *A. americanum* larval and nymphal RNAs using two different $MgCl_2$ concentrations. RT-PCR products were resolved on a 8% acrylamide gel and stained with ethidium bromide. F1, degenerate forward primer 1; F2, degenerate forward primer 2; C, control RNA; M, 1 kb DNA ladder.

A



B

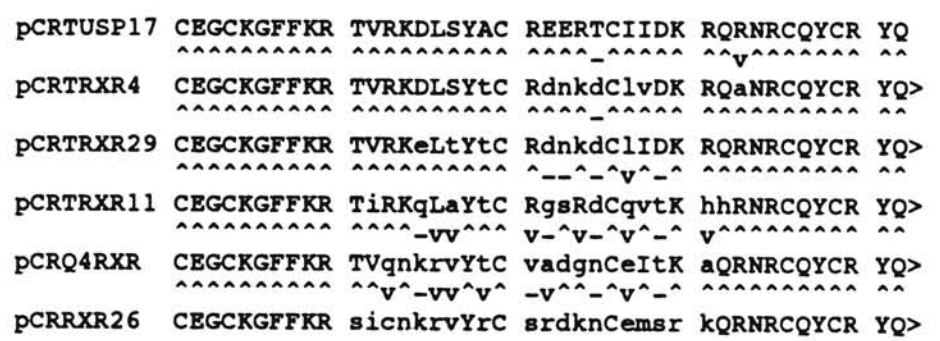


Figure 6. Alignment of the DNA (A) and predicted amino acid sequences (B) from pCRTUSP17 to clones in other classes (pCRTRXR4, pCRTRXR29, pCRQ4RXR, pCRTRXR11, and pCR26RC).

```
                P-box                D-box
pCRTUSP17*      CEGCKGFFKRTVRKDLsYACREertCIIDKRQRNRCQYCRYQ
DmUSP           CEGCKGFFKRTVRKDLTYACREnrnCIIDKRQRNRCQYCRYQ
BmCF1USP        CEGCKGFFKRTVRKDLTYACREdknCIIDKRQRNRCQYCRYQ

pCRTRXR4*       CEGCKGFFKRTvRKdLsYtCRDNKDClvDKRQaNRCQYCRYQ
pCRTRXR29*      CEGCKGFFKRTvRKeLtYtCRDNKDCliDKRQrNRCQYCRYQ
RXRα            CEGCKGFFKRTvRKdLtYtCRDNKDCliDKRQrNRCQYCRYQ
RXRβ            CEGCKGFFKRTiRKdLtYsCRDNKDCtvDKRQrNRCQYCRYQ
RXRγ            CEGCKGFFKRTiRKdLiYtCRDNKDCIiDKRQrNRCQYCRYQ

pCRTXRXR26*     CEGCKGFFKRSIcnkrVYrCsRDKNCemsrkqRNRCQYCRYQ
RARα            CEGCKGFFrRSIqKnmVYtChRDKNCiinkvtRNRCQYCRlQ
RARβ            CEGCKGFFrRSIqKnmIYtChRDKNCvinkvtRNRCQYCRlQ
RARγ            CEGCKGFFRRQIqKnmVYtChRDKNCiinkvtRNRCQYCRlQ

pCRQ4RXR*       CEGCKGFFKRTVqnkrvYTCvadgnCeItKaQRNRCQYCRYQ
pCRTRXR11*      CEGCKGFFKRTirKaLaYTCRgsRdCqvtKhhRNRCQYCRYQ
                ..........********.*****.*******.........
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Figure 7. Alignment of putative translational products from 6 classes of *A. americanum* RT-PCR clones to USP, RXR, RAR sequences. Dm = *Drosophila melanogaster*, Bm = *Bombyx mori*, RXR = murine retinoic X receptor, RAR = murine retinoic acid receptor. Dots indicate common amino acids among sequences. Stars indicate different amino acids among sequences.

A

	I TRXR4	II TRXR29	III TRXR11	IV TUSP17	V QRXR4	VI TRXR26
RXRα	84.0	83.3	73.8	76.8	69.6	77.0
RXRβ	79.0	73.8	74.6	80.0	68.8	71.4
RXRγ	71.4	80.9	70.6	72.0	67.2	69.0
DmUSP	78.6	72.2	69.8	77.6	70.4	78.6

B

	I TRXR4	II TRXR29	III TRXR11	IV TUSP17	V QRXR4	VI TRXR26
RXRα	97.6/93.0	100/97.6	95.2/73.8	95.2/81.0	78.6/69.0	78.6 /59.5
RXRβ	95.2/88.1	97.6/88.1	95.2/76.2	92.9/76.2	81.0/61.9	78.6/61.9
RXRγ	95.2/90.5	97.6/95.2	92.9/76.2	92.9/78.6	81.0/66.7	81.0/61.9
DmUSP	97.6/81.0	100/85.7	92.9/71.4	95.2/88.1	78.6/69.0	78.6/59.5

Figure 8. Percentage of DNA (A) and amino acid sequence (B) similarity/ identity of the six classes of *A. americanum* RXR DNA-binding domains to vertebrate RXRs (RXRα, β, and γ) and *Drosophila* USP (DmUSP).

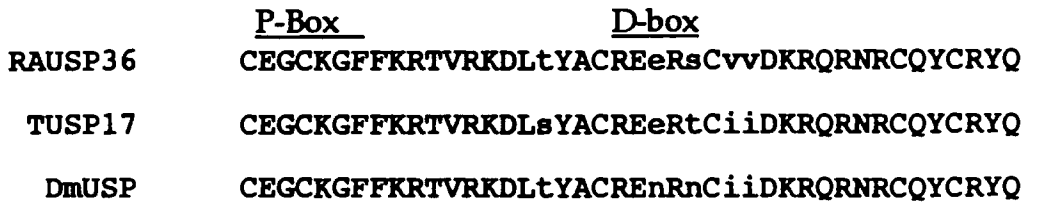

Figure 9. Alignment of amino acid sequences of RAUSP36, a clone from the *R. appendiculatus* cell line RAE25 to *A. americanum* TUSP17 and *D. melanogaster*,USP.

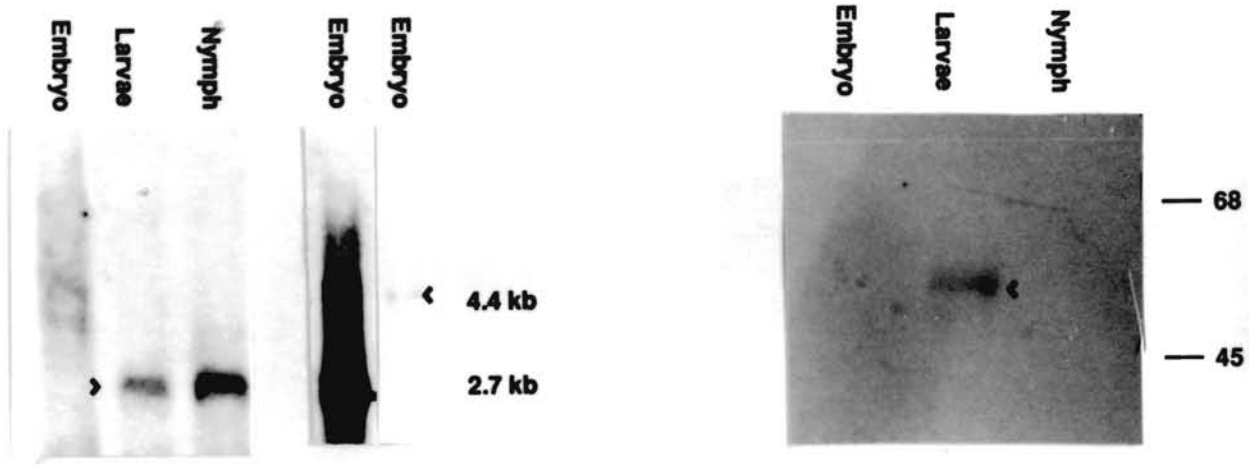

Figure 10. Northern and Western blot analysis of *A. americanum* . The left panel shows Northern blot analysis of mRNA isolated from *A. americanum* embryos, molting larvae and nymphs using TRXR11, TRXR4, and TUSP17 as probes. The right panel shows Western blot analysis of crude extracts of *A. americanum* embryos, molting larvae and nymphs, using a polyclonal antibody direct against the amino terminus of the *Drosophila* USP protein.

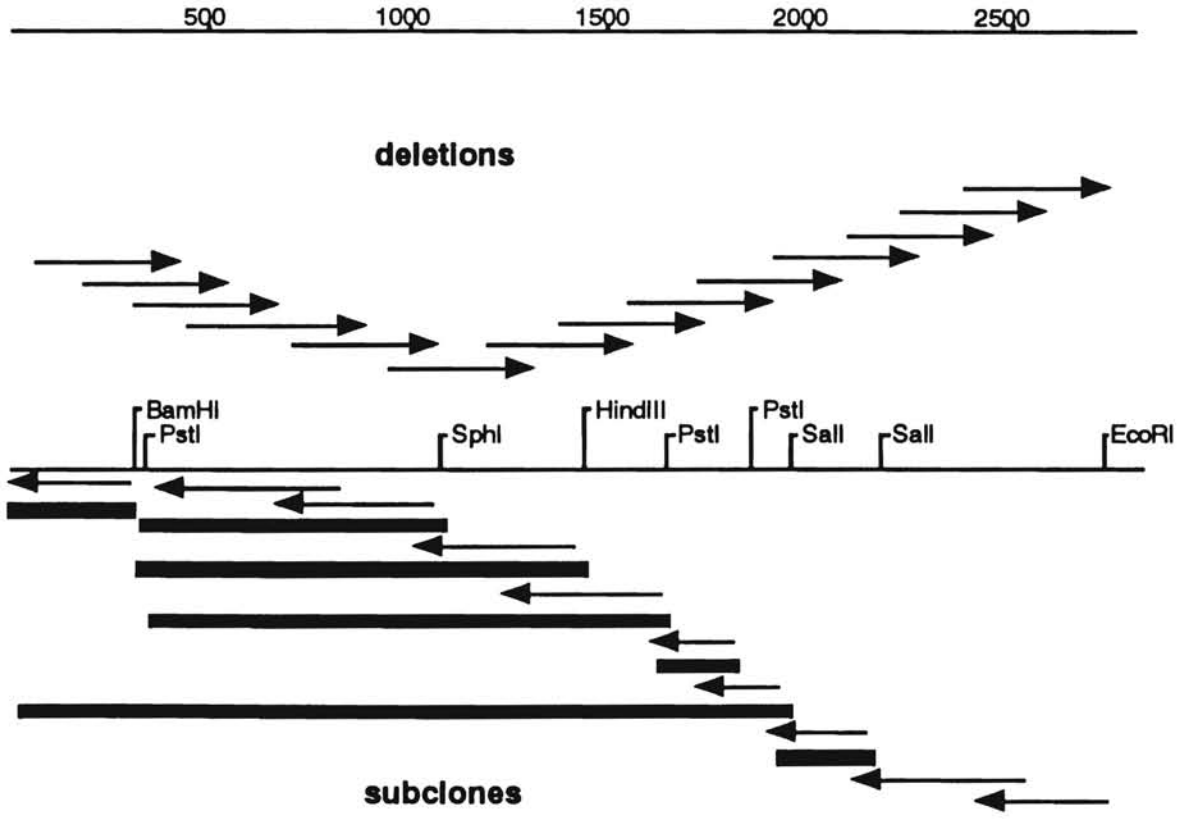

Figure 11. Schematic representation of DNA sequence generated from RXRE6 deletions, subclones and oligonucleotide primers.

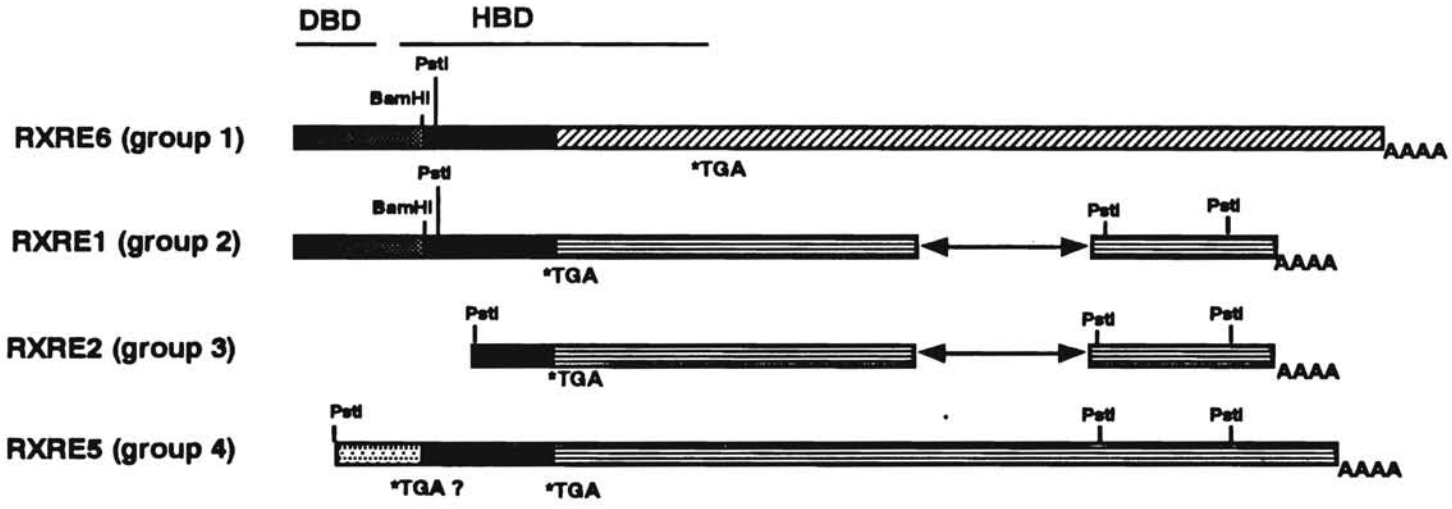

Figure 12. Schematic representation of the 4 groups of RXR-like cDNAs isolated from the *A. americanum* embryonic cDNA library. Lines indicate regions of the cDNA sequence corresponding to the DNA-binding domain (DBD) and the hormone binding domain (HBD). *TGA, stop codon in ORF; AAAA, poly-A+ tail. Arrows indicate gaps in the DNA sequence of RXRE1 and RXRE2.

MOLECULAR WEIGHT (Kb)	RESTRICTION ENZYME	RXRE 1, 2, 5	RXRE 6
9.7	*Hind*III	+	-
8.7	*Eco*RI	+	-
7.3	*Hind*III	+	+
4.2	*Hind*III	+	+
3.2	*Eco*RI+*Hind*III	+	-
3.0	*Eco*RI	-	+
1.75	*Hind*III	+	-
1.4	*Hind*III	+	-
1.37	*Eco*RI+*Hind*III	-	+
1.35	*Eco*RI+*Hind*III	+	+
1.25	*Hind*III	+	-

Table 2. Southern blot analysis of *A. americanum* genomic DNA using RXRE1, 2, 5, and 6 as probes. The genomic DNA was digested with *Eco*RI, *Hind*III, or double digested with *Eco*RI and *Hind*III.

```
   1  GGGATGCAAGGGCTTCTTCAAGCGTACAGTGCGGAAGGACCTGAGCTATGCCTGCCGAGA   60
   1    G   C   K   G   F   F   K   R   T   V   R   K   D   L   S   Y   A   C   R   E    20

  61  AGAGCGCACGTGTATAATAGACAAGCGCCAGCGCAACCGCTGCCAGTACTGCCGCTACCA   120
  21    E   R   T   C   I   I   D   K   R   Q   R   N   R   C   Q   Y   C   R   Y   Q     40

 121  AAAATGCCTCGCATGTGGCATGAAGCGGGAGGCTGTCCAGGAAGAACGGCAGCGCACCAA   180
  41    K   C   L   A   C   G   M   K   R   E   A   V   Q   E   E   R   Q   R   T   K     60

 181  GGACCGTGCCGACAGTGAGGTGGAAAGCACTAGTGGTGGAGCACCTCCTGAGATGCCTCT   240
  61    D   R   A   D   S   E   V   E   S   T   S   G   G   A   P   P   E   M   P   L     80

 241  GGAGCGCATACTGGAGGCAGAGCTGCGGGTTGAGTCACAGACGGGGACCCTCTCGGAAAG   300
  81    E   R   I   L   E   A   E   L   R   V   E   S   Q   T   G   T   L   S   E   S    100

 301  CGCACAGCAGCAGGATCCAGTGAGCAGCATCTGCCAAGCTGCAGACCGACAGCTGCACCA   360
 101    A   Q   Q   Q   D   P   V   S   S   I   C   Q   A   A   D   R   Q   L   H   Q    120

 361  GCTAGTTCAATGGGCCAAGCACATTCCACATTTTGAAGAGCTTCCCCTTGAGGACCGCAT   420
 121    L   V   Q   W   A   K   H   I   P   H   F   E   E   L   P   L   E   D   R   M    140

 421  GGTGTTGCTCAAGGCTGGCTGGAACGAGCTGCTCATTGCTGCTTTCTCCCACCGTTCTGT   480
 141    V   L   L   K   A   G   W   N   E   L   L   I   A   A   F   S   H   R   S   V    160

 481  TGACGTGCGTGATGGCATTGTGCTCGCTACAGGTCTTGTGGTGCAGCGGCATAGTGCTCA   540
 161    D   V   R   D   G   I   V   L   A   T   G   L   V   V   Q   R   H   S   A   H    180

 541  TGGGGCTGGCGTTGGGGCCATATTTGATAGGGTTCTCACTGAACTGGTAGCAAAGATGCG   600
 181    G   A   G   V   G   A   I   F   D   R   V   L   T   E   L   V   A   K   M   R    200

 601  TGAGATGAAGATGGACCGCACTGAGCTTGGATGCCTGCTTGCTGTGGTACTTTTTAATCC   660
 201    E   M   K   M   D   R   T   E   L   G   C   L   L   A   V   V   L   F   N   P    220

 661  TGAGGCCAAGGGGCTGCGGACCTGCCCAAGTGGAGGCCCTGAGGGAGAAAGTGTATCTGC   720
 221    E   A   K   G   L   R   T   C   P   S   G   G   P   E   G   E   S   V   S   A    240

 721  CTTGGAAGAGCACTGCCGGCAGCAGTACCCAGACCAGCCTGGGCGCTTTGCCAAGCTGCT   780
 241    L   E   E   H   C   R   Q   Q   Y   P   D   Q   P   G   R   F   A   K   L   L    260

 781  GCTGCGGTTGCCAGCTCTGCGCAGTATTGGCCTCAAGTGCCTCGAACATCTCTTTTTCTT   840
 261    L   R   L   P   A   L   R   S   I   G   L   K   C   L   E   H   L   F   F   F    280

 841  CAAGCTCATCGGGGACACGCCCATCGACAACTTTCTTCTTTCCATGCTGGAGGCCCCCTC   900
 281    K   L   I   G   D   T   P   I   D   N   F   L   L   S   M   L   E   A   P   S    300

 901  TGACCCCTAAAGCCCTGCACAGCATGGCCTTCGGTACAGTGGTACACCCCCATGACTTTT   960
 301    D   P   *                                                                        320

 961  CTGTGTTTCACATCTGGTTACCTCAGGCTAGGAGGGCTTTGAGAATATGAGCCTGTGCCT  1020
1021  ACGCCGTGGAAAAAAGCTCTGTGATGCCATATCTAGCACCTCCTGCTGACTTGCATGCAG  1080
1081  GTAAACCTTTCTTTAATACTTGCATTAATCTGAGCCAATTACAGTGTGGCCAGAGGTCTG  1140
1141  TTGGGACACATTTAAAGAGGGAACTTTCTGAAAGCGAAAATAGCCAGTCCTTTAATATCA  1200
1201  GTCATTATTTTAGATGCTTCTTTTAGCATGGTGGTTTTCTTGCGGAGTTCCCAGTGGTTT  1260
1261  TAACTTTTAAGAGACATTCTTATGGAGGAGTCAATTTTAGAATACATCAGTTTTAAGTTT  1320
1321  GCATATGTAATCCTCACATACATATTTAAATGAATAAGGCTAAGCATAAATTCTACCAAG  1380
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1381  CATAAAATTCTGAAGAATGCGGATGATTTATTTTTAGTGTGAAAGAGTAAGCTTACTACG 1440
1441  TGTCTTTTCTGGTCAGTTGTCACTCAGACCACATTCTTACAAGAAACCCTGAAAATGGCG 1500
1501  GCATTAGGTTGGCAAATTATAATACTAAGGTGAAATAGTCAAATCATTGATGAGAATTGC 1560
1561  CTTGAATTATGTAGCTGAAGCACATGGAAGAACATGCCACTTGCGTTCATATTATGAGAA 1620
1621  GATGTGGTTGCAGTATCTGCAGTCCATTGAACTATAGCCACTTTCTTTCAGGATGTTCAC 1680
1681  TTTTGGAACTCATAAATGAACCAGTGTTTTCGTGTGGATGTAAACTTAAGAGAATGGAAG 1740
1741  TTCCCATTCAACGTCATGGTCTCCACCATAGGTGCTCTGCCTTTTAACCTTGTGTCGTCA 1800
1801  TTGAATGGTCCTTTTGGTAGGGCAGGCATTAAGGTTGCAACAACCTGCAGGTGAAAGAGC 1860
1861  ATACGTGAAGGTATGTTAGCTCACTGGGTACTCAGGAAGTTGCTTTTTTTGTGTATAAAT 1920
1921  GGCCCTACATGTTTAAGATGTGGCTGTGTCTGTCGACCCTGAAAATTTTCTTTATAACGA 1980
1981  GAATGTTGAAAAGACAGTGCACTGAATTTAACAAAATATGCCAGCCTATTTTCTGTGATG 2040
2041  GTTTTAAGTATACACATTACTGAATGTTCTTTTCACACTGATCGGGTGTTTCACACTACT 2100
2101  TTGCCTGAGCACCAGCACCGAGTGAGAAAGTTCACATCCTAATATGCCACTTCTCTTGGC 2160
2161  TATCACCAGTTCAAGTCGACTTGTATACGGGTAAATATTTACTTGAATTACTTTGATTAA 2220
2221  ACTCTTTGGTATAGCTCTTGGCTCTTGCATTCTACAGAAAAATCTTTATCGTTACCTTTC 2280
2281  CCCTTTGAAAAGTTATGTGCTGAATGTATGCATACAAGGTGTGCTTTTAGAGTAGTTGGC 2340
2341  TAACACTGTCACTTGGCTGCATTTTTTGTTTCCAAAATAAAATTAATTGCTATTCATTTT 2400
2401  TTTTTTTTGACCTGATTGATGCAGTGTAGGGTGCTTGTAACAGCTAAATGTATATTATAT 2460
2461  TCCTTACAAAGCAGTCTTATATACTATCAGTTTTACTGCGGTTTATCGTGCCAAAACATG 2520
2521  ACTGACCGCGGTGTAAAGCACGGTGGTTTTATATTGAGCGGTTAAGGCTGGCGGCAGCTT 2580
2581  GCTTAATATGTTTAGCTTGTTTTCTCGGTAAGGTGAGGATGCACAATCACTTGAGCTTGT 2640
2641  GGTGTGCCTGTGCGATTCCATATTCAAACATAAATTTCATTCTGAGGTATACGCAGTGGA 2700
2701  CTCCCTTCAAGCCAAACTTGGTTAAGTGGAATTCTGTGGTAGGCTGAACCGTTTAAGGAC 2760
2761  ATTTGTTTAATTTCACATGAGCACGGTGGAAAAAAAAAAAAAAAAA              2808
```

Figure 13. cDNA Sequence and Predicted Translation of the RXRE6 Open Reading Frame. Amino acids in the DNA- and hormone-binding domains, respectively, are underlined. One putative polyadenylation signal is underlined.

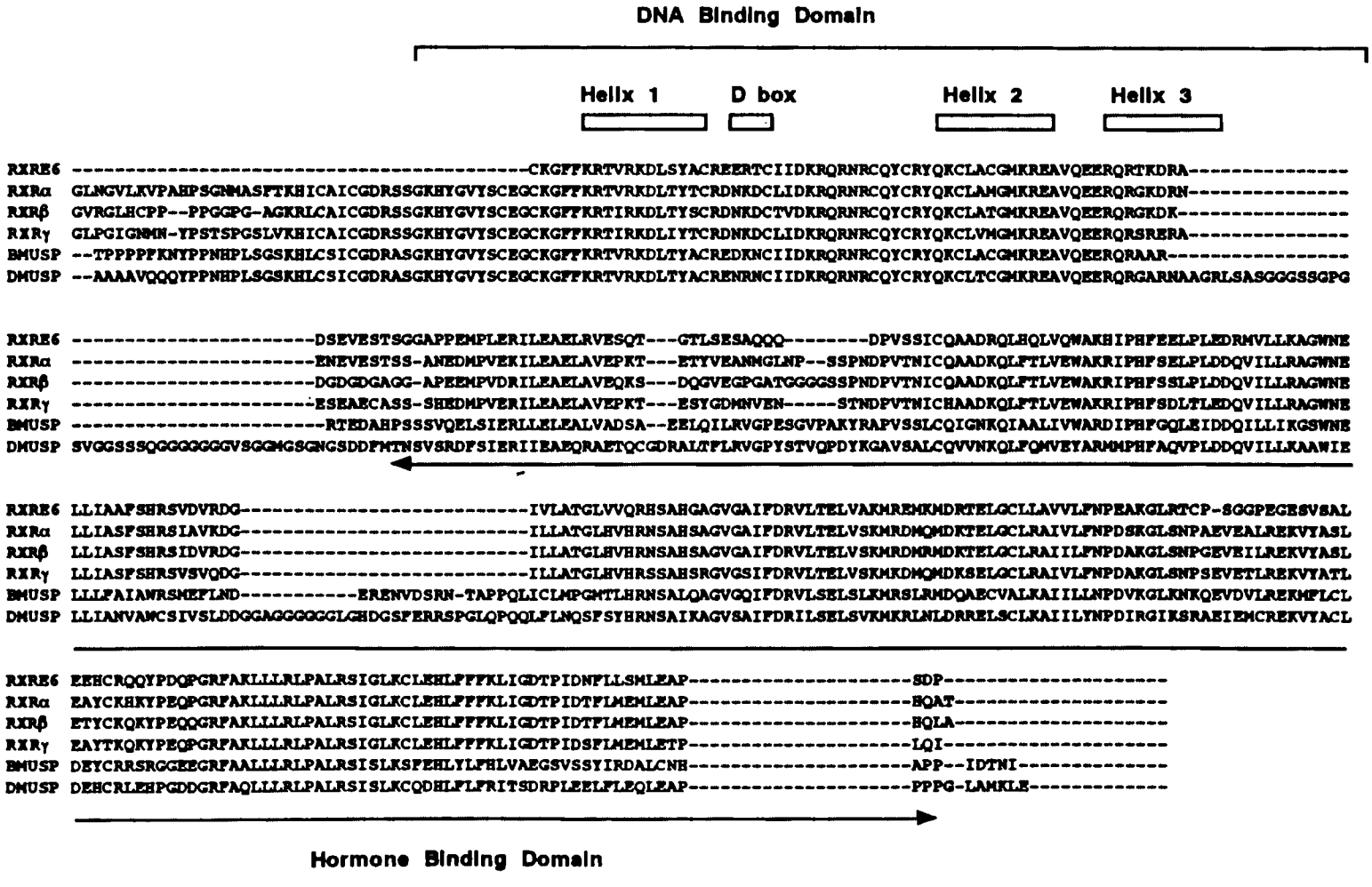

Figure 14. Alignment of the translated RXRE6 sequence to vertebrate RXR and insect USP amino acid sequences.

Gene	Forward Primers	Reverse Primers	Internal oligonucleotide
EcR	CATCAAGCGGGAGTCTAAG	GCTGGTTATTGGCAAACAC	GAGGGACCTCATCAACAAG
Actin	CGCAGATGATGTTTGAGACC	GGGCGGTGATTTCCTTCTGC	AACGAGGGCTGGAAGAGG
RXR	CAGTGAGGTGGAAAGCACTAG	TGTCCCCGATGAGCTTGAAG	

Figure 15. Gene specific primers for amplification of *A. americanum* EcR, actin and RXR mRNAs. Internal oligonucleotides used for detection of EcR and actin products by hybridization are also shown.

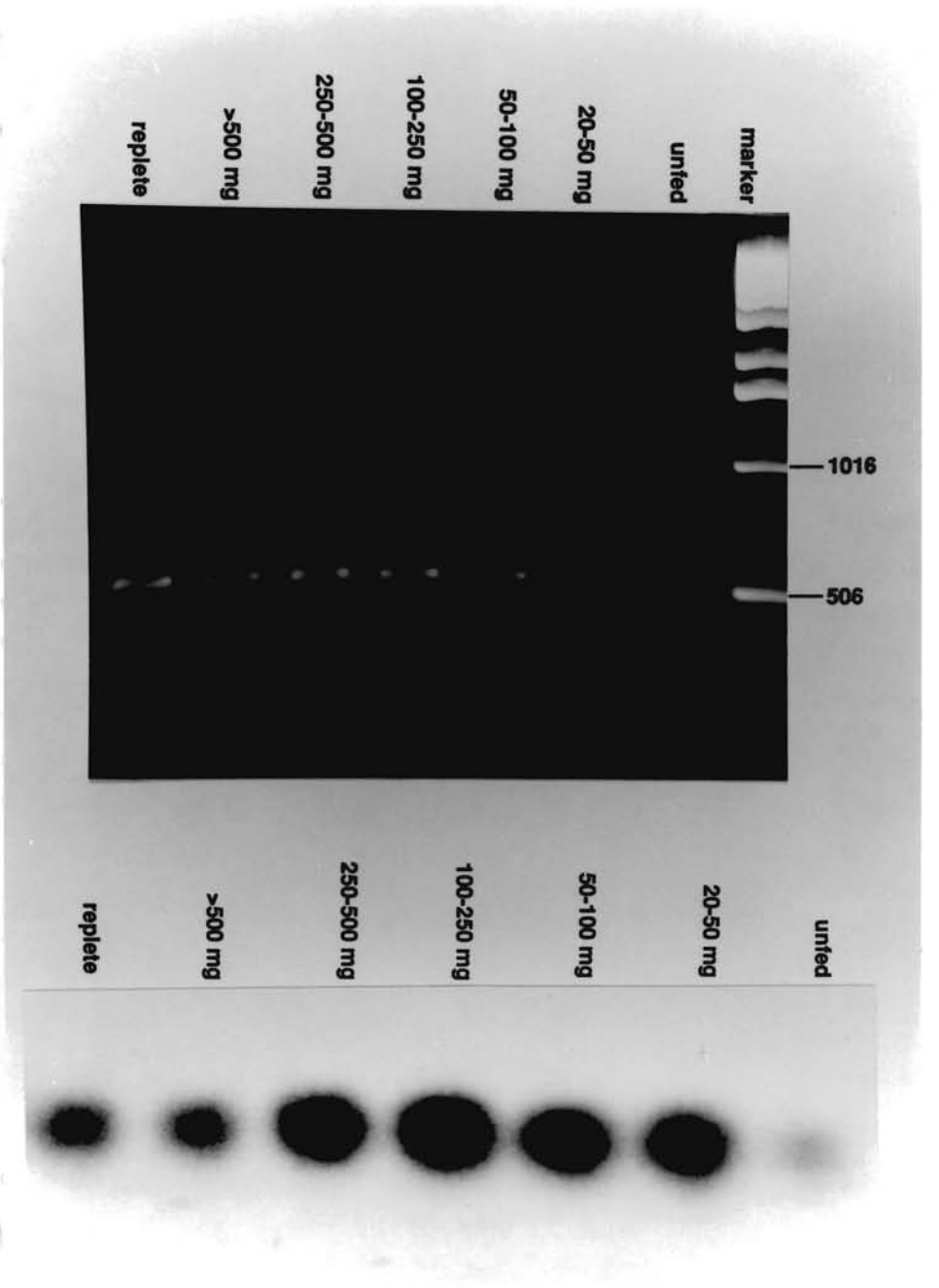

Figure 16. RT-PCR analysis of EcR transcripts in salivary glands from *A. americanum* of different weights. EcR-specific RT-PCR products are visualized by ethidium bromide staining of 1.5 % agarose gel (upper panel) and by hybridization with an EcR-specific internal oligonucleotide (lower panel). A major product of ~530 bp is seen in all stages.

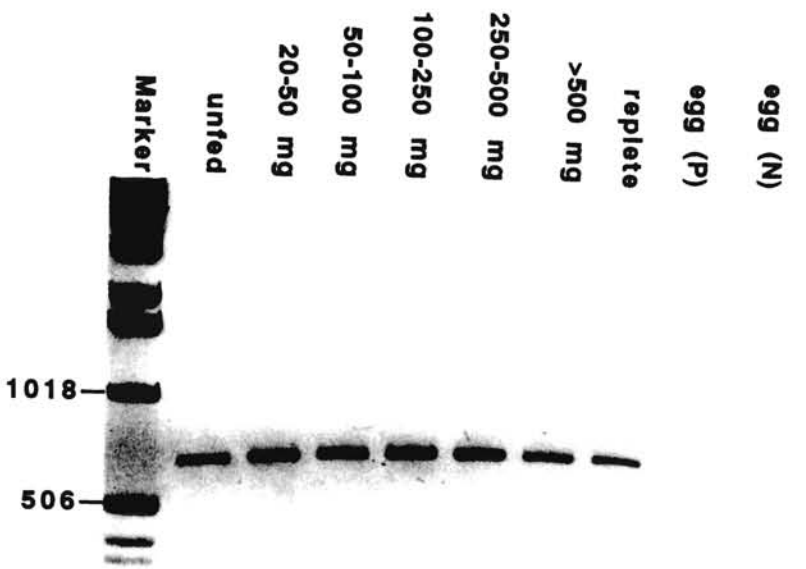

Figure 17. RT-PCR analysis of RXRE6 transcript in salivary glands from *A. americanum* of different weight. RT-PCR products are visualized by ethidium bromide staining of 1.5 % agarose gel. A major product of ~660 bp is seen in all stages and the positive control reaction (P). A negative control reaction (N), which did not include the reverse transcriptase was also performed.

VITA

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