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GRADUATE COLLEGE

CRUSTACEAN HORMONE NUCLEAR RECEPTORS: CHARACTERIZATION, GENE EXPRESSION, AND EVOLUTION

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1998

CRUSTACEAN HORMONE NUCLEAR RECEPTORS: CHARACTERIZATION, GENE EXPRESSION, AND EVOLUTION

A Dissertation APPROVED FOR THE
DEPARTMENT OF ZOOLOGY

BY

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LIST OF ABBREVIATIONS

	Abbreviations	Full terms
Molt stages		
	A	Early metecdysis
	B	Late metecdysis
	C	Anecdysis
	C_4	Late Anecdysis
	D	Proecdysis
	D_0	Early proecdysis
	D_{1-4}	Late proecdysis
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	AgrEcR	*Anthonomus grandis* ecdysteroid receptor
	AaeEcR	*Aedes aegypti* ecdysteroid receptor
	AaeUSP	*Aedes aegypti* ultraspiracle protein
	ANOVA	Analysis of variance
	BmeEcR	*Bombyx mori* ecdysteroid receptor
	BmoUSP	*Bombyx mori* ultraspiracle protein
	CfuEcR	*Choristoneura fumiferana* ecdysteroid receptor
	cRNA	Complement RNA
	CteEcR	*Chironomus tentans* ecdysteroid receptor
	COUP-TF	Chicken ovalbumin upstream promotor-transcription factor
	DBD	DNA binding domain
	D-box	Distal box
	DmeEcR	*Drosophila melangaster* ecdysteroid receptor
	DmeUSP	*Drosophila melangaster* ultraspiracle protein
	DreRXR	*Danio rerio* retinoid-X receptor
	EcR	Ecdysteroid receptor
	HsaRXR	*Homo sapiens* retinoid-X receptor
	HviEcR	*Heliothis virescens* ecdysteroid receptor
	LBD	Ligand binding domain
	LcuEcR	*Lucilia cuprina* ecdysteroid receptor
	NLS	Nuclear localization signal
	OliEcR	*Orconectes limosus* ecdysteroid receptor
	P-box	Proximal box
	Pipes	Piperazine-N,N'-bis[2-ethanesulfonic acid]
	RA	Retinoic acid
	RAR	Retinoic acid receptor
	rRNA	Ribosomal RNA
	RXR	Retinoid-X receptor
	MseUSP	*Manduca sexta* ultraspiracle protein
	MseEcR	*Manduca sexta* ecdysteroid receptor

TmoEcR	*Tenebrio molitor* ecdysteroid receptor
TR	Thyroid hormone receptor
UpuEcR	*Uca pugilator* ecdysteroid receptor
UpuRXR	*Uca pugilator* retinoid-X receptor
USP	*ultraspiracle* protein
VDR	Vitamin D_3 receptor

CHAPTER II

cRNA	Complement RNA
DBD	DNA binding domain
Dm	*Drosophila melangaster*
EcR	Ecdysteroid receptor
EDTA	Ethylenediamine tetraacetic acid
ER	Growth rate of limb bud (Experimental rate)
LBD	Ligand binding domain
RXR	Retinoid-X receptor
MA	Multiple autotomy
MIH	Molt inhibiting hormone
Ms	*Manduca sexta*
Pipes	Piperazine-N,N'-bis[2-ethanesulfonic acid]
SA	Single autotomy
SDS	Sodium dodecyl sulfate
SSC	Saline sodium citrate
Tris.HCl	Tris[hydroxymethyl]aminomethane, buffered with hydrochloric acid
UpuEcR	*Uca pugilator* ecdysteroid receptor
UpuRXR	*Uca pugilator* retinoid-X receptor
USP	Ultraspiracle protein

CHAPTER III

20-OH ecdysone	20-hydroxyecdysone
cRNA	Complement RNA
EcR	Ecdysteroid receptor
ER	Growth rate of limb bud (Experimental rate)
FGF	Fibroblast growth factor
LAF	Limb autotomy factor
LAFan	Anecdysial limb autotomy factor
LAFpro	Proecdysial limb autotomy factor
LGIF	Limb growth inhibiting factor
MIH	Molt inhibiting hormone
R_3	Regeneration Index of third limb bud
RXR	Retinoid-X receptor
UpuEcR	*Uca pugilator* ecdysteroid receptor

	UpuRXR	*Uca pugilator* retinoid-X receptor
CHAPTER IV		
	AaeEcR	*Aedes aegypti* ecdysteroid receptor
	AaeUSP	*Aedes aegypti* ultraspiracle protein
	AamEcR	*Amblyomma americanum* ecdysteroid receptor
	AamRXR	*Amblyomma americanum* retinoid-X receptor
	BmeEcR	*Bombyx mori* ecdysteroid receptor
	BmoUSP	*Bombyx mori* ultraspiracle protein
	CfuEcR	*Choristoneura fumiferana* ecdysteroid receptor
	CteEcR	*Chironomus tentans* ecdysteroid receptor
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	COUP-TF	Chicken ovalbumin upstream promotor-transcription factor
	DBD	DNA binding domain
	D-box	Distal box
	DmeEcR	*Drosophila melangaster* ecdysteroid receptor
	DmeUSP	*Drosophila melangaster* ultraspiracle protein
	DreRXR	*Danio rerio* retinoid-X receptor
	EcR	Ecdysteroid receptor
	FXR	Farnesoid-X receptor
	GgaRXR	*Gallus gallus* retinoid-X receptor
	HsaCOUP-TF	*Homo sapiens* Chicken ovalbumin upstream promotor-transcription factor
	HsaER	*Homo sapiens* estrogen receptor
	HsaFXR	*Homo sapiens* farnesoid-X receptor
	HsaLXR	*Homo sapiens* Liver-X receptor
	HsaRAR	*Homo sapiens* retinoic acid receptor
	HsaRXR	*Homo sapiens* retinoid-X receptor
	HsaTR	*Homo sapiens* thyroid hormone receptor
	HsaVDR	*Homo sapiens* Vitamin D_3 receptor
	HviEcR	*Heliothis virescens* ecdysteroid receptor
	LBD	Ligand binding domain
	LcuEcR	*Lucilia cuprina* ecdysteroid receptor
	LXR	Liver-X receptor
	MmuRXR	*Mus musculus* retinoid-X receptor
	MseUSP	*Manduca sexta* ultraspiracle protein
	MseEcR	*Manduca sexta* ecdysteroid receptor
	NGFI-B	Nerve growth factor induction protein - B
	P-box	Proximal box
	RA	Retinoic acid
	RAR	Retinoic acid receptor
	RnoFXR	*Rattus norvegicus* farnesoid-X receptor

RnoRXR	*Rattus norvegicus* retinoid-X receptor
RXR	Retinoid-X receptor
ScrEcR	*Sarcophaga crassipalpis* ecdysteroid receptor
Svp	Sevenup protein
TmoEcR	*Tenebrio molitor* ecdysteroid receptor
TR	Thyroid hormone receptor
UpuEcR	*Uca pugilator* ecdysteroid receptor
UpuRXR	*Uca pugilator* retinoid-X receptor
USP	*ultraspiracle*
VDR	Vitamin D_3 receptor
XlaRXR	*Xenopus laevis* retinoid-X receptor

APPENDIX

EcR	Ecdysteroid receptor

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Abstract

Ecdysteroids are the arthropod molting hormones that control growth and development in insects and crustaceans. Molting and limb regeneration in crustaceans are closely related to ecdysteroid titers. During the molt cycle of the fiddler crab, *Uca pugilator*, levels of ecdysteroids fluctuate significantly. Like vertebrate steroid hormone receptors, the actions of ecdysteroids are mediated through their nuclear receptor, ecdysteroid receptor (EcR). Studies in insect EcRs show the EcR needs to dimerize with another nuclear receptor before it becomes a functional dimer. Its dimer partner is the ultraspiracle (USP) protein, an insect homolog of retinoid-X receptor (RXR).

Cloning of *EcR* and *RXR* gene homologs in *U. pugilator* provides tools to investigate the ecdysteroid actions in different crustacean tissues. The deduced amino acid sequence of *Uca* EcR (UpuEcR = UpEcR) most closely resembles insect EcRs within both the DNA binding domain (DBD) and ligand binding domain (LBD) . Interestingly, the amino acid sequence of the DBD of *Uca* RXR (UpuRXR = UpRXR) shares greatest identity to insect USPs while the sequence of LDB is more similar to vertebrate RXRs. This feature is supported by the phylogenetic analyses.

Antisense RNA probes have been developed from the cDNA clones of these two crustacean receptors to investigate the expression of the receptor transcripts in *Uca* tissues during the molt cycle. Regenerating limb buds, gills, eyestalks, hepatopancreas, hypodermis, and muscle from non-regenerating walking legs and the large cheliped express both receptor transcripts and they may represent the ecdysteroid target tissues. At any given time during

the molt cycle, steady-state concentrations of the *UpuEcR* transcript show marked variation between tissues, indicating differences in the potential to respond to ecdysteroids. Furthermore, there is no correlation between high levels of *UpuEcR* transcripts in some tissues and total ecdysteroid titers, suggesting that *UpuEcR* expression in these tissues is not dependent on high ecdysteroid titers. Simultaneous expression of *UpuEcR* and *UpuRXR* mRNA in tissues supports the possibility of heterodimerization for EcR and RXR *in vivo*.

Both *UpuEcR* and *UpuRXR* transcripts are detected in the *Uca* blastemas during the early stages of limb regeneration. Immersion of the crab in the sea water containing *all-trans* retinoic acid (RA) elevates the steady-state concentrations of *UpuRXR* transcript and alters the pattern of circulating ecdysteroids. These changes may correlate with the disruptive effects of RA on limb regeneration observed in earlier studies.

Removal of several limbs (multiple autotomy) can trigger faster limb regeneration in crustaceans but the removal of limb buds often retards the limb regeneration. The underlying mechanism is still unclear. Contralateral removal of half of the limb buds during basal growth decreases the growth rate of the primarily regenerating limb buds in *U. pugilator*, but does not affect the total ecdysteroid titers initially. The limb bud removal delays the appearance of large peaks of total circulating ecdysteroids in late proecdysis and extends the molt cycle.These results suggest that the decrease of growth rate in the primarily regenerating limb buds is not dependent on the drop of total ecdysteroid titers. Some factor(s), other than ecdysteroids, may exist and regulate the limb regeneration. Steady-state concentrations of both *UpuEcR* and *UpuRXR* mRNAs are higher in secondarily regenerating limb buds than in the primarily regenerating limb buds when the proecdysial growth rate of

the secondary limb buds is high. The increase of *UpuEcR* transcript levels may be related to the growth rate of proecdysial limb buds and does not depend on the high titers of total circulating ecdysteroids.

EcRs and USPs are the only two invertebrate nuclear receptors that have been shown to possess ligand-binding ability. Ligand binding ability of the steroid hormone receptor /nuclear receptor superfamily has been suggested to have evolved after the evolutionary separation of the invertebrates and vertebrates. Results from phylogenetic analyses suggest that the ligand binding ability of EcRs and USPs may have been acquired independently during the arthropod evolution. In addition, the ligand binding ability of insect USPs may be different from other RXRs, including other invertebrate RXRs.

INTRODUCTION

Molt cycle and limb regeneration

Exoskeleton is a characteristic property of arthropods, including crustaceans. This highly calcified, heavy armor in crustaceans not only provides support to the animals but also protects the animals from being attacked by predators. It, however, limits continuous body growth. In order to grow, crustaceans need to shed the old exoskeleton, and replace it with a new and larger one regularly. This event is called molting. The time between two successive molts is called the molt cycle. In crustaceans, the molt cycle is generally divided into five major stages (Drach, 1939). At stage E ecdysis (or molting) occurs, which is the actual shedding of the old exoskeleton. Stages A and B immediately follow ecdysis and are called metecdysis. Stage B of metecdysis is characterized by the initiation of synthesis of endocuticle (Skinner, 1985). The metecdysis is the time for expansion and hardening of the new exoskeleton, which has been synthesized underneath the old exoskeleton. Stage C is anecdysis which is the time of feeding, reproduction, and storage of organic reserves. In the fiddler crab, *U. pugilator*, this stage can last more than 2 months, depending on physiological conditions (Hopkins, 1982). Stage D is proecdysis, which is the time for preparation for molting. The first visible sign of late proecdysis is apolysis, the separation of hypodermis from the old exoskeleton. Synthesis of a new cuticle from the hypodermis and the retrieval of useful substances from old exoskeleton then continue until ecdysis.

During the preparation for molting, crustaceans are able to regenerate the lost limbs (reviewed in Hopkins, 1988). When the animals respond to threat or injury (Juanes and

Smith, 1995), they can cast off a limb at a predetermined position proximal to the injury through a reflex action, autotomy, and regenerate a new limb in its place (McVean, 1984). In the fiddler crab, *U. pugilator*, limb regeneration can be assigned into two different growth processes, basal and proecdysial growths (reviewed in Hopkins, 1993). After healing of the wound, the basal growth starts with the formation of a blastema and is followed by the emergence of a limb bud. The basal growth is an epimorphic growth process which includes the migration of epidermal cells underneath the wound, influx of cells during the blastema formation and rapid mitosis (Hopkins, 1993). Immersion of crabs in *all-trans* retinoic acid (RA) during early basal growth disrupts the pattern of regenerating limb buds significantly (Hopkins and Durica, 1995). It interferes with the pattern of cuticle secretion in limb buds and often causes limb buds to grow slowly and to be malformed.

Proecdysial growth of limb buds starts when the crabs prepare to molt. This growth process represents a hypertrophic growth which involves protein synthesis and water uptake (reviewed in Hopkins, 1988). During late proecdysis, several days prior to molt, the limb buds cease to grow and this period is called terminal plateau. At molt (ecdysis) the newly regenerated limb is withdrawn from the cuticular sac, fills with blood, and expands into a functional limb.

In addition, multiple autotomy during anecdysis (i.e. removal of four to six limbs) can cause a quick onset of proecdysis with the regeneration of lost limbs (Skinner and Graham, 1972; Hopkins, 1982). The removal of many limb buds during proecdysis, however, has an opposite effect and can retard limb regeneration (Holland and Skinner, 1976; McCarthy and Skinner, 1977; Weis 1976, 1977).

Hormonal control of molt cycle and limb regeneration

In the fiddler crab, *U. pugilator*, molting and limb regeneration are well coordinated so that the lost limb(s) can be regenerated in a single molt cycle. This tight coordination is under hormonal control (Hopkins, 1992). The molt inhibiting hormone (MIH) from the X-organ-sinus gland in eyestalks inhibits ecdysteroid release from the Y-organs (Mattson and Spaziani, 1985, Naya et al., 1988). Ecdysteroids are the arthropod molting hormones which are responsible for many physiological events related to molt cycle, including limb regeneration (reviewed by Chang, 1989). In addition, some other factors are proposed to be involved in this coordination such as the limb growth inhibiting factor (LGIF) and the limb autotomy factor (LAF) (Hopkins, et al., 1979; Skinner, 1985).

In *U. pugilator*, the levels and ratios of circulating ecdysteroids change in a molt cycle-related pattern (Hopkins, 1983, 1986, 1989, 1992). The levels of total ecdysteroids in blood start at a low level in anecdysis. During the transition from anecdysis to proecdysis, a small peak of total circulating ecdysteroids is observed. This small peak is necessary for proecdysial regeneration of lost limbs (Hopkins, 1989). The total ecdysteroid titers then drop to a lower level until late proecdysis. At late proecdysis (D_{1-4}) which is limited to the time just prior to molting, a great increase of total ecdysteroids appears in blood. This increase in total ecdysteroid titers may be responsible for regulating many physiological and biochemical processes related to the molting event, such as protein synthesis in regenerating limbs (Hopkins, 1989), proliferation of epidermal cells, secretion of layers of new cuticle, withdrawal and storage of calcium salt from the old cuticle and construction of new exoskeleton underneath the old one (reviewed in Chang, 1989). Multiply autotomy can result

in the truncation of the molt cycle and disruption of the pattern of circulating ecdysteroids (Hopkins, 1982, 1992).

Regeneration in arthropods is closely linked to the molt cycle and, in part, depends on ecdysteroids. Basal growth requires extensive mitotic activity (Adiyodi, 1972). Low levels of 20-OH ecdysone appear to elevate mitotic activity in insects and crabs (Bressac, 1978; Kunieda et al., 1997). In the fiddler crab, *U. pugilator*, a small peak of total circulating ecdysteroids appears during the transition from anecdysis to proecdysis (Hopkins, 1989). Without this transition peak, the regenerating limb buds cannot proceed to the proecdysial growth. In addition, during the transition from basal growth to proecdysial growth in multiply autotomized *U. pugilator*, a switch of relative concentrations of four ecdysteroids is observed (Hopkins, 1992). The ratio of ecdysone and 20-OH ecdysone to Ponasterone A and 25-deoxyecdysone is higher than that during basal growth. Then Ponasterone A and 25-deoxyecdysone dominate when the limb buds reach the terminal plateau and late proecdysis starts. *In vitro* incubation of proecdysial limb buds in ecdysone and 20-OH ecdysone has been shown to increase protein synthesis in the limb buds (Hopkins, 1989). Furthermore, the proecdysial growth of limb buds depends on the presence of the Y-organs which produce ecdysteroids (Suzuki, 1985). Injection of 20-OH ecdysone in the crabs deprived of Y-organs induces the proecdysial limb bud growth (Suzuki, 1985). In the land crab, *Gecarcinus lateralis*, removal of half of the regenerating limb buds during early proecdysial growth decreases total circulating ecdysteroids rapidly within 24 hrs (McCarthy and Skinner, 1977).

Ecdysteroid and retinoid-X receptors

Like vertebrate steroid hormone receptors, the action of ecdysteroids is mediated through their nuclear receptor, ecdysteroid receptor (EcR) (Koelle et al., 1991; Talbot et al., 1993; Bender et al., 1997). Studies in insect EcRs show that the EcR must dimerize with another nuclear receptor before it becomes a functional receptor (Thomas et al., 1993; Yao et al., 1993; Swevers et al., 1996). Its dimer partner is the ultraspiracle (USP) protein, an insect homolog of the vertebrate retinoid-X receptor (RXR) (Oro et al., 1990). Most vertebrate RXRs are activated only by *9-cis* RA, one of the Vitamin A metabolites (Chambon, 1996). Two of zebra fish RXRs (DreRXRδ and ϵ) and insect USPs do not bind to RAs (Yao et al., 1993; Jones et al., 1995). Recently, DmeUSP has been shown to bind to a terpenoid hormone, insect juvenile hormone, although with low affinity (Jones and Sharp, 1997).

Both EcRs and RXRs (or USPs) are members of the steroid hormone receptor /nuclear receptor superfamily. The superfamily includes many ligand-dependent transcription factors, such as glucocorticoid receptors, retinoic acid receptor, and thyroid hormone receptor (Tsai and O'Malley, 1994; Mangelsdorf and Evans, 1995). In addition, some members in this superfamily can function without ligands. Together with the ones whose ligands have not yet been found, they are called orphan receptors. Proteins in this superfamily consist of six functional domains, including a ligand independent transactivation domain (A/B domain) at the amino terminal, followed by the DNA binding domain (C domain or DBD), a hinge region (D domain), and ligand binding domain (E domain or LBD) (Tsai and O'Malley, 1994). The distinguishing characteristic of this superfamily is possession of a conserved

DBD with two Cys_2-Cys_2 zinc-finger motifs (Deter-Wadleigh and Fanning, 1994). In addition to binding to a specific DNA sequence, called the hormone response element, to regulate specific gene expression, the DBD provides a dimerization interface (Zalluacus et al., 1995). The LBD is the second conserved domain within the superfamily and has several functions, including ligand binding, dimerization and ligand-dependent transactivation. Results of crystal structure analysis of three nuclear receptors suggest that the LBDs in the steroid hormone receptor /nuclear receptor superfamily should contain twelve helices and a beta-turn region (Bourguet et al., 1995; Renaud et al., 1995; Wagner et al., 1995; Wurtz et al., 1996). Helices three, five and seven, as well as the beta extended sheet regions are suggested to form the putative ligand binding pocket while helix 12 is shown to contain an important ligand-dependent transactivation subdomain, the AF-2 subdomain.

The formation of an EcR-USP heterodimer is not only required for DNA binding but also for ecdysone binding (Thomas et al., 1993; Yao et al., 1993). The requirement of heterodimerization prior to ligand binding has not been reported in other nuclear receptors. Information about the distribution of *EcR* and *USP* transcripts and isoforms during larval development have been reported for several insects (Henrich et al., 1994; Talbot et al., 1993; Cho et al., 1995; Jindra et al., 1996; Kapitskaya et al., 1996; Jindra et al., 1997; Mouillet et al., 1997). In tobacco hornworm, *Manduca sexta*, one of the *EcR* isoforms is induced by small amounts of ecdysone and 20-OH ecdysone (Hiruma et al., 1997). Exposure to 20-OH ecdysone induces the expression of total *USP* (*MseUSP*) mRNA in the 2nd day of fifth instar larval epidermis of *M. sexta* (Jindra et al., 1997). MseUSP has two isoforms, MseUSP-1 and MseUSP-2. During both larval and pupal molts when the titers of circulating

ecdysteroids are high, *MseUSP-1* mRNA disappears as *MseUSP-2* mRNA increases. In the fruit fly, *Drosophila melanogaster*, low ecdysteroid titers (2 nM) can induce the expression of *EcR* mRNA but the *Drosophila USP* (*DmeUSP*) gene is not ecdysone inducible (reviewed by Russell, 1996). That the expression of *EcR* and *USP* is inducible by ecdysteroid(s) is still unclear.

Hypothesis

Ecdysteroids are the arthropod molting hormone. Studies in other arthropod EcRs show that the actions of ecdysteroids are mediated through the EcRs and a functional EcR requires dimerization with a RXR. Cloning the crustacean EcR and RXR gene homologs will provide not only the information about the properties of these two receptors in crustaceans, but also tools for further investigation of the effects of ecdysteroids *in vivo*. Cloning and characterization of the crustacean EcR and RXR gene homologs were the first goal of my research.

Molting and limb regeneration have been shown to be under ecdysteroid control in crustaceans. In order to determine the relationship between these two events and total ecdysteroid titers, the second aim of my research was to establish potential ecdysteroid targets by measuring *UpuEcR* mRNA expression in a variety of tissues.

Some members of the vertebrate steroid hormone receptor superfamily, such as vitamin D receptor and estrogen receptor alpha, are expressed in response to rising titers of hormone (Carson-Jurica et al., 1990). The third goal of this research was to establish correlations between patterns of *UpuEcR* and *UpuRXR* expression and circulating

ecdysteroids.

As mentioned above, multiple autotomy accelerates the molt cycle and changes circulating ecdysteroid levels. The fourth question addressed by this research was whether the expression of *UpuEcR* in tissues was changed following multiple autotomy.

Immersion of crabs in sea water with *all-trans* RA disrupts the pattern of limb regeneration (Hopkins and Durica, 1995). It interferes with the events during early stages of limb regeneration and causes limb buds to grow slowly or not at all. The limb buds that do grow are frequently malformed. Preliminary studies showed nuclear extracts from the blastemas during early stages of limb regeneration possessed ecdysteroid and retinoic acid binding abilities (Hopkins et al., 1994). The expression of *UpuEcR* and *UpuRXR* mRNAs has been found in blastemas (Durica and Hopkins, 1996). The fifth goal was to examine transcript levels in blastemas and titers of total circulating ecdysteroids during early limb regeneration, and the effects of immersion of crabs in sea water with *all-trans* retinoic acid on the transcript levels of *UpuEcR* and *UpuRXR* during blastemal differentiation.

In the land crab, *G. lateralis*, removal of half of the regenerating limb buds during early proecdysial growth (R_3 values at 15) results in a rapid significant decrease of circulating ecdysteroids within 24 hrs (McCarthy and Skinner, 1977). During basal growth in *U. pugilator*, the titers of total circulating ecdysteroids are low. The next aim was to determine whether the titers of total circulating ecdysteroids at this stage (R_3 values at 10) in *U. pugilator* were affected by contralateral removal of limb buds.

Some previous studies showed that the growth rate of the secondary regenerating limb buds was faster than that of the primarily regenerating limb buds (Holland and Skinner,

1976; McCarthy and Skinner, 1977; Weis 1976, 1977). During limb regeneration of *U. pugilator*, the transcript levels of *UpuEcR* and *UpuRXR* in limb buds are high when the growth rate of proecdysial limb buds is high (Chung et al., 1998). Thus I examined whether the different growth rates in the primarily and secondarily regenerating limb buds were related to the steady-state concentrations of the *UpuEcR* and *UpuRXR* mRNAs in limb buds.

Finally, after characterization of the deduced amino acid sequences of UpuEcR and UpuRXR, some distinct features have been found in these crustacean receptors, especially the UpuRXR. The DBD of UpuRXR shares greatest identity with insect USPs but the LBD is more similar to those in vertebrate RXRs. In addition, UpuRXR has a distinct AF-2 subdomain which is not observed in other RXRs. These differences raise a question as to how these receptors evolved. The last goal was to use phylogenetic analysis to test a null hypothesis that both the EcRs and USPs gained ligand binding independently after the evolutionary separation of the invertebrates and the vertebrates. In addition, I examined whether RXRs evolved differently within arthropods.

Organization of the dissertation

This dissertation is organized into four chapters and an appendix. Chapter I and II are reprinted from the published papers in General and Comparative Endocrinology, and Molecular and Cellular Endocrinology, respectively. Some modifications have been done to improve the consistency throughout the dissertation. Chapter III and IV are written as manuscripts that are to be submitted to General and Comparative Endocrinology, and Molecular Biology and Evolution, respectively. The Appendix contains an early poster

presentation that may be helpful to future western blot analyses.

Literature cited

Adyodi, R. G. (1972). Wound healing and regeneration in the crab, *Paratelphusa hydrodromous*. *Int. Rev. Cytol.* **32,** 257-289.

Bender, M., Imam, F. B., Talbot, W. S., Ganetzky, B., Hogness, D. S. (1997). Drosophila ecdysone receptor mutations reveal functional differences among receptor isoforms. *Cell* **91,** 777-788.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., and Moras, D. (1995). Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. *Nature* **375,** 377-382.

Bressac, C. (1978). L'activite mitotique an niveau de l'organe y du Crabe *Pachygrapsus marmoratus*. *C. R. Acad. Sci. Paris* **285,** 289-291.

Carson-Jurica, M. A., Schrader, W. T., and O'Malley, B. W. (1990). Steroid receptor family: Structure and functions. *Endo. Rev.* **11,** 201-219.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10,** 940-954.

Chang, E. S. (1989). Endocrine regulation of molting in Crustacea. *Rev. Aquat. Sci.* **1,** 131-157.

Cho, W.-L., Kapitskaya, M. Z., and Rackhel, A. S. (1995). Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. *Insect Biochem. Molec. Biol.* **25,** 19-27.

Chung, A. C.-K., Durica, D. S., and Hopkins, P. M. (1998). Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator*. *Gen.. Comp. Endocrinol.***109,** 375-389.

Detera-Wadleigh, S. D., and Fanning, T. G. (1994). Phylogeny of the steroid receptor superfamily. *Molec. Phylogen. Evol.* **3,** 192-205.

Drach, P. (1939). Mue et cycle d'intermue chez les Crustacés Décapodes. *Ann. Inst. Oceanogr. Monaco* **19,** 103-391.

Durica, D. S. and Hopkins, P. M. (1996). Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator*. *Gene* **171**, 237-241.

Henrich, V. C., Szekely, A. A., Kim, S. J., Brown, N. E., Antoniewski, C., Hayden, M. A., Lepesant, J.-A., and Gilbert, L. I. (1994). Expression and function of the *ultraspiracle* (*usp*) gene during development of *Drosophila melanogaster*. *Dev. Biol.* **165**, 38-52.

Hiruma, K, Böcking, D., Lafont, R., and Riddiford, L.M. (1997). Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* **107**, 84-97.

Holland, C. A. and Skinner, D. M. (1976). Interactions between molting and regeneration in the land crab. *Biol. Bull.* **150**, 222-240.

Hopkins, P. M. (1982). Growth and regeneration patterns in the fiddler crab, *Uca pugilator*. *Biol. Bull.* **163**, 301-319.

Hopkins, P. M. (1983). Patterns of serum ecdysteroids during induced and uninduced proecdysis in fiddler crab, *Uca pugilator*. *Gen. Comp. Endocrinol.* **63**, 362-373.

Hopkins, P. M. (1986). Ecdysteroid titers and Y-organ activity during late anecdysis and proecdysis in the fiddler crab, *Uca pugilator*. *Gen. Comp. Endocrinol.* **63**, 362-373.

Hopkins, P. M. (1988). Control of regeneration in crustaceans. *In* "Endocrinology of Selected Invertebrate Types" (R. Downer and H. Laufer, Eds.) Vol. 2, pp.327-340, A.R. Liss, New York.

Hopkins, P. M. (1989). Ecdysteroids and regeneration in the fiddler crab, *Uca pugilator*. *J. Exp. Zool.* **252**, 293-299.

Hopkins, P. M. (1992). Hormonal control of the molt cycle in the fiddler crab, *Uca pugilator*. *Am. Zool.* **32**, 450-458.

Hopkins, P. M. (1993). Regeneration of walking legs in the fiddler crab, *Uca pugilator*. *Am. Zool.* **33**, 348-356.

Hopkins, P. M., and Durica, D. S. (1995). Effects of *all-trans* retinoic acid on regenerating limbs of the fiddler crab, *Uca pugilator*. *J. Exp. Zool.* **272**, 455-463.

Hopkins, P. M., Durica, D. S., and Carter, K. L. (1994). Ecdysteroid and retinoid receptors in the early blastema of regenerating limbs in the fiddler crab, *Uca pugilator*. *Am. Zool.* **34**,

82A.

Hopkins, P. M., Bliss, D. M., Sheehan, S. W., and Boyer, J. R. (1979). Limb growth-controling factors in the crab *Gecarcinus lateralis*, with special reference to the limb growth-inhibiting factor. *Gen. Comp. Endocrinol.* **39,** 192-207.

Jindra, M., Malone, F., Hiruma, K., and Riddiford, L. M. (1996). Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. *Dev. Biol.* **180,** 258-272.

Jindra, M., Huang, J.-Y., Malone, F., Asahina, M., and Riddiford, L. M. (1997). Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta*. *Insect Molec. Biol.* **6,** 41-53.

Jones, G., and Sharp, P. A. (1997). Ultraspiracle: An invertebrate nuclear receptor for juvenile hormones. *Proc. Natl. Acad. Sci. USA* **94,** 13499-13503.

Jones, B. B., Ohno, C. K., Allenby, G., Boffa, M. B., Levin, A. A., Grippo, J. F., and Petkovich, M. (1995). New retinoid X receptor subtypes in zebra fish (*Danio rerio*) differentially modulate transcription and do not bind *9-cis* retinoid acid. *Mol. Cell. Biol.* **15,** 5226-5234.

Juanes, F., and Smith, L. D. (1995). The ecological consequences of limb regeneration of limb damage and loss in decapod crustaceans. *J. Exp. Mar. Biol. Ecol.* **195,** 197-223.

Kapitskaya, M., Wang, S., Cress, D. E., Dhadialla, T. S., and Raikel. A. S. (1996). The mosquito *ultraspiracle* homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell. Endocrinol.* **121,** 119-132.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P., and Hogness, D. S. (1991). The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67,** 59-77.

Kunieda, T., Kurata, S., and Natori, S. (1997). Regeneration of *Sarcophaga* imaginal discs *in vitro*: Implication of 20-hydroxyecdysone. *Dev. Biol.* **183,** 86-94.

Mangelsdorf, D. J., and Evans, R. M. (1995). The RXR heterodimers and orphan receptors. *Cell* **83,** 841-850.

Mattson, M.P., and Spaziani, E. (1985). Characterization of molt-inhibiting hormone (MIH) action on crustacean Y-organ segments and dispersed cells in culture and a bioassay for MIH activity. *J. Exp. Zool.* **236,** 93-101.

McCarthy, J. F., and Skinner. D. M. (1977). Interruption of proecdysis by autotomy of partially regenerated limbs in the land crab, *Gecarcinus lateralis*. *Gen. Comp. Endocrinol.* **61,** 299-310.

McVean, A. (1984). Autotomy. *In* "The Biology of Crustacea" (Bliss, D. E., Ed.), vol. 4, pp. 107-132. Academic Press, New York.

Mouillet, J. F., Delbecque, J. P., Quennedey, B., and Delachambre, J. (1997). Cloning of two putative ecdysteroid receptor isoforms from *Tenebrio molitor* and their developmental expression in the epidermis during metamorphosis. *Eur. J. Biochem.* **248,** 856-860.

Naya, Y., Kishida, K., Sugiyama, M., Murata, M., Miki, W., Ohnishi, M., and Nakanishi, K. (1988). Endogenous inhibitor of ecdysone synthesis in crabs. *Experienta* **44,** 50-52.

Oro, A.E., McKeown, M., and Evans, R.M. (1990). Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature* **347,** 298-301.

Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H.,and Moras, D. (1995). Crystal structure of the RAR-γ ligand-binding domain bound to all-*trans* retinoic acid. *Nature* **378,** 681-689.

Russell, S. R. H. (1996). Nuclear hormone receptors and the *Drosophila* ecdysone response. *Biochem. Soc. Symop.* **62,** 111-121.

Swevers, L., Cherbas, L., Cherbas, P., and Iatrou, K. (1996). *Bombyx* EcR (BmEcR) and *Bombyx* USP (BmCF1) combine to form a functional ecdysone receptor. *Insect Biochem. Molec. Biol.* **26,** 217-221.

Skinner, P. M. (1985). Molting and regeneration *In* "The Biology of Crustacea" (Bliss, D. E., Ed.), vol. 9, pp. 43-146. Academic Press, New York.

Skinner, D. M., and Graham, D. E., (1972). Loss of limbs as a stimulus to ecdysis in Brachyura (True crab). *Biol. Bull.* **143,** 222-233.

Suzuki, S. (1985). Effect of Y-organ removal on limb regeneration and molting in the terrestrial crab, *Sesarma haematocheir*. *Gen. Comp. Endocrinol.* **58,** 202-210.

Talbot, W. S., Swyryd, E. A., and Hogness, D. S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73,** 1323-1337.

Thomas, H. E., Stunnenberg, H. G., and Steward, A. F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. *Nature* **362,** 471-

475.

Tsai, M.-J., and O'Malley, B. W. (1994). Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annu. Rev. Biochem.* **63,** 451-486.

Wagner, R. L., Aprilette, J. W., McGrath, M. E., West, B. L., Baxer, J. D., and Fletterick, R. J. (1995). A structural role of hormone in the thyroid hormone receptor. *Nature* **378,** 690-697.

Weis, J. S. (1976). Effects of environmental factors on regeneration and molting in fiddler crabs. *Biol. Bull.* **150,** 152-162.

Weis, J. S. (1977). Regeneration in the fiddler crab, *Uca pugilator*, after injury or removal of limb buds. *J. Exp. Zool.* **197,** 21-30.

Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Chambon, P., Moras, D., and Gronemeyer, H. (1996). A canonical structure for the ligand-binding domain of nuclear receptors. *Nature Struc. Biol.* **3,** 87-94.

Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P., and Evans, R.M. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366,** 476-479.

Zilliacus, J., Wright, A. P. H., Carlstedt-Duke, J., and Gustafsson, J.-A. (1995). Structural determinants of DNA-binding specificity by steroid receptors. *Mol. Endocrinol.* **9,** 389-400.

CHAPTER I

Cloning of Crustacean EcR and RXR Gene Homologs and Elevation of RXR mRNA by Retinoic Acid

Abstract

We report the cloning and analysis of ecdysteroid receptor (*UpuEcR*) and retinoid-X receptor (*UpuRXR*) cDNA homologs from the fiddler crab, *Uca pugilator*. The deduced amino acid sequence of this crustacean EcR most closely resembles the insect EcRs within the DNA binding and ligand binding domains. For UpuRXR, the DNA binding domain shares greatest identity to the insect USPs. The ligand binding domain, however, is closer to vertebrate RXRs but may have a nonfunctional AF-2 domain. Probes derived from these clones were used to examine transcript levels in blastemas during early limb regeneration. Both *UpuEcR* and *UpuRXR* transcripts were detected in low amounts one day following limb loss, but increased during the next four days. Immersion of crabs in sea water containing *all-trans* retinoic acid increased the steady state concentrations of *UpuRXR* transcript and altered the pattern of circulating ecdysteroids. These effects correlate with the disruptive effects of retinoic acid on blastemal differentiation observed in earlier studies.

Introduction

Many crustaceans possess a remarkable ability to regenerate lost or damaged limbs as adults. Similar to neotenized urodele amphibians, crustaceans are able to regenerate entire limbs. When the limb of the fiddler crab, *Uca pugilator*, is injured, the animal can cast it off at a predetermined point proximal to the injury by a reflex behavior called autotomy (McVean, 1984). A new limb will regenerate at the point of autotomy through two distinct stages, basal and proecdysial growth (reviewed in Hopkins, 1988). Basal growth follows autotomy and involves the formation of a blastema, rapid mitosis, and differentiation. Later in the molt cycle, as the crab prepares to shed its old exoskeleton, the limb bud enters proecdysial growth, which primarily is hypertrophic growth due to protein synthesis and water uptake (reviewed in Hopkins, 1988). At molt (ecdysis) the new limb is withdrawn from the cuticular sac and is expanded with blood.

Crustacean limb regeneration, in part, appears to be under the control of the arthropod steroid hormones, the ecdysteroids (Hopkins, 1989, 1993). The actions of ecdysteroids are similar to vertebrate steroid hormones since they are mediated through a nuclear receptor, the ecdysteroid receptor (EcR). The *EcR* genes of several insects have been cloned and shown to belong to the steroid hormone/nuclear receptor superfamily (Koelle et al., 1991, Imhof et al., 1993; Cho et al., 1995; Fujiwara et al., 1995; Kothapalli et al., 1995; Swevers et al., 1995; Dhadialla and Tzertzinis, 1997; Hannan and Hill, 1997; Mouillet et al., 1997). Insect EcR dimerizes with the ultraspiracle (USP) protein, a *Drosophila* homolog of the vertebrate retinoid-X receptor (RXR), to form a functional receptor (Thomas et al., 1993; Yao et al., 1993; Kapitskaya et al., 1996; Swevers et al., 1996). Information

about the distribution of *EcR* and *USP* transcripts and isoforms during larval development have been reported for several insects (Henrich et al., 1994; Talbot et al., 1993; Cho et al., 1995; Jindra et al., 1996; Kapitskaya et al., 1996; Jindra et al., 1997; Mouillet et al., 1997). We recently have reported the expression of *EcR* and *RXR* mRNAs in several tissues of *Uca pugilator* (Chung et al., 1998). Although both *EcR* and *RXR* transcripts have been detected in early blastemal tissues (Durica and Hopkins, 1996), the role of ecdysteroids and these receptors in early limb regeneration is unknown.

Retinoic acid (RA) is derived from vitamin A and exists in several forms, such as *all-trans* RA, *9-cis* RA, and *13-cis* RA. Exogenous retinoic acids can respecify the proximodistal axis in vertebrate limb regeneration (reviewed in Brockes, 1997) and high doses of *all-trans* RA are teratogenic in a number of species (reviewed in Niazi, 1996). In *U. pugilator*, the immersion of the whole crabs in sea water containing *all-trans* RA affects the regeneration of lost limbs. When crabs are kept in a sea water emulsion of *all-trans* retinoic acid at concentrations identical to levels which caused teratogenic effects in vertebrates (Maden, 1983), the pattern of regenerating limb buds is significantly disrupted (Hopkins and Durica, 1995). Immersion of crabs in *all-trans* RA interferes with the pattern of cuticle secretion in limb buds and causes limb buds to grow slowly or not at all. The limb buds that do grow are frequently malformed. The cellular mechanism(s) underlying these effects is unknown.

In vertebrates, the effects of the retinoic acids are mediated via interactions with cytoplasmic retinoic acid binding proteins (Denker et al., 1991; reviewed in Paulsen, 1994) and specific nuclear receptors (Brockes, 1992; Kastner et al., 1997). The binding of RA to these receptors will modify the expression of specific genes that specify, either directly or

indirectly, the axis of regeneration (Viviano and Brockes, 1996). The nuclear RA receptors comprise two different members of the steroid hormone/nuclear receptor superfamily, the retinoic acid receptor (RAR) and the retinoid-X receptor (RXR). In vertebrates, the RAR family (RAR α, β, γ, and δ) is activated by *all-trans* RA and *9-cis* RA, whereas RXR α, β, and γ are activated only by *9-cis* RA (Chambon, 1996). RXR δ and ϵ in zebra fish, and USP, the *Drosophila* RXR homolog, do not bind to RAs (Yao et al., 1993; Jones et al., 1995).

At least three mechanisms of action are proposed for the vertebrate RXRs. First, unliganded RXR can act as a silent partner when it forms a heterodimer by binding to other members of the nuclear receptor family such as RAR, vitamin D_3 receptor (VDR), and thyroid receptor (TR). The pairing of RXR in these dimers stabilizes DNA binding (Forman et al., 1995; Mangelsdorf and Evans, 1995; Minucci and Ozato, 1996). Second, RXR can form homodimers in the presence of *9-cis* RA (Pfahl et al., 1994) and may regulate gene expression via different binding affinities or sequestration of RXR from heterodimers (Zhang et al., 1992; Lehmann et al., 1993; MacDonald et al., 1993). Third, liganded RXR appears to enhance the teratogenic effects of liganded partners, such as RAR, TR, or VDR (Li et al., 1997; Lu et al., 1997; Minucci et al., 1997; Puzianowska-Kuznicka et al., 1997; Zou et al., 1997). These results support a model in which RXR functions as a transcriptionally active receptor/partner alone or with other nuclear receptors in either a ligand-dependent or ligand-independent manner.

This is the first report of crustacean *EcR* and *RXR* cDNA homologs containing intact sequences of DNA binding, hinge, ligand binding, and terminal domains (C-F domains). Probes derived from these clones were used to simultaneously examine the steady state

levels of *UpuEcR* and *UpuRXR* transcripts in blastemas during early stages of limb regeneration. We also report that immersion of whole crabs in sea water containing *all-trans* retinoic acid affects the relative transcript levels of these two potential dimer partners during blastema formation. Our results suggest that the pattern of receptor expression may play an important role during the early stages of limb regeneration of *U. pugilator*.

Materials and Methods

Animals

Male *Uca pugilator* were purchased from Gulf Specimen Company, Florida, USA. As soon as the crabs were acclimated to laboratory conditions, we autotomized seven limbs from each crab by pinching the limb with forceps.

cDNA library construction

Total RNA was isolated from late proecdysial regenerating limb buds (Chomczynski and Sacchi, 1987). Poly $(A)^+$ RNA was enriched by oligo-dT cellulose column chromatography and was collected by ultracentrifugation (Sambrook et al., 1989). About 5 micrograms of poly $(A)^+$ RNA was used to construct an oligo(dT)-primed cDNA library in the vector Lambda ZAP II (Stratagene, La Jolla, CA). The procedures followed the manufacturer's instructions. The libraries were screened using DNA probes which encoded the *UpuEcR* or *UpuRXR* DNA binding domains (described in Durica and Hopkins, 1996). Representative isolates of *UpuEcR* and *UpuRXR* were sequenced on an ABI 373A sequencer (Chissoe et al., 1991; Bodenteich et al., 1994). Analyses of nucleotide and deduced amino acid sequences of the putative *UpuEcR* and *UpuRXR* clones were performed using BLAST

searches of the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/) (Altschul et al., 1990; Gish and States, 1993) and GCG (Genetic Computer Group, University of Wisconsin, Madison) software. The sequence data of other receptors were obtained from DDBJ/EMBL/GenBank databases (Table 1). Multiple sequence alignment was performed using the ClustalW program (version 1.7) with default parameters (Thompson et al., 1994) at Baylor's search launcher for Biologists website (http://dot.imgen.bom.tmc.edu:9331/multi-align/multi-align.html). The ClustalW program used pairwise similarity scores to arrange the order of the sequences. Percentage similarity and identity were calculated using the ToPLign program with default parameters at ToPLign: Toolbox for protein alignment website (http://cartan.gmd.de/ToPLign.html) (Thiele et al., 1995).

In vivo experiments

Following autotomy of six walking legs and a cheliped, crabs were immersed in *all-trans* retinoic acid (RA). *All-trans* RA (Sigma, St. Louis, MO) was dissolved in 5 ml ethanol. This solution was then emulsified by sonication in 800 ml artificial sea water, Instant Ocean (Aquarium System Inc., OH) to a concentration of 50 μM. The solution was mixed in subdued light and the crabs were kept in the dark during incubation. The animals were kept in plastic shoe boxes and were partially immersed in either 50 μM *all-trans* RA sea water or sea water containing 0.6% ethanol. For five days following autotomy, at 24 hour intervals, the blastemas from experimental and control crabs were removed. Each tissue sample represents blastemas pooled from either ten or twenty-five animals. Total RNA was extracted from blastemas and subjected to ribonuclease protection assay (see below). Blood

was drawn at the same time that the blastemas were removed. Total ecdysteroid titers in blood were measured by radioimmunoassay (Hopkins, 1983).

Plasmid constructs and probe synthesis

Subclones used for probe synthesis were constructed from *UpuEcR* and *UpuRXR* cDNA clones isolated from the oligo-dT primed cDNA library. The *EcR 162* plasmid encodes a 162 bp fragment of the UpuEcR ligand binding domain (LBD). The *RXR 307* plasmid contains a 307 bp fragment of the UpuRXR DNA binding domain (DBD). The *Uca 29* plasmid contains an insert which encodes 195 nt of 18S ribosomal RNA (rRNA). Prior to *in vitro* transcription, *Uca 29* was cut by *StuI* to generate a 59 bp antisense RNA probe. The construction of these plasmids has been previously described (Chung et al., 1998). Antisense radiolabeled RNA probes were produced by *in vitro* transcription (Promega, Madison, WI) with [α-^{32}P]-UTP (800 Ci/mmol, Amersham, Arlington, IL). The antisense probe was transcribed by either T3 or T7 RNA polymerase and purified on Select RT sepharose spin columns (5 Prime- 3 Prime, Boulder, CO).

Ribonuclease protection assay

To quantitate *UpuEcR* and *UpuRXR* transcripts from small amounts of tissue, a ribonuclease protection assay was used. Total RNA was extracted (Chomczynski and Sacchi, 1987) from pooled blastemal tissue. Samples (10 to 20 μg) were resuspended in hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM PIPES (pH 6.0), 1 mM EDTA). Anti-sense ^{32}P-UTP-labeled RNA probes were synthesized from *EcR* 162 and *RXR 307* plasmids, and the *Uca 29* probe was used as an internal control for quantifying total RNA concentration. Hybridizations of RNA samples with excess RNA probes were performed

at 45°C for 16 to 18 hrs, and unhybridized templates were digested by RNase A/T1 (Ambion, Austin, TX) at 37°C for 30 min. RNases were removed by protease K digestion and organic extraction, and the protected fragments were separated by 6% PAGE under denaturing conditions. The amount of radioactivity in the protected fragments was determined on a Packard Instantimager® electronic autoradiography system (Packard, Meriden, CT). Background subtraction and data quantification were performed by the associated image analysis software package.

For example, in Fig. 1a, results of ribonuclease protection assays of pooled total RNA samples taken from blastemas at one day to five days after autotomy showed *UpuRXR* and *UpuEcR* mRNA protected fragments of 307 nt and 162 nt in both experimental (RA treated) and control (ethanol carrier) groups, respectively (Fig. 1a: Lanes 2-11). The transcript levels of each receptor were quantified by comparison with standards of protected sense-strand complementary RNA (cRNA) for each receptor (Fig. 1a: Lanes 13-15; 17-19), synthesized from the plasmid vectors using T7 RNA polymerase. The absolute amounts of each receptor mRNA were calculated as the fraction of radioactivity bound to the standard cRNA and expressed as pg cRNA. The amount of total RNA loaded in each assay was normalized by a second ribonuclease protection assay. Portions of pooled RNA samples (10^{-4} dilutions) were assayed with the *Uca* 29 probe which protects a 59 nt fragment of *Uca* 18S rRNA (Fig. 1b). The amount of total RNA loaded for each pooled tissue sample was quantified by comparing the radioactivity of the 59 nt protected fragment (Fig. 1b: Lanes 2-11) with the radioactivity of a protected standard (Fig. 1b: Lane 1). The standard was a single RNA isolate from late proecdysial limb buds, where the RNA concentration was quantified by

ultraviolet spectrophotometry. The concentration of each transcript was then calculated from the absolute amount of receptor mRNA divided by the amount of the total RNA, and expressed as pg cRNA per μg total RNA. Error bars on data represent standard errors which were calculated from the results of four independent pooled samples. Each of the first two sets of pooled samples contained RNA from 10 crabs and the rest contained RNA from 25 crabs.

Statistical analysis

UpuEcR and *UpuRXR* mRNA levels from different time points were compared by Students' t-test (Microsoft Excel, Cambridge, MA). Comparisons of control and experimental groups were done by 2-way ANOVA (SigmaStat, San Rafael, CA). $P \leq 0.05$ was considered to be significant.

Results:

cDNA Library Screenings for *EcR* and *RXR* Sequences and DNA Sequence Analysis of Recovered Clones

We constructed an oligo(dT)-primed cDNA library with mRNA isolated from late proecdysial regenerating limb buds, a stage known to contain relatively high levels of both *UpuEcR* and *UpuRXR* transcripts (Chung et al., 1998). The library (1×10^6 primary clones) was screened using probes spanning the *UpuEcR* and *UpuRXR* DBDs, derived from clones previously generated by PCR (Durica and Hopkins, 1996). Five *EcR* and two *RXR* cDNA clones were subsequently isolated and sequenced. Composites of the *UpuEcR* and *UpuRXR* sequences are presented in Fig. 2 and 3. None of these clones represented full-length

transcripts with complete 5' termini. The sizes of *UpuEcR* and *UpuRXR* mRNA transcripts are approximately 7 kb and 5 kb (Durica and Hopkins, 1996). The longest cloned inserts recovered were 5 kb for *UpuEcR* and 4.5 kb of *UpuRXR*. We estimate that approximately 2000 nt of 5' non-coding and N-terminal coding sequence for *UpuEcR* and 500 nt of *UpuRXR* sequence were not recovered from the oligo-dT primed library.

Analyses of the deduced UpuEcR and UpuRXR amino acid sequences revealed that each contained a DNA binding domain (DBD) with Cys_2Cys_2 zinc finger motifs, a hinge region, and a ligand binding domain (LBD) representative of the steroid hormone/nuclear receptor superfamily. Comparisons to previously described PCR clones encoding the UpuEcR and UpuRXR DBDs revealed two internal nucleotide substitutions in *UpuEcR* which could not be assigned to the degenerate primers used in synthesis; these substitutions did not lead to a change in the UpuEcR DBD amino acid sequence. A BLAST search of the NCBI database indicated that, among superfamily members, *UpuEcR* has greatest amino acid identity to insect *EcR*s. All five *UpuEcR* clones began upstream of, or within, the DBD. Although no variation was found in the *UpuEcR* DBD, among five individual isolates that were sequenced, three different hinge regions were identified (Fig. 2).

The deduced sequence of UpuEcR showed more than 88% amino acid identity to the DBD and more than 53% identity to the LBD of insect EcRs (Fig. 4). The DBD of UpuEcR was identical in length (66 amino acids) to other EcRs, and showed 97% amino acid identity with that of the coleopteran, *Tenebrio molitor* (TmoEcR) (Mouillet et al., 1997). UpuEcR exhibited about 90% identity with the DBD of EcRs from the dipterans *Aedes aegypti* (AaeEcR), *Chironomus tentans* (CteEcR), *Drosophila melanogaster* (DmeEcR), and *Lucilia*

cuprina (LcuEcR), and the lepidopterans *Bombyx mori* (BmoEcR), *Choristoneura fumiferana* (CfuEcR), *Heliothis virescens* (HviEcR), and *Manduca sexta* (MseEcR) (Koelle et al., 1991; Imhof et al., 1993; Cho et al., 1995; Fujiwara et al., 1995; Kothapalli et al., 1995; Swevers et al., 1995; Hannan and Hills, 1997; GenBank accession no: Y09009). The UpuEcR proximal box (P-box), a short stretch of five amino acids in the first zinc finger, was 100% identical to the P-boxes of other EcRs and members of the thyroid hormone (TR)/ retinoic acid receptor (RAR) subfamily (data not shown). The UpuEcR distal box (D-box), found in the second zinc finger, was 100% identical to TmoEcR, and 60% identical to other EcRs (Fig. 5).

The hinge regions of UpuEcR showed about 40% amino acid identity to insect EcRs (Fig. 4). Like TmoEcR, the hinge region of the UpuEcR composite sequence was shorter than other EcRs but contained a potential nuclear localization signal (NLS) which shared a high degree of identity with those in DmeEcR and AaeEcR (Koelle et al., 1991; Cho et al., 1995) (Fig. 5).

The sequence of the UpuEcR LBD showed 69% identity with that of TmoEcR, and about 60% identity with that of the dipteran EcRs and lepidopteran EcRs (Fig. 4). In addition, UpuEcR showed conservation in the helix-turn-zipper motif and regulatory zippers, which are important for receptor dimerization and transcriptional activation in members of the steroid hormone receptor superfamily (Forman and Samuels, 1990; Maksymowych et al., 1993) (Fig. 5). A 35 amino acid stretch at the end of the LBD, which is believed to be the ninth heptad repeat and AF-2 activation domain (or τc) (Durand et al., 1994; Perlmann et al., 1996), exhibited a high level of conservation with other EcRs (Fig.5). By contrast, the

terminal region F domain of UpuEcR (like TmoEcR) is much shorter than that of other insect EcRs (Fig. 4).

Compared with a partial sequence of EcR from crayfish, *Orconectes limosus* (D. Böcking, personal communication), UpuEcR showed 96% amino acid identity to the DBD and 87% identity to the LBD (Fig. 5). In addition, the hinge region of OliEcR displayed a high degree of conservation with one of the variants of UpuEcR (Fig. 5).

For *UpuRXR*, two cDNA clones were isolated. One of the clones (designated 3B) encoded a protein containing canonical DBD, hinge, and LBD regions while the second cDNA clone (designated 11C) contained a DBD and hinge region that was identical to those of clone 3B, but terminated within the LBD (Fig. 3). BLAST searches of the DBD indicated that it shared close similarity to insect USPs and vertebrate RXRs.

We compared the deduced amino acid sequence from clone 3B with four insect USPs, *D. melanogaster* (DmeUSP), *A. aegypti* (AaeUSP), *B. mori* (BmoUSP), *M. sexta* (MseUSP), three human RXRs (HsaRXR α, β, and γ), and four zebra fish, *Danio rerio*, RXRs (DreRXR α, γ, δ, and ϵ) (Mangelsdorf et al., 1990; Oro et al., 1990; Leid et al., 1992; Mangelsdorf et al., 1992; Tzertzinis et al., 1994; Jones et al., 1995; Kapitskaya et al., 1996; Jindra et al., 1997). Although the A/B transactivation domain in UpuRXR was incomplete, a nine amino acid stretch upstream of the DBD contained 100% identity to insect USPs (Fig. 6, 7). The DBD of UpuRXR exhibited greater than 90% identity to insect USPs (Fig. 6). The identity between UpuRXR DBD and vertebrate RXRs was between 83% and 86%. The UpuRXR P-box was 100% identical to the P-boxes of USPs and RXRs (Fig. 7) and also identical to members of the TR/RAR subfamily (not shown). The UpuRXR D-box, found

in the second zinc finger, had two or three amino acids identical to insect USPs, but had only one amino acid identity to the D-boxes of vertebrate RXRs (Fig. 7).

The short UpuRXR hinge region exhibited between 48% and 67% amino acid identity to the hinge region of USPs or other RXRs (Fig. 6). It did not contain the long stretch of glycine residues as in DmeUSP (data not shown). A 10 amino acid span adjacent to the DBD and containing the eight-residue long T-box was 100% conserved between the insect USPs and vertebrate RXRs, except that DreRXRϵ had an amino acid substitution and a eight amino acid insertion within the T-box (Fig. 7).

The length of the LBD of UpuRXR was similar to other USPs and RXRs (Fig. 6). The LBD of UpuRXR exhibited about 63% to 68% identity with those of vertebrate RXRs while the identity of UpuRXR with insect USPs was lower than 50% (Fig. 6). As with EcR, the UpuRXR LBD exhibited conservation of putative structural motifs common to the steroid hormone receptor family (helix-turn zippers and regulatory zippers) (Fig. 7). High amino acid conservation occurred at the ninth heptad repeat in all USPs and RXRs. In UpuRXR, the β-turn region, which is part of the putative ligand binding site (Bourguet et al., 1995), had higher amino acid identity to vertebrate RXRs than insect USPs (Fig. 7). In addition, the putative AF-2 domain and 14 amino acids upstream of this activation domain showed 75% amino acid identity to vertebrate RXRs, and 33% to 42% identity to insect USPs (Fig. 7). Interestingly, unlike the AF-2 domain in other RXRs where a highly conserved glutamic acid is found, a lysine was observed at position 356 in UpuEcR (Fig. 7). This substitution occurred in a residue believed to be critical for ligand-induced conformational changes in vertebrate retinoid receptors (Renaud et al., 1995).

Effects of *all-trans* retinoic acid incubations on steady state *UpuEcR* and *UpuRXR* transcript levels during early limb regeneration

Figure 1 shows a representative ribonuclease protection assay designed to quantify steady state concentrations of *UpuRXR* and *UpuEcR* mRNAs in the small amounts of tissue present in early limb blastemas. In these experiments, the two probes protected 307 nt and 162 nt fragments of *UpuRXR* and *UpuEcR* mRNA, respectively. To determine whether the immersion of the whole crabs in sea water containing RA has an effect on *UpuEcR* and *UpuRXR* transcript abundance during early blastemal organization, experimental animals were exposed to a 50 μM emulsion of *all-trans* RA over a five day interval and compared to control animals incubated in sea water with the ethanol carrier. Following normalization to the total amount of RNA assayed (Fig. 1; see materials and methods), the results are given in Figure 8.

In the control groups, the average steady state transcript level of *UpuEcR* was low but detectable 24 hours following autotomy (0.03 ± 0.01 pg cRNA per μg total RNA; open bars in Fig. 8a). Over the next four days, *UpuEcR* transcript levels increased to 0.52 ± 0.12 pg cRNA per μg total RNA. This increase was significant relative to the first day after autotomy ($P<0.01$). When the crabs were immersed in sea water containing 50 μM RA, *UpuEcR* transcripts were also low but detectable on the first day after autotomy (0.06 ± 0.01 pg cRNA per μg total RNA; filled bars in Fig. 8a). After four days, they increased to 0.26 ± 0.08 pg cRNA per μg total RNA. Comparison of *UpuEcR* expression in both control and experimental groups showed no significant difference between the groups ($P>0.05$, 2-way ANOVA), nor was there any significant differences between controls and experimentals on

any given day (P>0.05, Students' t-test, Fig. 8a).

The pattern of *UpuRXR* transcript accumulation in control animals paralleled the pattern observed for *UpuEcR*. *UpuRXR* transcripts in control blastemas on the first day after autotomy were 0.04 ± 0.02 pg cRNA per μg total RNA (open bars in Fig. 8b). The amount of *UpuRXR* then gradually increased on the following days. The concentration of *UpuRXR* transcript was 0.16 ± 0.01 pg cRNA per μg total RNA at five days after autotomy. This increase was significantly higher than levels on the first day after autotomy (P<0.001).The transcript levels of *UpuRXR* in blastemas from crabs immersed in sea water with RA also increased significantly four days after autotomy (0.37 ± 0.03 pg cRNA per μg total RNA) when compared with one day after autotomy (0.09 ± 0.01 pg cRNA per μg total RNA) (P<0.001) and stayed at comparable levels. In addition, *UpuRXR* transcript levels in RA treated animals (filled bars in Fig. 8b) were significantly higher than the controls on the first, third and fifth days after autotomy (P<0.05, Students' t-test, Fig. 8b). The ratios of *UpuRXR* transcript levels to *UpuEcR* transcript levels in blastemas from the crabs immersed in sea water with RA were approximately two fold higher than the ratios in blastemas from the control animals. The pattern of expression of *UpuRXR* was the same in control and experimental animals, but the levels of expression were significantly higher in animals immersed in sea water with RA relative to the controls (P<0.05, 2-way ANOVA).

Effects of *all-trans* RA on titers of circulating ecdysteroids during early stages of limb regeneration

The circulating levels of ecdysteroids in control crabs averaged 15.7 ± 2.94 pg of 20-hydroxyecdysone equivalents per μl of hemolymph (pg/μl) for the five days after autotomy

(open bars in Fig. 9). When the crabs were immersed in sea water with RA (filled bars in Fig. 9), circulating ecdysteroid levels transiently increased to 42.2 ± 6.4 pg/μl two days after autotomy ($P<0.01$), then decreased to control levels (22.6 ± 7.5 pg/μl) over the following three days. Comparison of both groups showed a significant difference between the control and experimental animals ($P<0.05$, 2-way ANOVA).

Discussion

Uca EcR sequence analysis

The UpuEcR homolog exhibits more than 88% amino acid identity (more than 97% similarity) to insect EcRs in the conserved DNA-binding domain (DBD). In the DBD, there are two sequences in the zinc finger region, called the P-box and D-box, which affect the receptor's ability to bind to a particular hormone response element (HRE) (Zilliacus et al., 1995). The UpuEcR P-box is 100% identical to all EcRs, suggesting that UpuEcR will have DNA recognition specificity similar to insect EcRs. In addition, the D-box governs receptor dimerization and specifies a spacer length between HRE half-sites. The D-box of UpuEcR is 100% identical to TmoEcR, and 60% identical to other EcRs. This suggests that UpuEcR and TmoEcR may interact with similar HREs.

Unlike most insect EcRs, the hinge regions of UpuEcR are shorter. A potential nuclear localization signal (NLS) sequence (Picard and Yamamoto, 1987) appears in the hinge region of UpuEcR and other EcRs (Fig. 5) although the hinge region is not highly conserved in nuclear receptors (Pfahl et al., 1994). Insect EcRs contain an intron splicing site within the hinge region and two variant sequences in this region were reported in MsEcR

(Fujiwara et al., 1995). In the hinge region of UpuEcR, three variant sequences have been identified among the five individual cDNA clones (Fig. 2). The sequence of one clone (7A) is similar to the hinge region in crayfish EcR (OliEcR) (Fig. 5). In crustaceans different intron processing may occur in at least two different sites to generate the variant hinge regions observed.

The LBDs of all members of the steroid hormone superfamily have helix-turn-zipper and regulatory zipper subdomains which are important for transcriptional activation and receptor dimerization (Forman and Samuels, 1990; Maksymowych et al., 1993). Furthermore, two conserved regions at the C-terminal occur in the LBD of nuclear receptors. The ninth heptad repeat of the LBD is another dimerization interface which is important for heterodimer formation and selection of HREs (Perlmann et al., 1996). An AF-2 activation domain (or τc), situated in the last α-helix, is a ligand-dependent transcriptional activation domain in nuclear receptors (Danielian et al., 1992; Durand et al., 1994; Schulman et al., 1996). Sequences characteristic of these subdomains are located in the LDB of UpuEcR (Fig. 5). Their existence supports our hypothesis that UpuEcR may form heterodimers in ways that may be similar to other EcRs.

The F domain is located at the C-terminal end of some steroid hormone and nuclear receptors. No specific function for this domain has been identified. Deletion of the F domain in human estrogen receptor (hERα) does not affect any known functions of this receptor (Kumar et al., 1987). The fact that UpuEcR lacks a F domain suggests that this domain may be a dipteran- and lepidopteran-EcR-specific adaptation.

Comparison of the functional domains of UpuEcR to other EcRs shows that it is most

similar to a partial sequence of EcR from another crustacean (*O. limosus*) and to a coleopteran EcR (*T. moiltor*) and less similar to EcRs in dipterans or lepidopterans. A partial amino acid sequence of another coleopteran, *Anthonomous grandis*, also shows 96% amino acid identity with the DBD of UpuEcR and 57% identity with the LBD of UpuEcR (Dhadialla and Tzertzinis, 1997). Examination of the ribosomal RNAs suggests that crustaceans are a sister group of insects (Friedrich and Tautz, 1995). Phylogenetic analyses of α-amylase and hemocyanins also show close evolutionary relationships between the crustaceans and the dipterans and lepidopterans (Van Wormhoudt and Sellos, 1996; Durstewitz and Terwilliger, 1997). The evolutionary relationship between different classes within Arthropoda is, however, not clear. Additional sequence data from evolutionarily conserved crustacean genes may provide a tool to examine these relationships.

Uca RXR sequence analysis

When compared to insect USPs and vertebrate RXRs, the deduced amino acid sequence of UpuRXR is very interesting. The DBD of UpuRXR possesses characteristic features of insect USPs. The LBD of UpuRXR, however, shows greater identity to some vertebrate RXRs than to insect USPs. In the conserved DBD, the UpuRXR P-box is 100% identical among USPs and RXRs and members of the TR/RAR subfamily (Fig. 7). The UpuRXR D-box shows a lower level of conservation with insect USPs and vertebrate RXRs. As discussed above, differences in this region relate to potential dimerization and DNA binding properties.

The length of the hinge region of UpuRXR is similar to most insect USPs and vertebrate RXRs and exhibits 48% to 67% amino acid identity to USPs and RXRs. A

conserved T-box sequence appears immediately downstream of the DBD of USPs and RXRs, which is necessary for DNA binding (Zilliacus et al., 1995). The T-box of UpuRXR is identical to T-boxes of insect USPs and most vertebrate RXRs (Fig. 7), suggesting that the T-box of UpuRXR may assist DNA binding in a manner similar to other RXRs. From the DBD to the beginning of the LBD, the amino acid sequences of two *UpuRXR* cDNA clones are identical (Fig. 3). One of the *UpuRXR* cDNA clones, 11C, terminates within the LBD at a putative splice junction. This clone may represent a transcript intermediate.

The LBD of UpuRXR also contains several conserved subdomains, such as helix-turn-zippers and regulatory zippers (Fig. 7). In addition, a 19 amino acid stretch at the C-terminal of the LBD, called the ninth repeat region, is responsible for dimerization of RXRs and is highly conserved in USPs and RXRs (Zhang et al., 1995; Perlmann et al., 1996). The β-turn region in RXR is the putative ligand-binding site and combines with several helices to form a putative ligand-binding pocket (Bourguet et al., 1995). UpuRXR shows a higher amino acid identity in the putative ligand-binding site to vertebrate RXRs than to insect USPs. In the β-turn, UpuRXR does not have an insertion of amino acids common to insect USPs and UpuRXR does not contain extra amino acids downstream of the β-turn region as seen in DreRXR δ and ϵ. The conserved basic and hydrophobic amino acid residues which are believed important in ligand entry and anchoring of *all-trans* retinoic acid to the putative ligand-binding pocket in retinoid receptors appear in the LBD of UpuRXR (Bourguet et al., 1995; Renaud et al., 1995). These results suggest that UpuRXR may have a similar conformation in the putative ligand-binding pocket as vertebrate RXRs. DreRXR δ and ϵ, as well as USPs, do not bind to *9-cis* RA (Yao et al., 1993; Jones et al., 1995). Both have

been classified as orphan receptors although a recent report has implicated juvenile hormone as a potential natural ligand for USPs (Jones and Sharp, 1997)

The UpuRXR AF-2 domain of helix 12 shows significant divergence relative to insect USPs and vertebrate RXRs. In human RARγ, x-ray crystallographic studies indicate that ligand binding induces a large conformational change, where helix 12 is repositioned to form a "lid" on the ligand-binding pocket. In the holo-LBD, two conserved glutamic acid residues in the AF-2 domain participate in the formation of a salt bridge with a lysine residue in helix 4 (Renaud et al., 1995; Wurtz et al., 1996). Mutational studies have shown that alterations of these charged residues in nuclear receptors, either in helix 12 or 4, compromise the AF-2 activation function, but not ligand binding (Saatcioglu et al., 1993; Barettino et al., 1994; Durand et al., 1994; Tone et al., 1994; Baniahmad et al., 1995; Renaud et al., 1995; LeDouarin et al., 1996). These studies suggest that the formation of a salt bridge and the associated conformational change appears critical for the ligand-dependent transactivation ability of all the vertebrate retinoid receptors that bind ligands. The putative UpuRXR AF-2 domain and 14 amino acids upstream of this region are similar to the vertebrate RXRs, but the conserved acidic acid residues in the AF-2 domain are changed in UpuRXR to lysine and valine, while helix 4 retains the conserved basic residue. These substitutions may inhibit salt bridge formation between the AF-2 domain and helix 4 of the LBD, suggesting that UpuRXR may have different transconformation and transactivation properties than vertebrate RXRs. A similar substitution in the conserved acidic residues has recently been reported for two RXR receptors in the tick, *Amblyomma americanum* (M. Palmer, personal communication). The crustacean AF-2 domain may be nonfunctional or subject to other

conformational changes which may mediate interactions with co-regulatory molecules.

Effects of *all-trans* retinoic acid

Earlier studies showed that *all-trans* RA affects normal limb regeneration of *U. pugilator* (Hopkins and Durica, 1995). Malformation and retarded growth of limb buds are observed when the newly autotomized crabs are immersed in a sea water emulsion of RA. During the first five days of RA immersion, there are no obvious histological differences in the blastemas between RA-treated and control crabs. Similar results are observed in amphibian blastemas (Niazi, 1996). Malformed and histologically abnormal limb buds are observed in later stages of regeneration, even after RA treatment is withdrawn. No effect of RA is seen when the immersion of crabs in sea water containing RA is restricted to stages following blastemal organization (P. Hopkins, unpublished data). Thus, the effects of exogenous RA on regenerating limbs seem to be limited to a specific window during limb organization and differentiation.

During amphibian limb regeneration, treatment with exogenous RA results in respecification along the proximodistal and anteroposterior axes (Niazi and Saxena, 1978; Maden, 1983). *All-trans* RA may affect limb development and regeneration either directly or indirectly. *All-trans* RA is able to directly stimulate the expression of its receptors, RARs and RXRs, in mouse embryos and several human cell lines (Jiang et al., 1994; Gianini et al., 1997; Copper et al., 1997). Recently, the results from a retinoid receptor knock-out experiment indicate RAR/RXR heterodimers are essential for retinoic acid signaling *in vivo* (Kastner et al., 1997). Exogenous *all-trans* RA can alter the normal expression pattern of downstream regulatory genes, such as *Hox* genes, in regenerating amphibian limbs (Gardiner

et al., 1995; reviewed in Tsonis, 1996). Furthermore, *all-trans* RA is able to induce the expression of another nuclear receptor, COUP-TF (reviewed in Tsai and Tsai, 1997). Our results suggest that *all-trans* RA elevates *UpuRXR* transcript levels after autotomy although the mechanism(s) underlying this change is unknown.

Ecdysteroids are necessary for proecdysial limb regeneration in *U. pugilator* (Hopkins, 1989; 1993). In insect larvae, low titers of ecdysteroids are necessary for the imaginal discs to regenerate (Kunieda et al., 1997). In *Drosophila*, EcR requires USP, a *Drosophila* homolog of RXR, to form a functional receptor that can bind effectively to an ecdysteroid HRE (Yao et al., 1993; Antoniewski et al., 1996). The binding of ecdysteroids to EcR governs the early events of the ecdysteroid signal cascade (reviewed in Thummel, 1996). We hypothesize that a liganded UpuEcR/UpuRXR heterodimer may exist and be involved in the early events of limb regeneration. In this study, both *UpuEcR* and *UpuRXR* transcript levels in blastemas increase after autotomy and support the hypothesis that they participate in the early stages of limb regeneration. The ratio of *UpuRXR* transcript levels to *UpuEcR*, however, increases after crabs are immersed in sea water with RA. Disturbance of receptor expression may interfere with the normal signaling pathway by disrupting normal dimerization and signaling. Ectopic expression of seven-up, a *Drosophila* homolog of human COUP-TF, interferes with the normal USP signaling pathway during *Drosophila* eye development by pairing with either USP or DmeEcR (Zelhof et al., 1995). By analogy, if other pairing partners of either UpuEcR or UpuRXR are stimulated by RA exposure, competition for DNA binding, or protein-protein interaction could compromise normal limb regeneration.

Indirectly, *all-trans* RA may affect the normal growth of limb buds through its metabolites: *9-cis* RA or *13-cis* RA can isomerize rapidly and nonenzymatically from *all-trans* RA (reviewed in Napoli, 1996). *9-cis* RA binding to RXR favors homodimer formation over heterodimer formation (reviewed in Pfahl et al., 1994). The addition of *9-cis* RA can diminish the effects of VDR/RXR and TR/RXR heterodimers and turn on the RXR homodimer signaling pathway (Lehmann et al., 1993; Tsonis et al., 1996; Collingwood et al., 1997; Mangelsdorf et al., 1990; Mak et al., 1994; Medin et al., 1994). Higher steady state *UpuRXR* transcript levels are observed in *Uca* blastemas from crabs immersed in sea water with *all trans* RA. *All trans* RA may be isomerized to *9-cis* RA inside the blastema. If, in analogy to vertebrate RXRs, the presence of *9-cis* RA favors RXR homodimer formation, then RXR may be sequestered into RXR homodimers during blastema formation, resulting in a decrease of EcR/RXR heterodimers. Such changes could affect normal EcR/RXR signaling during the early stages of limb regeneration.

Injection of ecdysone or 20-hydroxyecdysone into crabs inhibits early stages of limb regeneration (blastema formation through papilla emergence) (Bazin, 1977; Rao, 1978; Hopkins et al., 1979). The immersion of whole crabs in sea water containing *all-trans* RA increases total circulating ecdysteroids transiently two days after limb loss. The role that this transient increase may play in blastema differentiation is unclear at this time. No immediate changes in histology or *UpuEcR* transcript levels are observed. The increase of total circulating ecdysteroids may be due to the increase of ecdysteroid synthesis in the Y-organs or the reduction of ecdysteroid clearing rate in gills. Although we have not examined the Y-organs, we detect both *UpuEcR* and *UpuRXR* transcripts in gills (Chung et al., 1998).

Exogenous RA may affect the circulating titers of ecdysteroids via the alteration of gene expression in these tissues. In addition, there are four major circulating ecdysteroids in crustaceans. The changes of total circulating ecdysteroid titers are the sum of changes of each of these individual ecdysteroids. In this experiment, the antiserum used in RIAs has higher binding affinity to 20-hydroxyecdysone than to the other three ecdysteroids and the total circulating ecdysteroid titers are expressed in 20-hydroxyecdysone equivalents. The changes in titers of other ecdysteroids may be obscured because of the higher affinity of our antiserum for 20-hydroxyecdysone.

Vertebrate RXRs are able to bind *9-cis* RA but insect USPs do not. Divergence in ligand requirements for transactivation of these receptors may be due to structural differences in the LBDs. Nuclear receptors have been proposed to gain ligand binding activity independently during evolution and the insect USPs have been classified as orphan receptors (Escriva et al., 1997). Invertebrate and vertebrate RXRs may have evolved different transactivation mechanisms. While vertebrate RXRs require ligand to activate the AF-2 domain, invertebrate RXRs may be active in the absence of ligand or through other pathways. A recent report suggests that juvenile hormone is a USP ligand (Jones and Sharp, 1997). Whether RA or other classes of terpenoids may serve as ligands for the crustacean receptors remains to be determined. Preliminary experiments using nuclear extracts from *Uca* limb buds show binding to *all-trans* RA or its metabolites (unpublished data). Thus, UpuRXR may provide insight into the functional differences between insect USPs and vertebrate RXRs. The production of UpuEcR and UpuRXR through recombinant expression vectors will allow us to evaluate their interactions with each other and potential ligands and

provide more information about structure-function relationships within the nuclear receptor family.

Literature cited

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., Lipman, D. J., 1990. Basic local alignment tool. J. Mol. Biol. 215, 403-410.

Antoniewski, C., Mugat, B., Delbac, F., and Lepesant, J.-A., 1996. Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster*. Mol. Cell. Biol. 16, 2977-2986.

Baniahmad, A., Leng, X., Burris, T. P., Tsai, S., Tsai, M.-J., O'Malley, B. W., 1995. The τ4 activation domain of the thyroid hormone receptor is required for release of a putative corepressor(s) necessary for transcriptional silencing. Mol. Cell. Biol. 15, 76-86.

Barettino, D., Vivanco Ruiz, M., Stunnenberg, H. G., 1994. Characterization of the ligand-dependent transactivation domain of thyroid hormone receptor. EMBO J. 13, 3039-3049.

Bazin, F., 1977. Action inhibitrice de l'ecdysterone sur la regeneration chez le Crabe *Carcinus maenas*. C. R. Acad. Sci. Paris 284, 1211-1214.

Bodenteich, A., Chissoe, S. L., Wang, Y. F., Roe, B. A., 1994. Shotgun cloning of the strategy of choice to generate templates for high throughout dideoxynucleotide sequencing. In: Adam, M. D., Fields, C., Venter, C., (eds.), Automated DNA sequencing and analysis techniques. Academic Press, London, pp. 42-50.

Brockes, J. P., 1992. Introduction of a retinoid reporter gene into the urodele limb blastema. Proc. Natl. Acad. Sci. USA. 89, 11386-11390.

Brockes, J. P., 1997. Amphibian limb regeneration: Rebuilding a complex structure. Science 276, 81-87.

Bourguet, W., Ruff, M., Chambon, P., Gronemeyer, H., Moras, D., 1995. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. Nature 375, 377-382.

Chambon, P., 1996. A decade of molecular biology of retinoic acid receptors. FASEB J. 10, 940-954.

Chissoe, S. L., Wang, Y. F., Chifton, S. W., Ma, N., Sun, H. J., Lobsinger, J. S., Kenton, S.

M., White, J. D., Roe, B. A., 1991. Strategies for rapid and accurate DNA sequencing. Methods 3, 55-65.

Cho, W.-L., Kapitskaya, M. Z., Rackhel, A. S., 1995. Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. Insect Biochem. Molec. Biol. 25, 19-27.

Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162, 156-159.

Collingwood, T. N., Butler, A., Tone, Y., Clifton-Bligh, R. J., Parker, M. G., Chatterjee, K. K., 1997. Thyroid hormone-mediated enhancement of heterodimer formation between thyroid hormone receptor β and retinoid X receptor. J. Biol. Chem. 272, 13060-13065.

Copper, M. P., Klaassen, I., Brakenhoff, R. H., Cloos, J., Snow, G. B., Braakhuis, B. J. M., 1997. *All-trans* retinoic acid induced gene expression and growth inhibition in head and neck cancer cell lines. Oral Oncology 33, 270-274.

Chung, A. C.-K., Durica, D. S., Hopkins, P. M. 1998. Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator*. Gen.. Comp. Endocrinol. 109, 375-389.

Danielson, P. S., White, R., Lees, J. A., Parker, M. G., 1992. Identification of a conserved region required for hormone dependent transcriptional activation by steroid hormone receptors. EMBO J. 11, 1025-1033.

Denker, L., Gustafson, A. L., Annerwell, E., Bush, C., Eriksson, U., 1991. Retinoid-binding proteins in craniofacial development. J. Craniof. Dev. Biol. 11, 303-314.

Dhadialla, T. S., Tzertzinis, G., 1997. Characterization and partial cloning of ecdysteroid receptor from a cotton boll weevil embryonic cell line. Arch. Insect. Biochem. Physiol. 35, 45-57.

Durand, B., Saunders, M., Gaudon, Roy, B., Losson, R., Chambon, P., 1994. Activation function 2 (AF-2) of retinoic acid receptor and *9-cis* retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. EMBO J. 13, 5370-5382.

Durica, D. S., Hopkins, P. M., 1996. Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator*. Gene 171, 237-241.

Durstewitz, G., Terwilliger, N. B., 1997. cDNA cloning of a developmentally regulated

hemocyanin subunit in the crustacean *Cancer magister* and phylogenetic analysis of the hemocyanin gene family. Mol. Biol. Evol. 14, 266-276.

Escriva, H., Safi, R., Hanni, C., Langois, M.-C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R., Laudet, V., 1997. Ligand binding was acquired during evolution of nuclear receptors. Proc. Natl. Acad. Sci. USA 94, 6803-6808.

Forman, B. M., Samuels, H. H., 1990. Interactions among a subfamily of nuclear hormone receptors: The regulatory zipper model. Mol. Endocrinol. 4, 1293-1301.

Forman, B. M., Umesono, K., Chen, J., Evans, R. M., 1995. Unique response pathways are established by allosteric interactions among nuclear hormone receptors. Cell 81, 541-550.

Friedrich, M., Tautz, D., 1995. Ribosomal DNA phylogeny of the major extant arthropod classes and the evolution of myriapods. Nature 376, 165-167.

Fujiwara, H., Jindra, M., Newitt, R., Palli, S. R., Hiruma, K., Riddiford, L. M., 1995. Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. Insect Biochem. Molec. Biol. 25, 845-856.

Gardiner, D. M., Blumberg, B., Komine, Y., Bryant, S. V., 1995. Regulation of *HoxA* expression in developing and regenerating axolotl limbs. Development 121, 1731-1741.

Gianini, F., Maestro, R., Vukosavljevic, T., Pomponi, F., Boicchi, M., 1997. All-*trans*, *13-cis* and *9-cis* retinoic acids induce a fully reversible growth inhibition in HNSCC cell lines: implications for *in vivo* retinoic acid use. Int. J. Cancer 70, 194-200.

Gish, W., States, D. J. 1993. Identification of protein coding regions by database similarity search. Nature Genet. 3, 266-272.

Henrich, V. C., Szekely, A. A., Kim, S. J., Brown, N. E., Antoniewski, C., Hayden, M. A., Lepesant, J.-A., Gilbert, L. I., 1994. Expression and function of the *ultraspiracle* (*usp*) gene during development of *Drosophila melanogaster*. Dev. Biol. 165, 38-52.

Hannan, G. N., Hills, R. J., 1997. Cloning and characterization of LcEcR: a functional ecdysone receptor from the sheep blowfly, Lucilia cuprina. Insect Biochem. Mol. Biol. 27, 479-488.

Hopkins, P. M., 1983. Patterns of serum ecdysteroids during induced and uninduced proecdysis in fiddler crab, *Uca pugilator*. Gen. Comp. Endocrinol. 63, 362-373.

Hopkins, P. M., 1988. Control of regeneration in crustaceans. In: Downer, R., Laufer, H., (eds.), Endocrinology of selected invertebrate types. vol. 2, Alan R. Liss, New York, pp.

327-340.

Hopkins, P. M., 1989. Ecdysteroids and regeneration in the fiddler crab, *Uca pugilator*. J. Exp. Zool. 252, 293-299.

Hopkins, P. M., 1993. Regeneration of walking legs in the fiddler crab, *Uca pugilator*. Amer. Zool. 33, 348-356.

Hopkins, P. M., Durica, D. S., 1995. Effects of *all-trans* retinoic acid on regenerating limbs of the fiddler crab, *Uca pugilator*. J. Exp. Zool. 272, 455-463.

Hopkins, P. M., Bliss, D. E., Sheehan, S. W., Boyer, J. R., 1979. Limb growth-controlling factors in the crabs *Gencarcinus lateralis* with special reference to the limb growth-inhibiting factor. Gen. Comp. Endocrinol. 39, 192-207.

Imhof, M. O., Rusconi, S., Lezzi, M., 1993. Cloning of a *Chironomis tentans* cDNA encoding a protein (cEcRH) homologous to the *Drosophila melanogaster* ecdysteroid receptor (dEcR). Insect Biochem. Molec. Biol. 23, 115-124.

Jiang, H., Gyda III, M., Harnish, D. C., Chandraratna, R. A., Soprano, K. J., Kochhar, D. M., Soprano, D. R., 1994. Teratogenesis by retinoic acid analogs positively correlates with elevation of retinoic acid receptor-β2 mRNA levels in treated embryos. Teratology 50, 38-43.

Jindra, M., Malone, F., Hiruma, K., Riddiford, L. M., 1996. Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta*. Dev. Biol. 180, 258-272.

Jindra, M., Huang, J.-Y., Malone, F., Asahina, M., Riddiford, L. M., 1997. Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta*. Insect Molec. Biol. 6, 41-53.

Jones, G., Sharp, P. A., 1997. Ultraspiracle: An invertebrate nuclear receptor for juvenile hormones. Proc. Natl. Acad. Sci. USA 94, 13499-13503.

Jones, B. B., Ohno, C. K., Allenby, G., Boffa, M. B., Levin, A. A., Grippo, J. F., Petkovich, M., 1995. New retinoid X receptor subtypes in zebra fish (*Danio rerio*) differentially modulate transcription and do not bind *9-cis* retinoid acid. Mol. Cell. Biol. 15, 5226-5234.

Kapitskaya, M., Wang, S., Cress, D. E., Dhadialla, T. S., Raikel. A. S., 1996. The mosquito *ultraspiracle* homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. Mol. Cell. Endocrinol. 121, 119-132.

Kastner, P., Mark, M., Ghyselinck, N., Krezel, W., Dupe, V., Grondona, J. M., Chambon, P., 1997. Genetic evidence that retinoic signal is transduced by heterodimeric RXR/RAR functional units during mouse development. Development 124, 313-326.

Koelle, M. R., Talbot, W. S., Segraves, W. A., Bender, M. T., Cherbas, P., Hogness, D. S., 1991. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67, 59-77.

Kothapalli, R., Palli, S. R., Ladd, T. R., Sohi, S. S., Cress, D., Dhadialla, T. S., Tzertzinis, G., Retnakaran, A., 1995. Cloning and developmental expression of the ecdysone receptor gene from the spruce budworm, *Choristoneura fumiferana*. Dev. Genet. 17, 319-330.

Kumar, V., Green, S., Stack, G., Berry, M., Jin, J., Chambon, P., 1987. Functional domains of the human estrogen receptor. Cell 51, 941-951.

Kunieda, T., Kurata, S., Natori, S., 1997. Regeneration of *Sarcophaga* imaginal discs *in vitro*: Implication of 20-hydroxyecdysone. Dev. Biol. 183, 86-94.

LeDouarin, B., VomBaur, E., Zechel, C., Heery, D., Heine, M., Vivat, V., Gronemeyer, H., Losson, R., Chambon, R., 1996. Ligand-dependent interaction of nuclear receptors with potential transcriptional intermediary factors (mediators). Phil. Trans. R. Soc. Lond. B. 351, 569-578.

Lehmann, J. M., Zhang, X.-K., Graupner, G., Lee, M.-O., Hermann, B., Hoffmann, T., Pfahl, M., 1993. Formation of RXR homodimers leads to repression of T3 response: Hormonal cross-talk by ligand induced squelching. Mol. Cell. Biol. 13, 7698-7707.

Leid, M., Kastner, P., Lyons, R., Nakshatri, H., Saunders, M., Zacharewsi, T., Chen, J.-Y., Staub, A., Garnier, J.-M., Mader, S., Chambon, P., 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell 68, 377-395.

Li, X.-Y., Xiao, J.-H., Feng, X., Qin, L., Voorhees, J. J., 1997. Retinoid X receptor-specific ligands synergistically upregulate 1, 25-dihydroxyvitamin D_3-dependent transcription in epidermal keratinocytes *in vitro* and *in vivo*. J. Invest. Dermatol. 108, 506-512.

Lu, H.-C., Eichele, G., Tahaller, C., 1997. Liganded bounded RXR can mediate retinoid signal transduction during embryogenesis. Development 124, 195-203.

MacDonald, P. N., Dowd, D. R., Nakajima, S., Galligan, M. A., Reeder, M. C., Haussler, C. A., Ozato, K., Haussler, M., 1993. Retinoid X receptors stimulate and *9-cis* retinoic acid inhibits 1,25-dihydroxyvitamin D3-activated expression of the rat osteocalcin gene. Mol. Cell. Biol. 13, 5907-5917.

Maden, B. K., 1983. The effects of vitamin A on the regenerating axolotl limb. J. Embryol. Exp. Morphol. 77, 273-295.

Madin, J. A., Minucci, S., Driggers, P. H., Lee, I. J., Ozato, K., 1994. Quantitative increases in DNA binding affinity and positional effects determine *9-cis* retinoic acid induced activation of the retinoid X receptor β homodimer. Mol. Cell. Endocrinol. 105, 27-35.

Mak, P., Fuernkranz, H. A., Ge, R., Karathanasis, S. K., 1994. Retinoid X receptor homodimers function as transcriptional activators in yeast. Gene 145, 129-133.

Maksymowysch, A. B., Hsu, T.-C., Litwack, G., 1993. A novel, highly conserved structural motif is present in all members of the steroid receptor superfamily. Receptor 2, 225-240.

Mangelsdorf, D. J., Evans, R. M., 1995. The RXR heterodimers and orphan receptors. Cell 83, 841-850.

Mangelsdorf, D. J., Ong, E. S., Dyck, J. A., Evans, R. M., 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature 345, 224-229.

Mangelsdorf, D. J., Borgmeyer, U., Heyman, R. A., Zhou, J. Y., Ong, E. S., Oro, A. E., Kakizuka, A., Evans, R. M. 1992. Characterization of three RXR genes that mediate the action of *9-cis* retinoic acid. Genes Dev. 6, 329-344.

McVean, A., 1984. Autotomy. In: Bliss, D. E., (ed.), The biology of Crustacea. Academic Press, New York, pp. 107-132.

Minucci, S., Ozato, K., 1996. Retinoid receptors in transcriptional regulation. Curr. Opin. Genet. Dev. 6, 564-574.

Minucci, S., Leid, M., Toyama, R., Siant-Jeannet, J.-P., Peterson, V. J., Horn, V., Ishmael, J. E., Bhattacharyya, N., Dey, A., Sawid, I. B., Ozato, K., 1997. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. Mol. Cell. Biol. 17, 644-655.

Mouillet, J. F., Delbecque, J. P., Quennedey, B., Delachambre, J. 1997. Cloning of two putative ecdysteroid receptor isoforms from *Tenebrio molitor* and their developmental expression in the epidermis during metamorphosis. Eur. J. Biochem. 248, 856-860.

Napoli, J. L., 1996. Retinoic acid biosynthesis and metabolism. FASEB J. 10, 993-1001.

Niazi, I. A., 1996. Background to work on retinoids and amphibian limb regeneration: Studies on anuran tadpoles - a retrospect. J. Biosci. 21, 273-297.

Niazi, I. A., Saxena, S., 1978. Abnormal limb regeneration in tadpoles of the toad, *Bufo anderosonii* exposed to vitamin A excess. Folia Biol. 26, 3-8.

Oro, A. E., McKeown, M., Evans, R. M., 1990. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. Nature 347, 298-301.

Paulsen, D. F., 1994. Retinoic acid in limb-bud outgrowth: review and hypothesis. Anat. Embryol. 190, 399-415.

Perlmann, T., Umesono, K., Rangarajan, P. N., Forman, B. M., Evans, R. M., 1996. Two distinct dimerization interfaces differentially modulate target gene specificity of nuclear hormone receptors. Mol. Endocrinol. 10, 958-966.

Pfhal, M., Apfel, R., Bendik, I., Fanjul, A., Graupner, G., Lee, M.-O., La-Vista, N., Lu, X.-P., Piedrafita, J., Ortiz, M. A., Salbert, G., Zhang, X.-K., 1994. Nuclear retinoid receptors and their mechanism of action. In: Litwack, G., (ed.), Vitamins and hormone, vol. 49, Academic Press, San Diego, pp. 327-382.

Picard, D., Yamamoto, R., 1987. Two signals mediate hormone-dependent nuclear localization of the gluccorticoid receptor. EMBO J. 6, 3333-3340.

Puzianowska-Kuznicka, M., Damjanovski, S., Shi, Y.-B., 1997. Both thyroid hormone and *9-cis* retinoic acid receptors are required to efficiently mediate the effects of thyroid hormone on embryonic development and specific gene regulation in *Xenopus laevis*. Mol. Cell. Biol. 17, 4738-4749.

Rao, K. R., 1978. Effects of ecdysterone, inokosterone and eyestalk ablation on limb regeneration in the fiddler crab, *Uca pugilator*. J. Exp. Zool. 203, 257-270.

Renaud, J.-P., Rochel, N., Ruff, M., Vivat, V., Chambon, P., Gronemeyer, H., Moras, D., 1995. Crystal structure of the RAR-γ ligand-binding domain bound to all-*trans* retinoic acid. Nature 378, 681-689.

Saatcioglu, F., Bartunek, P., Deng, T., Zenke, M., Karin, M., 1993. A conserved C-terminal sequence that is deleted in v-Erb A is essential for the biological activities of c-Erb A (the thyroid hormone receptor). Mol. Cell. Biol. 13, 3675-3685.

Sambrook, J., Fritsch, E. F., Maniatis, T., 1989. Molecular cloning: A laboratory manual. Second edition, Cold Spring Harbor Laboratory Press, New York.

Schulman, I. G., Juguilon, H., Evans, R. M., 1996. Activation and repression by nuclear hormone receptors: Hormone modulates an equilibrium between active and repressive states. Mol. Cell. Biol. 16, 3807-3813.

Swevers, L., Drevet, J. R., Lunke, M. D., Iatrou, K., 1995. Cloning of the ecdysone receptor of the silkmoth *Bombyx mori* and analysis of its expression during follicular cell differentiation. Insect Biochem. Molec. Biol. 25, 857-866.

Swevers, L., Cherbas, L., Cherbas, P., Iatrou, K., 1996. *Bombyx* EcR (BmEcR) and *Bombyx* USP (BmCF1) combine to form a functional ecdysone receptor. Insect Biochem. Molec. Biol. 26, 217-221.

Talbot, W. S., Swyryd, E. A., Hogness, D. S., 1993. *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73, 1323-1337.

Thiele, R., Zimmer, R., Lengauer, T., 1995. Recursive dynamic programming for adaptive sequence and structural alignment. In: Rawlings, C., (ed.), Third international symposium on intelligent systems for molecular biology. AAAI Press, Menlo Park, pp. 384-392.

Thomas, H. E., Stunnenberg, H. G., Steward, A. F., 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. Nature 362, 471-475.

Thompson, J. D., Higgins, D. G., Gibson, T. J., 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. 22, 4673-4680.

Thummel, C. S., 1996. Flies on steroids - *Drosophila* metamorphosis and the mechanisms of steroid hormone action. Trends Genet. 12, 306-310.

Tone, Y., Collingwood, T. N., Adams, M., Chatterjee, V. K., 1994. Functional analysis of a transactivation domain in the thyroid hormone β receptor. J. Biol. Chem. 269, 31157-31161.

Tsai, S. Y., Tsai, M.-J., 1997. Chick ovalbumin upstream promoter-transcription factors (COUP-TFs): Coming of age. Endocrine Rev. 18, 229-240.

Tsonis, P. A., 1996. Hox gene and limb regeneration (Limb regeneration), pp. 179-187, Cambridge University Press, Melbourne.

Tsonis, P. A., Sargent, M. T., Del Rio-Tsonis, K., Jung, J. C., 1996. 9-cis retinoic acid antagonizes the stimulatory effect of 1,25-dihydroxyvitamin D_3 on chondrogenesis of chick limb bud mesenchymal cells: Interactions of their receptors. Int. J. Dev. Biol. 40, 1053-1059.

Tzertzinis, G., Malecki, A., Kafato, F. C., 1994. BmCF1, a *Bombyx mori* RXR-type receptor related to the *Drosophila* ultraspiracle. J. Mol. Biol. 258, 479-486.

Van Wormhoudt, A., Sellos, D., 1996. Cloning and sequencing analysis of three amylase cDNAs in the shrimp *Penaeus vannamei* (Crustacea decapoda): Evolutionary aspects. J. Mol. Evol. 42, 543-551.

Viviano, C. M., Brockes, J. P., 1996. Is retinoic acid an endogenous ligand during urodele limb regneration? Int. J. Dev. Biol. 40, 817-822.

Wurtz, J.-M., Bourguet, W., Renaud, J.-P., Vivat, V., Chambon, P., Moras, D., Gronemeyer, H., 1996. A canonical structure for the ligand-binding domain of nuclear receptors. Nature Struc. Biol. 3, 87-94.

Yao, T.-P., Forman, B. M., Jiang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P., Evans, R. M., 1993. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. Nature 366, 476-479.

Zelhof, A. C., Yao, T.-P., Chen, J. D., Evans, R. M., McKeown, M., 1995. Seven-up inhibits ultraspiracle-based signaling pathways *in vitro* and *in vivo*. Mol. Cell. Biol. 15, 6736-6745.

Zhang, X.-K., Hoffmann, B., Tran, P., Graupner, G., Pfahl, M., 1992. Retinoid X receptor is an auxiliary protein for thyroid hormone and retinoic acid receptors. Nature 355, 441-446.

Zhang, X.-K., Salbert, G., Lee, M.-O., Pfahl, M., 1995. Mutations that alter ligand-induced switches and dimerization activities in the retinoid X receptor. Mol. Cell. Biol. 14, 4311-4323.

Zilliacus, J., Wright, A. P. H., Carlstedt-Duke, J., Gustafsson, J.-A., 1995. Structural determinants of DNA-binding specificity by steroid receptors. Mol. Endocrinol. 9, 389-400.

Zou, A., Elgort, M. G., Allegretto, E. A., 1997. Retinoid X receptor (RXR) ligands activate the human 25-hydroxyvitamin D_3-24-hydroxylase promotor via RXR heterodimer binding to two vitamin D-responsive elements and elicit additive effects with 1,25-dihydroxyvitamin D_3. J. Biol. Chem. 272, 19027-19034.

Table 1 List of EcRs, USPs and RXRs used in the sequence analysis

Species		Abbreviation *	GenBank Acession Number	Literature cited
EcR				
Crustacean	*Uca pugilator*	UpuEcR	AF034086	this paper
	Orconectes limosus	OliEcR		D. Böcking personal comm.
Coleopteran	*Tenebrio molitor*	TmoEcR	Y11533 (n)	Mouillet et al., 1997
	Anthonomus grandis	AgrEcR		Dhadialla and Tzertzinis, 1997
Dipteran	*Aedes aegypti*	AaeEcR	P49880 (a)	Cho et al., 1995
	Chironomus tentans	CteEcR	S60739 (a)	Imhof et al., 1993
	Drosophila melanogaster	DmeEcR	A41055 (a)	Koelle et al.,1991
	Lucilia cuprina	LcuEcR	U75355 (n)	Hannan and Hill, 1997
Lepidopteran	*Bombyx mori*	BmeEcR	P49881 (a)	Swevers et al., 1995
	Choristoneura fumiferana	CfuEcR	U29531 (n)	Kothapalli et al., 1995
	Heliothis virescens	HviEcR	Y09009 (n)	Martinez et al., unpublished
	Manduca sexta	MseEcR	P49883 (a)	Fujiwara et al., 1995
RXR				
Crustacean	*Uca pugilator*	UpuRXR	AF032983	this paper
Insect	*Aedes aegypti*	AaeUSP		Kapitskaya et al., 1996
	Drosophila melanogaster	DmeUSP	P20153 (a)	Oro et al., 1990
	Bombyx mori	BmoUSP	P49700 (a)	Tzertzinis et al., 1994
	Manduca sexta	MseUSP	U44837 (a)	Jindra et al., 1997
Zebra fish	*Danio rerio*	DreRXRα	U29940 (n)	Jones et al., 1995
		DreRXRγ	U29894 (n)	Jones et al., 1995
		DreRXRδ	U29941 (n)	Jones et al., 1995
		DreRXRϵ	U29942 (n)	Jones et al., 1995
Human	*Homo sapiens*	HsaRXRα	P19793 (a)	Mangelsdorf et al., 1990
		HsaRXRβ	M84820 (a)	Leid et al., 1992
		HsaRXRγ	U38480 (n)	Mangelsdorf et al., 1992

* The abbreviations used here are the same as in the text and figures.
(a) amino sequence in GenBank
(n) nucleotide sequence in GenBank

FIG. 1 Ribonuclease protection assay measuring *UpuEcR* and *UpuRXR* steady state transcript levels during the first five days after autotomy in RA treated and control crabs. (a) Results of a ribonuclease protection assay of blastemal total RNA isolated from 25 animals hybridized to antisense RNA probes encoding the *UpuEcR* LBD and the *UpuRXR* DBD. 307 nt protected fragments of *UpuRXR* transcript and 162 nt protected fragments of *UpuEcR* transcript were seen (lanes 2 through 11). No protected fragment was observed using yeast transfer RNA (tRNA) which served as a negative control (lane 12). Lane 1: DNA size marker; lane 2 through 6: RNA samples from blastemas of control animals taken one day to five days after autotomy respectively; Lane 7 through 11: RNA samples from blastemas of crabs immersed in sea water with 50 μM RA taken one day to five days after autotomy respectively; Lane 12: 5 μg of yeast tRNA; Lane 13 through 15: 3.6, 1.8, 0.9 pg of *UpuRXR* cRNAs respectively; Lane 16: undigested *UpuRXR* RNA probe; Lane 17 through 19: 3.6, 1.8, 0.9 pg of *UpuEcR* cRNAs respectively; Lane 20: undigested *UpuEcR* RNA probe. (b) Results of ribonuclease protection assay of the diluted fraction (10^{-4} dilution) of blastemal total RNA isolated from 25 animals hybridized to an antisense RNA probe encoding *Uca* 18S rRNA. 59 nt protected fragments of 18S rRNA were seen in all experimental samples (lane 1 through 11). No signal was observed when yeast tRNA was used as a negative control (lane 12). Lane 1: total RNA calibration standard; lane 2 through 6: diluted RNA samples from blastemas of control animals taken one day to five days after autotomy respectively; Lane 7 through 11: diluted RNA samples from blastemas of crabs immersed in sea water with RA taken one day to five days after autotomy respectively; Lane 12: 5 μg of yeast tRNA.

FIG. 2 Nucleotide and deduced amino acid sequences of *UpuEcR* cDNA clones isolated from the oligo-(dT) primed cDNA library. Deduced amino acids are represented by one letter symbols below the nucleotide sequence. The putative DNA binding and ligand binding domains are underlined with single and double lines, respectively. Clones 7A and 13B sequences diverge at nt 417 and 444, respectively, then resume identity at nt 448 of the composite sequence (indicated by arrows).

Fig. 3 Nucleotide and deduced amino acid sequences of *UpuRXR* cDNA clones isolated from the oligo-(dT) primed cDNA library. Deduced amino acids are represented by one letter symbols below the nucleotide sequence. The putative DNA binding and ligand binding domains are underlined with single and double lines, respectively. Clone 11C sequence diverges at nt 486 of the composite sequence (indicated by arrow).

FIG. 4 Comparison of functional domains of the UpuEcR homolog with other EcRs. The species are given in Table 1. Letters above the boxes indicate the receptor domains while the numbers below show the length (number of amino acids) of the domains. The UpuEcR A/B domain is incomplete (jagged line). Percentages of amino acid identity and similarity of each functional domain are indicated inside the boxes. Percentage similarity and identity were calculated using TopLign pairwise sequence analysis software (Thiele et al., 1995).

FIG. 5 Alignment of the UpuEcR homolog amino acid sequence with other EcRs. The

species are given in Table 1. The order of EcRs is assigned by the ClustalW program, relative to degree of sequence similarities (Thompson et al., 1994). The DNA binding (DBD) and ligand binding (LBD) domains, P-box, D-box, nuclear localization signal (NLS), and the subdomains in LBD are marked by the arrow heads (▸ and ◂) above the sequences. Heptad repeat units of helix-turn-zippers and regulatory zippers are marked by short dashed arrows below the sequences. Asterisks indicate amino acids identical in all sequences. Gaps are represented by hyphens while the dots indicate that the sequence is not available. The extreme C-terminal regions of the dipteran and lepidopteran EcRs are not shown. The numbers correspond to the amino acid position in the original sequences. The amino acid sequence of the UpuEcR 7A variant is indicated (in italic) above the UpuEcR composite sequence at aa 148-155.

FIG. 6 Comparison of functional domains of the UpuRXR homolog with insect USPs and vertebrate RXRs. The species are given in Table 1. Letters above the boxes indicate the receptor domains while the numbers below show the length (number of amino acids) of the domains. The UpuRXR A/B domain is incomplete (jagged line). Percentages of amino acid identity and similarity of each functional domain are indicated inside the boxes. Percentage similarity and identity were calculated using TopLign pairwise sequence analysis software (Thiele et al., 1995).

FIG. 7 Alignment of the UpuRXR homolog amino acid sequence with insect USPs and vertebrate RXRs. The species are given in Table 1. The order of RXRs is assigned by

the ClustalW program, relative to degree of sequence similarities (Thompson et al., 1994). The regions of DNA binding (DBD) and ligand binding (LBD) domains, P-box, D-box, T-box and the subdomains of LBD are marked by the arrow heads (▸ and ◂) above the sequences. Twelve helices (H1 to H12) are marked by the arrow heads above the sequences and open diamonds (◇) represents the s1 and s2 segments in β-turn (Bourguet et al., 1995; Wurtz et al., 1996). The arrows (↑) in the AF-2 domain indicate the conserved glutamic acid residues in vertebrate RXRs. The open circles (○) indicate the amino acid residues which are important in anchoring *all-trans* RA to the ligand-binding pocket (Renaud et al., 1995). Heptad repeat units of helix-turn-zippers and regulatory zippers are marked by short dashed arrows below the sequences. Asterisks indicate amino acids identical in all sequences. Gaps are represented by hyphens while the dots indicate that the sequence is not available. The numbers correspond to the amino acid position in the original sequences. The hinge regions (e.g. residues 90-101 for UpuRXR) are not shown.

FIG. 8 Steady state concentrations of receptor mRNA in blastemas during early stages of limb regeneration as detected by ribonuclease protection assay: (a) *UpuEcR* and (b) *UpuRXR*. Receptor transcript abundance from control blastemas (crabs were immersed in sea water with 0.6% ethanol) is represented by closed circles and solid lines. Receptor transcript abundance from experimental blastemas (crabs were immersed in sea water with 50 μM *all-trans* RA) is represented by closed squares and dashed lines. Time of RNA isolation following autotomy is given on the x-axis. The y-axis represents

abundance of receptor mRNA as pg cRNA per μg of total RNA loaded (see Materials and Methods). Error bars represent standard errors of the mean calculated from the results of four independent pooled samples. Each pooled sample contained RNA isolated from ten or twenty-five crabs. Asterisks indicate significant differences between control and experimental groups at the same time point by Student's t-test ($P \leq 0.05$).

FIG. 9 Titers of total circulating ecdysteroids during early stages of limb regeneration determined by radioimmunoassay (RIA). The titer of total circulating ecdysteroids from the crabs which were immersed in sea water with 0.6% ethanol after autotomy is represented by closed circles and solid lines. The level of total circulating ecdysteroids from the crabs which were immersed in sea water with 50 μM *all-trans* RA after autotomy is represented by closed squares and dashed lines. Days after multiple autotomy are on the x-axis. RIA-active material in hemolymph is represented as pg of 20-hydroxyecdysone equivalents per μl of hemolymph on the y-axis. Error bars represent standard errors of the mean calculated from the results of 40 individual samples. Asterisks indicate significant differences between control and experimental groups at the same time point by Student's t-test ($P \leq 0.05$).

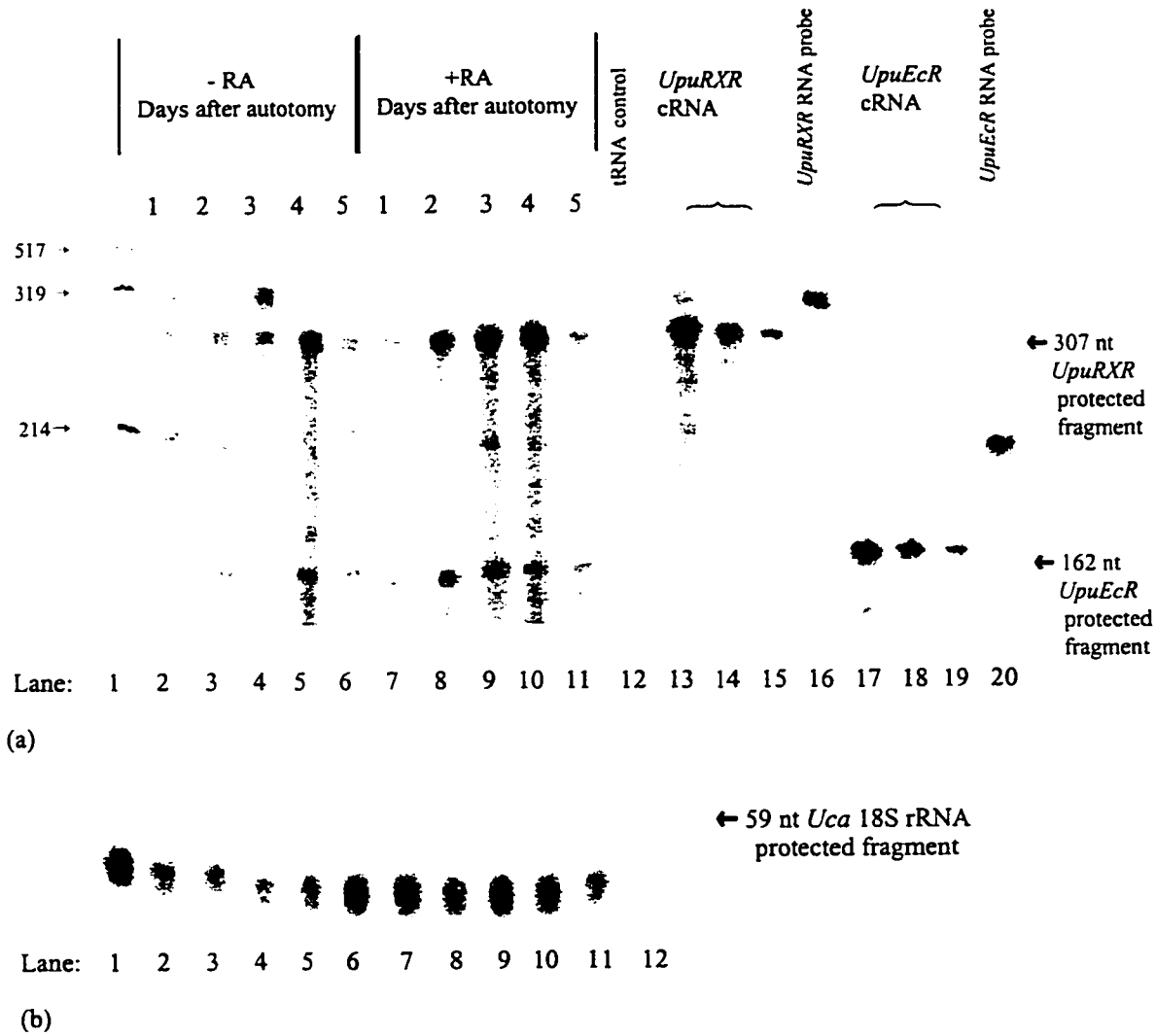

- RA
Days after autotomy
+RA
Days after autotomy
tRNA control
UpuRXR cRNA
UpuRXR RNA probe
UpuEcR cRNA
UpuEcR RNA probe
1 2 3 4 5 1 2 3 4 5
517
319
214
← 307 nt UpuRXR protected fragment
← 162 nt UpuEcR protected fragment
Lane: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20
(a)
← 59 nt Uca 18S rRNA protected fragment
Lane: 1 2 3 4 5 6 7 8 9 10 11 12
(b)

FIG. 1

Uca pugilator EcR homolog: Composite sequence
.....estimated ~2000 nt to 5'end of transcript

```
   1 CGA GAT AAC ATG TCG CCG CCC TCT TCG CTG AGT AAC TTC GGT GCC GAC TCC TAC GGC GAC   60
     R   D   N   M   S   P   P   S   S   L   S   N   F   G   A   D   S   Y   G   D
                                                                 | begin DBD
  61 CTG AAG AAG AAG AAA GGC CCT ATC CCA CGC CAG CAG GAG GAG ATG TGT CTG GTG TGT GGC  120
     L   K   K   K   K   G   P   I   P   R   Q   Q   E   E   M   C   L   V   C   G
 121 GAC AGG GCA TCC GGC TAC CAC TAC AAC GCC CTC ACC TGT GAA GGA TGC AAA GGT TTC TTC  180
     D   R   A   S   G   Y   H   Y   N   A   L   T   C   E   G   C   K   G   F   F
 181 CGG AGG TCC ATC ACT AAG AAT GCC GTG TAC CAG TGT AAA TAT GGC AAC AAC TGT GAG ATG  240
     R   R   S   I   T   K   N   A   V   Y   Q   C   K   Y   G   N   N   C   E   M
 241 GAC ATG TAT ATG CGA CGC AAG TGT CAG GAG TGT CGC CTC AAA AAG TGT CTC AAC GTG GGC  300
     D   M   Y   M   R   R   K   C   Q   E   C   R   L   K   K   C   L   N   V   G
   | end DBD
 301 ATG CGG CCA GAA TGT GTC GTG CCC GAG TCT CAG TGT CAG GTG AAG AGA GAG CAG AAG AAG  360
     M   R   P   E   C   V   V   P   E   S   Q   C   Q   V   K   R   E   Q   K   K
                                                         13B sequence diverges |
 361 GCA CGA GAC AAG GAC AAA ACT TAC CCA AGC CTA GGT TCC CCT ATT GCT GAG GAC AAG GCT  420
     A   R   D   K   D   K   T   Y   P   S   L   G   S   P   I   A   E   D   K   A
               7A sequence diverges |   | begin common sequence
 421 GCT CCC ATT AGT CCA GTG AGT AAA GAT ATG TCA GCC GCA CCC CGG CTA AAT GTC AAG CCA  480
     A   P   I   S   P   V   S   K   D   M   S   A   A   P   R   L   N   V   K   P
                                                                 | begin LBD
 481 CTC ACG CGA GAG CAA GAG GAG CTG ATC AAC ACT CTA GTC TAC TAT CAA GAA GAG TTT GAG  540
     L   T   R   E   Q   E   E   L   I   N   T   L   V   Y   Y   Q   E   E   F   E
 541 CAG CCA ACT GAA GCA GAT GTC AAG AAG ATC AGA TTT AAC TTC GAT GGT GAA GAT ACA AGT  600
     Q   P   T   E   A   D   V   K   K   I   R   F   N   F   D   G   E   D   T   S
 601 GAC ATG AGA TTC AGG CAC ATA ACC GAG ATG ACG ATC CTC ACA GTT CAG CTC ATA GTG GAA  660
     D   M   R   F   R   H   I   T   E   M   T   I   L   T   V   Q   L   I   V   E
 661 TTC TCC AAG CAA CTA CCA GGT TTC GCC ACA CTT CAG CGA GAA GAC CAG ATT ACC CTG CTC  720
     F   S   K   Q   L   P   G   F   A   T   L   Q   R   E   D   Q   I   T   L   L
 721 AAG GCT TGC TCA TCC GAG GTC ATG ATG CTT CGA GCA GCC CGG CGT TAT GAT GCC AAG ACA  780
     K   A   C   S   S   E   V   M   M   L   R   A   A   R   R   Y   D   A   K   T
 781 GAT TCC ATT GTG TTT GGA AAC AAC TAC CCC TAC ACA CAG GCC TCC TAT GCA CTG GCT GGC  840
     D   S   I   V   F   G   N   N   Y   P   Y   T   Q   A   S   Y   A   L   A   G
 841 TTA GGA GAA TCA GCA GAG ATA CTC TTC CGT TTC TGC CGT AGC CTT TGT AAA ATG AAG GTG  900
     L   G   E   S   A   E   I   L   F   R   F   C   R   S   L   C   K   M   K   V
 900 GAC AAT GCA GAG TAT GCA CTA CTA GCT GCC ATA GCT ATT TTC TCA GAG AGG CCA AAC CTA  960
     D   N   A   E   Y   A   L   L   A   A   I   A   I   F   S   E   R   P   N   L
 961 AAG GAA CTC AAA AAG GTA GAA AAA CTT CAG GAA ATC TAC CTT GAA GCA CTG AAG TCT TAT 1020
     K   E   L   K   K   V   E   K   L   Q   E   I   Y   L   E   A   L   K   S   Y
1021 GTG GAG AAT CGA CGG CTG CCT CGA TCT AAC ATG GTG TTT GCG AAG TTG CTT AAT ATC CTC 1080
     V   E   N   R   R   L   P   R   S   N   M   V   F   A   K   L   L   N   I   L
1081 ACA GAG TTG CGA ACC CTC GGA AAC ATA AAC TCA GAG ATG TGC TTC TCC CTC ACA CTC AAG 1140
     T   E   L   R   T   L   G   N   I   N   S   E   M   C   F   S   L   T   L   K
                                                             | end LBD
1141 AAC AAA AGA CTC CCG CCC TTC CTG GCT GAG ATC TGG GAC GTT TCT GGA TAC TGA         1194
     N   K   R   L   P   P   F   L   A   E   I   W   D   V   S   G   Y   STOP
```

variant 7A sequence

```
TGT AAA TCC AAA GGT CCA TCA ACT GCG TGT GCT ATG CCG TTC AAA AAT CTT GTT GAC AGC
  C   K   S   K   G   P   S   T   A   C   A   M   P   F   K   N   L   V   D   S
TCT AGC ACC GTT CAG TCT CCT
  S   S   T   V   Q   S   P
```

variant 13B sequence

```
GTT GCT CCC ATT AGT CCA
  V   A   P   I   S   P
```

FIG. 2

Uca pugilator RXR homolog: Composite sequence
.....estimated -500 nt to 5'end of transcript

```
     ! begin similarity to Drosophila USP ! begin DBD
   1 CAC CCA CTG TCT GGC TCC AAG CAC CTC TGC TCT ATC TGT GGT GAC CGC GCC TCA GGC AAA   60
       H   P   L   S   G   S   K   H   L   C   S   I   C   G   D   R   A   S   G   K
  61 CAC TAT GGC GTG TAC AGC TGT GAG GGG TGC AAG GGG TTC TTC AAG CGG ACA GTG CGC AAG  120
       H   Y   G   V   Y   S   C   E   G   C   K   G   F   F   K   R   T   V   R   K
 121 GAC CTG ACA TAT GCC TGC CGT GAG GAG CGG TCA TGC ACC ATT GAC AAA CGG CAG AGG AAC  180
       D   L   T   Y   A   C   R   E   E   R   S   C   T   I   D   K   R   Q   R   N
                                                          end DBD!
 181 CGC TGC CAA TAC TGC CGC TAC CAA AAA TGC TTG ACC ATG GGG ATG AAG AGA GAA GCG GTC  240
       R   C   Q   Y   C   R   Y   Q   K   C   L   T   M   G   M   K   R   E   A   V
 241 CAG GAG GAG CGC CAG CGG ACG AAG GGT GAC AAA GGC GAC GGA GAT ACA GAG TCA TCC TGC  300
       Q   E   E   R   Q   R   T   K   G   D   K   G   D   G   D   T   E   S   S   C
         ! begin LBD
 300 GGC GCC ATC TCA GAC ATG CCA ATT GCC AGC ATA CGG GAG GCA GAG CTC AGC GTG GAT CCC  360
       G   A   I   S   D   M   P   I   A   S   I   R   E   A   E   L   S   V   D   P
 361 ATA GAT GAG CAG CCG CTG GAC CAA GGG GTG AGG CTT CAG GTT CCA CTC GCA CCT CCT GAT  420
       I   D   E   Q   P   L   D   Q   G   V   R   L   Q   V   P   L   A   P   P   D
 421 AGT GAA AAG TGT AGC TTT ACT TTA CCT TTT CAT CCC GTC AGT GAA GTA TCC TGT GCT AAC  480
       S   E   K   C   S   F   T   L   P   F   H   P   V   S   E   V   S   C   A   N
           ! 11C sequence diverges
 481 CCT CTG CAG GAT GTG GTG AGC AAC ATA TGC CAG GCA GCT GAC AGA CAT CTG GTG CAG CTG  540
       P   L   Q   D   V   V   S   N   I   C   Q   A   A   D   R   H   L   V   Q   L
 541 GTG GAG TGG GCC AAG CAC ATC CCA CAC TTC ACA GAC CTT CCC ATA GAG GAC CAA GTG GTA  600
       V   E   W   A   K   H   I   P   H   F   T   D   L   P   I   E   D   Q   V   V
 601 TTA CTC AAA GCC GGG TGG AAC GAG TTG CTT ATT GCC TCA TTC TCA CAC CGT AGC ATG GGC  660
       L   L   K   A   G   W   N   E   L   L   I   A   S   F   S   H   R   S   M   G
 661 GTG GAG GAT GGC ATC GTG CTG GCC ACA GGG CTC GTG ATC CAC AGA AGT AGT GCT CAC CAG  720
       V   E   D   G   I   V   L   A   T   G   L   V   I   H   R   S   S   A   H   Q
 721 GCT GGA GTG GGT GCC ATA TTT GAT CGT GTC CTC TCT GAG CTG GTG GCC AAG ATG AAG GAG  780
       A   G   V   G   A   I   F   D   R   V   L   S   E   L   V   A   K   M   K   E
 781 ATG AAG ATT GAC AAG ACA GAG CTG GGC TGC CTT CGC TCC ATC GTC CTG TTC AAC CCA GAT  840
       M   K   I   D   K   T   E   L   G   C   L   R   S   I   V   L   F   N   P   D
 841 GCC AAA GGA CTA AAC TGC GTC AAT GAT GTG GAG ATC TTG CGT GAG AAG GTG TAT GCT GCC  900
       A   K   G   L   N   C   V   N   D   V   E   I   L   R   E   K   V   Y   A   A
 901 CTG GAG GAG TAC ACA CGA ACC ACT TAC CCT GAT GAA CCT GGA CGC TTT GCC AAG TTG CTT  960
       L   E   E   Y   T   R   T   T   Y   P   D   E   P   G   R   F   A   K   L   L
 961 CTG CGA CTT CCT GCA CTC AGG TCT ATA GGC CTG AAG TGT CTT GAG TAC CTC TTC CTG TTT 1020
       L   R   L   P   A   L   R   S   I   G   L   K   C   L   E   Y   L   F   L   F
1021 AAG CTG ATT GGA GAC ACT CCC CTG GAC AGC TAC TTG ATG AAG ATG CTC GTA GAC AAC CCA 1080
       K   L   I   G   D   T   P   L   D   S   Y   L   M   K   M   L   V   D   N   P
1081 AAT ACA AGC GTC ACT CCC CCC ACC AGC TAG
       N   T   S   V   T   P   P   T   S  STOP

variant 11C sequence
     GTA AGA CAA CCA ACT GAC CTG TAT TGA
      V   R   Q   P   T   D   L   Y  STOP
```

FIG. 3

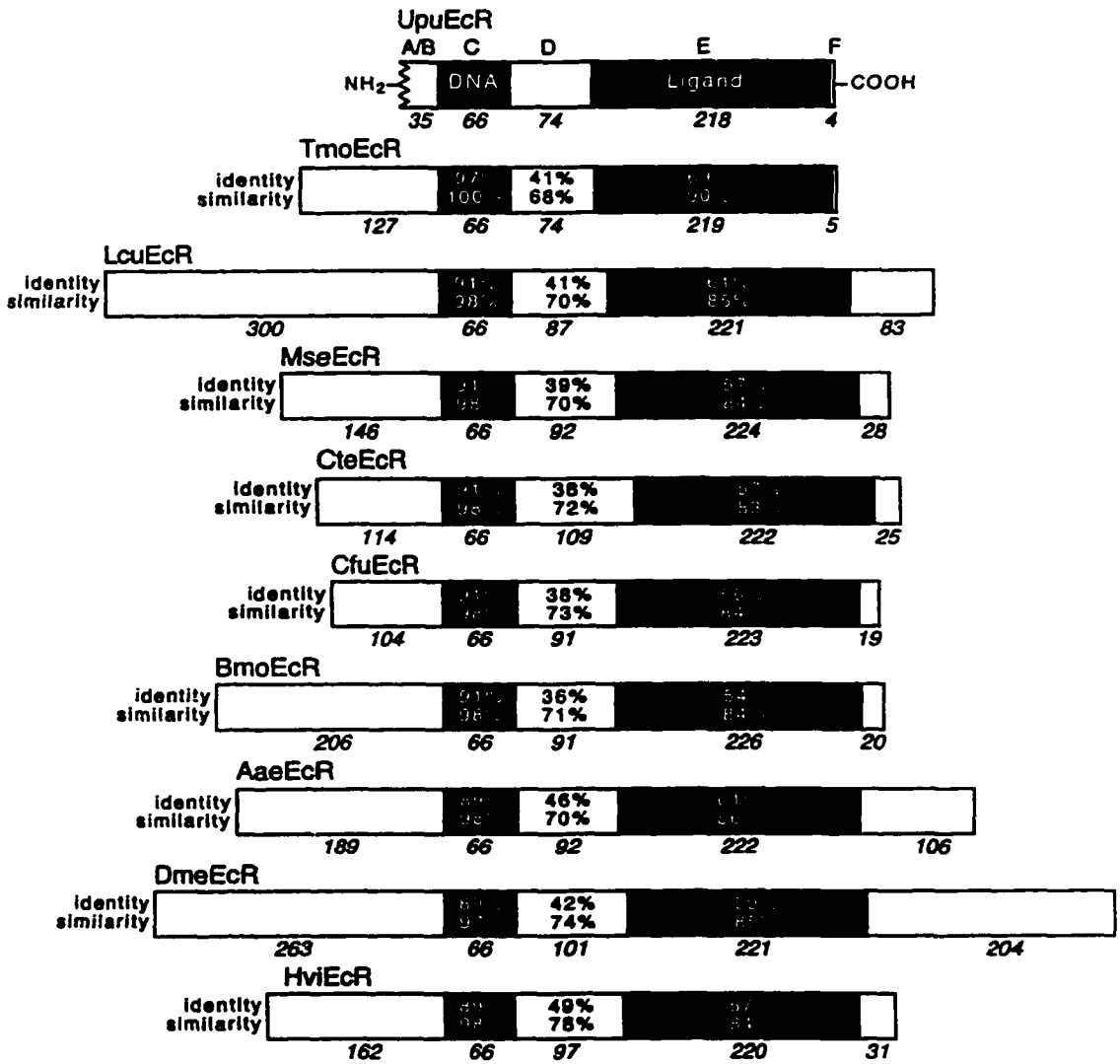

UpuEcR
A/B
C
D
E
F
NH2
DNA
Ligand
COOH
35
66
74
218
4
TmoEcR
identity
similarity
41%
68%
127
66
74
219
5
LcuEcR
identity
similarity
41%
70%
300
66
87
221
83
MseEcR
identity
similarity
39%
70%
146
66
92
224
28
CteEcR
identity
similarity
38%
72%
114
66
109
222
25
CfuEcR
identity
similarity
38%
73%
104
66
91
223
19
BmoEcR
identity
similarity
36%
71%
206
66
91
226
20
AaeEcR
identity
similarity
46%
70%
189
66
92
222
106
DmeEcR
identity
similarity
42%
74%
263
66
101
221
204
HviEcR
identity
similarity
49%
78%
162
66
97
220
31

FIG. 4

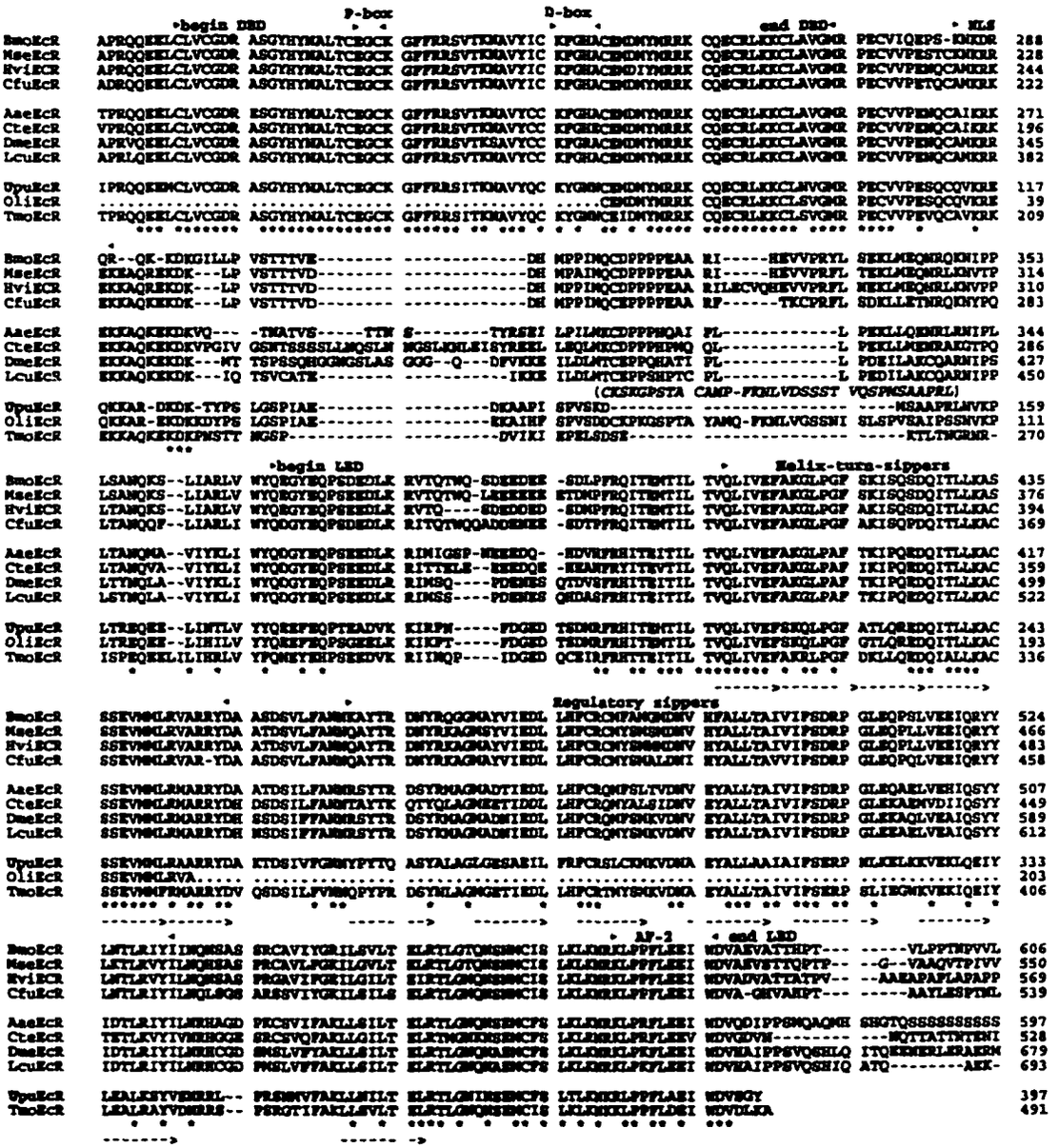
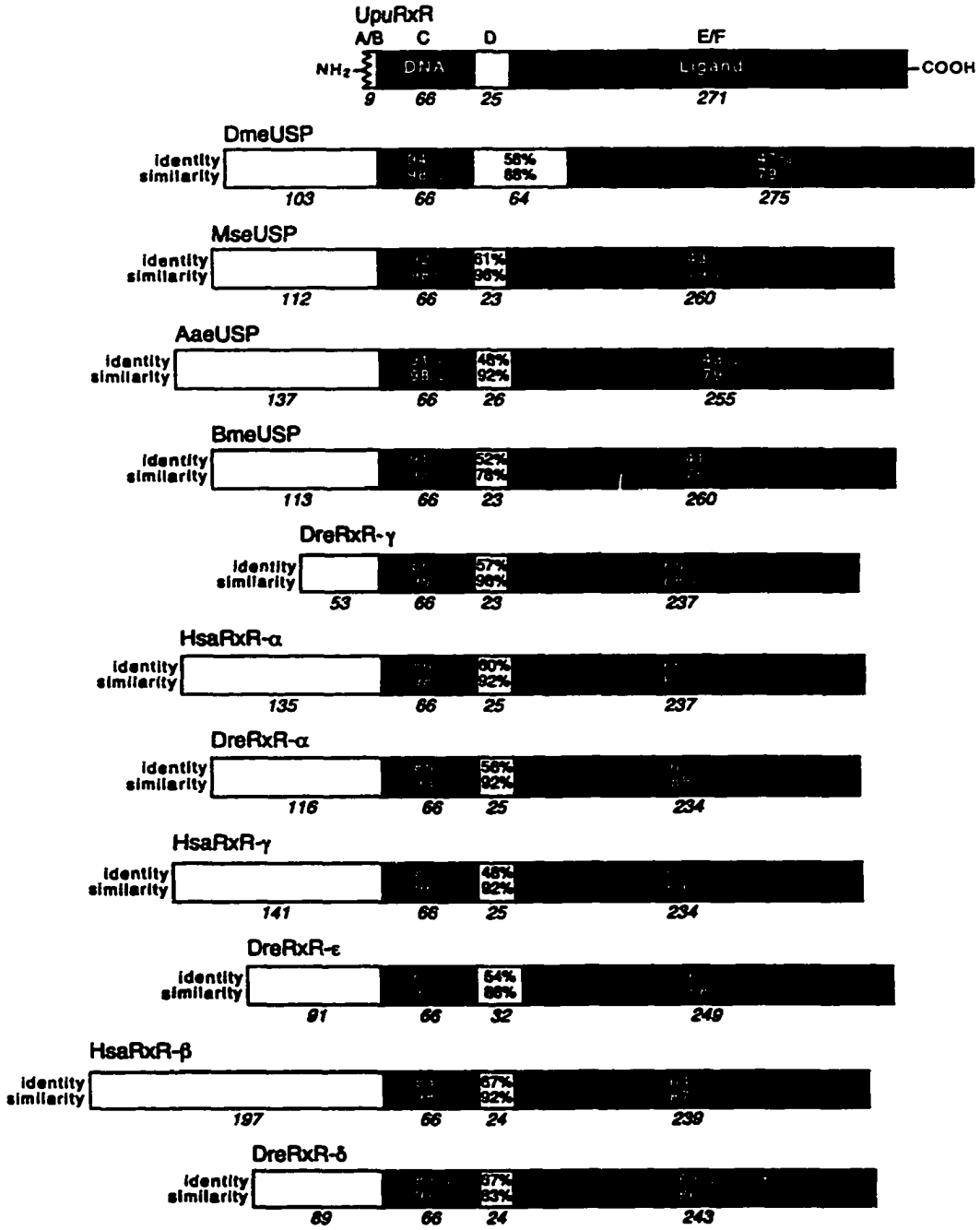


FIG. 5


UpuRxR
A/B
C
D
E/F
NH2
DNA
Ligand
COOH
9
66
25
271
DmeUSP
identity
similarity
58%
88%
103
66
64
275
MseUSP
identity
similarity
61%
96%
112
66
23
260
AaeUSP
identity
similarity
48%
92%
137
66
26
255
BmeUSP
identity
similarity
52%
78%
113
66
23
260
DreRxR-γ
identity
similarity
57%
96%
53
66
23
237
HsaRxR-α
identity
similarity
60%
92%
135
66
25
237
DreRxR-α
identity
similarity
56%
92%
116
66
25
234
HsaRxR-γ
identity
similarity
48%
92%
141
66
25
234
DreRxR-ε
identity
similarity
64%
88%
91
66
32
249
HsaRxR-β
identity
similarity
67%
92%
197
66
24
239
DreRxR-δ
identity
similarity
67%
83%
89
66
24
243

FIG, 6

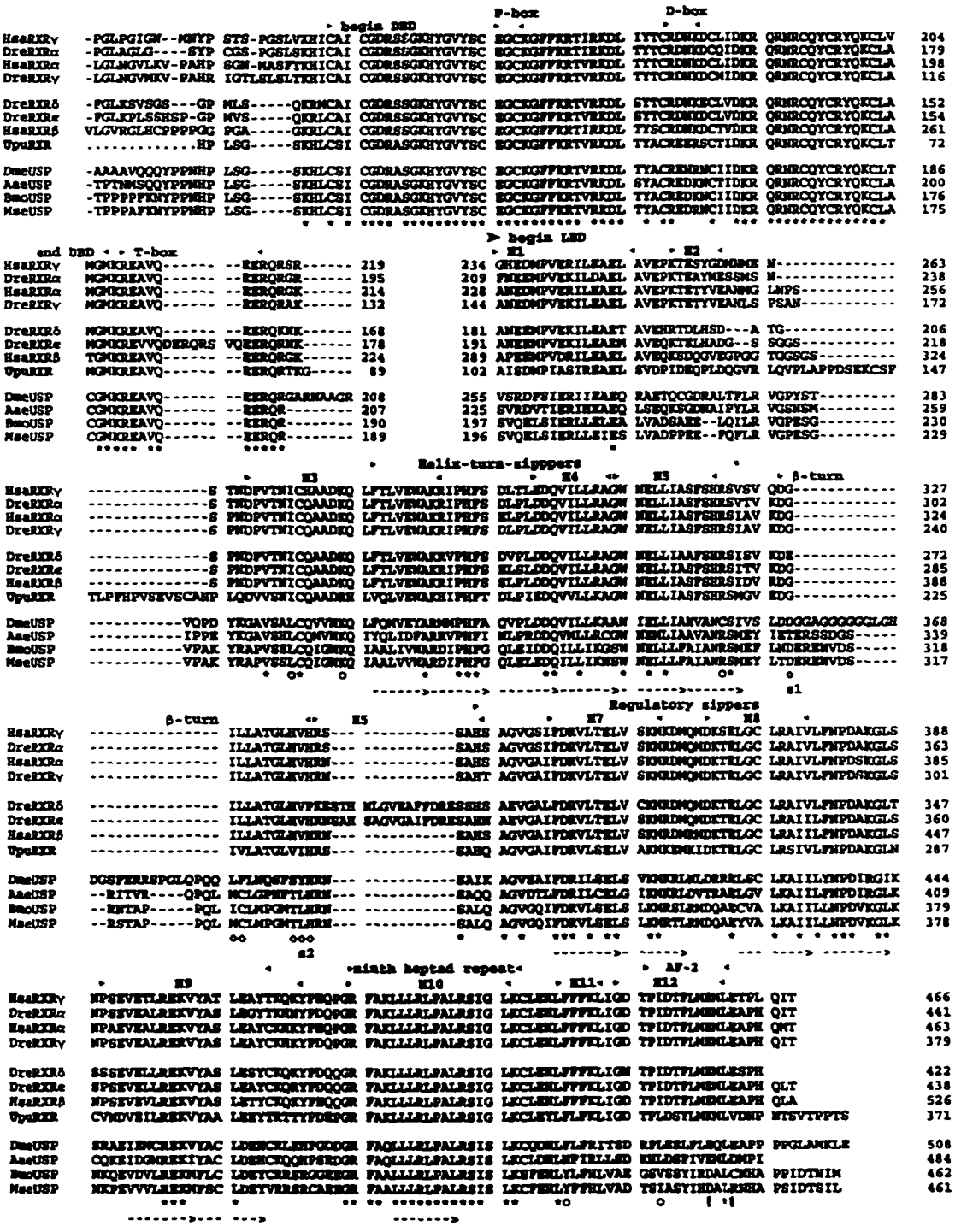


FIG. 7

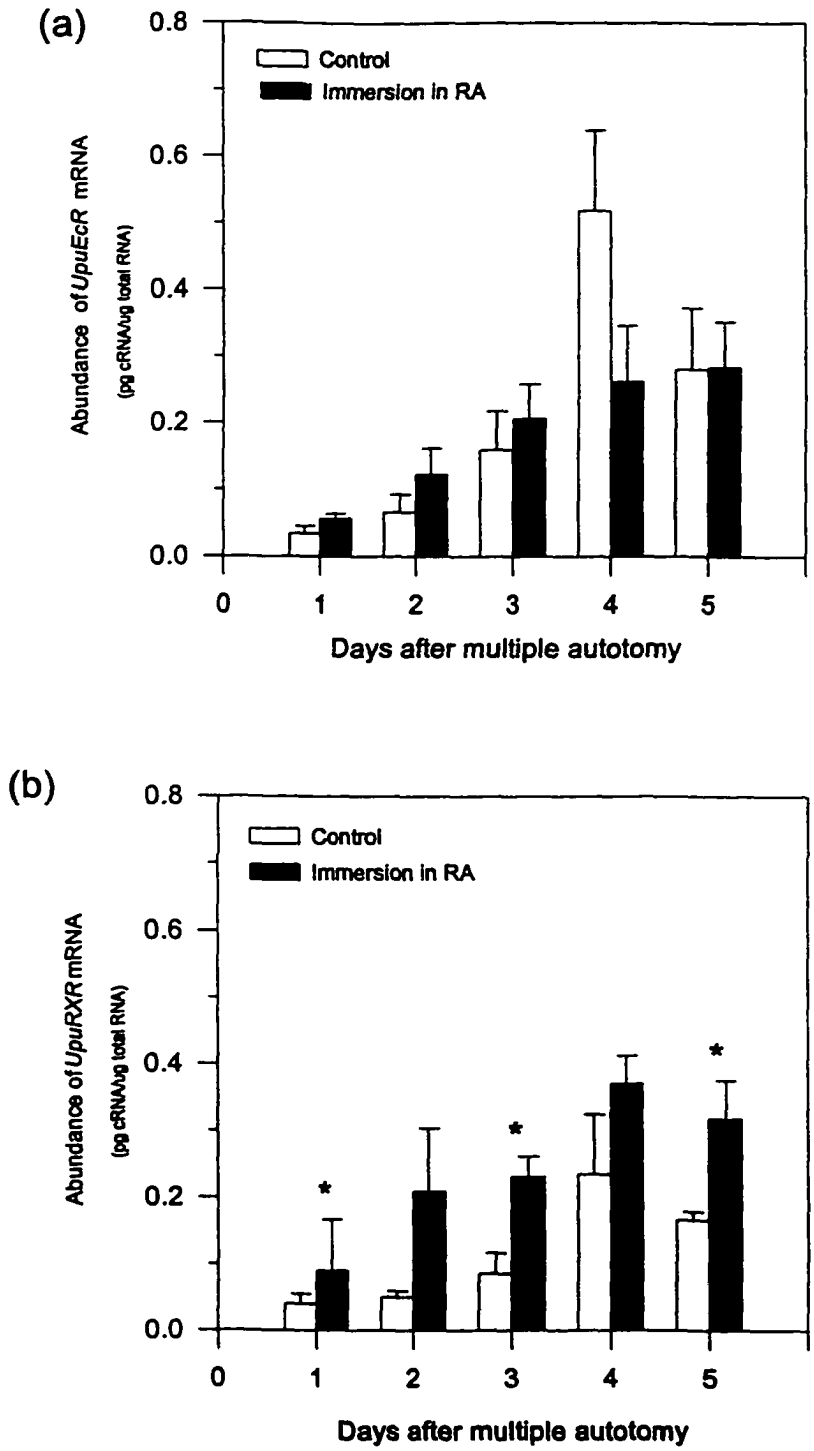

(a)
Control
Immersion in RA
Abundance of UpuEcR mRNA
(pg cRNA/ug total RNA)
0.8
0.6
0.4
0.2
0.0
0
1
2
3
4
5
Days after multiple autotomy
(b)
Control
Immersion in RA
Abundance of UpuRXR mRNA
(pg cRNA/ug total RNA)
0.8
0.6
0.4
0.2
0.0
*
*
*
0
1
2
3
4
5
Days after multiple autotomy

FIG. 8

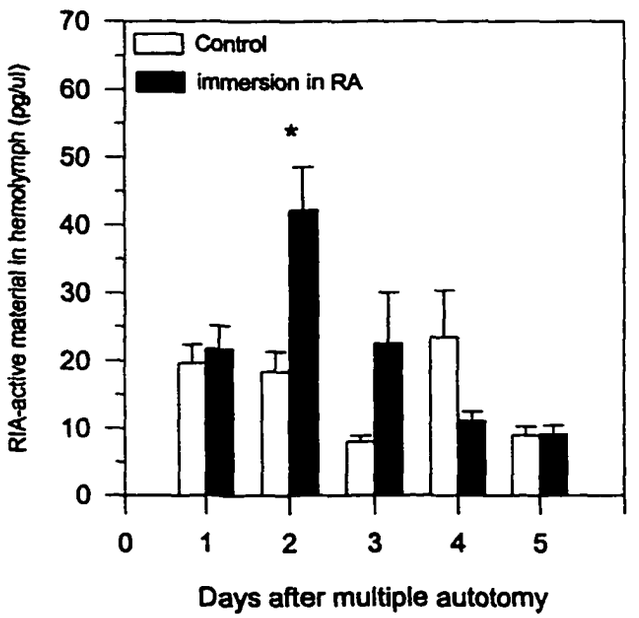

70
60
50
40
30
20
10
0
Control
immersion in RA
*
RIA-active material in hemolymph (pg/ul)
0
1
2
3
4
5
Days after multiple autotomy

FIG. 9

CHAPTER II

Tissue-Specific Patterns and Steady-State Concentrations of Ecdysteroid Receptor and Retinoid-X-Receptor mRNA during the Molt Cycle of the Fiddler Crab, *Uca pugilator*

Abstract

In the fiddler crab, *Uca pugilator*, we have investigated the temporal expression of receptors in various tissues using probes that encode *Uca* ecdysteroid receptor (*UpuEcR*) and retinoid-X-receptor (*UpuRXR*) gene homologs. During molt stages C_4 through $D_{1\text{-}4}$, *UpuEcR* and *UpuRXR* transcripts are expressed in regenerating limb buds, gills, eyestalks, hypodermis, hepatopancreas, muscle from nonregenerating walking legs, and the large cheliped. Some of these tissues have not previously been recognized as ecdysteroid-target tissues. Levels of ecdysteroids in the hemolymph fluctuate significantly during the molt cycle of *U. pugilator*. The variation in steady-state concentrations of *UpuEcR* transcripts in tissues from C_4 to $D_{1\text{-}4}$ implies molt cycle-related differences in the potential of these tissues to respond to changing titers of ecdysteroids in the hemolymph. In singly autotomized crabs, the highest concentrations of *UpuEcR* transcript in some tissues did not coincide with the highest levels of circulating ecdysteroids, suggesting that *UpuEcR* expression in these tissues is not dependent on high ecdysteroid titers and may be induced by low or rising concentrations of ecdysteroids. *UpuEcR* and *UpuRXR* genes were expressed simultaneously in tissues, supporting the possibility of heterodimerization for EcR and RXR *in vivo*. In some tissues, however, levels of transcripts differed, suggesting other possible receptor

interactions. Moreover, *UpuEcR* expression in tissues from multiply autotomized crabs differed from the expression patterns in tissues from singly autotomized crabs.

Introduction

Ecdysteroids are polyhydroxylated steroids that control growth and development in insects and crustaceans (Lachaise et al., 1993; Thummel, 1996). Although much is known about the synthesis and circulating levels of ecdysteroids in both insects and crustaceans (Lachaise et al., 1993; Lafont, 1997), relatively little is known in crustaceans about the physiological effects or target tissues under ecdysteroid control.

In insects, the ecdysteroids, like vertebrate steroid hormones, modulate gene transcription after binding to a nuclear receptor, the ecdysteroid receptor (EcR) (Evans, 1988; Thomas et al., 1993; Yao et al., 1993). The gene for EcR in insects has been cloned and shown to be a member of the steroid hormone/nuclear receptor superfamily, containing conserved DNA and ligand binding domains (Koelle et al., 1991; Imhof et al., 1993; Cho et al., 1995; Fujiwara et al., 1995; Swevers et al., 1995). Moreover, the *EcR* gene encodes three different forms of the receptor in *Drosophila melanogaster* (Dme) and two isoforms in the tobacco hornworm, *Manduca sexta* (Mse) (Talbot et al., 1993; Jindra et al., 1996). Each isoform has a different N-terminal region. The distribution of these receptor isoforms is significantly different in various ecdysteroid responsive tissues during different metamorphic stages in insects (Talbot et al., 1993; Truman et al., 1994; Jindra et al., 1996). *EcR* has been shown to be expressed in a crustacean (Durica and Hopkins, 1996) but neither the tissue distribution nor the existence of EcR isoforms in crustaceans have been described.

DmEcR forms a functional heterodimer with the *ultraspiracle* protein (USP) (Thomas et al., 1993; Yao et al., 1993). USP is the *Drosophila* homolog of the vertebrate retinoid-X-receptor (RXR) and, like RXR, is a member of the steroid hormone/nuclear

receptor superfamily (Oro et al., 1990). The *USP* gene has been cloned in several insects and isoforms have been detected (Oro et al., 1990; Tzertzinis et al., 1994; Kapitskaya et al., 1996; Jindra et al., 1997). Despite the fact that the ligand for the vertebrate RXR isoforms is *9-cis* retinoic acid, a ligand for the insect receptor has not been characterized (Yao et al., 1993; Chambon, 1996). *In crustaceans*, a RXR homolog has been identified (Durica and Hopkins, 1996) but nothing is known of its distribution or function.

Hormone binding and immunological cross-reactivity have been used to detect the presence of putative receptors in crustaceans. Characterization of a putative crustacean EcR protein in hypodermis and hepatopancreas was first reported in crayfish (Spindler-Barth et al., 1981; Kuppert and Spindler, 1982; Londershausen et al., 1982). Using a heterologous *Drosophila* anti-EcR antibody, a cross reacting protein was detected in tissues of *Uca pugilator* and the lobster, *Homarus americanus* (Chung et al., 1994; El Haj et al., 1994). *EcR* or *RXR* expression in crustacean tissues, however, has not been examined using a homologous probe. We have recently reported the isolation of RT-PCR clones representing the DNA binding domain (DBD) of *Uca EcR* (*UpuEcR*) and *RXR* (*UpuRXR*) homologs (Durica and Hopkins, 1996). These clones have subsequently been used to screen *Uca* cDNA libraries (Chung et al., 1998, in press). Among the members of the nuclear receptor family, the deduced amino acid sequence of the *UpuEcR* gene homolog is closest to that of insect EcRs. The DBD sequence of the *UpuRXR* gene homolog is closest to that of DmeUSP while the LBD is most similar to zebra fish RXRγ. This paper reports for the first time the use of homologous nucleic acid probes derived from these clones to monitor the expression of *UpuEcR* and *UpuRXR* in a crustacean, the fiddler crab, *U. pugilator*.

In order to grow, crustaceans must shed (or molt) the old exoskeleton and replace it with a new and larger one. The time between two successive molts is called the molt cycle. In crustaceans, the molt cycle is generally divided into five stages (Drach, 1939). At stage E ecdysis (or molting) occurs, which is the actual shedding of the old exoskeleton. Stages A and B immediately follow ecdysis and are called metecdysis. This is the time for expansion and hardening of the new exoskeleton, which has been synthesized underneath the old exoskeleton. Stage C is anecdysis which is the time of feeding, reproduction, and storage of organic reserves. In the fiddler crab, *U. pugilator*, this stage can last more than 2 months, depending on physiological conditions (Hopkins, 1982). Stage D is proecdysis, which is the time for preparation for shedding of the old exoskeleton and the synthesis of new exoskeleton.

In addition to shedding of the old exoskeleton (somatic growth), *U. pugilator* is able to regenerate limbs lost during the molt cycle (epimorphic growth) (reviewed in Hopkins, 1988a). When a limb of *U. pugilator* is injured, the animal can cast off the injured limb at a predetermined point proximal to the injury and regenerate a new limb in its place. The reflex behavior of casting off the injured limb is called autotomy (McVean, 1984). The loss of three or more limbs is called multiple autotomy (MA) and can result in the truncation of the molt cycle and disruption of the pattern of circulating ecdysteroids (Hopkins, 1982, 1992). Our laboratory has shown that the regeneration of lost limbs is, in part, dependent on ecdysteroids (Hopkins, 1989, 1993).

In *U. pugilator*, the levels and ratios of circulating ecdysteroids change in a molt cycle-related pattern (Hopkins, 1983, 1986, 1989, 1992). These fluctuations may be

responsible for regulating many physiological and biochemical processes related to the molting event, such as protein synthesis in regenerating limbs (Hopkins, 1989), proliferation of epidermal cells, secretion of layers of new cuticle, withdrawal and storage of calcium salt from the old cuticle and construction of new exoskeleton underneath the old one (reviewed in Chang, 1989). Which of these events are under direct ecdysteroid control in crustaceans is not known. In order to determine this, the first aim of the research reported here is to establish potential ecdysteroid targets by measuring *UpuEcR* mRNA expression in a variety of tissues. Some members of the vertebrate steroid hormone receptor superfamily, such as vitamin D and estrogen receptors, are expressed in response to rising titers of hormone (Carson-Jurica et al., 1990). The second goal of this research is to establish correlations between patterns of *UpuEcR* and *UpuRXR* expression and circulating ecdysteroids. As mentioned above, multiple autotomy will disrupt the molt cycle and change circulating ecdysteroid levels. The third question addressed by this research is whether the expression of *EcR* in tissues is changed following multiple autotomy.

This paper reports for the first time that several tissues in *U. pugilator* express *EcR* and *RXR* mRNAs. These tissues include regenerating limb buds, gills, eyestalks, hypodermis, hepatopancreas, muscle from nonregenerating legs, and the large cheliped. In addition, we also report the relationship between the expression of *UpuEcR* and *UpuRXR* genes and changes in titers of circulating ecdysteroids. Furthermore, we have shown that in some tissues from singly autotomized crabs the pattern of expression of *UpuEcR* is different from that in multiply autotomized crabs. We suggest that ecdysteroids and their receptors have significant role(s) in many tissues of the crab and that the effects of the ecdysteroids

and EcR(s) may be dependent on the presence of RXR.

Materials and Methods

Animals

Male *U. pugilator* were purchased from Gulf Specimen Company (Florida). After laboratory acclimation, crabs had either the third right walking leg autotomized (single autotomy, SA) or seven limbs autotomized (multiple autotomy, MA). After the limb buds emerged, the length of the right third limb bud (R_3) was measured, divided by the width of the carapace, and expressed as an R value (Bliss, 1956). Blood was collected at the time tissues were collected for RNA isolation. The blood was subjected to radioimmunoassay to detect the amount of total circulating ecdysteroids (Hopkins, 1983). The Drach's molt stage of all crabs was determined by a composite of R-value, growth rate of the limb buds (Experimental Rate, ER) (see Bliss and Hopkins, 1974), and circulating levels of ecdysteroids (Table 1). Following MA, there was a lag period of little or no external growth of the limb buds. This lag period was assigned to Drach's stage C_4.

RNA Extraction

Total RNA was isolated from crab tissues at different molt stages following autotomy (Chomczynski and Sacchi, 1987). Poly(A)$^+$ RNA was enriched by oligo-dT cellulose column chromatography (Sambrook et al., 1989).

Plasmid Constructs and Probe Synthesis

DNA probes. Clones containing the *UpuEcR* (GenBank U31817) and *UpuRXR* (GenBank

U31832) DNA binding domains (DBD) were generated by RT-PCR, as previously described (Durica and Hopkins, 1996). Materials for probe synthesis were first generated by PCR with appropriate primers, and then radiolabeled by random priming (Megaprime DNA labeling system, Amersham, Arlington, IL) with [α-^{32}P]dCTP (3000 Ci/mmol, Du Pont-NEN, Boston, MA).

RNA probes. Two subclones were constructed from *UpuEcR* (GenBank AF034086) and *UpuRXR* (GenBank AB032983) cDNA clones isolated from a oligo-dT primed library representing D_0 regenerating limb bud mRNA (Chung et al., 1998, in press). The *EcR 162* plasmid was constructed by the ligation of a 162-bp *Acc*I-*Eco*RI restriction fragment, encoding a portion of the UpuEcR ligand binding domain (LBD), into the corresponding sites of the plasmid pBluescript II KS multiple cloning site (Stratagene, La Jolla, CA). The *RXR 307* plasmid is a derivative of a 2.8-kb *UpuRXR* cDNA introduced into a pBluescript II SK phagemid (Chung et al., 1998, in press). A 2.5-kb *Sty*I-*Xho*I restriction fragment, containing sequences 3' to the *UpuRXR* DBD, was removed. The plasmid containing the DBD was purified, end-filled by T4 DNA polymerase, and religated. The resulting subclone contained a 307-bp insert, comprising the most highly conserved region of the *UpuRXR* DBD.

The *Uca 1* plasmid (Durica and Hopkins, 1996) contains an insert which encodes 189-bp of the *UpuEcR* DBD. The *Uca 29* plasmid contains an insert which encodes 195-nt of 18S ribosomal RNA (rRNA) (Durica, unpublished). The insert was generated by RT-PCR and introduced into the *Sma*I site of pBluescript II KS (Stratagene). Prior to *in vitro* transcription, *Uca 29* was cut by *Stu*I to generate a 59-bp antisense RNA probe.

Antisense radiolabeled RNA probes were produced by *in vitro* transcription (Promega, Madison, WI) with [α-^{32}P]UTP (800 Ci/mmol, Amersham, Arlington, IL). The antisense probe was transcribed by either T3 or T7 RNA polymerase and purified on Select RT sepharose spin columns (5 Prime- 3 Prime, Boulder, CO).

Northern Blot Analysis

We used Northern blot analysis to examine the presence and size of *UpuEcR* and *UpuRXR* transcripts in poly(A)$^+$ RNA from regenerating limb buds, muscle from nonregenerating legs, and gills. Poly(A)$^+$ mRNA was glyoxylated, separated on a 0.8% agarose gel, and transferred to nitrocellulose filters (Sambrook et al., 1989). Hybridization was then performed using ^{32}P-labeled DNA probes representing the *UpuEcR* and *UpuRXR* DBDs, and a *Drosophila* RP49 probe (nt 447-764) which covers a highly conserved region of the ribosomal protein coding sequence (O'Connell and Rosbash, 1984). For the hybridization with *UpuEcR* and *UpuRXR* DNA probes, the filters were hybridized at 42°C for 48 h in hybridization solution (50% formamide, 5× SSC, 0.02% Tris·HCl (pH 7.6), 1× Denhardt's solution, 10% dextran sulfate, 0.1% SDS). The filters were washed twice with 2× SSC, 0.1% SDS for 15 min at 42°C, then once for 15 min in 0.1× SSC, 0.1% SDS at 42°C and once more at 50°C. For the hybridization with the RP49 DNA probe, the filters were hybridized with the probe at 37°C for 48 h in hybridization solution (50% formamide, 5× SSC, 0.02% Tris·HCl (pH 7.6), 1× Denhardt's solution, 10% dextran sulfate, 0.1% SDS). The filters were washed twice with 2× SSC, 0.1% SDS for 15 min at 37°C, and then twice for 15 min at 42°C. Finally, the filters were exposed to X-ray film.

Ribonuclease Protection Assay

To improve the quantification of *UpuEcR* and *UpuRXR* transcripts from a variety of molt stages using smaller amounts of tissue, a ribonuclease protection assay was developed. With this assay, we were able to simultaneously detect both transcripts in smaller quantities of total RNA, and minimize the variability associated with larger pooled samples. Several crab tissues were examined in order to determine potential ecdysteroid target tissues. These tissues were limb buds, gills, hypodermis, eyestalks, hepatopancreas, muscle from nonregenerating limbs and the large cheliped. Total RNA (3 to 50 μg) was extracted from pooled tissue of five crabs. Samples were resuspended in hybridization buffer (80% formamide, 0.4 M NaCl, 40 mM Pipes (pH 6.0), 1 mM EDTA). For singly autotomized crabs, antisense [^{32}P]UTP-labeled RNA probes were synthesized from *EcR* 162 and *RXR 307* plasmids, and the *Uca 29* probe was used as a control for quantifying total RNA concentrations. For multiply autotomized crabs, *Uca 1* plasmid was used for RNA probe synthesis. Hybridizations of RNA samples with excess RNA probes were performed at 45°C for 16 to 18 h, and unhybridized templates were digested by RNases A/T1 (Ambion, Austin, TX) digestion at 37°C for 30 min. RNases were removed by protease K digestion and organic extraction, and the protected fragments were separated by 6% PAGE under denaturing conditions. The amount of radioactivity in the protected fragments was determined on a Packard Instantimager® electronic autoradiography system (Packard, Meriden, CT). Background subtraction and data quantification were performed by the associated image analysis software package.

The transcript levels of each receptor were determined by ribonuclease protection

assay with *UpuEcR* and *UpuRXR* antisense RNA probes. In this assay, 307-nt fragments of *UpuRXR* mRNA and 162-nt fragments of *UpuEcR* mRNA were protected. The quantification of these protected fragments was performed by comparing to standards of protected sense-strand complementary RNA (cRNA) for each receptor, synthesized from the plasmid vectors using T7 RNA polymerase. The absolute amounts of each receptor mRNA were quantified relative to the fraction of radioactivity bound to a known amount of the standard cRNA and expressed as picograms cRNA. A linear relationship between input cRNA standards and recovered counts was observed from 0.5 to 128 picograms cRNA. The amount of total RNA loaded in each assay was normalized by a second ribonuclease protection assay. Portions of pooled RNA samples (10^{-4} dilutions) were assayed with the *Uca* 29 probe, which protects a 59-nt fragment of a *Uca* 18S rRNA. The amount of total RNA loaded for each pooled tissue sample was quantified by comparing the radioactivity of the 59-nt protected fragment with the radioactivity of a protected standard. This standard was a single RNA isolate from D_{1-4} limb buds, quantified by ultraviolet spectrophotometry. The concentration of each transcript was then calculated from the absolute amount of protected receptor mRNA divided by the amount of the total RNA, and expressed as picograms cRNA per microgram total RNA. Error bars on data represent standard errors which were calculated from the results of independent pooled samples. For each tissue, there were at least three pooled samples for each time point.

Statistical Analysis

UpuEcR and *UpuRXR* mRNA levels in different tissues were compared by Student's t-test (Microsoft Excel, Cambridge, MA); $P \leq 0.05$ was considered to be significant.

Results:

Detection of *UpuEcR* and *UpuRXR* Transcripts by Northern Blot Analysis

Using a DNA probe which encoded the *UpuEcR* DBD, a single *UpuEcR* transcript band of 7 kb was observed in regenerating limb buds on Northern blot analysis (Fig. 1). The same transcript size was observed in gills (Fig. 1) and muscle from nonregenerating legs (data not shown). Owing to variation in background and saturation of the film, levels of *UpuEcR* transcript in different molt stages were difficult to quantitate. Comparison of band density relative to the RP49 control suggested transcript levels consistent with the ribonuclease protection assay (see below).

When the blots were probed with a DNA fragment which encoded the *UpuRXR* DBD, a doublet of bands migrating at approximately 5 kb was observed (Fig. 1). These two bands appeared in RNA samples from regenerating limb buds, gill tissues (Fig. 1), and muscle from nonregenerating limbs (data not shown). Relative to tissues or molt cycle stages, for both *UpuEcR* and *UpuRXR*, no differences in transcript size were observed in Northern blots.

Detection of *UpuEcR* Transcripts by Ribonuclease Protection Assay in Singly Autotomized Crab Tissues

To facilitate the quantification of transcripts from a variety of molt stages, using smaller amounts of tissue, a ribonuclease protection assay was developed (Materials and Methods; Fig. 2). The ribonuclease protection assays showed that the steady-state concentrations of *UpuEcR* transcript varied between tissues at any given time during C_4 through $D_{1\text{-}4}$. Steady-state concentrations were highest in regenerating limb buds during D_0 and in muscle from the large cheliped during $D_{1\text{-}4}$. Lowest levels were found in all tissues

during C_4 and remained low in muscle from nonregenerating limbs throughout the molt cycle (Fig. 3).

In regenerating limb bud (Fig. 3b), the concentration of *UpuEcR* increased from stage C_4/D_0 transition to stage D_0 ($P<0.05$). The increase in concentration of *UpuEcR* mRNA during D_0 occurred before the levels of circulating ecdysteroids rise (Table 1 and Fig. 3a). In the gills (Fig. 3c), the levels of *UpuEcR* transcript remained low during stage C_4. The transcript levels slightly increased during stage D_0 and remained at this level during D_{1-4} ($P<0.1$). In the eyestalks (Fig. 3d), the *UpuEcR* transcript levels remained low during stage C_4 and early D_0. At late D_0, transcript levels slightly increased ($P<0.1$) and did not change significantly during D_{1-4}. In the hypodermis that secretes the dorsal carapace (Fig. 3e), the concentrations of *UpuEcR* transcript did not vary much at the early C_4 stages but increased significantly at late D_0 and D_{1-4} ($P<0.01$). In *U. pugilator*, there is a second type of hypodermis which separates the brachial chamber from the body cavity. This hypodermis lies next to the gills and the cuticle of this hypodermis remains soft and flexible throughout the molt cycle. There were no detectable *UpuEcR* transcripts in this soft hypodermis (data not shown). In the hepatopancreas (Fig. 3f), the *UpuEcR* transcript levels were low during the early C_4 stages and increased significantly at early D_0 ($P<0.001$). At late D_0, the transcript levels decreased significantly ($P<0.001$), and stayed at low levels during stage D_{1-4}. In the muscle from nonregenerating legs (Fig. 3g), the *UpuEcR* transcript levels did not vary significantly during C_4 or D ($P<0.5$). In muscle from the large cheliped (Fig. 3h), the concentrations of *UpuEcR* transcript did not vary significantly during C_4 and D_0 ($P<0.2$) but did increase significantly at D_{1-4} ($P<0.05$).

Detection of *UpuRXR* Transcripts by Ribonuclease Protection Assay in Singly Autotomized Crab Tissues

UpuRXR was found in every tissue in which *UpuEcR* was found. Similar to *UpuEcR*, the steady-state concentrations of *UpuRXR* transcript varied among tissues from C_4 through $D_{1\text{-}4}$ (Fig. 4). Concentrations were highest in the hepatopancreas during D_0 and cheliped muscle during $D_{1\text{-}4}$. Lowest levels were found in most tissues during C_4, and remained low in eyestalks and muscle from nonregenerating limbs throughout the molt cycle (Fig. 4) The amounts of these two receptor mRNAs varied in different tissues and during different molt cycle stages (Table 2). The average amount of *UpuRXR* mRNA in hepatopancreas at any given stage was more than twice that of *UpuEcR* mRNA. Gills and muscle from nonregenerating limbs had similar levels of both receptor mRNAs at any given stage. In the rest of the tissues examined, the amounts of *UpuRXR* mRNA were variable across the molt cycle.

In regenerating limb buds (Fig. 4b), the concentrations of *UpuRXR* transcript increased markedly from C_4 to C_4/D_0 transition ($P<0.01$), and remained elevated in the limb buds in subsequent stages. In the gills (Fig. 4c), the *UpuRXR* transcript levels remained low following autotomy and increased significantly at D_0 and $D_{1\text{-}4}$ ($P<0.02$). In the eyestalks (Fig. 4d), there was a slight, but not statistically significant, increase in *UpuRXR* transcript levels at late D_0 when compared with the transcript levels during C_4($P<0.1$). In the hypodermis that secretes the dorsal carapace (Fig. 4e), the concentrations of *UpuRXR* transcript were low during stage C_4 and early D_0, increased significantly at late D_0 ($P<0.05$), and stage $D_{1\text{-}4}$. As with *UpuEcR*, there were no detectable *UpuRXR* transcripts in soft hypodermis (data not

shown). In hepatopancreas (Fig. 4f), high *UpuRXR* transcript levels were observed during stage C_4, and increased significantly during D_0 ($P<0.05$), fell slightly at late D_0, and remained at comparable levels during D_{1-4}. In muscle from non- regenerating legs (Fig. 4g), *UpuRXR* transcript levels did not vary significantly during C_4-D_{1-4} ($P<0.5$). In muscle from the large cheliped (Fig. 4h), the concentrations of *UpuRXR* transcript did not vary from C_4 through D_0. Like *UpuEcR* in large cheliped (Fig. 3h), however, levels increased significantly at D_{1-4} ($P<0.01$) (Fig. 4h).

Detection of *UpuEcR* Transcripts by Ribonuclease Protection Assay in Multiply Autotomized Crab Tissues

Ribonuclease protection assays showed that the steady-state concentrations of *UpuEcR* transcript from MA crabs varied between tissues at any given time from C_4 through D_{1-4} (Fig. 5) but showed less intra-tissue variation between stages than seen in SA crab tissues (Fig. 4). Relative abundance was highest in regenerating limb buds and hepatopancreas during D_0. Intermediate levels were found in gills and eyestalks, and lowest levels in muscle from nonregenerating limbs and hypodermis (Fig. 5). The absence of significant variations of *UpuEcR* transcript within tissues from MA crabs during C_4 to D_0 is in striking contrast to the pattern of increases and decreases seen in the SA crab tissues. The levels of *UpuEcR* transcript in the hepatopancreas, however, did increase significantly at late D_0 when compared to C_4 ($P<0.01$). This increase was later than the increase seen in the SA crab tissues, which increased at mid D_0 and decreased at late D_0 (Figs. 3e and 5e). Northern blot data (Fig. 1) showed some indication of a *UpuEcR* transcript increase in D_0 regenerating limb buds of MA crab tissues. No transcript increase at this stage, however, was detected in

ribonuclease protection assays ($P<0.2$) (Fig. 5b).

Discussion

The ecdysteroid receptor and retinoid-X-receptor gene homologs are expressed in regenerating limb buds, gills, eyestalks, hypodermis, hepatopancreas, muscle from nonregenerating walking legs and the large cheliped of the fiddler crab, *U. pugilator* during molt stages C_4 through D_4 . The variation in steady-state concentrations of *UpuEcR* transcripts in these tissues implies molt cycle-related differences in the potential of these tissues to respond to changing titers of ecdysteroids in the blood. Moreover, multiple autotomy, which disrupts the molt cycle, also disrupts the pattern of *UpuEcR* expression.

In limb buds, highest concentrations of *UpuEcR* transcript occur at the same time as an ecdysone-sensitive period of protein synthesis (Hopkins, 1989). These occur prior to late proecdysial peaks of circulating ecdysteroids. Likewise in wing discs of the tobacco hornworm, *M. sexta*, (Fujiwara et al., 1995), and the silkworm, *Bombyx mori* (Kamimura et al., 1996), increases in *EcR* expression occur prior to peaks of ecdysteroid in the blood. *DmEcR* is induced in *Drosophila* mid-third instar larvae by low and rising titers of circulating ecdysone (Karim and Thummel, 1992). In *M. sexta*, one of the *EcR* isoforms is induced by small amounts of ecdysone and 20-OH ecdysone (Hiruma et al., 1997). Our results suggest that low levels of ecdysteroids may induce *UpuEcR* expression in regenerating limb bud.

In eyestalks of SA crabs, we observed high concentrations of *UpuEcR* mRNA during proecdysis. The X-organ-sinus gland of the lobster eyestalk shows immunoactivity to a

Drosophila anti-EcR antibody (El Haj et al., 1994). These results indicate that ecdysteroids may act in feedback control of the release of molt inhibiting hormone (MIH) from the X-organ-sinus gland of the eyestalks. Secretion of ecdysteroids is inhibited by MIH (Mattson and Spaziani, 1985; Naya et al., 1988). RIA active ecdysteroids accumulate in the eyestalks of *U. pugilator* during proecdysis (Hopkins, 1988b). The highest levels of circulating ecdysteroids are seen during stage D_{1-4}. If ecdysteroids (via EcR) stimulate MIH release then we would expect ecdysteroid levels to fall during D_{1-4}. Indeed, there is a precipitous drop in circulating ecdysteroids at the very end of D_{1-4}. The presence of *UpuEcR* transcripts in eyestalks at this time supports the hypothesis of a feedback loop.

The increase of *UpuEcR* mRNA in the hypodermis may be associated with the physiological changes that occur in this tissue during late proecdysis. At the beginning of stage D_{1-4}, apolysis occurs in the crustacean hypodermis (Jenkin, 1966). The separated hypodermis secretes a new cuticle underneath the old one. In *U. pugilator*, both apolysis and cuticle secretion occur at the time of a dramatic rise in circulating ecdysteroids. Injection of 20-OH ecdysone can accelerate apolysis in some crustaceans, and *in vitro* incubation of crayfish hypodermis in 20-OH ecdysone increases protein synthesis (Krishnakumaran and Schneiderman, 1968, 1969; Gilgan and Burns, 1976; Gilgan and Farquharson, 1977; Traub et al., 1987). The accumulation of organic reserves (Chandumpai et al., 1991), the increase of oxygen uptake (Mangum, 1992), and high specific activities of Na^++K^+-ATPase and chitinolytic enzymes (Spindler-Barth et al., 1990; Mangum, 1992) are structural and functional changes that occur in crustacean hypodermis during late proecdysis (D_{1-4}). The increase of *UpuEcR* transcript levels at late proecdysis in *Uca* hypodermis supports the

hypothesis that these physiological events in the hypodermis may be subjected to ecdysteroid regulation.

The increase of *UpuEcR* transcripts in the gills and hepatopancreas of SA *U. pugilator* during proecdysis may be related to proecdysial physiological changes in these tissues (O'Connor and Gilbert, 1969; Yamaoka and Scheer, 1970; Kanazawa et al., 1976; Towle and Mangum, 1985; Lautier and Lagarrigue, 1987; Chandumpai et al., 1991; Mangum, 1992; Andrews and Dillaman, 1993). The proecdysial increase of *UpuEcR* transcript levels in the gills and hepatopancreas suggests that ecdysteroids play a role in molt-related events in these tissues.

In the muscle from the large cheliped of SA crabs, the increase of *UpuEcR* during late proecdysis may be relevant to atrophy of cheliped muscle prior to molt. At molt, the male *U. pugilator* must pull the extremely large cheliped muscle through a narrow opening at the basiishial joint of the cheliped. Molt-induced atrophy in the large cheliped of the fiddler crab *Uca pugnax* occurs when the titers of circulating ecdysteroids increase significantly during proecdysis (Ismail and Mykles, 1992). Earlier research suggests that ecdysteroids are involved in the muscle atrophy that accompanies metamorphosis in some insects (Schwarz and Truman, 1984; Kimura and Truman, 1990). Ismail and Mykles (1992) suggest that in *U. pugnax*, the protein catabolism in cheliped muscle during proecdysis may be initiated by 20-OH ecdysone. Our results show that during D_{1-4}, the concentrations of *UpuEcR* transcript increase significantly in muscle from the large cheliped. This supports the hypothesis that ecdysteroids are involved in the protein catabolism observed in the cheliped muscle during late proecdysis.

In the muscle from nonregenerating legs of SA crabs, *UpuEcR* transcript levels are low and remain constant. Our results using homologous nucleic acid probes show that the *UpuEcR* transcript levels in *U. pugilator* do not vary significantly in the muscle from nonregenerating legs during C_4 through $D_{1\text{-}4}$ (Figs. 3g and 5g).

In all tissues examined from MA crabs, levels of *UpuEcR* transcripts vary less than in SA crabs. This may be related to the disrupted proecdysis that follows MA. In *U. pugilator*, MA will shorten the molt cycle, alter the pattern of circulating ecdysteroids, and affect the growth of the carapace (Hopkins, 1982, 1992). Lower levels of *UpuEcR* transcript in hypodermis from MA crabs at $D_{1\text{-}4}$ may account for the smaller growth of carapace after MA-induced ecdysis (Hopkins, 1982). The higher levels of *UpuEcR* mRNA seen in stage C_4 limb buds may also account for the quicker onset of proecdysial growth of limb buds that follows MA. In most tissues, however, the levels of *UpuEcR* transcript from SA crab tissues were not significantly different from *UpuEcR* levels in MA crab tissues. Likewise, total amounts of circulating ecdysteroids do not differ significantly between these two treatments. It is the patterns that vary. The significance of these variations in patterns is not clear at this time.

The *Drosophila* RXR homolog, USP, binds to DmEcR to form a heterodimer before binding to DNA. USP enhances the binding of EcR to DNA (Yao et al., 1993). USP is, therefore, important for modulation of gene expression. In *U. pugilator*, *UpuRXR* is expressed in every tissue where we detected *UpuEcR* expression. Similar expression patterns of *UpuRXR* and *UpuEcR* in most crab tissues may indicate that dimerization of UpuRXR and UpuEcR occurs.

During the larval development of *D. melanogaster*, the expression pattern of *USP* transcript does not correlate with changes of ecdysteroid titers (Andres et al., 1993; Henrich et al., 1994) suggesting that *DmeUSP* may be expressed constitutively and not in response to ecdysteroids. In *M. sexta*, a small transient increase of *MseUSP* mRNA appears when a small peak of circulating ecdysteroids occurs on the 3rd day of fifth larval development (Jindra et al., 1997). Exposure to 20-OH ecdysone induces the expression of total *MseUSP* mRNA in the 2nd day of fifth instar larval epidermis (Jindra et al., 1997). MseUSP has two isoforms, MseUSP-1 and MseUSP-2. During both larval and pupal molts when the titers of circulating ecdysteroids are high, *MseUSP-1* mRNA disappears as *MseUSP-2* mRNA increases. In *U. pugilator*, the transcript levels of *UpuRXR* were low during stage C_4 in most tissues examined except hepatopancreas (Fig. 4). After the small transient peak of circulating ecdysteroids at C_4/D_0 transition, the concentrations of *UpuRXR* increase in regenerating limb buds, gills, eyestalks, hypodermis and hepatopancreas (Figs. 4b-f).

The isoforms of insect EcRs and USPs differ from each other in the N-terminal transactivation domain (Talbot et al., 1993; Jindra et al., 1996; Kapitskaya et al., 1996; Jindra et al., 1997). Since we used probes to the common region of the receptor genes, these results represent changes of total *UpuEcR* and *UpuRXR* transcripts during the molt cycle. If isoforms of these two receptors exist in *U. pugilator*, the expression pattern of individual isoforms may be obscured by the determination of total transcript levels (Jindra et al., 1996).

The ratios of *UpuEcR* and *UpuRXR* mRNAs are different in different crab tissues. In vertebrates, RXR can be the partner of other nuclear receptors, such as the vitamin D_3 receptor, peroxisome proliferator-activated receptor, and thyroid hormone receptor, or it can

form a homodimer in some cases (Chambon, 1996). Additionally, there is evidence which indicates other pairing partners for *Drosophila* USP (Sutherland et al., 1995; Zelhof et al., 1995). The presence of higher amounts of *UpuRXR* mRNA in the hepatopancreas could mean that in this tissue UpuRXR may have binding partner(s), other than UpuEcR. UpuRXR may participate in a variety of signaling pathways in the hepatopancreas. In some tissues, the levels of *UpuEcR* detected were higher than *UpuRXR* levels. This may be a result of EcR isomers in the tissues that dimerize with partners other than RXR (Zelhof et al., 1995). The patterns of *UpuEcR* and *UpuRXR* expression in different tissues, however, reinforce the possibility that UpuEcR and UpuRXR function together *in vivo*.

Literature Cited

Andres, A.J., Fletcher, J.C., Karim, F.D., and Thummel, C.S. (1993). Molecular analysis of the initiation of insect metamorphosis: A comparative study of *Drosophila* ecdysteroid-regulated transcription. *Dev. Biol.* **160,** 388-404.

Andrews, S.C., and Dillaman, R.M. (1993). Ultrastructure of the gill epithelia in the crayfish *Procambarus clarkii* at different stages of the molt cycle. *J. Crust. Biol.* **13,** 77-86.

Bliss, D.E. (1956). Neurosecretion and the control of growth in a decapod crustacean. *In* "Bertil Hanstrom. Zoological Papers in Honour of His Sixty-Fifth Birthday" (K. G. Wingstrand, Ed.), pp. 56-75. Zoological Institute, Lund.

Bliss, D.E. and Hopkins, P.M. (1974). Bioassay and characterization of crustacean limb growth-controlling factors. *In* "Neurosecretion - The final Neuroendocrine Pathway, VI International Symposium Neurosecretion, London, 1973" pp. 104-114, Springer-Verlag, New York.

Carson-Jurica, M.A., Schrader, W.T., and O'Malley, B.W. (1990). Steroid receptor family: Structure and functions. *Endo. Rev.* **11,** 201-219.

Chambon, P. (1996). A decade of molecular biology of retinoic acid receptors. *FASEB J.* **10,** 940-954.

Chandumpai, A., Dall, W., and Smith, D. M. (1991). Lipid-class composition of organs and tissues of the tiger prawn *Penaeus esculentus* during the moulting cycle and during starvation. *Mar. Biol.* **108,** 235-345.

Chang, E.S. (1989). Endocrine regulation of molting in Crustacea. *Rev. Aquat. Sci.* **1,** 131-157.

Cho, W.-L., Kapitskaya, M.Z., and Rackhel, A.S. (1995). Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. *Insect Biochem. Mol. Biol.* **25,** 19-27.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156-159.

Chung, A. C.-K., Durica, D.S., and Hopkins, P.M. (1994). The ecdysone receptor (EcR) during proecdysis in the fiddler crab, *Uca pugilator*. *Am. Zool.* **34,** 24A.

Chung, A. C.-K., Clifton, S. W., Roe, B. A., Durica, D. S. and Hopkins, P. M. (1998). Cloning of crustacean EcR and RXR gene homologs and elevation of RXR mRNA by

retinoic acid. *Mol. Cell. Endocrinol.* In press

Drach, P. (1939). Mue et cycle d'intermue chez les Crustacés Décapodes. *Ann. Inst. Oceanogr. Monaco* **19,** 103-391.

Durica, D.S. and Hopkins, P.M. (1996). Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator. Gene* **171,** 237-241.

El Haj, A.J., Harrison, P., and Chang, E.S. (1994). Localization of ecdysteroid receptor immunoreactivity in eyestalk and muscle tissue of the American lobster, *Homarus americanus. J. Exp. Zool.* **270,** 343-349.

Evans, R.M. (1988). The steroid and thyroid hormone receptor superfamily. *Science* **240,** 889-895.

Fujiwara, H., Jindra, M., Newitt, R., Palli, S.R., Hiruma, K., and Riddiford, L.M. (1995). Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. *Insect Biochem. Mol. Biol.* **25,** 845-856.

Gilgan, M.W., and Burns, B.G. (1976). The successful induction of moulting in the adult male lobster (*Homarus americanus*) with a slow-release form of ecdysterone. *Steroids* **27,** 571-580.

Gilgan, M.W., and Farquharson, T.E. (1977). A change in the sensitivity of adult male lobster (*Homarus americanus*) to ecdysterone on changing from intermolt to active premolt development. *Comp. Biochem. Physiol.* **58A,** 29-32.

Henrich, V.C., Szekely, A.A., Kim, S.J., Brown, N.E., Antoniewski, C., Hayden, M.A., Lepesant, J.-A., and Gilbert, L.I. (1994). Expression and function of the *ultraspiracle* (*usp*) gene during development of *Drosophila melanogaster. Dev. Biol.* **165,** 38-52.

Hiruma, K, Böcking, D., Lafont, R., and Riddiford, L.M. (1997). Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta. Gen. Comp. Endocrinol.* **107,** 84-97.

Hopkins, P.M. (1982). Growth and regeneration patterns in the fiddler crab, *Uca pugilator. Biol. Bull.* **163,** 301-319.

Hopkins, P.M. (1983). Patterns of serum ecdysteroids during induced and uninduced proecdysis in fiddler crab, *Uca pugilator. Gen. Comp. Endocrinol.* **63,** 362-373.

Hopkins, P.M. (1986) Ecdysteroid titers and Y-organ activity during late anecdysis and proecdysis in the fiddler crab, *Uca pugilator. Gen. Comp. Endocrinol.* **63,** 362-373.

Hopkins, P.M. (1988a) Control of regeneration in crustaceans. *In* "Endocrinology of Selected Invertebrate Types" (R. Downer and H. Laufer, Eds.) Vol. 2, pp.327-340, A.R. Liss, New York.

Hopkins, P.M. (1988b). Localization of ecdysteroids in the eyestalk ganglia of the fiddler crab, *Uca pugilator. J. Exp. Zool.* **248,** 160-167.

Hopkins, P.M. (1989). Ecdysteroids and regeneration in the fiddler crab, *Uca pugilator. J. Exp. Zool.* **252,** 293-299.

Hopkins, P.M. (1992). Hormonal control of the molt cycle in the fiddler crab, *Uca pugilator. Am. Zool.* **32,** 450-458.

Hopkins, P.M. (1993). Regeneration of walking legs in the fiddler crab, *Uca pugilator. Am. Zool.* **33,** 348-356.

Imhof, M.O., Rusconi, S., and Lezzi, M. (1993). Cloning of a *Chironomis tentans* cDNA encoding a protein (cEcRH) homologous to the *Drosophila melanogaster* ecdysteroid receptor (dEcR). *Insect Biochem. Mol. Biol.* **23,** 115-124.

Ismail, S. Z. M., and Mykles, D. (1992). Differential molt-induced atrophy in the dimorphic claws of male fiddler crabs, *Uca pugnax. J. Exp. Zool.* **263,** 18-31.

Jenkin, P.M. (1966). Apolysis and hormones in the moulting of Arthropoda. *Ann. Endocrinol.* **27**(Suppl. 3), 331-341.

Jindra, M., Malone, F., Hiruma, K., and Riddiford, L.M. (1996). Developmental profiles and ecdysteroid regulation of the mRNAs for two ecdysone receptor isoforms in the epidermis and wings of the tobacco hornworm, *Manduca sexta. Dev. Biol.* **180,** 258-272.

Jindra, M., Huang, J.-Y., Malone, F., Asahina, M., and Riddiford, L.M. (1997). Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta. Insect Mol. Biol.* **6,** 41-53.

Kamimura, M., Tomita, S., and Fujiwara, H. (1996). Molecular Cloning of an ecdysone receptor (B1 isoform) homologue from the silkworm, *Bombyx mori*, and its mRNA expression during wing disc development. *Comp. Biochem. Physiol.* **113B,** 341-347.

Kanazawa, A., Teshima, S., and Sakamoto, Y. (1976). The variation of lipids and cholesterol contents in the tissues of prawn, *Penaeus japonicus*, during the moulting cycle. *Bull. Jap.*

Soc. Sci. Fisher. **42,** 1003-1007.

Kapitskaya, M., Wang, S., Cress, D.E., Dhadialla, T.S., and Raikel, A.M. (1996). The mosquito *ultraspiracle* homologue, a partner of ecdysteroid receptor heterodimer: Cloning and characterization of isoforms expressed during vitellogenesis. *Mol. Cell. Endocrinol.* **121,** 119-132.

Karim, F.D., and Thummel, C.S. (1992). Temporal coordination of regulatory gene expression by the steroid hormone ecdysone. *EMBO J.* **11,** 4083-4093.

Kimura, K., and Truman, J.W. (1990). Postmetamorphic cell death in the nervous and muscular systems of *Drosophila melanogaster. J. Neurosci.* **10,** 403-411.

Koelle, M.R., Talbot, W.S., Segraves, W.A., Bender, M.T., Cherbas, P., and Hogness, D. S. (1991). The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. *Cell* **67,** 59-77.

Krishnakumaran, A., and Schneiderman, H.A. (1968). Chemical control of molting in arthropods. *Nature* **220,** 955-956.

Krishnakumaran, A., and Schneiderman, H.A. (1969). Control of molting in Crustacea by an insect molting hormone. *Gen. Comp. Endocrinol.* **12,** 515-518.

Kuppert, P., and Spindler, K.-D. (1982). Characterization of nuclear ecdysteroid receptor from crayfish integument. *J. Steroid Biochem.* **17,** 205-210.

Lachaise, F., Le Roux, A., Hubert, M., and Lafont, R. (1993). The molting gland of crutaceans: Localization, activity, and endocrine control. *J. Crust. Biol.* **13,** 198-234.

Lafont, R. (1997). Ecdysteroids and related molecules in animals and plants. *Arch. Insect Biochem. Physiol.* **35,** 3-20.

Lautier, J., and Lagarrigue, J.-G. (1987). Lipid metabolism of the female crab *Pachygrapsus marmoratus* during the moulting cycle. *Biochem. Syst. Ecol.* **15,** 611-619.

Londershausen, M., Kuppert, P., and Spindler, K.-D. (1982). Ecdysteroid receptors: A comparison of cytoplasmic and nuclear receptor from crayfish hypodermis. *Hoppe-Seyler's Z. Physiol. Chem.* **363,** 797-802.

Mangum, C. (1992). Physiological aspects of molting in the blue crab, *Callinectes sapidus. Am. Zool.* **32,** 459-469.

Mattson, M.P., and Spaziani, E. (1985). Characterization of molt-inhibiting hormone (MIH)

action on crustacean Y-organ segments and dispersed cells in culture and a bioassay for MIH activity. *J. Exp. Zool.* **236,** 93-101.

McVean, A. (1984). Autotomy. *In* "The Biology of Crustacea" (Bliss, D. E., Ed.), pp. 107-132. Academic Press, New York.

Naya, Y., Kishida, K., Sugiyama, M., Murata, M., Miki, W., Ohnishi, M., and Nakanishi, K. (1988). Endogenous inhibitor of ecdysone synthesis in crabs. *Experienta* **44,** 50-52.

O'Connell, P.O., and Rosbash, M. (1984). Sequence, structure, and codon perference of the *Drosophila* ribosomal protein 49 gene. *Nucleic Acids Res.* **12,** 5495-5513.

O'Connor, J.D., and Gilbert, L.I. (1969). Alteration in lipid metabolism associated with premolt activity in a crab and freshwater crayfish. *Comp. Biochem. Physiol.* **29,** 889-904.

Oro, A.E., McKeown, M., and Evans, R.M. (1990). Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. *Nature* **347,** 298-301.

Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989). "Molecular Cloning: A Laboratory Manual," 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Schwartz, L.M., and Truman, J.W. (1984). Hormonal control of muscle atrophy and degeneration in the moth *Antheraea polyphemus*. *J. Exp. Biol.* **111,** 13-30.

Spindler-Barth, M., Kuppert, P., and Spindler, K.-D. (1981). Isolation of nuclei from crayfish tissues and demonstration of nuclear ecdysteroid receptors. *Z. Naturforsch.* **36,** 326-332.

Spindler-Barth, M., Van Wormhoudt, A., and Spindler, K.-D. (1990). Chitinolytic enzymes in the integument and midgut gland of the shrimp *Palaemon serratus* during the moulting cycle. *Mar. Biol.* **106,** 49-52.

Sutherland, J.D., Kozolva, T., Tzertzinis, G., and Kafatos, F.C. (1995). DHR38: A new partner for *Drosophila* USP suggests a novel role for NGFI-B-type nuclear receptors. *Proc. Natl. Acad. Sci. USA* **92,** 7966-7970.

Swevers, L., Drevet, J.R., Lunke, M.D., and Iatrou, K. (1995). Cloning of the ecdysone receptor of the silkmoth *Bombyx mori* and analysis of its expression during follicular cell differentiation. *Insect Biochem. Mol. Biol.* **25,** 857-866.

Talbot, W.S., Swyryd, E.A., and Hogness, D.S. (1993). *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. *Cell* **73,** 1323-1337.

Thomas, H.E., Stunnenberg, H.G., and Steward, A.F. (1993). Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. *Nature* **362,** 471-475.

Thummel, C.S. (1996). Flies on steroids - *Drosophila* metamorphosis and the mechanisms of steroid hormone action. *Trends Genet.* **12,** 306-310.

Towle, D.W., and Mangum, C. (1985). Ionic regulation and transport ATPase activity during the molt cycle in the blue crab, *Callinectes sapidus*. *J. Crust. Biol.* **5,** 216-222.

Traub, M., Gellissen, G., and Spindler, K.-D. (1987). 20(OH) ecdysone-induced transition from intermolt to premolt protein biosynthesis patterns in the hypodermis of the crayfish, *Astacus leptodactylus*, *in vitro*. *Gen. Comp. Endocrinol.* **65,** 469-477.

Truman, J.W., Talbot, W.S., Fahrbach, S.E., and Hogness, D.S. (1994). Ecdysone receptor expression in the CNS correlates with stage-specific responses to ecdysteroids during *Drosophila* and *Manduca* development. *Development* **120,** 219-234.

Tzertzinis, G., Malecki, A., and Kafato, F.C. (1994). BmCF1, a *Bombyx mori* RXR-type receptor related to the *Drosophila* ultraspiracle. *J. Mol. Biol.* **258,** 479-486.

Yamaoka, L.H., and Scheer, B.T. (1970). Chemistry of growth and development in crustaceans. *In* "Chemical Zoology" (M. Florkin and B. T. Scheer, Eds.), Vol. 5, Part A, pp. 321-341. Academic Press, New York.

Yao, T.-P., Forman, B.M., Jiang, Z., Cherbas, L., Chen, J.-D., McKeown, M., Cherbas, P., and Evans, R.M. (1993). Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. *Nature* **366,** 476-479.

Zelhof, A.C., Yao, T.-P., Chen, J.D., Evans, R.M., and McKeown, M. (1995). Seven-up inhibits ultraspiracle-based signaling pathways *in vitro* and *in vivo*. *Mol. Cell. Biol.* **15,** 6736-6745.

TABLE 1 R_3 Values and Total Circulating Ecdysteroids of *Uca pugilator* during Molt Cycle.

Stage	R_3 values (mean ± SEM)	Growth rate, ER (mean ± SEM)	Total circulating ecdysteroids (mean ± SEM)
Singly autotomized crabs			
4 days after single autotomy	0 (N=15)	-	15.9 ± 3.0 (N=15)
C_4	6.32 ± 0.9 (N=45)	25.8 ± 3.9 (N=45)	13.1 ± 1.0 (N=42)
C_4/D_0 transition (26 days after autotomy)	10.8 ± 0.4 (N=30)	39.3 ± 3.5 (N=30)	21.1 ± 1.9 (N=27)
D_0	15.4 ± 0.5 (N=40)	43.0 ± 3.6 (N=40)	31.0 ± 3.9 (N=36)
	18.6 ± 0.2 (N=35)	27.4 ± 5.9 (N=35)	98.0 ± 10.4 (N=30)
D_{1-4}	21.3 ± 1.2 (N=35)	3.28 ± 1.55 (N=35)	88.7 ± 9.7 (N=32)
Multiply autotomized crabs			
4 days after multiple autotomy	0 (N=15)	-	11.2 ± 0.7 (N=15)
C_4/D_0 transition (9 days after autotomy)	4.8 ± 0.3 (N=30)	32.3 ± 4.6 (N=30)	29.1 ± 2.4 (N=28)
D_0	10.5 ± 0.3 (N=20)	51.3 ± 2.2 (N=20)	35.3 ± 6.4 (N=20)
	15.8 ± 0.4 (N=15)	44.8 ± 3.5 (N=15)	22.9 ± 2.2 (N=13)
D_{1-4}	21.4 ± 0.3 (N=18)	23.2 ± 4.7 (N=18)	82.7 ± 11.2 (N=16)

Table 2 The ratios of the concentrations of *UpuRXR* mRNAs to that of *UpuEcR* mRNAs in different tissues during the molt stages of the fiddler crab, *Uca pugilator*.

	UpuRXR / UpuEcR					
Molt stages	C_4	C_4/D_0 transition	D_0	Late D_0	$D_{1\text{-}4}$	Average
Regenerating limb buds	0.620	1.02	0.554	0.544	0.733	0.694
Gills	1.35	1.00	1.27	1.33	0.94	1.18
Eyestalks	0.525	0.304	0.410	0.547	0.452	0.447
Hypodermis	0.683	0.436	0.463	0.826	1.00	0.678
Hepatopancreas	4.17	3.00	1.69	2.83	2.15	2.92
Muscle from walking legs	0.847	1.10	1.06	0.716	1.50	1.04
Muscle from large chelipeds	0.607	0.527	0.754	0.846	0.822	0.711
Average	1.26	1.06	0.88	1.09	1.08	1.10

FIG. 1 Northern blot assay of *UpuEcR* and *UpuRXR* expression in regenerating limb buds and gills of the multiply autotomized crab. Poly A^+ RNA isolated from limb buds and gills was run on agarose gels, transferred to nitrocellulose filters, and hybridized to the appropriate probe as described under Materials and Methods. (A) Hybridization to a 189-bp *UpuEcR* DBD DNA probe. (B) Hybridization to a 212-bp of *UpuRXR* DBD DNA probe. (C) Hybridization to a *Drosophila* RP49 DNA probe (nt 447-764). RP 49 served as a standard for loading of RNA. Lane 1, C_4/D_0 transition; lane 2, Early D_0; lane 3, D_0; and lane 4, D_{1-4}. Electrophoresis of limb bud and gill RNA samples performed with different gels, and run for different times, led to slightly greater separation of the RXR doublet in gill mRNA.

FIG. 2 Ribonuclease protection assay of *UpuEcR* and *UpuRXR* expression in regenerating limb buds at stage D_0 of singly autotomized crabs. (a) Results of a ribonuclease protection assay of total RNA from three independent pooled samples of stage D_0 regenerating limb buds hybridized to antisense RNA probes encoding the *UpuEcR* LBD and the *UpuRXR* DBD; 307-nt protected fragments of *UpuRXR* transcript and 162-nt protected fragments of *UpuEcR* transcript were seen in all three experimental samples (lanes 2 through 4). No protected fragment was observed using yeast transfer RNA (tRNA) which served as a negative control (lane 13). Lane 1, DNA marker; lanes 2 through 4, three pooled RNA samples from regenerating limb buds. Lanes 5 through 7, 0.9, 1.8, 3.6 pg of *UpuRXR* cRNAs, respectively. Lane 8, undigested *UpuRXR* RNA

probe. Lanes 9 through 11, 3.6, 1.8, 0.9 pg of *UpuEcR* cRNAs, respectively. Lane 12, undigested *UpuEcR* RNA probe. Lane 13, 5 μg of yeast tRNA. (b) Results of ribonuclease protection assay of the diluted fraction (10^{-4} dilution) of total RNA from three independent pooled samples of stage D_0 regenerating limb buds hybridized to an antisense RNA probe encoding *Uca* 18S rRNA. 59-nt protected fragments of 18S rRNA were seen in all experimental samples (lanes 1 through 3). No signal was observed when yeast tRNA was used as a negative control (lane 5). Lanes 1 through 3, three samples of diluted RNAs from regenerating limb buds shown in (a) lanes 2 through 4. Lane 4, a total RNA standard. Lane 5, 5 μg of yeast tRNA.

FIG. 3 Expression of *UpuEcR* in tissues from singly autotomized (SA) crabs by ribonuclease protection assay. (a) Total circulating ecdysteroids (solid line) and the growth curve of R_3 limb buds (dotted line) from the SA crabs are adapted from data reported in Hopkins (1983). Drach's molt stages are given at the bottom. Abundance of *UpuEcR* mRNA in various tissues (b through h) is represented as picograms cRNA per microgram of total RNA loaded (see Materials and Methods). The criteria used to align the transcript data with the growth curve and ecdysteroid levels in (a) are given in Table 1. Error bars represent standard errors of the mean calculated from the results of at least three independent pooled samples. Each pooled sample contained RNA isolated from five crabs. Asterisks indicate significant differences from the preceding stage ($p \leq 0.05$). nd, not determined.

FIG. 4 Expression of *UpuRXR* in tissues from singly autotomized (SA) crabs by ribonuclease protection assay. (a) Total circulating ecdysteroids (solid line) and the growth curve of R_3 limb buds (dotted line) from the SA crabs are adapted from data reported in Hopkins (1983). Drach's molt stages are given at the bottom. Abundance of *UpuRXR* mRNA in various tissues (b through h) is represented as picograms cRNA per mircogram of total RNA loaded (see Materials and Methods). The criteria used to align the transcript data with the growth curve and ecdysteroid levels in (a) are given in Table 1. Error bars represent standard errors of the mean calculated from the results of at least three independent pooled samples. Each pooled sample contained RNA isolated from five crabs. Asterisks indicate significant differences from the preceding stage ($p \leq 0.05$).

FIG. 5 Expression of *UpuEcR* in tissues from multiply autotomized (MA) crabs by ribonuclease protection assay. (a) Total circulating ecdysteroids (solid line) and the growth curve of R_3 limb buds (dotted line) from the MA crabs are adapted from data reported in Hopkins (1992). Drach's molt stages are given at the bottom. Abundance of *UpuEcR* mRNA in various tissues (b through g) is represented as picograms cRNA per microgram of total RNA loaded (see Materials and Methods). The criteria used to align the transcript data with the growth curve and ecdysteroid levels in (a) are given in Table 1. Error bars represent standard errors of the mean calculated from the results of at least three independent pooled samples. Each pooled sampie contained RNA isolated from five crabs.

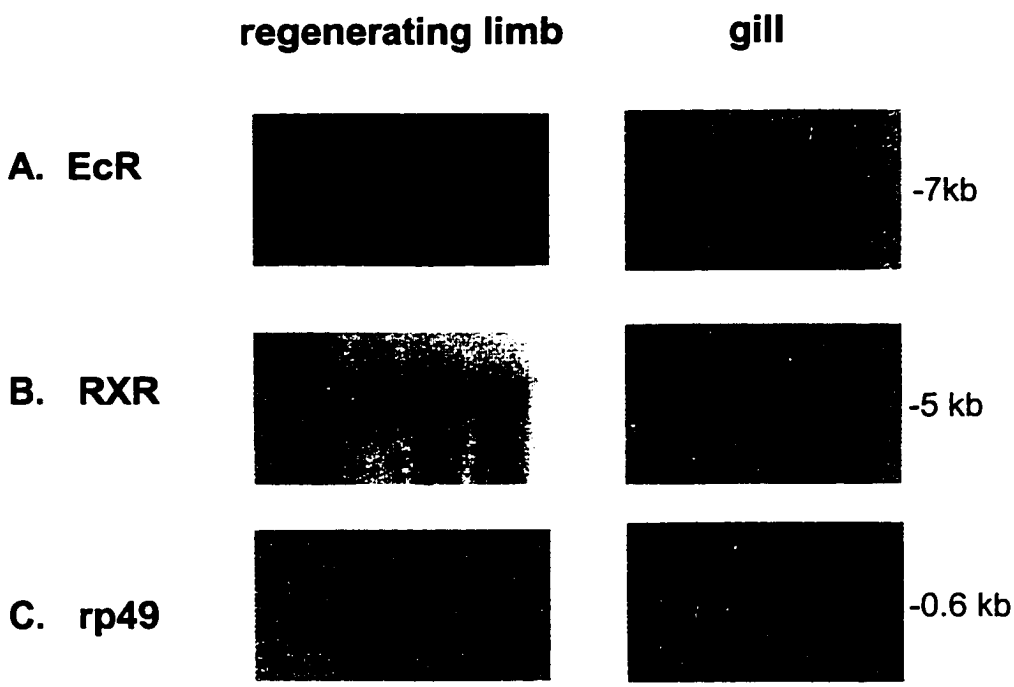


FIG. 1

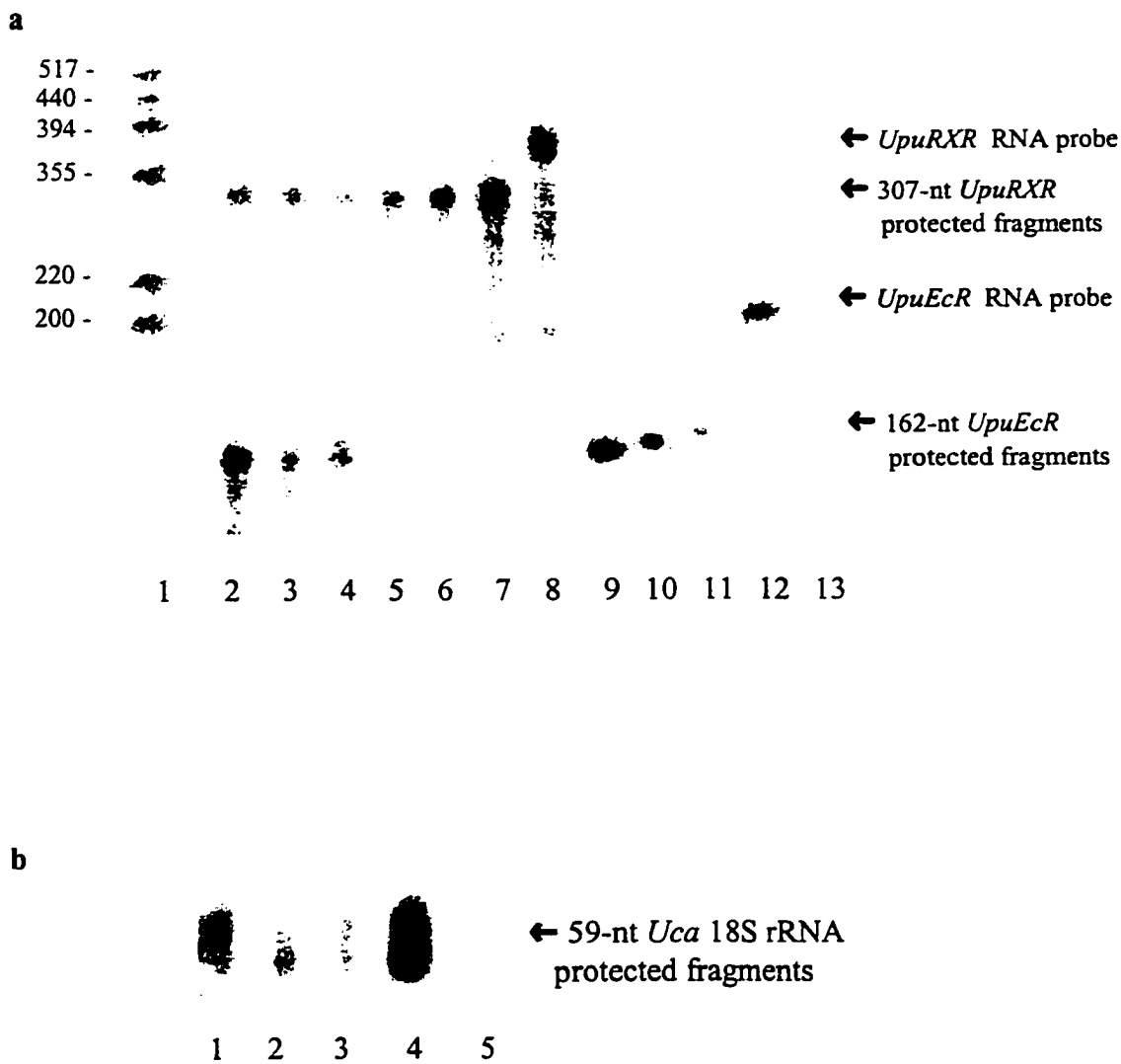

a
517 -
440 -
394 -
355 -
220 -
200 -
← UpuRXR RNA probe
← 307-nt UpuRXR protected fragments
← UpuEcR RNA probe
← 162-nt UpuEcR protected fragments
1 2 3 4 5 6 7 8 9 10 11 12 13
b
← 59-nt Uca 18S rRNA protected fragments
1 2 3 4 5

FIG. 2

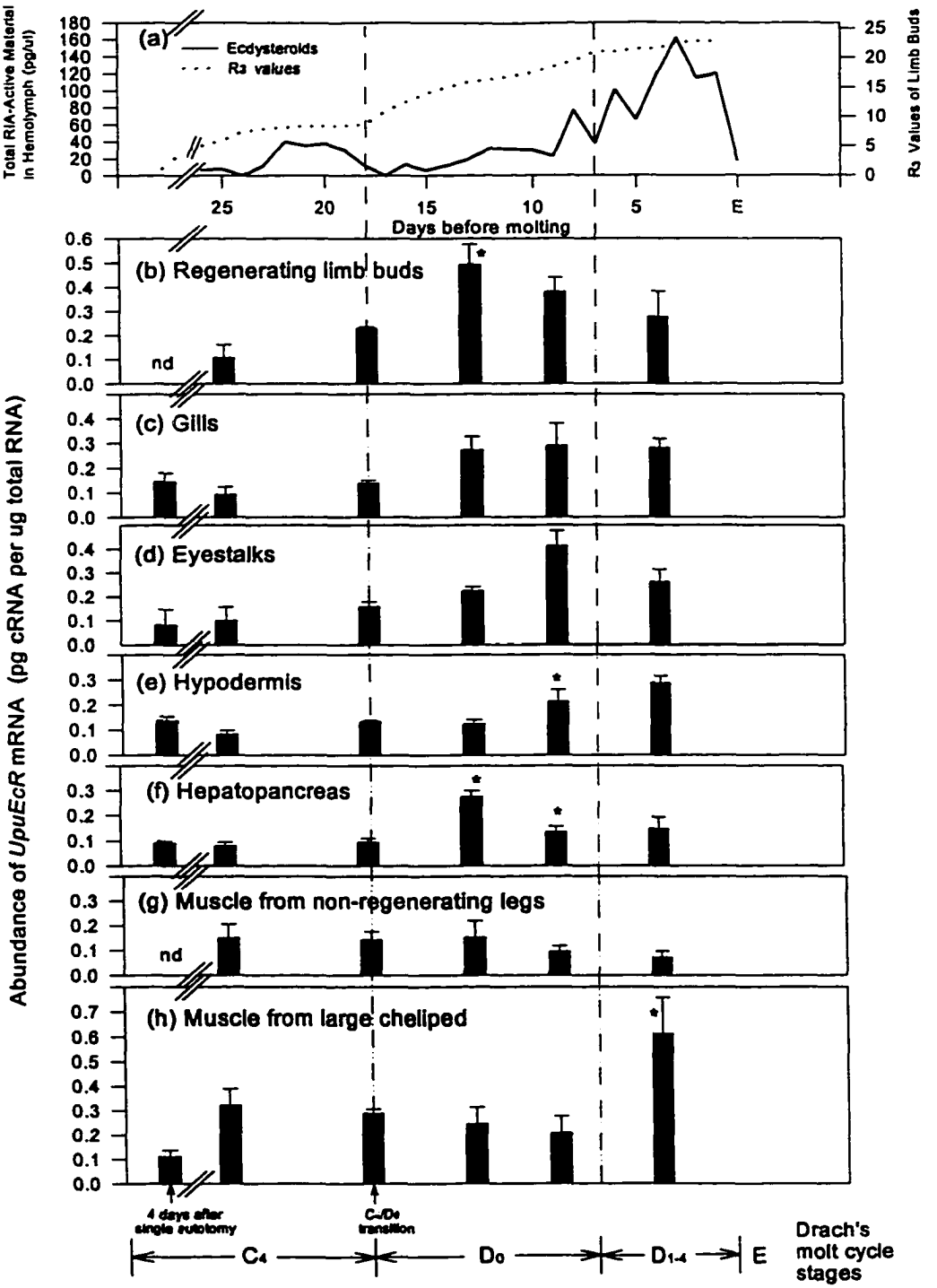

Total RIA-Active Material in Hemolymph (pg/ul)
(a)
Ecdysteroids
R3 values
R3 Values of Limb Buds
Days before molting
Abundance of UpuEcR mRNA (pg cRNA per ug total RNA)
(b) Regenerating limb buds
nd
(c) Gills
(d) Eyestalks
(e) Hypodermis
(f) Hepatopancreas
(g) Muscle from non-regenerating legs
nd
(h) Muscle from large cheliped
4 days after single autotomy
C4/D0 transition
C4
D0
D1-4
E
Drach's molt cycle stages

FIG. 3

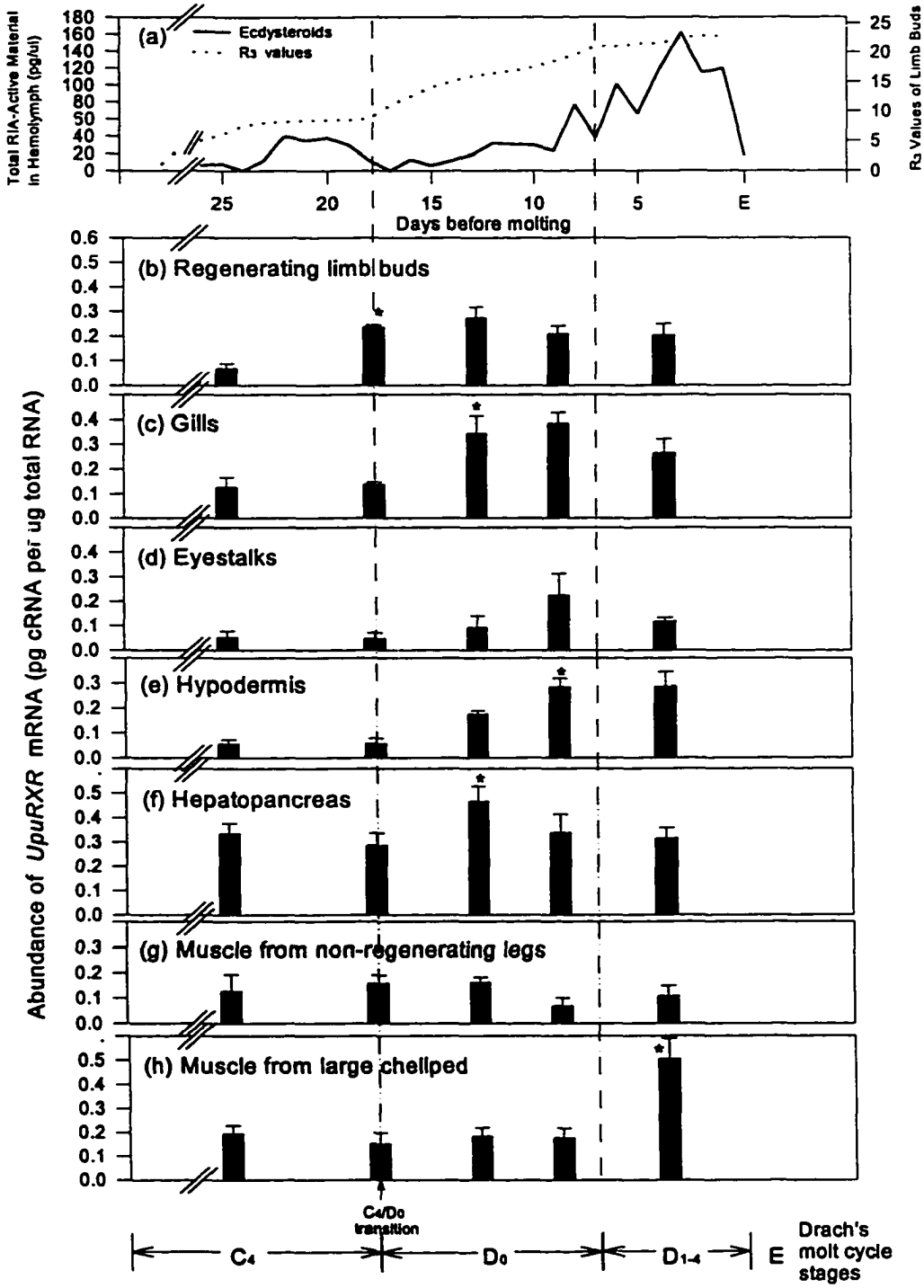

Total RIA-Active Material in Hemolymph (pg/ul)
(a)
Ecdysteroids
R3 values
R3 Values of Limb Buds
Days before molting
(b) Regenerating limb buds
(c) Gills
(d) Eyestalks
(e) Hypodermis
(f) Hepatopancreas
(g) Muscle from non-regenerating legs
(h) Muscle from large chelped
Abundance of UpuRXR mRNA (pg cRNA per ug total RNA)
C4/D0 transition
C4
D0
D1-4
E
Drach's molt cycle stages

FIG.4

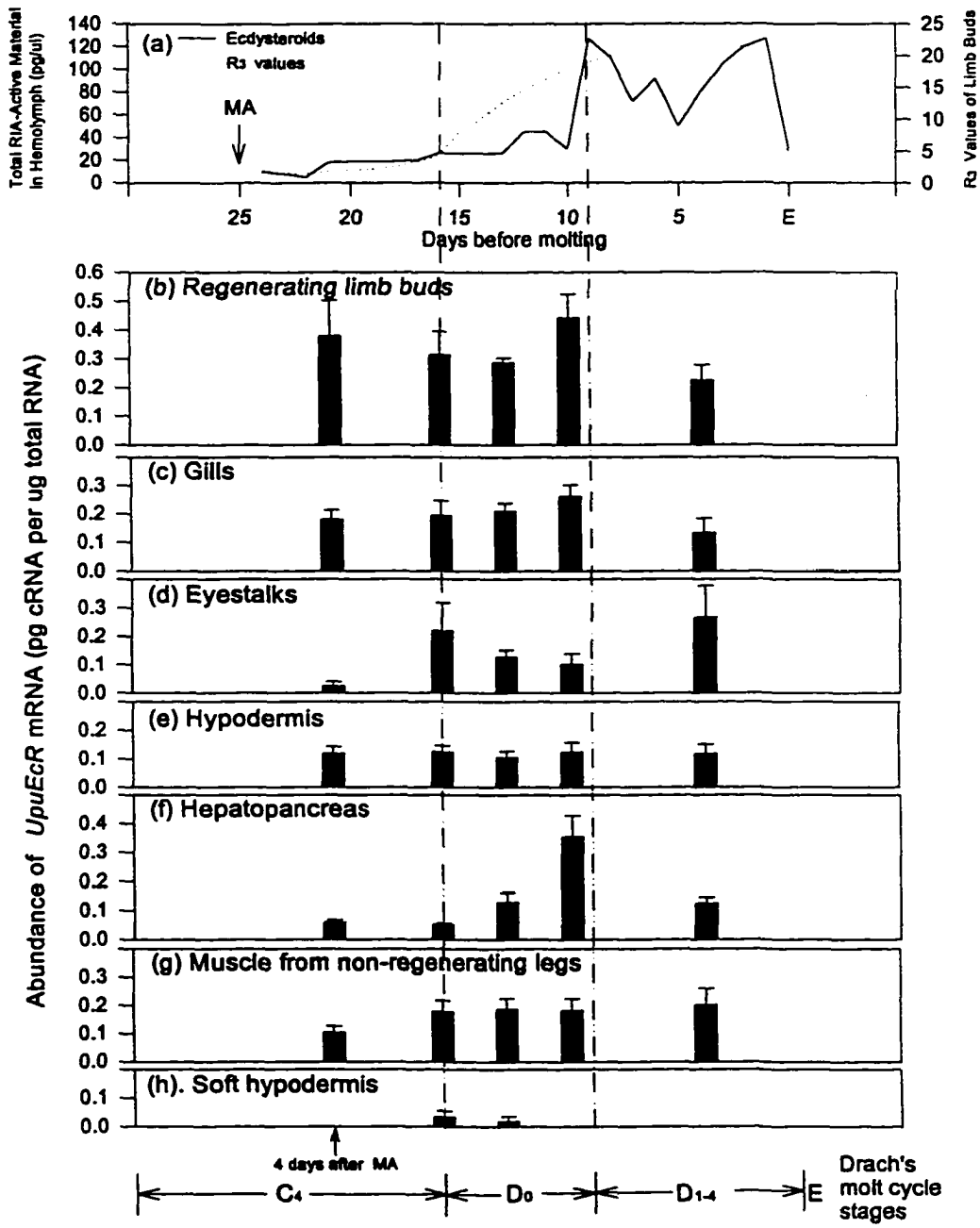

Total RIA-Active Material in Hemolymph (pg/ul)
(a)
Ecdysteroids
R3 values
MA
R3 Values of Limb Buds
Days before molting
(b) Regenerating limb buds
(c) Gills
(d) Eyestalks
(e) Hypodermis
(f) Hepatopancreas
(g) Muscle from non-regenerating legs
(h). Soft hypodermis
Abundance of UpuEcR mRNA (pg cRNA per ug total RNA)
4 days after MA
C4
D0
D1-4
E
Drach's molt cycle stages

FIG. 5

CHAPTER III

Effects of contralateral removal of regenerating limb buds on the growth rate of primarily and secondarily regenerating limb buds in the fiddler crab, *Uca pugilator*

Abstract

Following autotomy, the fiddler crab, *Uca pugilator*, is able to regenerate lost limbs. This regeneration process can be assigned into two growth stages, basal and proecdysial growth. The aim of this study was to test whether contralateral removal of limb buds during basal growth in *Uca pugilator* was able to decrease both growth rate of primarily regenerating limb buds and titers of total circulating ecdysteroids as shown in some earlier studies. In multiply autotomized crabs, contralateral removal of half of the limb buds during basal growth decreased the growth rate of the primarily regenerating limb buds and extended the molt cycle for 13 days. In contrast to earlier studies, the titers of total circulating ecdysteroids are not markedly altered immediately after the limb buds were contralaterally removed. In addition, the late proecdysial peaks of total circulating ecdysteroids were delayed for 16 days in the experimental animals. In these experimental crabs, the transcript levels of both ecdysteroid receptor (*UpuEcR*) and retinoid-X receptor (*UpuRXR*) were higher in the secondarily regenerating limb buds than in the primarily regenerating limb buds when the proecdysial growth rate of the secondarily regenerating limb buds was high. These results suggest that the titers of total circulating ecdysteroids may not be the main factor affecting the growth rate of the primarily regenerating limb buds. Other factor(s) may exist and alter

the growth rate of limb buds via the changes of ecdysteroid titers and/or ecdysteroid receptor transcript levels.

Introduction

Limb regeneration is a remarkable ability in many crustaceans. When animals respond to threat or injury (Juanes and Smith, 1995), they can cast off a limb at a predetermined position proximal to the injury through a reflex action, autotomy, and regenerate a new limb in its place (McVean, 1984). In the fiddler crab, *Uca pugilator*, limb regeneration can be assigned into two different growth processes, basal and proecdysial growth (reviewed in Hopkins, 1993). After healing of the wound, basal growth starts with the formation of a blastema and is followed by the emergence of a limb bud. These events are typical epimorphic growth which includes the migration of epidermal cells underneath the wound, influx of cells during blastema formation and rapid mitosis (Hopkins, 1993). Proecdysial growth of limb buds starts when the crabs prepare to molt and this process is a hypertrophic growth which is due to an increase in protein synthesis and water uptake in limb buds (reviewed in Hopkins, 1988). During late proecdysis, several days prior to molt, the limb buds cease to grow and this period is called terminal plateau. At molt (ecdysis) the newly regenerated limb is withdrawn from the cuticular sac, fills with blood, and expands into a functional limb. Furthermore, multiple autotomy (i.e. removal of four to six limbs) can cause the crab to molt and to regenerate lost limbs sooner than in non-autotomized crabs (Skinner and Graham, 1972; Hopkins, 1982).

Regeneration in arthropods is closely linked to the molt cycle and, in part, depends on ecdysteroids. Basal growth requires extensive mitotic activity (Adiyodi, 1972). Low levels of 20-OH ecdysone appear to elevate mitotic activity in insects and crabs (Bressac, 1978; Kunieda et al., 1997). In the fiddler crab, *U. pugilator*, a small peak of circulating

ecdysteroids appears to be important for the limb buds to proceed from basal growth to proecdysial growth (Hopkins, 1989). *In vitro* incubation of limb buds in two ecdysteroids, ecdysone and 20-OH ecdysone, increases the protein synthesis rate during proecdysis (Hopkins, 1989). In previous studies, we demonstrated that the transcripts of ecdysteroid receptor (EcR) and its putative dimer partner, retinoid-X receptor (RXR), appeared in both blastema and limb buds at various molt stages (Durica and Hopkins, 1996; Chung et al., 1998a, b). High levels of *UpuEcR* and *UpuRXR* transcripts are observed in limb buds when the overall growth rate of limb buds is high during early proecdysial growth (Fig. 3 and 4 in chapter II). This overlaps the period of ecdysteroid-sensitive protein synthesis of limb buds (Hopkins, 1989). The growth rate of the proecdysial limb buds may correspond to the transcript levels of these two receptors.

Previous studies showed that removal of some limb buds changed the pattern of normal limb regeneration (Holland and Skinner, 1976; McCarthy and Skinner, 1977; Weis, 1976; 1977). The effects of contralateral removal of limb buds may include changes in growth rate of the primarily regenerating limb buds, titers of total circulating ecdysteroids, transcript levels of *UpuEcR* and/or *UpuRXR*, and length of the molt cycle. In the land crab, *Gecarcinus lateralis*, removal of half of the regenerating limb buds during basal growth and early proecdysial growth decreases the growth rate and the synthesis of DNA in primarily regenerating limb buds (Holland and Skinner, 1976). For adult *Uca pugilator*, removal of regenerating limb buds during proecdysial growth causes a delay of growth in the primarily regenerating limb buds but the delay is not pronounced (Weis, 1976; 1977). In all of these studies, the growth rate of secondarily regenerating limb buds is faster than primarily

regenerating limb buds.

In *G. lateralis*, removal of half of the regenerating limb buds during early proecdysial growth (R_3 values at 15) results in a rapid significant decrease in titers of circulating ecdysteroids from 72.5 ($\pm$ 16) to 21.9 ($\pm$ 6.1) nanograms of ecdysone equivalent per milliliter of serum within 24 hrs (McCarthy and Skinner, 1977). During basal growth in *U. pugilator*, the titers of total circulating ecdysteroids are low. We determined whether the titers of total circulating ecdysteroids at this stage (R_3 values at 10) in *U. pugilator* were affected by contralateral removal of limb buds.

The previous studies mentioned above show that the growth rate of the secondarily regenerating limb buds is faster than that of the primarily regenerating limb buds (Holland and Skinner, 1976; McCarthy and Skinner, 1977; Weis, 1976; 1977). When limb regeneration in *U. pugilator* proceeds from basal growth to proecdysial growth, both transcript levels of *UpuEcR* and *UpuRXR* in limb buds and growth rate of limb buds increase (Chung et al., 1998a). We examined the relationship between the growth rates in the primarily and secondarily regenerating limb buds to the steady-state concentrations of the *UpuEcR* and *UpuRXR* mRNAs in limb buds.

We report here that the contralateral removal of half of the limb buds during basal growth in *U. pugilator* decreased the growth of the primarily regenerating limb buds during early stages of regeneration and delayed ecdysis for 13 days. In addition, contralateral removal of limb buds did not affect the titers of total circulating ecdysteroids 24 hours after the removal of limb buds. The first proecdysial peak of ecdysteroids in the experimental animals was delayed for 16 days relative to the control animals. Steady-state concentrations

of *UpuEcR* and *UpuRXR* mRNAs were significantly higher in secondarily regenerating limb buds than in primarily regenerating limb buds when the growth rate of secondarily regenerating limb buds was high eight to 14 days after contralateral limb bud removal.

Materials and Methods

Animals

Male *U. pugilator* were purchased from Gulf Specimen Company (Florida). After laboratory acclimation, crabs with a carapace width around 15 mm were selected and had seven limbs autotomized (multiple autotomy). Six walking legs and the large cheliped were removed. After the limb buds emerged, the length of the third limb bud was measured, divided by the maximum width of the carapace, and expressed as an R_3 value [=(length of limb bud x 100) /carapace width] (Bliss, 1956).

Interruption experiments

The period of basal growth of *U. pugilator* was designated as the first 10 days after multiple autotomy and the range of R_3 values during basal growth was from zero to about 10. When the R_3 values were about 10, three limb buds of walking legs were removed on either the left or right side of the experimental animals. For control animals, crabs were kept separately and their limb buds were allowed to undergo normal regeneration. At 24 hour intervals after contralateral removal of limb buds from experimental crabs, the size of limb buds in the experimental crabs was monitored. Growth rate of the limb buds (Experimental Rate, ER) was determined as follows: For two consecutive R_3 values, the slope of R_3 values against the day interval of measurement was calculated. The arc angle at the first R_3 value

was determined from the tangent of the slope [ER=Arc tangent (differences of R_3 values from two successive measurements /day interval of measurement)] (see Bliss and Hopkins, 1974). The blastemas or limb buds from experimental crabs were removed. Total RNA was extracted from blastemas and limb buds and subjected to ribonuclease protection assay to determine the concentrations of *UpuEcR* and *UpuRXR* transcripts (Chomczynski and Sacchi, 1987; Chung et al., 1998a). The details of this assay were described previously (Chung et al., 1998a). Blood was collected at the time of limb bud collection. The blood was subjected to radioimmunoassay to detect the amount of total circulating ecdysteroids (Hopkins, 1983).

Statistical analyses

Student's t-test was used for the statistical comparisons in this paper (Microsoft Excel, Cambridge, MA); $p \leq 0.05$ was considered to be significant. Linear regression was used to calculate the correlation coefficient for the ER values and the *UpuEcR* transcript levels.

Results:

Growth curves of regenerating limb buds

In the control crabs whose limb buds were kept intact, the overall growth rate (ER) of basal growth was 39.4 during five to 10 days after multiple autotomy. The limb buds continued to grow with high proecdysial growth rates after basal growth (Fig. 1a). The overall proecdysial growth rate was 41.3 until the terminal plateau was reached at 22 days after multiple autotomy. Then growth slowed and the overall ER was 5.1. The crabs molted at 35 days after multiple autotomy.

In experimental animals, the growth rate during basal growth prior to the removal of contralateral limb buds was 37.1. Half the limb buds were contralaterally removed when the average R_3 value of the buds was 10.05 (± 0.14) ten days after multiple autotomy. After contralateral removal of limb buds, the proecdysial growth rate of primarily regenerating limb buds in the experimental crabs slowed markedly (filled circles in Fig. 1b). The overall ER was reduced to 29.8. The primarily regenerating limb buds ceased to grow 15 days after the contralateral removal of limb buds and the average R_3 value at this time was 17.8 (± 0.39). The overall ER during the slow growth period was 6.7. This slow growth phase was designated as "interruption plateau". The growth resumed on the 28th day. The crabs molted 38 days after removal of contralateral limb buds or 48 days after original multiple autotomy. This was 13 days longer than the multiple autotomy-induced molt cycle of the controls.

The secondarily regenerating limb buds of these experimental animals emerged six days after the removal of contralateral limb buds (filled squares in Fig. 1b). The initial overall ER of basal growth was 29.8 within first four days after emergence. Proecdysial growth then started when the transition peak of total ecdysteroids appeared (Fig. 2b). The overall ER of these secondarily regenerating limb buds during proecdysial growth increased to 49.3. The secondarily regenerating limb buds reached the interruption plateau at an average R_3 value of 12.53 (±0.10) at 19 days after removal of contralateral limb buds. The overall ER during this plateau was 8.7. The growth of these secondarily regenerating limb buds resumed on the 28th day after interruption. At molt, the R_3 values of secondarily regenerating limb buds (19.5 ± 0.81) were lower than the primarily regenerating limb buds

(22.6 ± 0.96) (p<0.05). After molt, the length of the limb from secondarily regenerating limb buds was 13% shorter than the corresponding primarily regenerating limb bud.

Titers of total circulating ecdysteroids

The titer of total circulating ecdysteroids in control crabs was 25.9 (±0.98) picograms of 20-OH ecdysone equivalent per microliter of hemolymph at the transition peak 11 days after multiple autotomy (Fig. 2a). During late proecdysis, large increases of ecdysteroid titers occurred (56.2 ± 6.5 picograms of 20-OH ecdysone equivalent per microliter of hemolymph) at 21 days after multiple autotomy and two subsequent peaks of high total ecdysteroid titers (88.4 ± 8.2 and 105.7 ± 25.9 picograms of 20-OH ecdysone equivalent per microliter of hemolymph) were observed before the crabs molted 35 days after multiple autotomy.

Figure 2b represents the ecdysteroid titers of the crabs whose limb buds were contralaterally removed. On the day of limb bud removal, the average titer of total circulating ecdysteroids was 27.0 (±3.1) picograms of 20-OH ecdysone equivalent per microliter of hemolymph. Twenty four hours after removal of limb buds, the average titer remained at 27.2 (± 5.7) picograms per microliter. Twelve days after the removal of contralateral limb buds, a small transition peak of total ecdysteroid titers were observed during the proecdysial growth of secondarily regenerating limb buds. The first late proecdysial peak of total circulating ecdysteroid titers (79.5 ± 12.9 picograms per microliter) was observed 28 days after contralateral removal of limb buds. Two more peaks of total ecdysteroids (69.3 ± 14.3 and 68.4 ± 15.9 picograms per microliter) were observed during 33 and 35 days after limb bud removal. Compared to control animals, the first proecdysial peak of total circulating ecdysteroid titers was delayed for 16 days in experimental animals.

Steady-state concentrations of *UpuEcR* and *UpuRXR* mRNAs in limb buds from experimental crabs

When the growth rate of primarily regenerating limb buds slowed after the contralateral removal of limb buds, steady-state concentrations of *UpuEcR* transcripts from the primarily regenerating limb buds remained at about 0.1 picograms of complement RNA (cRNA) per microgram of total RNA on the first seven days after limb bud removal (open bars in Fig. 3a). *UpuEcR* mRNA concentrations then increased gradually to 0.2 picograms of cRNA per microgram of total RNA up to the 12th day and remained constant until 20 days after limb bud removal.

During blastema formation and differentiation, steady-state concentrations of *UpuEcR* mRNA in secondarily regenerating limb buds started at a low levels (0.0262 ± 0.0083 picograms of cRNA per microgram of total RNA) one day after the removal of contralateral limb buds (filled bars in Fig. 3a). Transcript levels increased markedly to higher levels (about 0.32 to 0.69 picograms of cRNA per microgram of total RNA) from eight days to 13 days after interruption. This period coincided with the fast proecdysial growth rate (ER = 49.3) of the secondarily regenerating limb buds. The correlation coefficient between ER values of the secondarily regenerating limb buds and *UpEcR* transcript levels was 0.64. The amount of the *UpEcR* transcript was about double that in primarily regenerating limb buds. The concentration of *UpuEcR* transcripts of the secondarily regenerating limb buds dropped to those of the primarily regenerating limb buds 15 days after limb bud removal when the primarily regenerating limb buds reached the interruption plateau.

Steady-state concentrations of *UpuRXR* mRNA in the primarily regenerating limb buds decreased slightly during the first four days after contralateral removal of limb buds (open bars in Fig. 3b). *UpuRXR* mRNA concentrations gradually increased to 0.15 picograms of cRNA per microgram of total RNA 10 days after removal of limb buds.

Steady-state concentrations of *UpuRXR* mRNA in secondarily regenerating limb buds started at a low level (0.0168 ± 0.0021 picograms of cRNA per microgram of total RNA) one day after the removal of limb buds (filled bar in Fig. 3b). Then the concentrations increased to higher levels (0.2 picograms of cRNA per microgram of total RNA) during eight days to 14 days after the removal of limb buds. Fifteen days after limb bud removal, the concentration of *UpuRXR* transcripts of the secondarily regenerating limb buds remained at similar transcript levels in the primarily regenerating limb buds when the primarily regenerating limb buds reached the interruption plateau.

Discussion

In the fiddler crab, *U. pugilator*, molting and limb regeneration are well coordinated so that the lost limb(s) can be regenerated in a single molt cycle. This tight coordination is under hormonal control (Hopkins, 1992). The molt inhibiting hormone (MIH) from the X-organ-sinus gland in eyestalks inhibits ecdysteroid release from the Y-organs (Mattson and Spaziani, 1985, Naya et al., 1988). Ecdysteroids are the arthropod molting hormones that are responsible for many physiological events related to molt cycle (reviewed by Chang, 1989). Injection of ecdysteroids can accelerate some molting events, such as apolysis and synthesis of new cuticle (Krishnakumaran and Schneiderman, 1968,

1969; Gilgan and Burns, 1976; Gigan and Farquharson, 1977).

At least two other factors are proposed to be involved in the coordination of molting and regeneration: limb growth inhibiting factor (LGIF) and the limb autotomy factor (LAF). LGIF is a heat-resistant peptide from eyestalks which may inhibit limb bud growth (Hopkins et al., 1979). Two LAFs have been proposed but have not been isolated and characterized (Skinner, 1985). Anecdysial LAF (LAFan) may be produced when multiple autotomy occurs. This factor may accelerate the molt cycle so the crab can molt and regenerate its lost limbs faster than an intact crab. In contrast, proecdysial LAF (LAFpro) should be an inhibiting factor which may be secreted after the removal of the primary regenerating limb buds. LAFpro may inhibit the growth of the primarily regenerating limb buds and may reduce the circulating ecdysteroid titers while blastema formation of the secondarily regenerating limb buds is performed.

Multiple autotomy is known to induce the onset of molting in many crustaceans (Bliss, 1956; Gomez, 1964; Skinner and Graham, 1972, Hopkins, 1982). The underlying mechanism is still unclear although the two factors mentioned above have been proposed by Skinner (1985). This precocious molt may be stimulated by the severing of a critical number of nerves in the lost limbs (Skinner and Graham, 1972).

Regeneration in the control animals

The growth curve of regenerating limb buds is similar to previous findings (Hopkins, 1982; 1992). A high proecdysial growth rate is observed just 10 days after multiple autotomy and the crabs molt 35 days after multiple autotomy. These results support the findings that multiple autotomy can accelerate the molt cycle and limb

regeneration (Skinner and Graham, 1972; Hopkins, 1982).

Bliss (1956) hypothesized that the basal limb growth is independent of the Y-organs while proecdysial growth is closely linked to the Y-organs. This hypothesis is first supported by the evidence that basal growth can occur in crustaceans deprived of their Y-organs (Passano and Jyssum, 1963; Charmantier-Daues and Vernet, 1974; Demeusy et la., 1988). In these animals, the limb buds cannot proceed to proecdysial growth. Small amounts of ecdysteroids have been shown to be required to initiate basal growth (Bazin, 1984). High ecdysteroid levels, however, inhibit the mitotic activities and growth at early stages of limb regeneration (from blastema formation to papilla emergence) (Bazin, 1977; Bressac, 1978; Rao, 1978; Hopkins et al., 1979). Similar to previous results from the multiply autotomized crabs (Hopkins, 1992), the titers of total circulating ecdysteroids during the basal growth of the control animals are low, supporting some of Bliss' hypothesis.

Stoffel and Hubschman (1974) suggest that multiple autotomy stimulates the neurosecretory cells of X-organ-sinus gland through nervous impulse to inhibit the release of MIH. It is presumed that this inhibition of MIH release activates the Y-organs to produce and/or release ecdysteroids which cause the onset of proecdysis and eventually ecdysis. In contrast, and consistent with previous results (Hopkins, 1992), large peaks of total ecdysteroid titers are observed in the control animals of this study only after 21 days following multiple autotomy. These results imply that multiple autotomy does not result in the immediate release of large amounts of ecdysteroids from the Y-organs.

The transcript levels of *UpuEcR* and *UpuRXR* may relate to the growth rate (ER) of the proecdysial limb buds. In *U. pugilator*, the transcript levels of *UpuEcR* and *UpuRXR*

in limb buds, as well as the growth rate of limb buds, increase when the proecdysial growth of limb buds begins (Chung et al., 1998a). The correlation between ER of proecdysial limb buds and *UpuEcR* transcript level during proecdysial growth is moderate (correlation coefficient = 0.63) (Fig. 5 in Chapter II), suggesting that the fast growth rate of the proecdysial limb buds may be a result of high steady-state concentrations of *UpuEcR* transcript.

Regeneration in the experimental animals

Similar to previous studies (Holland and Skinner, 1976; McCarthy and Skinner, 1977; Weis 1976, 1977), the contralateral removal of limb buds during basal growth (R_3 values at 10) of *U. pugilator* decreases the overall growth rate of the primarily regenerating limb buds from 41.3 to 29.8 during proecdysial growth. The cause of the retardation of the growth rate of the primarily regenerating limb buds is not well understood. In *G. lateralis*, the removal of early proecdysial limb buds (R_3 values at 15) causes total circulating ecdysteroid titers to decrease significantly from 72.5 (± 16) to 21.9 (± 6.1) nanograms of ecdysone equivalent per milliliter of serum within 24 hours (McCarthy and Skinner, 1977). It is proposed that low ecdysteroid titers caused by multiple autotomy may not be sufficient to support the continued growth of primarily regenerating limb buds. This drop of ecdysteroid titers therefore may be responsible for the growth retardation of primarily regenerating limb buds. In the present study, the removal of limb buds is performed during the basal growth of limb buds (R_3 values at 10). At this time, the average titer of total circulating ecdysteroids of *U. pugilator* is 27.0 (±3.1) picograms of 20-OH ecdysone equivalent per microliter of hemolymph, lower than proecdysis titers. Removal

of the limb buds does not elevate the levels and pattern of the ecdysteroid titers before the limb buds reach the interruption plateau. We therefore conclude that the decrease of growth rate in the primarily regenerating limb buds does not depend on a drop of total ecdysteroid titers.

Low total ecdysteroid titers have been shown to be important for basal growth of limb buds (Bazin, 1977, 1984; Bressac, 1978; Rao, 1978; Hopkins et al., 1979). Comparison of total ecdysteroid titers in the control and experimental animals in this study reveals that low total ecdysteroid titers are observed during basal growth and early proecdysial growth of secondarily regenerating limb buds in the experimental crabs, and the first proecdysial peak of total circulating ecdysteroids is delayed for 16 days (Fig 2b). The low titers of total circulating ecdysteroids in experimental animals during the basal growth of secondarily regenerating limb buds may allow secondarily regenerating limb buds to complete their basal growth.

The growth rate of proecdysial limb buds may correspond to transcript levels of *UpuEcR*. In this study, transcript levels of both *UpuEcR* and *UpuRXR* in the secondarily regenerating limb buds increase significantly when high overall growth rate (ER = 49.3) is observed during proecdysial growth of secondarily regenerating limb buds. The correlation between ER of the secondarily regenerating limb buds and steady-state concentration of *UpuEcR* mRNA in this period is moderate (correlation coefficient = 0.64) and similar to the previous study of multiply autotomized crabs (Fig. 5 in Chapter II). In addition, high concentrations of *UpuEcR* transcript occur prior to late proecdysial peaks of circulating ecdysteroids. Likewise, in wing discs of the tobacco hornworm,

Manduca sexta, and the silkworm, *Bombyx mori*, increases in *EcR* mRNA expression occur prior tc peaks of circulating ecdysteroids in the blood (Fujiwara et al., 1995; Kamimura et al., 1996). High titers of ecdysteroids are able to repress the expression of *Drosophila EcR* (reviewed in Thummel, 1996). These results suggest that the increase of *UpuEcR* transcript levels may correspond to the growth rate of proecdysial limb buds when titers of total circulating ecdysteroids are not high. An EcR isoform which is responsible for fast proecdysial growth of limb buds may be induced by low ecdysteroid titers.

Changes in *UpuEcR* and *UpuRXR* transcript levels during eight to 13 days after the limb bud removal are different in the primarily and secondarily regenerating limb buds. Specifically, more transcripts of these receptors are found in the secondarily regenera ting limb buds than in the primarily regenerating limb buds. Other factors may be p roduced in the limb buds and regulate the expression of *UpuEcR* and *UpuRXR* mRNAs differentially in the primarily and secondarily regenerating limb buds. In *G. lateralis*, an inhibitory factor that suspends the growth of primarily regenerating limb buds was recently shown (Yu and Mykles, 1998). Injection of the protein extract from secondarily regenerating limb buds inhibits the growth of normal limb buds. The appearance of this factor is consistent with LAFpro proposed by Skinner (1985). The source of the materials may come from the event of autotomy and these materials may affect the growth of the limb buds through the regulation on receptor transcript expression.

Proecdysial growth of limb buds depends, in part, on ecdysteroid titers. The Y-organs are shown to be required for the initiation of the proecdysial growth of limb buds from a terrestrial crab, *Sesarma haematocheir* (Suzuki, 1985). Injection of 20-OH

ecdysone into crabs deprived of the Y-organs induces proecdysial limb bud growth (Suzuki, 1985). In this study, both primarily and secondarily regenerating limb buds resume their growth as total ecdysteroid titers increase 28 days after limb bud removal. Growth then comes to a plateau on 34 days after limb bud removal. These results are similar to results from *G. lateralis* (Holland and Skinner, 1976). When limb buds are removed during basal growth (R_3 values at 5) in *G. lateralis*, the growth rate of the primarily regenerating limb buds first decreases and then increases rapidly as the R_3 values of secondarily regenerating limb buds come to about 12. This second phase of proecdysial growth may be correspond to the first proecdysial peak of total circulating ecdysteroid titers. High titers of total ecdysteroids may trigger the expression of another *EcR* isoform which complete the proecdysial growth of the limb buds.

Furthermore, similar to other studies (Holland and Skinner, 1976; McCarthy and Skinner, 1977), R_3 values of secondarily regenerating limb buds are significantly smaller than those of the primarily regenerating limb buds just prior to molt. After molt, limbs from secondarily regenerating limb buds are also smaller than those from primarily regenerating limb buds. Comparison of the R_3 values at the end of basal growth shows that the average R_3 value of secondarily regenerating limb limbs (2.46 ± 0.31) in the experimental animals (Fig. 1b) is lower than that of the normal limb buds (10.2 ± 0.59) in the control animals (Fig. 1a). These differences may be due to the insufficient allocation of energy to regenerate the secondarily regenerating limb buds during basal growth.

Finally, a model is proposed to explain the effects of contralateral removal of limb buds. During basal growth, titers of total circulating ecdysteroids are low and Ponasterone

A and 25-deoxyecdysone are the dominant components (Hopkins, 1992). Multiple autotomy may release LAFan to switch on the onset of proecdysis and to accelerate the molt cycle for limb regeneration. Proecdysis begins when the titers of circulating ecdysone and 20-OH ecdysone increase. Either one of these ecdysteroids may induce the transcript levels of *EcR* and *RXR* (Hiruma et al., 1997). As proposed by Ashburner et al., 1974, liganded functional EcR may turn on the downstream elements of the ecdysteroid regulatory cascade and stimulates the hypertrophic growth of the proecdysial limb buds.

Contralateral removal of the limb buds may release LAFpro in the position of secondarily regenerating limb regeneration. LAFpro switches off the progress of proecdysis by inhibit the synthesis or release of ecdysone from the Y-organs. Thus, the concentrations of circulating ecdysone and its metabolite, 20-OH ecdysone may decrease. This process may not be mediated through MIH because removal of limb buds at proecdysial growth from eyestalk ablated animals gives similar results as those with eyestalks (McCarthy and Skinner, 1977). In addition, LAFpro may also affect the expression of *UpuEcR* and *UpuRXR* mRNAs in primarily regenerating limb buds. This alternation may then decrease the growth rate of these limb buds. At the same time, the Y-organs may secrete low amounts of ecdysteroids, such as 25-deoxyecdysone, for basal growth of limb buds. As proecdysial growth starts in secondarily regenerating limb buds, more ecdysone and/or 20-OH ecdysone may be released from the Y-organs and these ecdysteroids may increase the transcript levels of *EcR* and *RXR*. The growth rate of secondarily regenerating limb buds becomes faster. LAFpro (or its downstream elements) may still have effects on the primarily regenerating limb buds and keeps the transcript levels of *EcR* and *RXR* low. During late proecdysis, the first proecdysial

peak of total ecdysteroids may trigger the expression of another *EcR* isoform in both primarily and secondarily regenerating limb buds and causes limb buds to resume the growth until the terminal plateau is reached.

In order to support this model, further investigation is necessary. To clarify the presence of isoforms of both EcR and RXR in crustaceans should be essential. In addition to isolation of putative LAFs in the crabs, tests of some putative LAFs, such as fibroblast growth factors (FGF), may shed some light about the mechanism of multiply autotomy and limb regeneration in crustaceans.

Literature cited

Ashburner, M., Chihara, C., Meltzer, P., and Richards, G. (1974). Temporal control of puffing activity in polytene chromosomes. *Cold Spring Harbor Symp. Quant. Biol.* **38,** 655-662.

Adyodi, R. G. (1972). Wound healing and regeneration in the crab, *Paratelphusa hydrodromous. Int. Rev. Cytol.* **32,** 257-289.

Bazin, F. (1977). Action inhibitrice de l'ecdysterone sur la regeneration chez le Crabe *Carcinus maenas. C. R. Acad. Sci. Paris* **284,** 1211-1214.

Bazin, F. (1984). La regeneration de la patte chex le crabe *Carcinus maenas*. These de Doctorat d'Etat, Caen, pp. 1-253.

Bliss, D.E. (1956). Neurosecretion and the control of growth in a decapod crustacean. *In* "Bertil Hanstrom. Zoological Papers in Honour of His Sixty-Fifth Birthday" (K. G. Wingstrand, Ed.), pp. 56-75. Zoological Institute, Lund.

Bliss, D.E. and Hopkins, P.M. (1974). Bioassay and characterization of crustacean limb growth-controlling factors. *In* "Neurosecretion - The final Neuroendocrine Pathway, VI International Symposium Neurosecretion, London, 1973" pp. 104-114, Springer-Verlag, New York.

Bressac, C. (1978). L'activite mitotique an niveau de l'organe y du Crabe *Pachygrapsus marmoratus. C. R. Acad. Sci. Paris* **285,** 289-291.

Chang, E.S. (1989). Endocrine regulation of molting in Crustacea. *Rev. Aquat. Sci.* **1,** 131-157.

Charmantier-Daures, M., and Vernet, G. (1974). Nouvelles donnees sur le role de l'organe y dans le deroulement de la mue chez *Pachygrapsus marmoratus. Influence de la regeneration intensive. C. R. Acad. Sci. Ser. Paris* **278,** 3367-3370.

Chomczynski, P., and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal. Biochem.* **162,** 156-159.

Chung, A. C.-K., Durica, D. S., and Hopkins, P. M. (1998a). Tissue-specific patterns and steady-state concentrations of ecdysteroid receptor and retinoid-X receptor mRNA during the molt cycle of the fiddler crab, *Uca pugilator. Gen.. Comp. Endocrinol.***109,** 375-389.

Chung, A. C.-K., Clifton, S. W., Roe, B. A., Durica, D. S. and Hopkins, P. M. (1998b). Cloning of crustacean EcR and RXR gene homologs and elevation of RXR mRNA by

retinoic acid. *Mol. Cell. Endocrinol.* In press.

Demusy, N., Moriniere, M., and Porcheron, P. (1988). Effects of ablation of Y organs and regenration on ecdysteroid levels and development of the crab, *Pilumnus hirtellus*. *Comp. Biochem. Physiol.* **91C,** 111-114.

Durica, D. S., and Hopkins, P. M. (1996). Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator*. *Gene* **171,** 237-241.

Fujiwara, H., Jindra, M., Newitt, R., Palli, S.R., Hiruma, K., and Riddiford, L.M. (1995). Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. *Insect Biochem. Mol. Biol.* **25,** 845-856.

Gilgan, M.W., and Burns, B.G. (1976). The successful induction of moulting in the adult male lobster (*Homarus americanus*) with a slow-release form of ecdysterone. *Steroids* **27,** 571-580.

Gilgan, M.W., and Farquharson, T.E. (1977). A change in the sensitivity of adult male lobster (*Homarus americanus*) to ecdysterone on changing from intermolt to active premolt development. *Comp. Biochem. Physiol.* **58A,** 29-32.

Gomez, R. (1964). Autotomy and regeneration in the crab, *Paratelphusa hydrodromous*. *J. Anim. Morphol. Physiol.* **11,** 97-104.

Hiruma, K, Böcking, D., Lafont, R., and Riddiford, L.M. (1997). Action of different ecdysteroids on the regulation of mRNAs for the ecdysone receptor, MHR3, dopa decarboxylase, and a larval cuticle protein in the larval epidermis of the tobacco hornworm, *Manduca sexta*. *Gen. Comp. Endocrinol.* **107,** 84-97.

Holland, C. A. and Skinner, D. M. (1976). Interactions between molting and regeneration in the land crab. *Biol. Bull.* **150,** 222-240.

Hopkins, P.M. (1982). Growth and regeneration patterns in the fiddler crab, *Uca pugilator*. *Biol. Bull.* **163,** 301-319.

Hopkins, P.M. (1983). Patterns of serum ecdysteroids during induced and uninduced proecdysis in fiddler crab, *Uca pugilator*. *Gen. Comp. Endocrinol.* **63,** 362-373.

Hopkins, P.M. (1988). Control of regeneration in crustaceans. *In* "Endocrinology of Selected Invertebrate Types" (R. Downer and H. Laufer, Eds.) Vol. 2, pp.327-340, A.R. Liss, New York.

Hopkins, P.M. (1989). Ecdysteroids and regeneration in the fiddler crab, *Uca pugilator*. *J. Exp. Zool.* **252,** 293-299.

Hopkins, P.M. (1992). Hormonal control of the molt cycle in the fiddler crab, *Uca pugilator*. *Am. Zool.* **32,** 450-458.

Hopkins, P.M. (1993). Regeneration of walking legs in the fiddler crab, *Uca pugilator*. *Am. Zool.* **33,** 348-356.

Hopkins, P. M., Bliss, D. M., Sheehan, S. W., and Boyer, J. R. (1979) Limb growth-controling factors in the crab *Gecarcinus lateralis*, with special reference to the limb growth-inhibiting factor. *Gen. Comp. Endocrinol.* **39,** 192-207.

Juanes, F., and Smith, L. D. (1995). The ecological consequences of limb regeneration of limb damage and loss in decapod crustaceans. *J. Exp. Mar. Biol. Ecol.* **195,** 197-223.

Kamimura, M., Tomita, S., and Fujiwara, H. (1996). Molecular Cloning of an ecdysone receptor (B1 isoform) homologue from the silkworm, *Bombyx mori*, and its mRNA expression during wing disc development. *Comp. Biochem. Physiol.* **113B,** 341-347

Krishnakumaran, A., and Schneiderman, H.A. (1968). Chemical control of molting in arthropods. *Nature* **220,** 955-956.

Krishnakumaran, A., and Schneiderman, H.A. (1969). Control of molting in Crustacea by an insect molting hormone. *Gen. Comp. Endocrinol.* **12,** 515-518.

Kunieda, T., Kurata, S., and Natori, S., (1997). Regeneration of *Sarcophaga* imaginal discs *in vitro*: Implication of 20-hydroxyecdysone. *Dev. Biol.* **183,** 86-94.

Mattson, M.P., and Spaziani, E. (1985). Characterization of molt-inhibiting hormone (MIH) action on crustacean Y-organ segments and dispersed cells in culture and a bioassay for MIH activity. *J. Exp. Zool.* **236,** 93-101.

McCarthy, J. F., and Skinner. D. M. (1977). Interruption of proecdysis by autotomy of partially regenerated limbs in the land crab, *Gecarcinus lateralis*. *Gen. Comp. Endocrinol.* **61,** 299-310.

McVean, A. (1984). Autotomy. *In* "The Biology of Crustacea" (Bliss, D. E., Ed.), vol. 4, pp. 107-132. Academic Press, New York.

Naya, Y., Kishida, K., Sugiyama, M., Murata, M., Miki, W., Ohnishi, M., and Nakanishi, K. (1988). Endogenous inhibitor of ecdysone synthesis in crabs. *Experienta* **44,** 50-52.

Passano, L. M., and Jyssum, S. (1963). The role of the Y-organ in crab proecdysis and limb regeneration. *Comp. Biochem. Physiol.* **9,** 195-213.

Rao, K. R. (1978). Effects of ecdysterone, inokosterone and eyestalk ablation on limb regeneration in the fiddler crab, *Uca pugilator*. *J. Exp. Zool.* **203,** 257-270.

Skinner, D. M. (1985). Molting and regeneration. *In* "The Biology of Crustacea" (Bliss, D. E., Ed.), vol. 9, pp. 43-146. Academic Press, New York.

Skinner, D. M., and Graham, D. E., (1972). Loss of limbs as a stimulus to ecdysis in Brachyura (True crab). *Biol. Bull.* **143,** 222-233.

Stoffel, L. A., and Hubschman, J. H. (1974). Limb loss and the molt cycle in the freshwater shrimp, *Palaemonetes kadiakensis*. *Biol. Bull.* **147,** 203-212.

Suzuki, S. (1985). Effect of Y-organ removal on limb regeneration and molting in the terrestrial crab, *Sesarma haematocheir*. *Gen. Comp. Endocrinol.* **58,** 202-210.

Weis, J. S. (1976). Effects of environmental factors on regeneration and molting in fiddler crabs. *Biol. Bull.* **150,** 152-162.

Weis, J. S. (1977). Regeneration in the fiddler crab, *Uca pugilator*, after injury or removal of limb buds. *J. Exp. Zool.* **197,** 21-30.

Yu, X. L., and Mykles, D. L. (1998). A factor in limb regenerates that suspends molting in the land crab, *Gecarinus lateralis*. *Am. Zool.* **37,** 154A.

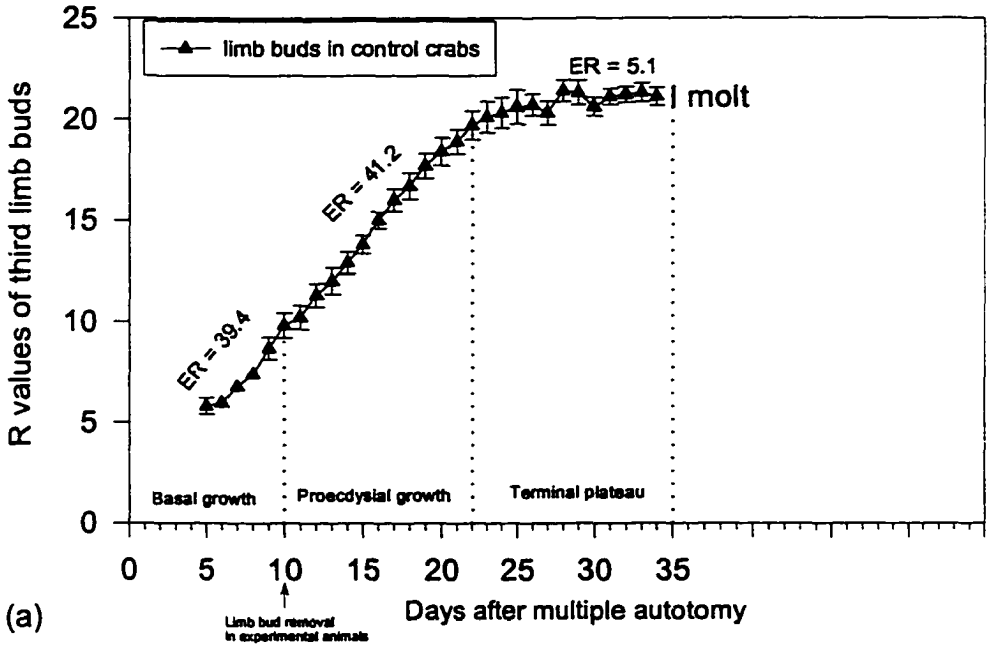
FIGURE LEGEND

FIG. 1 The growth curves of the regenerating limb buds. (a) Limb buds of the control animals whose limb buds remained intact during limb regeneration. Days after multiple autotomy of the control crabs are represented on the x-axis. The closed triangles represent the growth curve of limb buds from control crabs. (b) Primarily and secondarily regenerating limb buds of experimental animals whose limb buds were removed at R_3 values of 10. The closed circles represent the growth curve of the primarily regenerating limb buds and the closed squares represent the secondarily regenerating limb buds in experimental animals. Days before and after contralateral removal of limb buds in experimental crabs are represented on the x-axis. R values of third limb buds are represented on y-axis. The overall growth rate (ER) of limb buds at each stage of limb regeneration was placed near the growth curves. Error bars represent standard errors of the mean calculated from the results of 12 to 40 individual samples.

FIG. 2 Titers of total circulating ecdysteroids during limb regeneration determined by radioimmunoassay (RIA). (a) Ecdysteroid titers of the control animals whose limb buds remained intact during limb regeneration. Days after multiple autotomy of the control crabs are represented on the x-axis. (b) Ecdysteroids titers of experimental animals whose limb buds were removed at R_3 values of 10. Days before and after contralateral removal of limb buds in experimental crabs are represented on the x-axis. RIA-active material in hemolymph is represented as picograms of 20-hydroxyecdysone equivalent per microgram of hemolymph on the y-axis. Error bars represent standard

errors of the mean calculated from the results of 6 to 14 individual samples.

FIG. 3 Steady state concentrations of receptor mRNA in blastemas and limb buds as detected by ribonuclease protection assay: (a) *UpuEcR* and (b) *UpuRXR*. Receptor transcript abundance from primarily regenerating limb buds of experimental animals is represented by open bars. Receptor transcript abundance from secondarily regenerating limb buds is represented by filled bars. Days before and after contralateral removal of limb buds in experimental crabs are represented on the x-axis. The y-axis represents abundance of receptor mRNA as picograms of cRNA per microgram of total RNA loaded. Error bars represent standard errors of the mean calculated from the results of three to four independent pooled samples. Each pooled sample contained RNA isolated from three to five crabs. Asterisks indicate significant differences of transcript levels between the primarily and secondarily regenerating limb buds at the same time point by Student's t-test ($p \leq 0.05$).


limb buds in control crabs
ER = 5.1
molt
ER = 41.2
ER = 39.4
Basal growth
Proecdysial growth
Terminal plateau
R values of third limb buds
Days after multiple autotomy
Limb bud removal in experimental animals
(a)

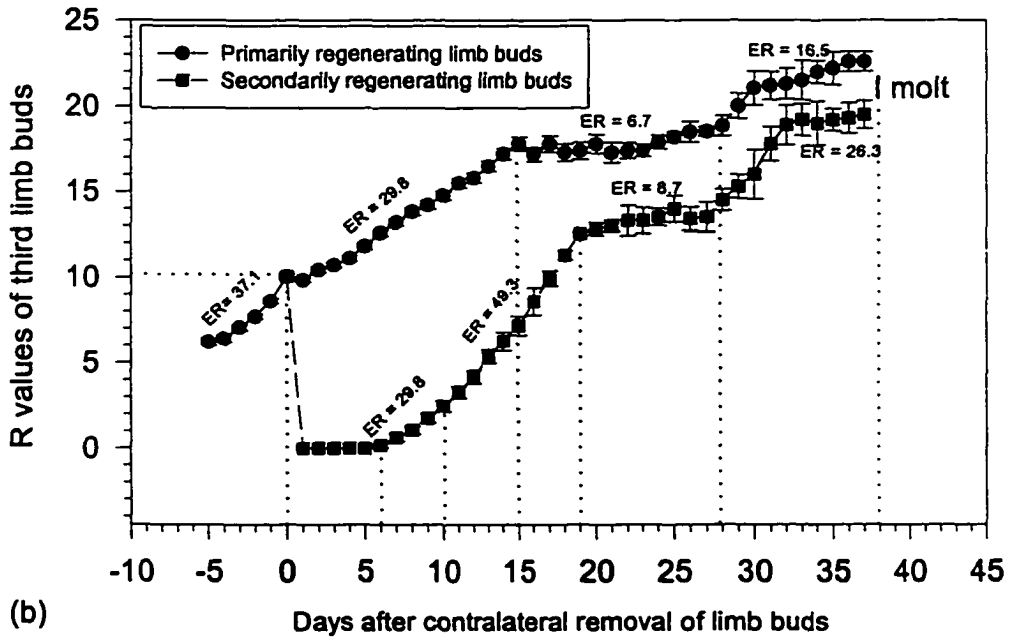
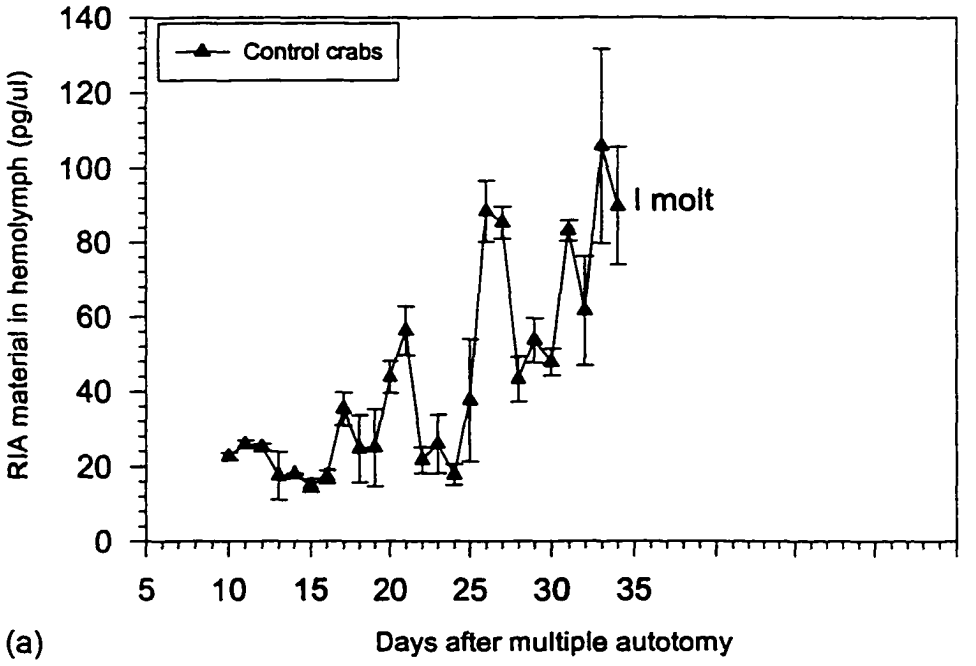
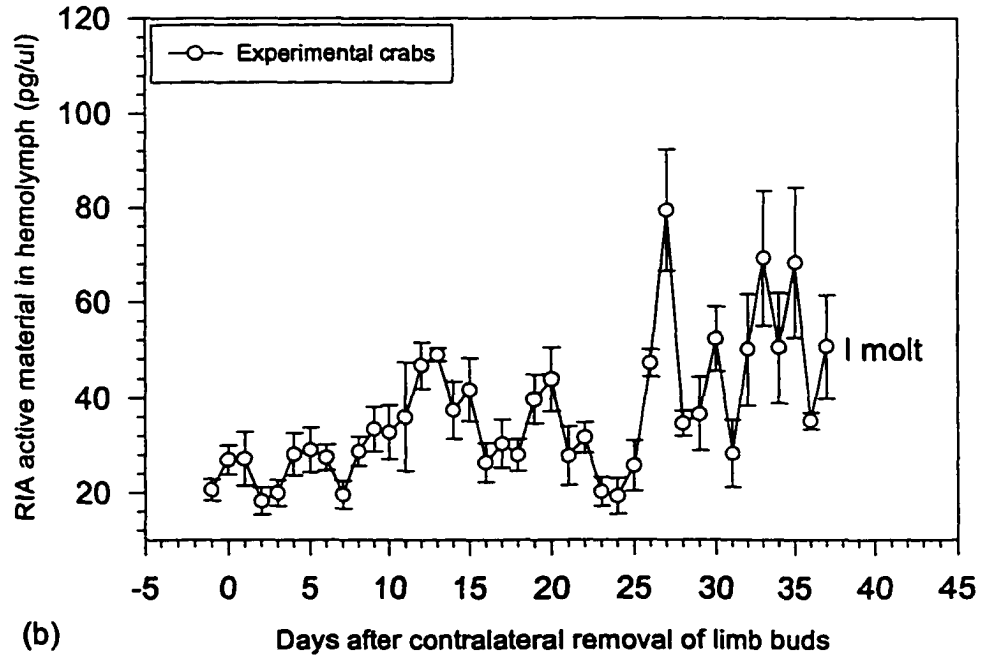

Primarily regenerating limb buds
Secondarily regenerating limb buds
ER = 16.5
molt
ER = 6.7
ER = 26.3
ER = 8.7
ER = 29.8
ER = 37.1
ER = 49.3
ER = 29.8
R values of third limb buds
Days after contralateral removal of limb buds
(b)

FIG. 1

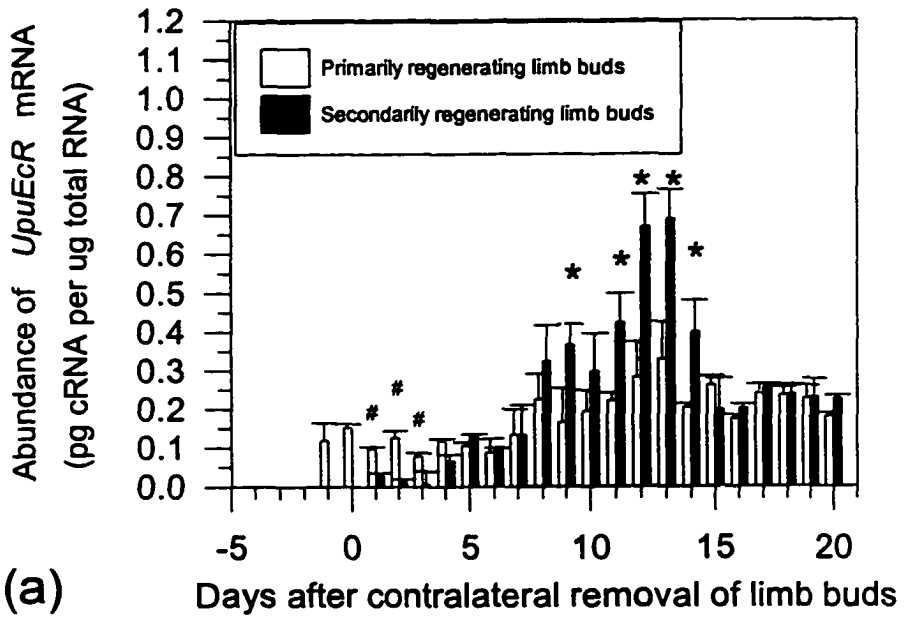

Control crabs
I molt
RIA material in hemolymph (pg/ul)
140
120
100
80
60
40
20
0
5
10
15
20
25
30
35
Days after multiple autotomy

(a)


Experimental crabs
I molt
RIA active material in hemolymph (pg/ul)
120
100
80
60
40
20
-5
0
5
10
15
20
25
30
35
40
45
Days after contralateral removal of limb buds

(b)

FIG. 2


Abundance of UpuEcR mRNA
(pg cRNA per ug total RNA)
1.2
1.1
1.0
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0.0
Primarily regenerating limb buds
Secondarily regenerating limb buds
-5
0
5
10
15
20
Days after contralateral removal of limb buds
(a)

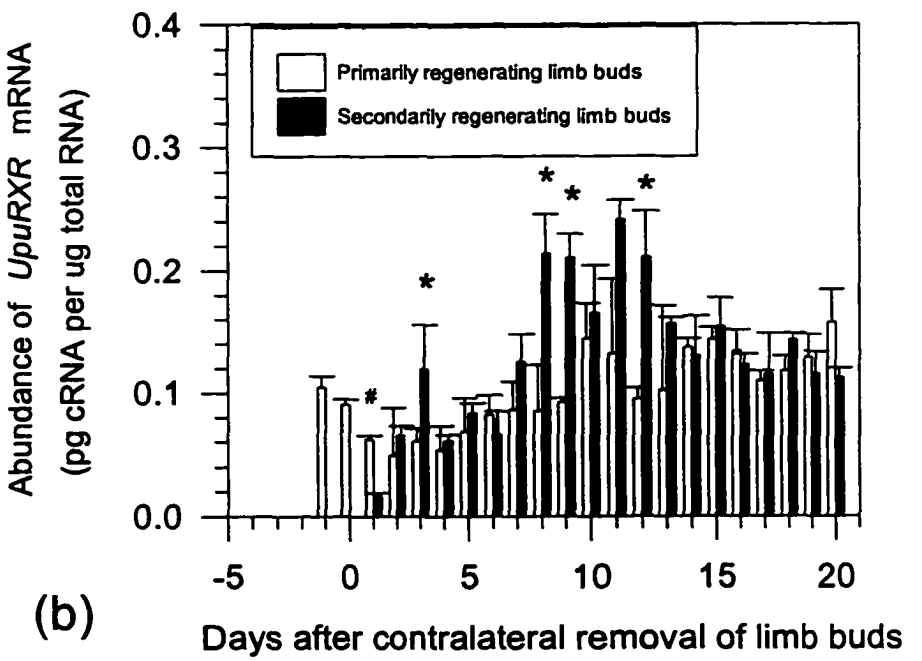

Abundance of UpuRXR mRNA
(pg cRNA per ug total RNA)
0.4
0.3
0.2
0.1
0.0
Primarily regenerating limb buds
Secondarily regenerating limb buds
-5
0
5
10
15
20
Days after contralateral removal of limb buds
(b)

FIG. 3

CHAPTER IV

Evolution of ecdysteroid and retinoid-X receptors

Abstract

Ecdysteroids are arthropod molting hormones which are responsible for induction of molting, development, and somatic growth. The actions of ecdysteroids are mediated through a nuclear receptor, ecdysteroid receptor (EcR). Insect EcRs must dimerize with ultraspiracle (USP), an insect homolog of the vertebrate retinoid-X receptor (RXR), to form a functional receptor. Ligand-binding abilities of steroid hormone receptors and nuclear receptors may have evolved after the evolutionary separation of the invertebrates and the vertebrates. EcR is the only invertebrate nuclear receptor which has ligand-binding ability to ecdysteroids. Most of the vertebrate RXRs are able to bind to *9-cis* retinoic acid (RA) while the insect USPs do not bind to *9-cis* RA. A recent report indicates that insect juvenile hormone, a terpenoid hormone, is a low affinity ligand of *Drosophila* USP but similar studies have not yet been applied to invertebrate RXRs. This study aims to investigate the evolution of the binding ability of the EcRs and RXRs. Results of phylogenetic analyses of the amino acid sequences of the EcRs indicated insect EcRs always clustered together while vertebrate paralogous homologs, FXR and the LXR, situated at a distant position. This suggests that the EcR may have acquired ecdysteroid-binding ability after the separation of arthropods and the vertebrates. Phylogenetic analysis of the C to F domains in RXRs showed that the USPs were separated from the vertebrate RXRs. Two invertebrate RXRs, chelicerate and crustacean RXRs, however, were grouped with vertebrate RXRs, and away from insect

USPs. Similar results were obtained when the analyses were performed on the amino acid sequences of the ligand binding domains. Interestingly, the analyses of the most conserved DNA binding domain clustered all invertebrate RXRs together. Together with substitutions observed within the ligand-dependent transactivation subdomain, the AF-2 core, these results suggest that the invertebrate RXRs may have an evolutionary pathway distinct from vertebrate RXRs and that insect USPs may have acquired a different ligand-binding ability during arthropod evolution.

Introduction

The steroid hormone receptor /nuclear receptor superfamily contains a vast variety of transcription factors found in both the invertebrates and vertebrates. These receptors regulate transcription under the influence of hormones, or other small ligands. Some nuclear receptors can function without ligands (Tsai and O'Mally, 1994; Mangeldorf and Evans, 1995; Enmark and Gustafsson, 1996). Each member generally contains several functional domains, including a ligand independent transactivation domain (A/B domain) at the amino terminal, followed by a DNA binding domain (C domain or DBD), a hinge region (D domain), and ligand-binding domain (E domain or LBD) (Tsai and O'Malley, 1994). The distinguishing characteristic of this superfamily is possession of a conserved DBD with two Cys_2-Cys_2 zinc-finger motifs (Deter-Wadleigh and Fanning, 1994). The DBD binds to a specific DNA sequence, called the hormone response element. In addition, the DBD provides a dimerization interface (Zalluacus et al., 1995). The LBD is the second conserved domain within the superfamily and has several functions in addition to ligand-binding, including dimerization and ligand-dependent transactivation. Structurally, this domain has two heptad repeat sequences that are proposed to be important to dimerization and transactivation (Forman and Sameul, 1990). Results of crystal structure analysis of three nuclear receptors suggest that the LBDs in this superfamily should contain twelve helices and a beta extended region (Bourguet et al., 1995; Renaud et al., 1995; Wagner et al., 1995; Wurtz et al., 1996). Helices three, five and seven, as well as the beta extended sheet regions are suggested to form the putative ligand-binding pocket while helix 12 is shown to contain an important ligand-dependent transactivation subdomain, the AF-2 subdomain.

Two hypotheses about the evolution of the receptors in this superfamily have been proposed. First, these receptors evolved from a combination of several genes of independent origins (Yamamoto, 1985). A second hypothesis suggested that these receptors evolved from a single gene which contained multiple functional domains and subsequently acquired more complex functions (O'Malley, 1989). More studies have supported the latter hypothesis (Amero, 1992, Laudet et al., 1992; Laudet, 1997). Laudet (1997) proposed that all members in this family evolved from an orphan receptor which did not bind to any ligand. This latter theory involves two waves of gene duplication. The first wave of gene duplication provided different families of receptors. Each family member subsequently diverged after the second wave of gene duplication. Steroid hormone receptors and most of the nuclear receptors whose ligands have been identified are found in the vertebrates while most invertebrate nuclear receptors are classified as orphan receptors. Ligand-binding ability of the vertebrate receptors was proposed to be acquired independently after the evolutionary separation of the invertebrates and the vertebrates (Escriva et al., 1997). Interestingly, two arthropod receptor subfamilies, ecdysteroid receptors (EcR) and retinoid-X receptors (RXR), have been shown to have ligands (Koelle et al., 1991; Jones and Sharp, 1997). How these two receptor subfamilies evolved still is unclear.

Ecdysteroids are the molting hormones which govern the molting events in arthropods (Lachaise et al., 1993; Thummel, 1996). The action of ecdysteroids is mediated through the nuclear receptors, the EcRs. Like other members in the nuclear receptor family, the insect EcR forms a functional receptor after it heterodimerizes with an insect RXR homolog, ultraspiracle (USP) (Thomas et al, 1993; Yao et al., 1993, Swevers et al., 1996).

Sequence analysis and phylogenetic analysis suggest that farnesoid X receptor (FXR) and liver-X receptor (LXR) are the vertebrate homologs of EcR (Enmark and Gustafsson, 1996; Laudet, 1997). FXRs may bind farnesoids and LXR binds oxysterols (Forman et al., 1995; Janowski et al., 1996) but they have not be shown to bind ecdysteroids. It is unknown why these structurally similar receptors bind to different ligands. It raises the question whether these receptors acquired binding ability to different ligands after the split of the invertebrates and the vertebrates.

The vertebrate RXRs are receptors of *9-cis* retinoic acid (RA), one of the Vitamin A metabolites (Chambon 1996). RXR often acts as a dimer partner of many vertebrate nuclear receptors, such as retinoic acid receptors (RAR), Vitamin D_3 receptors (VDR), and thyroid hormone receptors (TR) (Mangelsdorf and Evans, 1995; Minucci and Ozato, 1996). Vertebrate RXRs are well characterized and are encoded by five different genes (alpha to epsilon). Three RXR subtypes (alpha, beta and gamma) contain three different isoforms which result from alternative splicing within the A/B domain and different promoter usage (Chambon, 1996). Most vertebrate RXRs are able to bind to *9-cis* RA, except for two zebra fish-specific subtypes (delta and epsilon) (Jones et al., 1995).

Studies on invertebrate RXRs have been limited to the insect RXR homolog, ultraspiracle (USP). *Drosophila* USP is the dimer partner of EcR and *Drosophila* hormone receptor 38 (DHR38), a *Drosophila* homolog of rat nerve growth factor-induced protein B (NGFI-B) (Thomas et al., 1993; Yao et al., 1993; Sutherland et al., 1995). USP may interact with sevenup protein (svp), a *Drosophila* homolog of chicken ovalbumin upstream promotor-transcription factor (COUP-TF) (Zellof et al., 1995a, b). Unlike vertebrate RXRs,

USP does not bind to *9-cis* RA (Thomas et al., 1993; Yao et al., 1993). Recently, *Drosophila* USP was shown to bind a terpenoid hormone, insect juvenile hormone at low affinity (0.5 μM) (Jones and Sharp, 1997). So far, it is still uncertain if during arthropod evolution the invertebrate RXRs acquired binding ability to different ligands.

The AF-2 subdomain is important for ligand-dependent transactivation and interacts with several coregulators to mediate different transactivation abilities of the nuclear receptors (LeDouarin et al., 1996; Minucci and Ozato, 1996). After the ligands bind to the receptor, the helix containing the AF-2 subdomain closes the ligand-binding pocket and the conserved acidic amino acid residues in the AF-2 subdomain form a salt bridge with a basic amino acid residue in helix four (Renaud et al., 1995). Transcriptional activation can occur after the corepressor is released from the AF-2 subdomain (LeDouarin et al., 1996; Minucci and Ozato, 1996). In two recently cloned arthropod RXR genes, the most conserved acidic acid residue in the AF-2 domain of both RXRs is substituted by either a non-polar or a positive charged amino acid (Chung et al., 1998; Guo et al., 1998). This change has been suggested to account for the loss of transactivation ability of the chelicerate RXRs (Guo et al., 1998). Such an amino acid substitution has not yet been reported in insect USPs and other RXRs. This difference suggests that insect USPs may have a distinct evolutionary history from chelicerate and crustacean RXRs.

Laudet (1997) proposed that ligand-binding abilities of the vertebrate receptors in the steroid hormone /nuclear receptor superfamily have been acquired after separation of invertebrates and vertebrates during evolution. In these analyses, I test a null hypothesis that both invertebrate EcRs and USPs gained ligand-binding independently after the evolutionary

separation of the invertebrates and the vertebrates. In addition, I would like to know whether within the arthropods, the RXRs have evolved independently.

This study reports the results of the phylogenetic analysis of EcRs and RXRs. Since invertebrate EcRs are only distantly related to their vertebrate homologs, FXR and the LXR, invertebrate EcRs may have gained binding ability to ecdysteroids during evolution. Phylogenetic analyses of the LBD region suggest that insect USPs might have changed their ligand-binding ability during evolution and they might have an evolutionary history distinct from other invertebrate RXRs. The phylogenetic analysis of EcRs and RXRs also shows that these two receptors can be used to distinguish the different orders of insects and may be useful in future arthropod phylogenetic analyses.

Materials and Methods

Construction of the database

The sequences of EcRs, RXRs and other nuclear receptors were obtained from GenBank using the Entrez program (http://www.ncbi.nlm.nih.gov/Entrez/). Duplicates and partial sequences were not used from the analysis. The amino acid sequences of domains C to F, DBDs, and the LBDs were aligned separately using ClustalW 1.72 with default settings at European Bioinformatics Institute (EBI) (http://www2.ebi.ac.uk/clustalW/) (Thompson et al., 1994). The aligned sequences in PHYLIP format were used for the maximum parsimony method. The aligned sequences in GCG format were used for the neighbor-joining method and protein structure analysis. Pairwise sequence comparison was conducted with ClustalW 1.72 with default settings (gaps included).

Phylogenetic Analysis

The neighbor-joining (N-J) and the maximum parsimony (MP) methods were employed to infer the phylogenetic relationship between the members of the EcR and RXR subfamilies (Fitch, 1977; Saitou and Nei, 1987). The N-J method is a distance matrix method in which the evolutionary distances are computed for all pairs of taxa (Li, 1996). A phylogenetic tree is constructed by using an algorithm based on the distance values. The principle of the neighbor-joining method is to find neighbors sequentially that may minimize the total length of the tree (Saitou and Nei, 1987). The N-J trees based on the p-distance (fraction of sites that differ) were generated using the molecular evolutionary genetics analysis (MEGA) program (version 1.01) (Kumar et al., 1993). Other distance measure methods (number of differences and Possion correction) were used (Kumar et al., 1993), the topologies of the trees, however, were identical except with slightly different branch lengths. Gapped sites in the alignments were deleted in a pairwise fashion. 1000 replications of nonparametric bootstrap were performed to measure the statistical reliability of the tree topology (Felsenstein, 1985; Kumar et al., 1993). Greater than or equal to 90 % of bootstrap values in trees constructed by the N-J method were considered to be significant.

Maximum parsimony (MP) method is another common method to reconstruct phylogenetic relationships (Fitch, 1977). In this method, character-states (e.g. the nucleotide or amino acid at a site) are used and the optimal phylogenetic tree is the tree that requires the fewest number of character-state changes (Li, 1996). Gene trees were constructed by the programs in the phylogeny inference package (PHYLIP) (version3.572c) (Felsenstein, J. 1995). The Seqboot program resampled each data set 1000 times for bootstraps. Maximum

parsimony trees were generated with the Protpars program with 1000 resampled data sets. The Consense program was finally used to generate a consensus tree with bootstrap values. Greater than or equal to 90 % of bootstrap values in trees constructed by the MP method were considered to be a strong support. Bootstrap values from 50% to 89% were considered to be moderate supports.

The amino acid sequence of human retinoic acid receptor γ (HsaRARγ) was used as an outgroup. The obtained phylogenetic trees from PHYLIP programs were drawn with Treeview (version 1.5) (Page, 1996). The bootstrap values were listed beside the interior nodes of the tree.

Analysis of protein structure

Plots of the percentage of identity per site of amino acid sequences, and helical view of AF-2 domain were done using PlotSimilarity and Helicalwheel programs in the Genetics Computer Group (GCG) Sequence Analysis Packages (version 9.0) (University of Wisconsin, Madison). PlotSimilarity calculated the average identity among all members of a group of aligned sequences at each position in the alignment. The percentage of identity at each amino acid position in an alignment was the arithmetic average of the scores of all possible pairwise symbol comparisons among the sequence symbols at that position. The average percentage of amino acid identity across the entire alignment was indicated as a dotted line in the Figures 3 and 5.

The predictions of the secondary protein structures were performed using three different programs: DSC (King and Sternberg, 1997), PHDsec (Rost and Sander, 1993), and Predator (Frishman and Argos,1997).

Results

Sequence analysis

The amino acid sequences of 15 EcR and 24 RXR sequences from various species (Table 1) were aligned for comparison. Pairwise comparisons were done separately to the DBDs and LBDs of EcRs and RXRs (Table 2 and 3). The DBD of invertebrate EcRs showed more than 86% amino acid identity to each other (Table 2, above diagonal) and about 60% identity to the outgroup, HsaRARγ. Vertebrate EcR homologs, RnoFXR, HsaFXR, and HsaLXRα, exhibited from 68 to 86% amino acid identity to invertebrate EcRs. EcRs of dipterans (AaeEcR, CteEcR, DmeEcR, and LcuEcR) and lepidopterans (BmoEcR, CfuEcR, HviEcR, and MseEcR) showed a higher level of conservation (>94 %) to each other than to other EcRs. The aligned amino acid sequences showed that the differences in the DBD of EcRs appeared mainly around the Distal box (D-box), a region at the second zinc finger necessary for dimerization (Fig. 1a).

The LBDs of the EcR subfamilies showed similar results as the DBDs except for two major differences (Table 2, below diagonal). First, the overall level of identity was lower than those in the DBD, especially when insect EcRs were compared with vertebrate FXRs and the LXR. Second, in contrast to the results of the DBD above, the dipteran EcRs (AaeEcR, CteEcR, DmeEcR, LcuEcR, ScrEcR) did not share a high level of amino acid identity with lepidopteran EcRs (BmoEcR, CfuEcR, HviEcR, and MseEcR) (only about 60%). Furthermore, the average percentage of identity per site was 40% over the whole alignment of the EcR subfamily and no long insertion in the amino acid sequences of the LBD was observed (Fig. 2 and 3). The average percentage of identity per amino acid site of

invertebrate EcRs was about 50% (Fig. 3b).

The DBD of RXRs exhibited high levels of conservation (> 76% amino acid identity) (Table 3, above diagonal) and about 60% identity to the outgroup, HsaRARγ. Three vertebrate RXR subtypes (alpha, beta, and gamma), and invertebrate RXRs exhibited high levels of amino acid identity within each group. In addition, vertebrate RXR subtypes alpha and gamma shared higher percentages of amino acid identity than other RXRs. Like the EcR DBDs, most amino acid substitutions appeared around the D-box region of RXRs (Fig. 1b)

The comparison of the LBDs of the RXR subfamily showed large variation in the percentage of amino acid identity (Table 3, below diagonal). First, vertebrate RXRs maintained a high level of conservation (>76% amino acid identity), especially within each RXR subtype. DreRXRγ was more similar to RXR subtype alpha than subtype gamma. Second, all insect USPs had a lower percentage of identity to vertebrate, chelicerate and crustacean RXRs (<60%). Third, three invertebrate RXRs (AamRXRs and UpuRXR) shared higher amino acid identity to vertebrate RXRs (about 60%) than to insect USPs (36-44%).

In addition, the aligned amino acid sequences indicated that insect USPs had many substitutions and an insertion of about 20 amino acid residues in the beta turn region (Fig. 4). Two zebra fish RXRs (DreRXRδ and ϵ) had an insertion of 14 amino acid residues downstream of the beta turn region. In addition, the region around helix 10 exhibited a high level of amino acid conservation (Fig. 4). The plot of percentage of identity per amino acid clearly indicated the positions of these two insertions when all RXR sequences were compared (Fig 5a). Ignoring the gap produced by DreRXR δ and ϵ, the LBDs of the vertebrates showed high identity along the sequence and the average percentage of identity

per amino acid site was about 60% (Fig 5b). In contrast, the invertebrate RXRs exhibited a low average percentage of amino acid identity per site (35%) (Fig. 5c). When the comparisons were performed within insect USPs (Fig. 5d), the average percentage of amino acid identity per site of insect USPs was about 40% which was still lower than that of invertebrate EcR (about 50%) or vertebrate RXRs (about 60%) (Fig. 3b and 5b).

Using three different programs, twelve helices and a beta extended sheet region were consistently predicted in the LBDs of all EcRs and RXRs (Fig 2 and 4). These predictions were comparable to the structures from both HsaRARγ and HsaRXRα (Bourguet et al., 1995; Renaud et al., 1995). No specific secondary structure was predicted in the insertions of invertebrate RXRs. In contrast, an additional beta extended sheet was predicted in the amino acid insertion of DreRXRδ and ϵ (Fig. 4).

Phylogenetic analysis of the amino acid sequences of the entire C-F domains

Maximum parsimony and neighbor-joining methods were employed to investigate the phylogenetic relationships within the members of the EcR and RXR subfamilies. Most receptors investigated in this study contained isoforms which were different from each other only within the transactivation domain (A/B domain). In order to decrease the complexity, tree data of such isoforms were not presented in this paper. Bootstraps were performed to measure the statistical reliability of the tree topology (Felsenstein, 1985; Kumar et al., 1993) and the bootstrap values in percentage were listed beside the interior nodes of the tree.

The maximum parsimony analysis of the EcR subfamily showed that all arthropod EcRs formed a monophyletic group with strong support (100%) (Fig. 6a). Vertebrate FXRs and the LXR clustered with EcRs with strong support (100%) although they were at very

distant position. The two vertebrate FXRs were clustered together with a strong support (100%).Within the invertebrate group, although dipteran EcRs (AaeEcR, CteEcR, DmeEcR, and LcuEcR) and lepidopteran EcRs (BmoEcR, CfuEcR, HviEcR, and MseEcR) were significantly separated from others, they were clustered together and strongly supported by the bootstrap values (98% and 100%). Another insect EcR (TmoEcR) occupied the more distant position from dipterans and lepidopterans, contrary to expectation, clustering between the chelicerate EcR (AamEcRA1) and crustacean EcR (UpuEcR) with weak to moderate support. The neighbor-joining method showed a similar pattern. Chelicerate, coleopteran, and crustacean EcRs, however, were clustered as a sister group with a high bootstrap value (95%) (Fig. 6b).

The maximum parsimony analysis of the RXR subfamily showed that the insect USPs (AaeUSP, BmoUSP, CteUSP, DmeUSP, and MseUSP) were significantly separated from other invertebrate and all vertebrate RXRs (Fig. 6a). This separation was strongly supported by high bootstrap value (100%). High bootstrap values also support the lepidopteran USPs were different from dipteran USPs (100%). Two chelicerate RXRs (AamRXR1 and 2) and a crustacean RXR (UpuRXR) were strongly clustered with the vertebrate RXRs (99%) although they were situated in a distant position from vertebrate RXRs. Two AamRXR subtypes were strongly clustered together (100%). Most vertebrate RXRs were clustered consistently with their subtypes (alpha, beta, and gamma) with moderate bootstrap values (57 to 80%). Vertebrate RXR subtype beta branched off early from other subtypes with two zebra fish RXRs (DrRXR δ and ϵ) with moderate support (80%). RXR subtypes alpha and gamma were clustered separately with a moderate bootstrap

values (57 and 79%, respectively). Interesting, zebra fish RXRα (DreRXRα) was grouped with RXR subtype gamma and DreRXRγ was clustered with RXR subtype alpha. This situation was repeated in subsequent analyses. Within each vertebrate RXR subtype, the order of the receptors was identical to the phylogeny of the vertebrates. The neighbor-joining method showed similar results with slightly different bootstrap values (Fig. 6b).

Phylogenetic analysis of the amino acid sequences of C domain (DNA binding domain)

Although the DNA binding domain is the most conserved region within the steroid hormone receptor /nuclear receptor family, the EcR and RXR subfamilies were clearly separated from each other by phylogenetic analyses using DBD sequences (>98%) (Fig. 7a and b).

Results from the maximum parsimony analysis of the DBD of the EcR subfamily showed similar tree topology as with the entire C-F domains but with lower bootstrap values (Fig. 7a). Chelicerate EcR (AamEcRA1) and coleopteran EcR (TmoEcR) were, however, strongly clustered (92%). The neighbor-joining method also gave similar results (Fig. 7b).

Results from the maximum parsimony analysis of the DBD of the RXR subfamily (Fig. 7a) showed different results from the entire C-F domains (Fig. 7). First, all invertebrate RXRs were clustered with a moderate bootstrap support (89%). Deeper relationships within invertebrate RXRs were not robust because of low bootstrap support (< 51%). Second, the groupings of the vertebrate RXR subtypes were not strongly supported (<75%). Only RXR subtype beta was clustered with moderate support (77%). DreRXRδ and ϵ, were also clustered together (80%). Neighbor-joining method (Fig. 7b) and maximum likelihood method (Chung, unpublished data) supported the separation of vertebrate RXRs and

invertebrate RXRs (>80%) but these two methods were also unable to resolve any deeper relationship within invertebrate RXRs.

Phylogenetic analysis on amino acid sequences of E domain (ligand binding domain)

The ligand binding domain (LBD) is the second most conserved domain within the steroid hormone receptor /nuclear receptor superfamily. From the analyses of this domain, the EcR and RXR subfamilies were clustered separately with strong support (100%) (Fig. 8).

Maximum parsimony analysis of the LBD of the EcR subfamily (Fig. 8a) showed similar results as the C-F domains and the C domain alone (Fig. 6a and 7a). Both neighbor-joining (Fig. 8b) and maximum likelihood (Chung, unpublished data) methods still clustered chelicerate, coleopteran, and crustacean EcRs (AamEcRA1, TmoEcR, and UpuEcR) as an individual group although the bootstrap support value from maximum likelihood (64%) was lower than from neighbor-joining (89%).

Results from the maximum parsimony analysis of the LBD of the RXR subfamily (Fig. 8a) were similar to those of C-F domains (Fig. 6a), but different from the results of the DBD (Fig. 7a). The insect USPs were clustered separately from the rest of RXRs (100%). USPs of dipterans (AaeUSP, CteUSP, and DmeUSP) and lepidopterans (BmoUSP and MseUSP) were separated with strong support (> 97%). Chelicerate and crustacean RXRs occupied the most derived position, with vertebrate RXRs as sister groups (91%). Within the vertebrate RXRs, subtypes were separated from each other with moderate support. The cluster of mammalian RXR subtype beta were strongly supported (100%). Amphibian RXRβ (XlaRXRβ) was clustered with other RXR subtypes and clustered closely with two

zebra fish RXRs (DrRXR δ and ϵ) (73%). Vertebrate RXR subtypes alpha and gamma were clustered together with moderate support (60%). Results from both the neighbor-joining method (Fig. 8b) and maximum likelihood method (Chung, unpublished data) supported the separation of insect USPs from other RXRs (100%).

Analysis on the AF-2 subdomain

Two critical acidic residues in the AF-2 subdomain are necessary for the formation of a salt bridge with a basic residue in helix four after the nuclear receptor binds to ligand (Renaud et al., 1995). These two acidic residues are separated by two pairs of hydrophobic residues within an AF-2 core (Wurtz et al., 1996). Charged amino acids are located on one side while the hydrophobic amino acids are located on the other side to form an amphipathic alpha helix.

Amino acid sequences of the AF-2 subdomain from representative receptors were aligned in Figure 9a. As illustrated by both HsaRXRα and HsaRARγ (Fig 9b), these two acidic amino acids (Fig. 9a) are located on one side of an amphipathic alpha helix to form a functional ligand-dependent activation subdomain (Durand et al., 1994). From the alignment of amino acid sequences, the AF-2 cores of EcRs and RXRs were different from each other but the properties of critical positions were mostly conserved (Fig 9a). The AF-2 cores in the EcR subfamily were very similar while the AF-2 cores in RXRs had some variations in the sequences. First, all vertebrate RXRs showed identical sequences in the AF-2 cores (Fig. 4). Invertebrate RXRs, especially insect USPs, contained some amino acid substitutions although the physicochemical properties were similar (Fig. 9a). Second, the critical acidic residues in the AF-2 core were replaced by other residues in lepidopteran

USPs, and chelicerate /crustacean RXRs (AamRXRs and UpuRXR) (Fig. 9a).

Looking at the helical view, the AF-2 cores of all EcRs had similar conformations which were represented by BmoEcR, DmeEcR, UpuEcR and RnoFXR (Fig. 9c). Charged amino acids were located on one side while the hydrophobic amino acids were located on the other side. UpuEcR, vertebrate FXR and the LXR exhibited a similar amphipathic array as HsaRXRα. All insect EcRs contained extra charge residues in the AF-2 cores (Fig. 9c). Lepidopteran and coleopteran EcRs possessed three acidic residues while all dipteran EcRs contained a basic residue, clustering within three acidic residues in the AF-2 cores.

The AF-2 cores of all vertebrate RXRs produced identical amphipathic helices (represent by HsaRXRα in Fig. 9b). Although amphipathic conformation was seen in invertebrate RXRs, all invertebrate RXRs did not have a consistent arrangement. First, three dipteran USPs appeared slightly different from each other (Fig. 9d). The charge distribution of AaeUSP was identical to that of HsaRXRα (Fig. 9b). CteUSP had a similar charge distribution as HsaRARγ but the positions of these charged residues were shifted. DmeUSP possessed three negative charges similar to the AF-2 core of human estrogen receptor α (HsaERα) (Fig. 9a and d). In addition, lepidopteran USPs (Fig. 9e) lack the second acidic residue within the AF-2 core. Basic residues appeared within the lepidopteran AF-2 core and this arrangement was similar to the AF-2 cores in some orphan receptors, such as COUP-TF (Fig. 9a). Furthermore, the most critical acidic amino acid in the AF-2 domain was replaced by non-polar amino acid in two chelicerate RXRs (AamRXRs) (Fig. 9f). The crustacean RXR (UpuRXR) had an interesting arrangement (Fig. 9g). Both acidic amino acid residues in the AF-2 core were lost and one of these positions was replaced by a positively charged

lysine residue. There was no compensatory change in helix four of UpuEcR (Fig. 4).

Discussion

Comparative protein structure

The only invertebrate nuclear receptors which have reported ligand-binding ability are EcRs and USPs (Koelle, 1991, Jones and sharp, 1997). In insects, dimerization of EcR (with USP) is required not only for DNA binding but also for ecdysteroid binding (Thomas et al., 1993; Yao et al., 1993). Evolution of these two receptors may closely reflect arthropod evolution.

Both EcR and RXR proteins analyzed in this study show that they have highly conserved DBDs with two zinc fingers. Similar to other nuclear receptors, such as thyroid hormone receptors and retinoic acid receptors, EcR should dimerize with RXR in the form of a head-to-tail array when interacting with direct repeat (DR) hormone response elements (HRE) (Perlmann et al., 1993). An important area at the base of the second zinc finger is the D-box which governs the dimerization of the receptors and specifies a spacer length HRE half-sites (Zilliacus et. al., 1995). In this study, the majority of amino acid substitutions are found at the D-box of both EcRs and RXRs. These substitutions may be related to the different binding characteristics to hormone response elements, suggesting that each receptor may have developed different preferences on specific HREs during evolution. Several arrangements of hormone response elements with different spacings have been shown in EcRs and RXRs (reviewed in Pfahl et al., 1994; Cherbas and Cherbas, 1996). The canonical HRE half site of EcRs is predicted to be AGGTCA (Cherbas and Cherbas, 1996). From the

studies in DmeEcR, the hormone response elements contain directly repeated half sites as well as a palindrome (reviewed in Cherbas and Cherbas, 1996). Several laboratories have demonstrated that EcR/USP binds well *in vitro* to sequences of the form direct repeat with spacing 3 (DR3), DR4, and DR5 (D'Avino et al., 1995; Antoniewski et al., 1996).

In the EcR subfamily, the amino acid substitutions in the LBDs are not extensive within the invertebrate clade. The helices of the LBD in the steroid hormone/nuclear receptor superfamily should function at several levels. First, the helices should provide structures for some basic functions, such as dimerization, that are universally observed in this superfamily. Second, after the receptors acquired ligand-binding ability during evolution, the helices should allow for binding a specific ligand. In these studies, the substitutions in the LBDs of the EcR subfamily mainly appear between predicted helices, suggesting that the basic functions provided by these helices are maintained in the EcR subfamily. In contrast, the low percentage of identity of the LBDs between invertebrate EcRs and vertebrate homologs (FXRs and LXR) suggests that EcRs, FXRs and LXR may have different ligand abilities. Ligands of EcRs have been shown to be ecdysteroids (Koelle et al., 1991; Thomas et al., 1993; Yao et al., 1993), FXRs bind farnesoids and the LXR binds oxysterols (Forman et al., 1995; Janowski et al., 1996).

The LBDs of vertebrate RXRs show a very high level of amino acid conservation, except that two zebra fish RXRs (DreRXRδ and ϵ) have an insertion of 14 amino acid residues downstream of the putative ligand-binding site. An extra beta extended sheet is predicted at this insertion and may be related to the inability of DreRXRδ and ϵ to bind *9-cis* RA (Jones et al., 1995). In contrast, the LBDs of insect USPs have more substitutions than

the vertebrate RXRs. These substitutions may alter the overall architecture of invertebrate RXRs. In addition, an insertion of amino acids appears within the putative ligand bind site of USPs. These observations support the conclusion that the ligand-binding specificities of USPs are different from those of vertebrate RXRs.

Unlike helix 10 of the EcR subfamily, the helix 10 in the LBDs of the RXR subfamily is highly conserved. This region is also called the ninth heptad repeat and is important for dimerization (Forman and Samuel, 1990). Mutation within this region may inhibit the dimerization ability of RXRs (Leng et al., 1995; Zhang et al., 1995; Perlmann et al., 1996; Lee et al., 1998). Since RXRs are able to dimerize with receptors from different subfamilies, such as EcRs, thyroid hormone receptors, and retinoic acid receptors, it may be an advantage for RXRs to have a conserved dimerization interface in the LBD for dimerization with various partners. The high levels of amino acid conservation at helix 10 support this hypothesis.

Another major distinct difference is in the AF-2 subdomain, the ligand-dependent activation subdomain. Two acidic amino acid residues and four hydrophobic amino acid residues together with other amino acids form an amphipathic helix. The AF-2 cores of EcRs, FXRs and LXR have similar arrangements as other functional AF-2 cores, suggesting that the members in the EcR subfamily should have ligand-dependent transactivation ability as other nuclear receptors, such as retinoic acid receptor (RAR), thyroid hormone receptor (TR), and Vitamin D_3 receptor (VDR). Interestingly, all insect EcRs have extra charges within the AF-2 core, but the function of these extra charges is unknown. Unlike EcR, there are some variation in this region of RXRs. The AF-2 cores of vertebrate RXRs are highly

conserved in amino acid sequences and provide an amphipathic helix. In contrast, some substitutions are observed in invertebrate RXRs. The AF-2 cores of all invertebrate RXRs do not demonstrate a consistent pattern. Extra charges are seen in some USPs (as in the insect EcRs). Chelicerate and crustacean RXRs have amino acid substitutions of the acidic residues necessary for the formation of a salt bridge with a basic residue in helix four, suggesting that these three RXRs possess different transactivation ability, if at all (Chung et al., 1998; Guo et al., 1998).

Evolution of EcRs and RXRs

Multigene families are thought to be generated by a number of mechanisms, including genome duplication, tandem gene duplication, domain shuffling, and processed pseudogenes (Li, 1996). There is considerable evidence that the eukaryotic genome has undergone multiple genome duplication events, with the most recent duplication occurring about 500 million years ago (Nadeau, 1991; Philippe et al., 1994). This duplication occurred after the divergence of the lineages leading to the invertebrates and the vertebrates. The goal of molecular evolutionary studies is to explore the mechanisms by which genes and/or genomes have evolved. Furthermore, orthology and paralogy are two essential concepts related to homology (Fitch, 1970; Patterson, 1988). Two genes are said to be orthologous if they are derived from a speciation event, but paralogous if they are derived from a duplication event (Miyamoto and Cracraft, 1991). Phylogenetic analysis enables us to determine relationships between closely related genes or proteins. In spite of a relatively small data set, results from this study may provide several suggestions about how the

diversity of the EcRs and RXRs evolved.

Gene duplication that gave rise to the EcR and RXR subfamilies should have occurred long before the evolutionary separation of the invertebrates and the vertebrates in the Cambrian period more than 600 million years before the present time (Ayala et al., 1998). Results from phylogenetic analysis support the monophyletic origin of both EcRs and RXRs, in agreement with the results from prior studies (Amero, 1992, Detra-Wadleigh and Fanning, 1994; Escriva et al., 1997; Laudet et al., 1992; Laudet, 1997). It is, however, unclear how the two subfamilies are related to each other in terms of ancestor-descendant polarity because the trees generated are basically unrooted trees although HsaRARγ is assigned to the outgroup.

This study suggests that vertebrate FXR and LXR branch off early from arthropod EcRs although the previous phylogenetic studies clustered the DmeEcR with FXR and LXR, suggesting FXR and LXR to be vertebrate homologs of EcRs (Enmark and Gustafsson, 1996; Laudet, 1997). In vertebrate receptors, steroid hormone receptors are suggested to have evolved after nuclear receptors, which bind to non-steroid compounds (O'Malley, 1989; Baker, 1997). EcR binds to ecdysteroids, polyhydroxy steroids. There is no evidence that either FXR or LXR can bind ecdysteroids. FXR has been shown to bind farnesoids and the LXR binds oxysterols, such as 25-, 26-, or 27-hydoxycholesterol (Forman et al., 1995; Janowski et al., 1996). These findings suggest that FXR and LXR may have diverged early from EcRs. After the evolutionary separation of the invertebrates from the vertebrates, invertebrate EcRs may have gained binding ability to ecdysteroids independently during evolution.

In addition, the evolution of EcRs, as well as USPs, may be closely related to arthropod evolution. Within invertebrate EcRs, two clusters are robustly supported by bootstrapping, dipteran and lepidopteran EcRs. The dipteran and lepidopteran EcRs are always distinguishable from others and form a monophyletic group. Similar results are observed in insect USPs. These results are congruent with other arthropod phylogenies (Kristensen, 1991; Whiting et al., 1997), suggesting dipterans and lepidopterans may be closely related.

One of the puzzling results from this study is lack of assignment of the coleopteran EcR to the insect clade because it always situates between chelicerate and crustacean EcRs. The bootstrap support values of this cluster are often moderate. Similar situations have been discussed in another study (Guo et al., 1997). The low bootstrapping support on the topology formed by these three EcRs may be either due to an insufficient number of arthropod EcRs to provide a satisfactory resolution, or the possibility that coleopterans may be more closely related to chelicerates and crustaceans than to dipterans and lepidopterans.

The gene duplication that produced the currently observed RXR subtypes seems to have involved an evolutionary event after the vertebrates separated from the invertebrates in the Cambrian period (Carroll, 1988; Valentine, 1994; Philippe et al., 1994). This is best demonstrated by the case of vertebrate RXRs. Mammalian, avian, amphibian, and teleost RXRs fit well into each RXR subtype clade and are clearly separated from invertebrate genes. In addition, the history of the vertebrate RXRs appears to involve both paralogous and orthologous evolutionary events.

Results of phylogenetic analysis suggest that vertebrate RXR subtypes are an

example of paralogy. This study indicates that these three RXR subtypes may be generated by two gene duplications. First, subtype beta and the ancestor of subtypes alpha and gamma have been generated, followed by the duplication to produce subtypes alpha and gamma. Similar gene duplications were also reported in vertebrate steroid hormone receptors (Baker, 1997). If these duplications occurred in this superfamily, it would support the hypothesis that duplications involving most of the genome occurred when vertebrates evolved from simple chordates (Lundin, 1993; Ohno, 1993; Holland et al., 1994; Sidow, 1996; Endo et al., 1997). Searching for receptor genes in lower chordates, such as agnathans, urochordates, or cephalochordates should help to determine exactly when, on the evolutionary scale, the subtypes have been generated, and which subtype is close to the ancestor RXR.

Orthologous gene evolution, which by definition must be congruent with organismal systematics, can be observed on more than one level. Each vertebrate RXR subtype represents the phylogeny of vertebrate species. A puzzling result from these analyses is the designation of the DreRXRα and γ to the appropriate clades. The phylogenetic analyses always clustered DreRXRα with RXR subtype gamma, and vice versa. This incongruence may have been caused by the designation problem.

Furthermore, the designation of DreRXR δ and ϵ may give a cue about the evolution of vertebrate RXR subtypes. These two RXRs have not been reported in other vertebrates so far. In this study, they often cluster together with RXR subtype beta at the most distant position. These two RXRs may closely relate to an ancestor of RXR beta subtype. They may reflect that the RXR ancestor could be an orphan receptor, or an insertion, which may result in the inability to bind ligand, and could have occurred when these RXRs were generated

during gene duplication in teleost evolution. It will be interesting to know whether RXRβ exists in fish.

Orthology is also evidenced by the lack of overlap between genes from different organismal groups. In this study, the arthropod RXRs are separated from any of the vertebrate RXR subtypes. Investigation on the evolution of Paired box (*Pax*) genes also gives similar results (Balczarek et al., 1997). In the vertebrate lineage, gene duplication has doubled the number of *Pax* genes in each vertebrate *Pax* group. Similar gene duplications of other genes are not observed in the invertebrates (Endo et al., 1997), suggesting that these gene duplications may be absent in the invertebrates during evolution. So, far, it is not yet known whether there are multigenic subtypes of RXR in the invertebrates although two putative RXR subtypes are found in a chelicerate (Guo et al., 1998).

A model is proposed to describe the evolution of EcRs and RXRs. After gene duplications have produced these two families, arthropod EcRs might have acquired ecdysteroid-binding ability during arthropod evolution as the vertebrate FXR and LXR gained different ligand-binding abilities. The evolution of RXRs may be more complex. From the results of phylogenetic analysis on the conserved DBD region, all invertebrate RXRs reported in this study should have evolved together after the evolutionary separation of the invertebrates and the vertebrates. Vertebrate RXRs might have acquired retinoic acid-binding ability after this separation while the ancient invertebrate RXRs might still have been unliganded receptors. During arthropod evolution, insect USPs may have acquired a ligand-binding ability and acquired a functional ligand-dependent transactivation subdomain. Amino acid insertions in the putative ligand-binding site and the variations of AF-2 cores of

the LBD may correspond to the change in ligand-binding specificity of insect USPs. That DmeUSP can bind juvenile hormone also supports this speculation (Jones and Sharp, 1997). It may explain why many more mutations in the LBD are observed in USPs than other invertebrate RXRs. USPs became functional ligand-dependent transcription factors after divergence of insects from other arthropods. This change may be due to selective advantages of the mutated gene (Li, 1996). The underlying mechanism is unknown. It would be interesting to know whether the chelicerate and crustacean RXRs are able to bind retinoic acid since their amino acid sequences in the LBDs are similar to vertebrate RXRs, and whether they have a different ligand-dependent transactivation mechanism because of the changes in the AF-2 cores.

The topologies of the phylogenetic trees of insect EcRs and USPs were congruent with other ribosomal DNA phylogenies and agreed with traditional morphological classification in insects (Kristensen, 1991; Whiting et al., 1997). Insufficient data of EcRs from arthropods other than dipterans and lepidopterans, however, are available to support that coleopteran EcR is closer to insect EcRs than to the chelicerate or crustacean EcR. The EcR and RXR genes may be candidate genes for future phylogenetic analysis in invertebrates.

Finally, it is known that different genes can evolve at different rates, and recent analyses have shown that the different evolutionary rates may mislead the estimation of phylogenetic history (Yang, 1996 and references therein). It is dangerous to infer species phylogenies from a single-gene phylogeny. The EcR and RXR phylogenies presented here should be interpreted cautiously, as a guideline and a stimulus for future work with these and

other molecules. Future studies could improve the resolution by including more representatives from arthropods other than dipterans and lepidopterans. Increased resolution and confidence in the phylogenetic analysis would be expected by increasing the number of loci or amino acid sequences analyzed, rather than by increasing the length of the EcR and RXRs proteins analyzed.

Conclusion

The EcR and RXR subfamilies evolved from common ancestors before the evolutionary separation of the invertebrates and the vertebrates. Ligand-binding abilities may have been acquired later in the invertebrate and vertebrate clades of these two receptor families after the divergence of the invertebrates and the vertebrates.

Literature Cited

Antoniewski, C., B. Mugat, F. Delbac, and J.-A. Lepesant. 1996. Direct repeats bind the EcR/USP receptor and mediate ecdysteroid responses in *Drosophila melanogaster*. Mol. Cell. Biol. **16**:2977-2986.

Amero, S. A., R. H. Kertsinger, N. D. Moncrief, K. R. Yamamoto, and W. R. Pearson. 1992. The origin of nuclear receptor proteins: A single precursor distinct from other transcription factors. Mol. Endocrinol. **6**:3-7.

Ayala, F.J., A. Rzhetsky, F. J. Ayala. 1998. Origin of the metazoan phyla: molecular clocks confirm paleontological estimates. Proc. Natl. Acad. Sci. USA **95**:606-611.

Baker, M. E. 1997. Steroid receptor phylogeny and vertebrate origins. Mol. Cell. Endocrinol. **135**:101-107.

Baker, A. R., D. P. McDonnell, M. Hughes, T. M. Crisp, D. J. Mangelsdorf, M. R. Maussler, J. W.. Pike, J. Shine, and B. W. O'Malley. 1988. Cloning and expression of full-length cDNA encoding human vitamin D receptor. Proc. Natl. Acad. Sci. USA **85**:3294-3298.

Balczarek, K. A., Z-C. Lai, and S. Kumar. 1997. Evolution and functional diversification of the Paired Box (*Pax*) DNA-binding domains. Mol. Biol. Evol. **14**:829-842.

Blumberg, B.,D. J. Mangelsdorf, J. A. Dyck, D. A. Bittner, R. M. Evans, and E. M. De Robertis. 1992. Multiple retinoid-responsive receptors in a single cell: families of retinoid 'X' receptors and retinoic acid receptors in the Xenopus egg. Proc. Natl. Acad. Sci. U.S.A. **89**:2321-2325.

Bourguet, W., M. Ruff, P. Chambon, H. Gronemeyer, and D. Moras. 1995. Crystal structure of the ligand-binding domain of the human nuclear receptor RXR-α. Nature **375**:377-382.

Chambon, P., 1996. A decade of molecular biology of retinoic acid receptors. FASEB J. **10**:940-954.

Cherbas, P., and L. Cherbas. 1996. Molecular aspects of ecdysteroid hormone action. Pp. 175-221 *in* G. I. Gilbert, ed. Metamophosis/ Academic Press. San Diego.

Cho, W.-L., M. Z. Kapitskaya, and A. S. Rackhel. 1995. Mosquito ecdysteroid receptor: analysis of the cDNA and expression during vitellogenesis. Insect Biochem. Molec. Biol. **25**:19-27

Chung, A. C.-K., S. W. Clifton, B. A. Roe, D. S. Durica, and P. M. Hopkins. 1998. Cloning of crustacean EcR and RXR gene homologs and elevation of RXR mRNA by retinoic acid.

Mol. Cell. Endocrinol. In press.

D'Avino, P. P., S. Crispi, L. Cherbas, P. Cherbas, and M. Furia. 1995. The moulting hormone ecdysone is able to recognize target elements composed of direct repeats. Mol. Cell. Endocrinol. **113**:1-9.

Detera-Wadleigh, S. D., and T. G. Fanning. 1994. Phylogeny of the steroid receptor superfamily. Molec. Phylogen. Evol. **3**:192-205.

Durand, B., M. Saunders, M. Gaudon, B. Roy, R. Losson, and P. Chambon. 1994. Activation function 2 (AF-2) of retinoic acid receptor and *9-cis* retinoic acid receptor: presence of a conserved autonomous constitutive activating domain and influence of the nature of the response element on AF-2 activity. EMBO J. **13**: 5370-5382.

Endo, T., T. Imanishi, T. Gojobori, and H. Inoko. 1997. Evolutionary significance of intra-genome duplications on human chromones. Gene **205**:19-27.

Enmark, E., and J.-A. Gustafsson. 1996. Orphan nuclear receptors - the first eight years. Mol Endocrinol. **10**:1293-1307.

Escriva, H., R. Safi, C. Hanni, M.-C. Langois, P. Saumitou-Laprade, D. Stehelin, A. Capron, R. Pierce, and V. Laudet. 1997. Ligand binding was acquired during evolution of nuclear receptors. Proc. Natl. Acad. Sci. USA **94**:6803-6808.

Felsenstein, J. 1985. Confidence limits in phylogenies: An approach using the bootstrap. Evolution **39**:383-791.

Felsenstein, J. 1995. Phylogenetic inference program (PHYLIP) manual version 3.572c. Unversity of Washington, Seattle.

Fitch, W. 1970. Distinguishing homologous from analogous proteins. Systemat. Zool. **19**:99-113.

Fitch, W. 1977. On the problem of discovering the most parsimonious tree. Am. Nat. **111**:223-257.

Forman, B. M., and H. H. Samuels. 1990. Interactions among a subfamily of nuclear hormone receptors: The regulatory zipper model. Mol. Endocrinol. **4**:1293-1301.

Forman, B. M., E, Goode, J. Chen, A. E. Oro, D. J. Bradley, T. Perlmann, D. J. Noonan, L. T. Burka, T. McMorris, W. W. Lamph, R. M. Evans, and C. Weinberger. 1995. Identification of a nuclear receptor that is activated by farnesol metabolites. Cell **81**:687-693.

Frishman, D., and P. Argos. 1997. 75% accuracy with a new approach in utilizing related sequences for protein secondary structure prediction. Proteins **27**:329-335.

Fujiwara, H., M. Jindra, R. Newitt, S. R. Palli, K. Hiruma, and L. M. Riddiford. 1995. Cloning of an ecdysone receptor homolog from *Manduca sexta* and the developmental profile of its mRNA in wings. Insect Biochem. Molec. Biol. **25**:845-856.

Gearing, K.L., M. Gottlicher, M. Teboul, E. Widmark, and J. A. Gustafsson. 1993. Interaction of the peroxisome-proliferator-activated receptor and retinoid X receptor. Proc. Natl. Acad. Sci. U.S.A. **90**:1440-1444.

Giguere, V., E. S. Ong, P. Segui, and R. M. Evans. 1987. Identification of a receptor for the morphogen retinoic acid. Nature **330**:624-629.

Green, S., P. Walter, V. Kumar, A. Krust, J.-M. Bornet, P. Argos., and P. Chambon. 1986. Human oestrogen receptor cDNA: Sequence, expression and homology to v-erb A. Nature **320**:134-139.

Guo, X., M. A. Harmon, V. Laudet, D. J. Mangelsdorf, and M. J. Palmer. 1997. Isolation of a functional ecdysteroid receptor homologue from the ixodid tick Amblyomma americanum. Insect Biochem. Mol. Biol. **27**:945-962.

Guo, X., M. A. Harmon, X. Jin, V. Laudet, D. J. Mangelsdorf, and M. J. Palmer. 1998. Isolation of two functional retinoid X receptor subtypes from the ixodid tick Amblyomma americanum. Mol. Cell. Endocrinol. in press.

Hannan, G. N., and R. J. Hills. 1997. Cloning and characterization of LcEcR: a functional ecdysone receptor from the sheep blowfly, Lucilia cuprina. Insect Biochem. Mol. Biol. **27**:479-488.

Holland, P. W. H., J. Garcia-Fernadez, N. A. Williams, and A. Sidow. 1994. Gene duplications and the origins of vertebrate development. Dev. Suppl., 125-133.

Imhof, M. O., S. Rusconi, and M. Lezzi, M., 1993. Cloning of a *Chironomis tentans* cDNA encoding a protein (cEcRH) homologous to the *Drosophila melanogaster* ecdysteroid receptor (dEcR). Insect Biochem. Molec. Biol. **23**:115-124.

Jindra, M., J.-Y. Huang, F. Malone, M. Asahina, and L. M. Riddiford. 1997. Identification and mRNA developmental profiles of two ultraspiracle isoforms in the epidermis and wings of *Manduca sexta*. Insect Molec. Biol. **6**:41-53.

Janowski, B. A., P. J. Willy, T. RamaDevi, J. R. Falck, and D. J. Mangelsdorf. 1996. An oxysterol signalling pathway mediated by the nuclear receptor LXR alpha Nature **383**:728-

731.

Jones, G., and P. A. Sharp. 1997. Ultraspiracle: An invertebrate nuclear receptor for juvenile hormones. Proc. Natl. Acad. Sci. USA **94**:13499-13503.

Jones, B. B., C. K. Ohno, G. Allenby, M. B. Boffa, A. A. Levin, J. F. Grippo, and M. Petkovich. 1995. New retinoid X receptor subtypes in zebra fish (*Danio rerio*) differentially modulate transcription and do not bind *9-cis* retinoid acid. Mol. Cell. Biol. **15**:5226-5234.

Kapitskaya, M., S. Wang, D. E. Cress, T. S. Dhadialla, and A. S. Raikel. 1996. The mosquito *ultraspiracle* homologue, a partner of ecdysteroid receptor heterodimer: cloning and characterization of isoforms expressed during vitellogenesis. Mol. Cell. Endocrinol. **121**:119-132.

King, R. D., and M. J. E. Sternberg. 1997. DSC: public domain protein secondary structure prediction. Comput. Appl. Biosci. **13**:473-474.

Koelle, M. R., W. S. Talbot, W. A. Segraves, M. T. Bender, P. Cherbas, and D. S. Hogness, 1991. The *Drosophila EcR* gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell **67**:59-77.

Kothapalli, R., S. R. Palli, T. R. Ladd, S. S. Sohi, D. Cress, T. S. Dhadialla, G. Tzertzinis, and A. Retnakaran. 1995. Cloning and developmental expression of the ecdysone receptor gene from the spruce budworm, *Choristoneura fumiferana*. Dev. Genet. **17**:319-330

Kristensen, N. P. 1991. Phylogeny of extant hexapods. Pp. 125-140 *in* I. D. Naumann, P. B. Carne, J. F. Lawrence, E. S. Nielsen, J. P. Spradberry, R. W. Taylor, M, J. Whitten, and M. J. Littlejohn, eds. CSIRO, Melbourne University Press, Melbourne.

Krust A., P. Kastner, M. Petkovich, A. Zelent, and P. Chambon. 1989. A third human retinoic acid receptor, hRAR-gamma. Proc. Natl. Acad. Sci. USA **86**:5310-5314.

Kumar, S., K. Tamura, and M. Nei. 1993. MEGA: Molecular Evolutionary Genetics Analysis, version 1.01. The Pennsylvania State University, University Park, PA 16802.

Lachaise, F., A. Le Roux, M. Hubert, and R. Lafont. 1993. The molting gland of crutaceans: Localization, activity, and endocrine control. J. Crust. Biol. **13**:198-234.

Laudet, V. 1997. Evolution of the nuclear receptor superfamily: early diversification from an ancestral orphan receptor. J. Mol. Endocrinol.**19**:207-226.

Laudet, V, C. Hanni, J. Coll, F. Catzeflis, and D. Stehelin. 1992. Evolution of the nuclear receptor gene superfamily. EMBO J. **11**:1003-1013.

LeDouarin, B., E. VomBaur, C. Zechel, D. Heery, M. Heine, V. Vivat, H. Gronemeyer, R. Losson, and P. Chambon. 1996. Ligand-dependent interaction of nuclear receptors with potential transcriptional intermediary factors (mediators). Phil. Trans. R. Soc. Lond. B. **351**:569-578.

Lee, S. K., S. Y. Na, H. J. Kim, J. Soh, H. S. Choi, and J. W. Lee. 1998. Identification of critical residues for heterodimerization within the ligand-binding domain of retinoid X receptor. Mol. Endocrinol. **12**:325-332.

Leid, M., P. Kastner, R. Lyons, H. Nakshatri, M. Saunders, T. Zacharewski, J. Y. Chen, A. Staub, J. M. Garnier, S. Mader, and P. Chambon. 1992. Purification, cloning, and RXR identity of the HeLa cell factor with which RAR or TR heterodimerizes to bind target sequences efficiently. Cell **68**:377-395.

Leng, X., J. Blanco, S. Y. Tsai, K. Ozato, B. W. O'Malley, and M.-J. Tsai. 1995. Mouse Retinoid X receptor contains a separable ligand-binding and transactivation domian in its E region. Mol. Cell. Biol. **15**:255-263.

Li, W.-H. 1996. Molecular evolution. Sinauer Associate, Sunderland.

Li, X.-Y., J.-H. Xiao, X. Feng, L. Qin, and J. J. Voorhees. 1997. Retinoid X receptor-specific ligands synergistically upregulate 1, 25-dihydroxyvitamin D_3-dependent transcription in epidermal keratinocytes *in vitro* and *in vivo*. J. Invest. Dermatol. **108**:506-512.

Lu, H.-C., G. Eichele, and C. Tahaller. 1997. Liganded bounded RXR can mediate retinoid signal transduction during embryogenesis. Development **124**:195-203.

Lundin, L. G. 1993. Evolution of the vertebrate genome as reflected in paralogous chromosomal regions in man and the house mouse. Genomics **16**:1-19.

Mangelsdorf, D. J., and R. M. Evans, 1995. The RXR heterodimers and orphan receptors. Cell **83**:841-850.

Mangelsdorf, D. J., E. S. Ong, J. A. Dyck, and R. M. Evans, R. M., 1990. Nuclear receptor that identifies a novel retinoic acid response pathway. Nature **345**:224-229

Mangelsdorf, D. J., U. Borgmeyer, R. A. Heyman, J. Y. Zhou, E. S. Ong, A. E. Oro, A. Kakizuka, and R. M. Evans. 1992. Characterization of three RXR genes that mediate the action of 9-cis retinoic acid. Genes Dev. **6**:329-344

Marklew, S., D. P. Smith, C. S.Mason, and R. W. Old. 1994. Isolation of a novel RXR from Xenopus that most closely resembles mammalian RXR beta and is expressed throughout early development. Biochim. Biophys. Acta **1218**:267-272.

Minucci, S., and K. Ozato. 1996. Retinoid receptors in transcription regulation. Curr. Opin. Genet. Dev. 6:567-574.

Minucci, S., M. Leid, R. Toyama, J.-P. Siant-Jeannet, V. J. Peterson, V. Horn, J. E. Ishmael, N. Bhattacharyya, A. Dey, I. B. Sawid, and K. Ozato. 1997. Retinoid X receptor (RXR) within the RXR-retinoic acid receptor heterodimer binds its ligand and enhances retinoid-dependent gene expression. Mol. Cell. Biol. **17**:644-655

Miyamoto, M. M., and J. Crcraft. 1991. Phylogenetic analysis of DNA sequences. Oxford University Press, New York.

Mouillet, J. F., J. P. Delbecque, B. Quennedey, and J. Delachambre. 1997. Cloning of two putative ecdysteroid receptor isoforms from *Tenebrio molitor* and their developmental expression in the epidermis during metamorphosis. Eur. J. Biochem. **248**:856-860.

Nadeau, J. H. 1991. Pp. 269-296 *in* K. W. Adolph, ed. Advanced techniques in chromosome research. Marcell Dekker, New York.

O'Malley, B. W. 1989. Did eucaryotic steroid receptors evolve from intracrine gene regulator? Endocrinology **125**:1119-1120.

Ohno, S. 1993. Patterns in genome evolution. Curr. Opin. Genet. Dev. 3:911-914.

Oro, A. E., M. McKeown, and R. M. Evans. 1990. Relationship between the product of the *Drosophila ultraspiracle* locus and the vertebrate retinoid X receptor. Nature **347**:298-301.

Page, R. D. M. 1996. Treeview: An application to display phylogenetic trees on personal computers. Comput. Appl. Biosci. 12:357-358.

Patternson, C. 1988. Homolgy in classical and molecular biology. Mol. Biol Evol. **5**:603-625.

Perlmann, T., P. N. Rangarjan, K. Umesono, and R. M. Evans. 1993. Determinants for selective RAR and TR recognition of direct repeat HREs. Genes Dev. 7:1411-1422.

Perlmann, T., K. Umesono, P. N. Rangarajan, B. M. Forman, and R. M. Evans. 1996. Two distinct dimerization interfaces differentially modulate target gene specificity of nuclear hormone receptors. Mol. Endocrinol. **10**:958-966.

Pfhal, M., Apfel, R., Bendik, I., Fanjul, A., Graupner, G., Lee, M.-O., La-Vista, N., Lu, X.-P., Piedrafita, J., Ortiz, M. A., Salbert, G., Zhang, X.-K., 1994. Nuclear retinoid receptors and their mechanism of action. Pp. 327-382 *in* G. Litwack, ed. Vitamins and hormone, vol. 49, Academic Press, San Diego.

Philippe, H., A. Chenuil, and A. Adoutte. 1994. Can the Cambrian explosion be inferred through molecular phylogeny? Development Suppl. 15-25.

Renaud, J.-P., N. Rochel, M. Ruff, V. Vivat, P. Chambon, H. Gronemeyer, and D. Moras. 1995. Crystal structure of the RAR-γ ligand-binding domain bound to all-*trans* retinoic acid. Nature **378**:681-689.

Rost, B., and C. Sander. 1993. Prediction of protein structure at better than 70% accuracy. J. Mol. Biol. **232**:584-599.

Rowe, A., N. S. Eager, and P. M. Brickell. 1991. A member of the RXR nuclear receptor family is expressed in neural-crest-derived cells of the developing chick peripheral nervous system. Development **111**:771-778.

Saitou, N., and M. Nei, 1987. The neigbhor-joining method: a new method for reconstructing phylogenetic trees. Mol. Biol. Evol. **11**:341-356.

Sidow, A. 1996. Genome duplications in the evolution of early vertebrates. Curr. Opin. Genet. Dev. **6**:715-722.

Swevers, L., J. R. Drevet, M. D. Lunke, and K. Iatrou. 1995. Cloning of the ecdysone receptor of the silkmoth *Bombyx mori* and analysis of its expression during follicular cell differentiation. Insect Biochem. Molec. Biol. **25**:857-866.

Swevers, L., L. Cherbas, P. Cherbas, and K. Iatrou. 1996. *Bombyx* EcR (BmEcR) and *Bombyx* USP (BmCF1) combine to form a functional ecdysone receptor. Insect Biochem. Molec. Biol. **26**:217-221.

Sutherland, J.D., T. Kozolva, G. Tzertzinis, and F. C. Kafatos. 1995. DHR38: A new partner for *Drosophila* USP suggests a novel role for NGFI-B-type nuclear receptors. Proc. Natl. Acad. Sci. USA **92**:7966-7970.

Thomas, H. E., H. S. Stunnenberg, and A. F. Steward. 1993. Heterodimerization of the *Drosophila* ecdysone receptor with retinoid X receptor and *ultraspiracle*. Nature **362**:471-475.

Thompson, J. D., D. G. Higgins, and T. J. Gibson. 1994. Clustal W: improving the sensitivity of progressive multiple sequence alignment through sequence weighing, position-specific gap penalties and weight matrix choice. Nucleic Acids Res. **22**:4673-4680.

Thummel, C.S. 1996. Flies on steroids - *Drosophila* metamorphosis and the mechanisms of steroid hormone action. Trends Genet. **12**:306-310.

Tsai, M.-J., and B. W. O'Malley. 1994. Molecular mechanisms of action of steroid/thyroid receptor superfamily members. Annu. Rev. Biochem. **63**:451-486.

Tzertzinis, G., A. Malecki, and F. C. Kafato. 1994. BmCF1, a *Bombyx mori* RXR-type receptor related to the *Drosophila* ultraspiracle. J. Mol. Biol. **258**:479-486.

Willy, P. J., K. Umesono, E. S. Ong, R. M. Evans, R. A. Heyman, and D. J. Mangelsdorf. 1995. LXR, a nuclear receptor that defines a distinct retinoid response pathway. Genes Dev. **9**:1033-1045.

Wagner, R. L., J. W. Aprilette, M. E. McGrath, B. L. West, J. D. Baxer, and R. J. Fletterick. 1995. A structural role of hormone in the thyroid hormone receptor. Nature **378**:690-697.

Wang, L.-H., S. Y. Tsai, R. G. Cook, W. W. Beattie, M.-J. Tsai, and B. W. O'Malley. 1989. COUP transcription factor is a member of the steroid receptor superfamily. Nature **340**:163-166.

Weinberge, C., C. C. Thompson, E. S. Ong, R. Lebo, D. J. Gruol, and R. M. Evans. 1986. The *c-erb-A* gene encodes a thyroid homone reecptor. Nature **324**:641-646.

Whiting, M. F., J. C. Carpenter, W. D. Wheeler, and W. C. Wheeler. 1997. The strepsiptera problem: Phylogeny of the holometabolous insect orders inferred from 18S and 28S ribomosal DNA sequences and morphology. Syst. Biol. **46**:1-68.

Wurtz, J.-M., W. Bourguet, J.-P. Renaud, V. Vivat, P. Chambon, D. Moras, and H. Gronemeyer. 1996. A canonical structure for the ligand-binding domain of nuclear receptors. Nature Struc. Biol. **3**:87-94.

Yamamoto, K. R. 1985. Steroid receptor regulated transcription of specific genes and gene networks. Annu. Rev. Genet. **19**:209-252.

Yang, Z. 1996. Among-site rate variation and its impact on phylogenetic analysis. TREE **11**:367-372.

Yao, T.-P., B. M. Forman, Z. Jiang, L. Cherbas, J.-D. Chen, M. McKeown, P. Cherbas, and R. M. Evans. 1993. Functional ecdysone receptor is the product of *EcR* and *Ultraspiracle* genes. Nature **366**:476-479.

Yu, V. C., C. Delsert, B. Andersen, J. M. Holloway, O. V. Devary, A. M. Naar, S. Y. Kim, J. M. Boutin, C. K. Glass, and M. G. Rosenfeld. 1991. RXR beta: a coregulator that enhances binding of retinoic acid, thyroid hormone, and vitamin D receptors to their cognate response elements. Cell **67**:1251-1266.

Zelhof, A. C., T.-P. Yao, J. D. Chen, R. M.Evans, and M. McKeown. 1995a. Seven-up inhibits ultraspiracle-based signaling pathways *in vitro* and *in vivo*. Mol. Cell. Biol. **15**:6736-6745.

Zelhof, A. C., T.-P. Yao, R. M.Evans, and M. McKeown. 1995b. Identification and characterization of a *Drosophila* nuclear receptor with the ability to inhibit the ecdysone response. Proc. Natl. Acad. Sci. USA **92**:10477-10481.

Zhang, X.-K., G. Salbert, M.-O. Lee, and M. Pfahl. 1995. Mutations that alter ligand-induced switches and dimerization activities in the retinoid X receptor. Mol. Cell. Biol. **14**:4311-4323.

Zilliacus, J., A. P. H. Wright, J. Carlstedt-Duke, and J.-A. Gustafsson. 1995. Structural determinants of DNA-binding specificity by steroid receptors. Mol. Endocrinol. **9**:389-400.

Table 1 List of EcRs and RXRs used in the sequence analysis

	Species	Abbreviation *	GenBank Accession Number		References
			Amino acid	Nucleotide	
EcR					
Invertebrate					
Chelicerate	*Amblyomma americanum*	AamEcRA1		AF020187	Guo et al., 1997
Crustacean	*Uca pugilator*	UpuEcR		AF034086	Chung et al., 1998
Insect					
Coleopteran	*Tenebrio molitor*	TmoEcR	Y11533		Mouillet et al., 1997
Dipteran	*Aedes aegypti*	AaeEcR	P49880	U02021	Cho et al., 1995
	Chironomus tentans	CteEcR	S60739	P49882	Imhof et al., 1993
	Drosophila melanogaster	DmeEcR	A41055	M74078	Koelle et al.,1991
	Lucilia cuprina	LcuEcR		U75355	Hannan and Hill, 1997
	Sarcophaga crassipalpis	ScrEcR		AF023844	Flannagan et al., unpublished
Lepidopteran	*Bombyx mori*	BmoEcR	P49881	L35266	Swevers et al., 1995
	Choristoneura fumiferana	CfuEcR		U29531	Kothapalli et al., 1995
	Heliothis virescens	HviEcR		Y09009	Martinez et al., unpublished
	Manduca sexta	MseEcR	P49883		Fujiwara et al., 1995
Vertebrate homologs					
Human	*Homo sapiens*	HsaFXR		U68233	Papetti et al., unpublished
		HsaLXRα		U22662	Willy et al., 1995
Rat	*Rattus norvegicus*	RnoFXR		U18374	Forman et al., 1995
RXR					
Vertebrate					
Human	*Homo sapiens*	HsaRXRα	P19793	X52773	Mangelsdorf et al., 1990
		HsaRXRβ	M84820	M84820	Leid et al., 1992
		HsaRXRγ		U38480	Mangelsdorf et al., 1992
Rat	*Rattus norvegicus*	RnoRXRα	Q05343		Gearing et al., 1993
		RnoRXRβ	Ratrcor1a	M81766	Yu et al., 1991
Mouse	*Mus musculus*	MmuRXRα	P28700	X66223	Leid et al., 1992; Mangelsdorf et al., 1992
		MmuRXRβ	P28700	X66224	Leid et al., 1992; Mangelsdorf et al., 1992
		MmuRXRγ	P28705	X66225	Leid et al., 1992; Mangelsdorf et al., 1992
Chicken	*Gallus gallus*	GgaRXRγ	A43781	X58997	Rowe et al., 1991
Frog	*Xenopus laevis*	XlaRXRα	P51128	L11446	Blumberg et al., 1992
		XlaRXRβ	S47633	S73269	Marklew et la., 1994
		XlaRXRγ	P51129	L11443	Blumberg et al., 1992
Zebra fish	*Danio rerio*	DreRXRα		U29940	Jones et al., 1995
		DreRXRγ		U29894	Jones et al., 1995
		DreRXRδ		U29941	Jones et al., 1995
		DreRXRε		U29942	Jones et al., 1995
Invertebrate					
Chelicerate	*Amblyomma americanum*	AamRXR1			Guo et al., 1998
		AamRXR2			Guo et al., 1998
Crustacean	*Uca pugilator*	UpuRXR		AF032983	Chung et al., 1998
Insect	*Aedes aegypti*	AaeUSP			Kapitskaya et al., 1996
	Chironomus tentans	CteUSP		AF045891	Vogtl et al., unpublished
	Drosophila melanogaster	DmeUSP	P20153	X53417	Oro et al., 1990
	Bombyx mori	BmoUSP	P49700	U06073	Tzertzinis et al., 1994
	Manduca sexta	MseUSP	U44837	U44837	Jindra et al., 1997
Outgroup					
Human	*Homo sapiens*	HsaRARγ	P13631	M24857	Krust et al., 1989

* The abbreviations used here are the same as in the text and figures.

Table 2 Amino acid identity score between the DBDs and LBDs of EcRs (% amino acid identity)

Taxon	UpuEcR	AamEcR	TmoEcR	BmoEcR	MseEcR	HviEcR	CfuEcR	DmeEcR	ScrEcR	LcuEcR	AaeEcR	CteEcR	RnoFXR	HsaFXR	HsaLXR-α	HsaRARγ
DNA binding domain																
UpuEcR		95	96	90	90	89	90	89	-	90	89	90	84	86	69	59
AamEcR	64		98	87	87	86	87	86	-	57	86	87	83	84	68	60
TmoEcR	63	57		89	89	87	89	87	-	89	87	89	83	84	68	60
BmoEcR	50	50	57		100	98	100	95	-	98	96	96	81	83	72	56
MseEcR	54	51	58	88		98	100	95	-	98	96	96	81	83	72	56
HviEcR	55	52	57	86	89		98	93	-	96	95	95	80	81	72	56
CfuEcR	52	48	57	81	79	81		95	-	98	96	96	81	83	72	56
DmeEcR	55	52	61	62	61	63	65		-	96	95	95	80	81	71	54
ScrEcR	54	51	59	61	60	62	64	98		-	-	-	-	-	-	-
LcuEcR	56	54	62	62	63	64	63	83	91		98	98	81	83	71	56
AaeEcR	56	54	61	66	67	68	67	83	81	84		96	80	81	69	56
CteEcR	52	49	57	61	64	63	62	69	69	71	75		81	83	71	56
RnoFXR	30	33	31	26	27	26	28	28	27	29	29	27		96	72	59
HsaFXR	31	33	31	27	28	26	23	28	28	29	29	29	94		72	59
HsaLXRα	35	40	38	35	36	35	33	36	34	37	35	35	31	31		56
HsaRARγ	25	30	26	25	24	23	22	22	22	25	23	25	28	22	21	
Ligand binding domain																

Table 3 Amino acid identity score between the DBDs and LBDs of RXRs (% amino acid identity)

Taxon	HsaRXRα	RnRXRα	MmuRXRα	XlaRXRα	DreRXRα	HsaRXRβ	RnoRXRβ	MmuRXRβ	XlaRXRβ	DreRXRδ	DreRXRε	HsaRXRγ	MmuRXRγ	GgaRXRγ	XlaRXRγ	DreRXRγ	AamRXR1	AamRXR2	UpuRXR	DmeUSP	CteUSP	AaeUSP	BmoUSP	MseUSP	HsaRARγ
DNA binding domain																									
HsaRXRα		100	100	96	96	92	92	91	90	95	96	95	95	96	95	98	84	83	86	86	81	86	87	86	62
RnoRXRα	99		100	96	96	92	92	92	90	95	96	95	95	96	95	98	84	83	86	86	81	86	87	86	62
MmuRXR	99	99		96	96	92	92	92	90	95	96	95	95	96	95	98	84	83	86	86	81	86	87	86	62
XlaRXRα	96	96	96		95	90	90	90	92	92	93	92	92	93	95	98	84	83	86	84	81	86	87	86	62
DreRXRα	89	90	90	89		93	93	93	92	92	93	95	95	96	95	96	63	81	84	84	80	84	87	84	63
HsaRXRβ	87	87	87	86	84		100	100	93	90	92	90	90	92	90	92	81	83	83	83	78	84	86	83	60
RnoRXRβ	87	87	87	86	84	98		100	93	90	92	90	90	92	90	92	81	83	83	84	78	83	84	84	60
MmuRXR	87	87	87	86	84	98	100		93	90	92	90	90	95	90	92	81	83	83	83	78	84	84	83	60
XlaRXRβ	84	85	85	85	83	88	88	88		86	80	89	89	90	92	90	81	81	80	81	77	81	84	83	63
DreRXRδ	80	81	81	81	81	79	79	79	80		98	92	92	93	92	93	84	83	83	83	81	86	84	83	60
DreRXRε	87	88	88	87	87	87	87	87	87	85		93	93	95	93	95	84	83	83	83	81	86	84	83	60
HsaRXRγ	87	86	86	86	88	82	82	82	79	77	82		100	98	95	93	81	80	83	83	80	83	84	81	63
MmuRXR	86	85	85	85	88	82	81	81	79	77	82	98		98	95	93	81	80	83	83	80	83	83	81	63
GgaRXRγ	86	85	85	85	87	82	82	82	79	78	83	95	94		96	95	83	80	83	83	80	84	84	83	63
XlaRXRγ	84	83	83	83	87	82	81	81	80	77	80	80	90	89		93	83	80	83	81	80	84	84	83	65
DreRXRγ	94	94	94	93	91	86	85	85	84	81	87	86	85	85	84		84	83	86	86	81	83	87	86	62
AamRXR1	68	67	67	67	67	69	68	68	65	64	66	65	65	66	65	67		92	92	93	93	93	83	95	59
AamRXR2	67	66	66	66	66	65	65	65	63	59	63	66	66	66	66	67	75		92	92	92	89	90	92	56
UpuRXR	65	65	65	65	67	53	64	64	63	58	60	66	67	67	67	65	65	61		93	90	90	90	92	57
DmeUSP	47	47	47	46	41	45	45	45	42	43	40	43	43	44	40	46	44	41	36		92	92	95	96	60
CteUSP	39	39	39	39	36	40	40	40	36	34	35	38	38	40	37	39	38	35	36	47		92	90	92	59
AaeUSP	44	44	44	44	40	45	45	45	44	43	42	40	44	44	43	45	41	40	36	56	54		96	95	62
BmoUSP	42	42	42	42	41	42	42	42	42	37	40	42	41	39	40	41	40	41	36	44	38	49		98	63
MseUSP	44	44	44	44	40	44	44	44	44	39	41	42	44	42	44	43	42	43	37	43	39	49	87		62
HsaRARγ	25	25	25	25	25	24	24	24	24	22	25	25	25	27	25	25	23	25	22	17	18	20	25	24	
Ligand binding domain																									

FIGURE LEGEND

FIG. 1 Aligned amino acid sequences of the DBDs: (a) EcRs and (b) RXRs. The abbreviations are given in Table 1. The regions of DBD, P-box and D-box are marked by the arrows (⇦and ⇨) above the sequences. Identical amino acids to the first sequences are indicated by dots.

FIG. 2 Aligned amino acid sequences of the EcR LBDs. The abbreviations of the receptors are given in Table 1. Identical amino acids to the first sequences were indicated by dots while gaps are represented by hyphens. The amino acids involved in the twelve helices (H1 to H12) predicted by PHDsec program are marked by H above the sequences and those in the beta extended sheet are indicated by E. "EcRs" represents the structure prediction from the sequences of all invertebrate EcRs. "EcR+F/LXR" represents the structure prediction from the sequences of EcRs, FXRs and LXR. Asterisks indicate the region of the AF-2 subdomain at the carboxy terminal of the LBD.

FIG. 3 Percentage of amino acid identity over the aligned sequences of the EcR LBDs: (a) All EcR sequences and (b) Arthropod EcR sequences only. The average identity across the entire alignment is plotted as a dotted line. Y-axis represents the percentage of amino acid identity per amino acid residues in the aligned sequences. Position on x-axis is numbered arbitrarily according to the aligned sequences shown in Fig. 2.

FIG. 4 Aligned amino acid sequences of the RXR LBDs. The abbreviations of the receptors are given in Table 1. Identical amino acids to the first sequences were indicated by dots while gaps are represented by hyphens. HsaRXRa represents the twelve helices (H1 to H12) from results of the crystal structure analysis of human RXR alpha (Bourguet et al., 1995). Amino acids involved in the twelve helices (H1 to H12) predicted by PHDsec program are marked by H above the sequences and those in the beta extended sheet are indicated by E. "VertRXR", "DreXRde", "INV-RXR", and "USPs" represent the structure prediction from the sequences from all vertebrate RXRs, two zebra fish RXR (DrRXRδ and ϵ), all invertebrate RXRs, and all insect USPs, respectively. Asterisks indicate the region of the AF-2 subdomain at the carboxy terminal of the LBD.

FIG. 5 Percentage of amino acid identity over the RXR LBDs: (a) All RXR sequences, (b) vertebrate RXR sequences, (c) all invertebrate RXR sequences (including insect USPs), and (d) insect USP sequences. The average identity across the entire alignment is plotted as a dotted line. Y-axis represents the percentage of amino acid identity per amino acid residues in the aligned sequences. Position on x-axis is numbered arbitrarily according to the aligned sequences shown in Fig. 4. The letter G means that the gap should be absent when comparison is done within that group.

FIG. 6 Phylogenetic trees derived from the analysis of amino acid sequences from the entire C-F domains of EcRs and RXRs. (a) A consensus tree of maximum

parsimony method. (b) A Neighbor-joining tree. The abbreviations of the receptors are given in Table 1. The bootstrap values in percentage are shown next to the each internal node.

FIG. 7 Phylogenetic trees derived from the analysis of amino acid sequences of the DBDs only of EcRs and RXRs. (a) A consensus tree of maximum parsimony method. (b) A Neighbor-joining tree. The abbreviations of the receptors are given in Table 1. The bootstrap values in percentage are shown next to the each internal node.

FIG. 8 Phylogenetic trees derived from the analysis of amino acid sequences of the LBDs only of EcRs and RXRs. (a) A consensus tree of maximum parsimony method. (b) A Neighbor-joining tree. The abbreviations of the receptors are given in Table 1. The bootstrap values in percentage are shown next to the each internal node.

FIG. 9 AF-2 subdomains in EcRs and RXRs. (a) Sequence comparison of the AF-2 subdomains from representative EcRs and RXRs, and other members in the steroid hormone receptor /nuclear receptor superfamily. The assignment of the AF-2 core is according to Wurtz et al., 1996. Asterisk indicates the most critical acidic residues in AF-2 core and letters in bold represent the hydrophobic amino acids. HsaERα (human estrogen receptor alpha, Green et al., 1986); HsaVDR (human Vitamin D_3 receptor, Baker et al., 1988); HsaTRβ (human thyroid hormone receptor beta, Weinberg et al., 1986); HsaRARα (human retinoic acid alpha; Giguere et al., 1987); and HsaCOUP-TF

(human chicken ovalbumin upstream promotor-transcription factor; Wang et al., 1989) (b-g) The helical views of the AF-2 cores of EcRs and RXRs. The hydrophobic amino acids are boxed. Positive or negative charges are added onto the charged amino acids. Asterisk indicates the most critical acidic residues for the formation of a salt bridge with the basic residues in helix four of the LBD.

(a) The EcR subfamily

	P-box		D-box		
DmeEcR	CLVCGDRASG	YHYNALTCEG	CKGFFRRSVT	KSAVYCCKFG	RACEMDMYMR RKCQECRLKK CLAVGM
LcuEcR	....	....	....	.N......	H..... ....
AaeEcR	.......E..	....	....	.N......	H..... ....
CteEcR	....	....	....	.N......	HE.... ....
BmoEcR	....	....	....	.N...I....	H..... ....
MseEcR	....	....	....	.N...I....	H..... ....
HviEcR	....	....	....	.N...I....	H.....I... ....
CfuEcR	....	....	....	.N...I....	H..... ....
TmoEcR	....	....	........I.	.N...Q..Y.	NN..I..... ....
UpuEcR	....	....	........I.	.N...Q..Y.	NN..... ....N...
AamEcRA1	....	....	........I.	KNA..Q..Y.	NN.DI..... ....S...
RnoFXR	.V.....	....	........I.	.N...K..N.	GN.V..... ....D...R. .REM..
HsahFXR	.V.....	....	........I.	.N..QK.YN.	GN.V..... ....R. .KEM..
HsaLXRα	.S....K...	F...V.S...	I	.G.H.I.HS.	GH.PM.T... ....R. .RQA..

(b) The RXR subfamily

	P-box		D-box		
RnoRXRα	CAICGDRSSG	KHYGVYSCEG	CKGFFKRTVR	KDLTYTCRDN	KDCLIDKRQR NRCQYCRYQK CLAMGM
MmmRXRα	....	....	....	....	....
HsaRXRα	....	....	....	....	....
DreRXRγ	....	....	....	....	...M..... ....
XlaRXRα	....	....	....	.........S	...M..... ....
DreRXR5	....	....	....	...S.....	.E..V..... ....
DreRXRe	....	....	....	...S.....	....V..... ....
HsaRXRγ	....	....	........I.	...I.....	....
MmuRXRγ	....	....	........I.	...I.....	....
GgaRXRγ	....	....	........I.	...I.....	....
XlaRXRγ	....	....	........I.	...V.....S	....
DreRXRα	....	....	........I.	....	...Q..... ....
HsaRXRβ	....	....	........I.	....	...TV..... ....T..
RnoRXRβ	....	....	........I.	....	...TV..... ....T..
MmuRXRβ	....	....	........I.	....	...TV..... ....T..
XlaRXRβ	....	.....H....	........I.	.........S	...IV..... ....T..
UpuRXR	.S.....A..	....	....	.....A..EE	RS.T..... ....T...
AamRXR1	.S.....A..	....	....	...S.A..EE	RT.I..... ....C..
AamRXR2	.S.....A..	....	....	.....A..EE	RR.VV..... ....MC..
AaeUSP	.S.....A..	....	....	...S.A..ED	KN.T..... ....C..
CteUSP	.S.....A..	....	....	...S.A..EE	RN.V...K.. ....NC..
DmeUSP	.S.....A..	....	....	.....A..E.	RN.I..... ....TC..
BmoUSP	.S.....A..	....	....	.....A..ED	KN.I..... ....C..
MseUSP	.S.....A..	....	....	.....A..ED	RN.I..... ....C..

FIG. 1

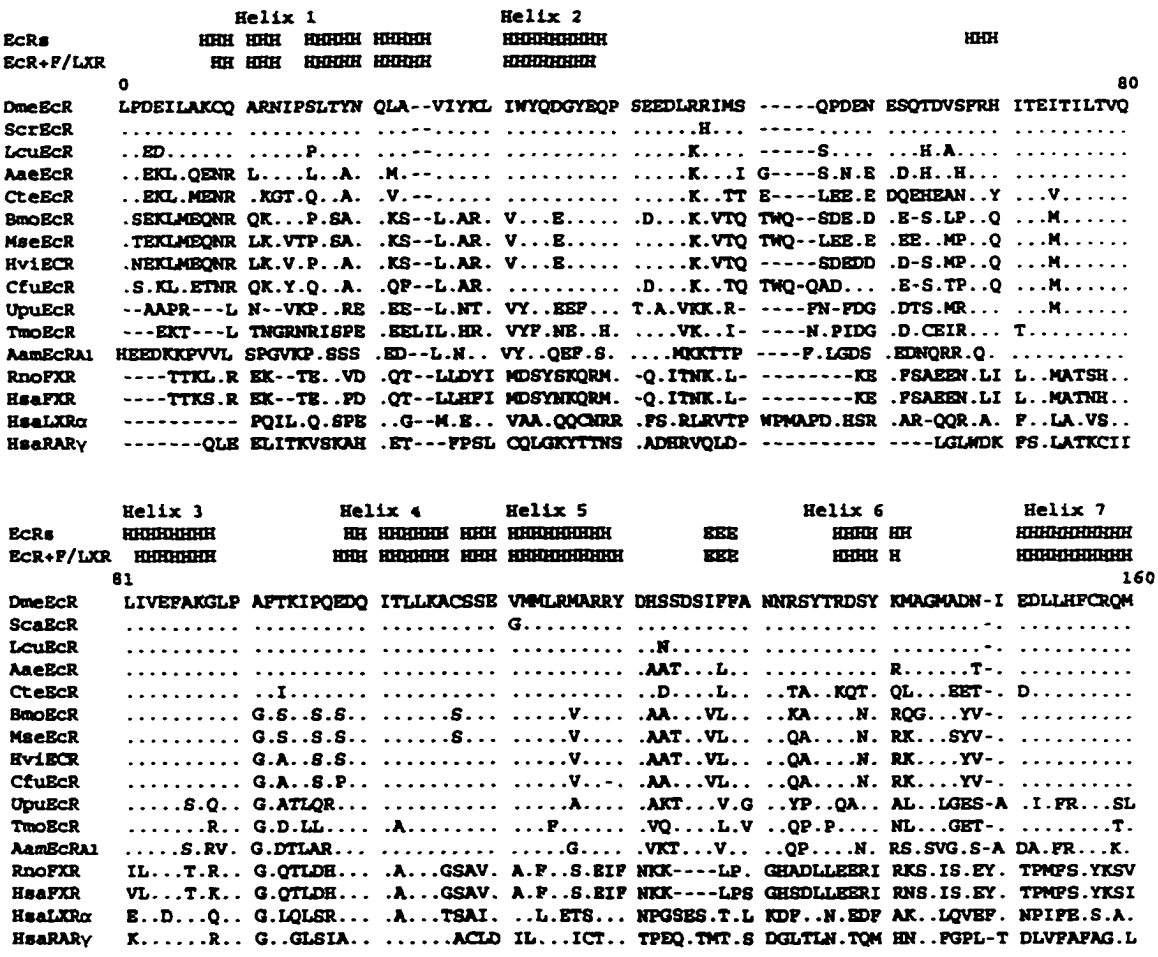

Helix 1
Helix 2
EcRs
EcR+F/LXR
0
80
DmeEcR LPDEILAKCQ ARNIPSLTYN QLA--VIYKL IWYQDGYEQP SEEDLRRIMS -----QPDEN ESQTDVSFRH ITEITILTVQ
ScrEcR
LcuEcR
AaeEcR
CteEcR
BmoEcR
MseEcR
HviECR
CfuEcR
UpuEcR
TmoEcR
AamEcRA1
RnoFXR
HsaFXR
HsaLXRα
HsaRARγ
Helix 3
Helix 4
Helix 5
Helix 6
Helix 7
81
160
DmeEcR LIVEFAKGLP APTKIPQEDQ ITLLKACSSE VMMLRMARRY DHSSDSIFFA NNRSYTRDSY KMAGMADN-I EDLLHFCRQM
ScaEcR
LcuEcR
AaeEcR
CteEcR
BmoEcR
MseEcR
HviECR
CfuEcR
UpuEcR
TmoEcR
AamEcRA1
RnoFXR
HsaFXR
HsaLXRα
HsaRARγ

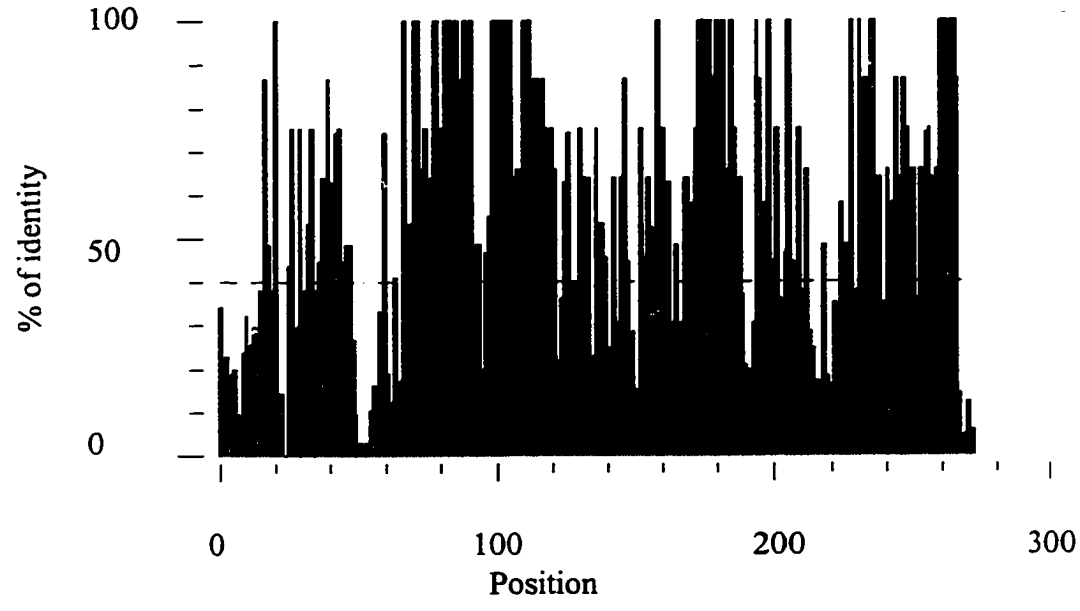
FIG. 2

```
                Helix 8                 Helix 9                                 Helix 10       Helix 11
EcRs       HH    HHHH HHHH8             HHH HHHHHHHHHH HHHHHHH9                 HHHHHHHHHH H10     HH
EcR+F/LXR HH    HHHH HHHHHH8             HH HHHHHHHHHH HHHHHHHH9             HH HHHHHHHHHH H10     HH
        161                                                                                        240
DmeEcR     FSMKVDNVEY ALLTAIVIFS -DRPGLEKAQ LVEAIQSYYI DTLRIYILNR HCGDSMSLVF YAKLLSILTE LRTLGNQNAE
ScrEcR     .......... .......... -......... .......... .......... .......... .......... ..........
LcuEcR     Y......... .......... -.......E.E .......... .......... ....P..... F......... ..........
AaeEcR     ..LT...... .......... -.......Q.E ...H...... .......... .A..P.CS.I F......... ........S.
CteEcR     YALSI..... .......... -......... M.DI.....T E..KV..V.. .G.E.RCS.Q F....G.... ......K.S.
BmoEcR     .A.GM...HF .......... -.......QPS ...E..R..L N......I.Q NSAS.RCA.I .GRI..V... .....T..SN
MseEcR     Y..SM...H. .......... -.......QPL ...E..R..L K...V....Q .SASPRCA.L PG.I.GV... .....T..SN
HviECR     Y..MM...H. .......... -.......QPL ...E..R..L N...V....Q NSASPRGA.I PGEI.G.... .....M..SN
CfuEcR     Y..AL..IH. .....V.... -.......QP. ...E..R..L N...I....Q LS.SAR.S.I .GKI....S. .....M..SN
UpuEcR     CK..V..A.. ...A..A... -E..N.KELK K..KL.EI.L EA.KS.VE.. R--LPR.NMV F....N.... ......I.S.
TmoEcR     Y...V..A.. .......... -E..S.IEGW K..K..EI.L EA..A.VD.. R--SPSRGTI F.....V... ........S.
AamEcRA1   CQLRV..A.. .......... -E..S.VDPH K..R..E... E...M.SE.H R--PPGKN-Y F.R....... ......M...
RnoFXR     GEL.MTQ... ........L. P..QYIKDRE A..KL.EPL. .V.QKLCKIY Q---PENPQH F.C..GR... ...FNHHH..
HsaFXR     GEL.MTQ... ........L. P..QYIKDRE A..KL.EPL. .V.QKLCKIH Q---PENPQH F.C..GR... ...FNHHH..
HsaLXRα    NELQLNDA.F ...I..S... A...NVQDQL Q..RL.HT.V EA.HA.VSIH .---PHDRLM FPRM.MK.VS ....SSVHS.
HsaRARγ    LPLEM.DT.T G..S..CLIC G..MD.EEPE K.DKL.EPLL EA..L.ARR. R---PSQPYM FPRM.MKI.D ..GISTKG..
```

```
                        Helix 12
EcRs       HH              HHHHHHH HH
EcR+F/LXR HH              HHHHHHH HH
                       ** ********** *
        241                                 268
DmeEcR     MCFSLKLK-NR KLPKFLEEIW DVH-AIPP
ScrEcR     .......T-.. T.S....... ........
LcuEcR     .........-.. .......... ...-....
AaeEcR     .........-.. ...R...... ..Q-D...
CteEcR     .......R-.. ...R....V. ..G-DVNN
BmoEcR     ..I.....-.. ...P...... ..A-EVAT
MseEcR     ..I.....-.. ...P...... ..A-EVST
HviECR     ..I.....-.. ...P...... ..A-DVAT
CfuEcR     ..I.....-.. ...P...... ..AGHVA-
UpuEcR     .....T...K R..P..A... ..SGY---
TmoEcR     ..I.....-.K ...P..D... ..DLKA--
AamEcRA1   ......VQ-.K ...P..A... .IQE----
RnoFXR     .LM.WRVN-DH .FTPL.C... ..Q-----
HsaFXR     .LM.WRVN-DH .FTPL.C... ..Q-----
HsaLXRα    QV.A.R.Q-DK ...PL.S... ...E----
HsaRARγ    RAIT..MEIPG PMPPLIR.ML ENPEMPE-
```

FIG. 2 (con't)

(a) EcRs+ FXRs +LXR



(b) Invertebrate EcRs

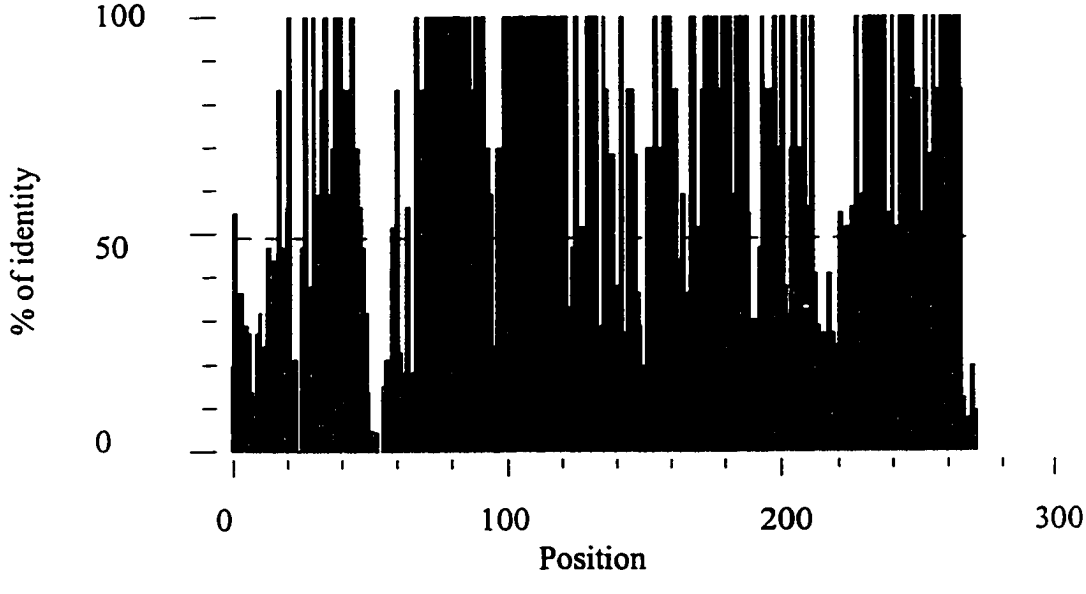
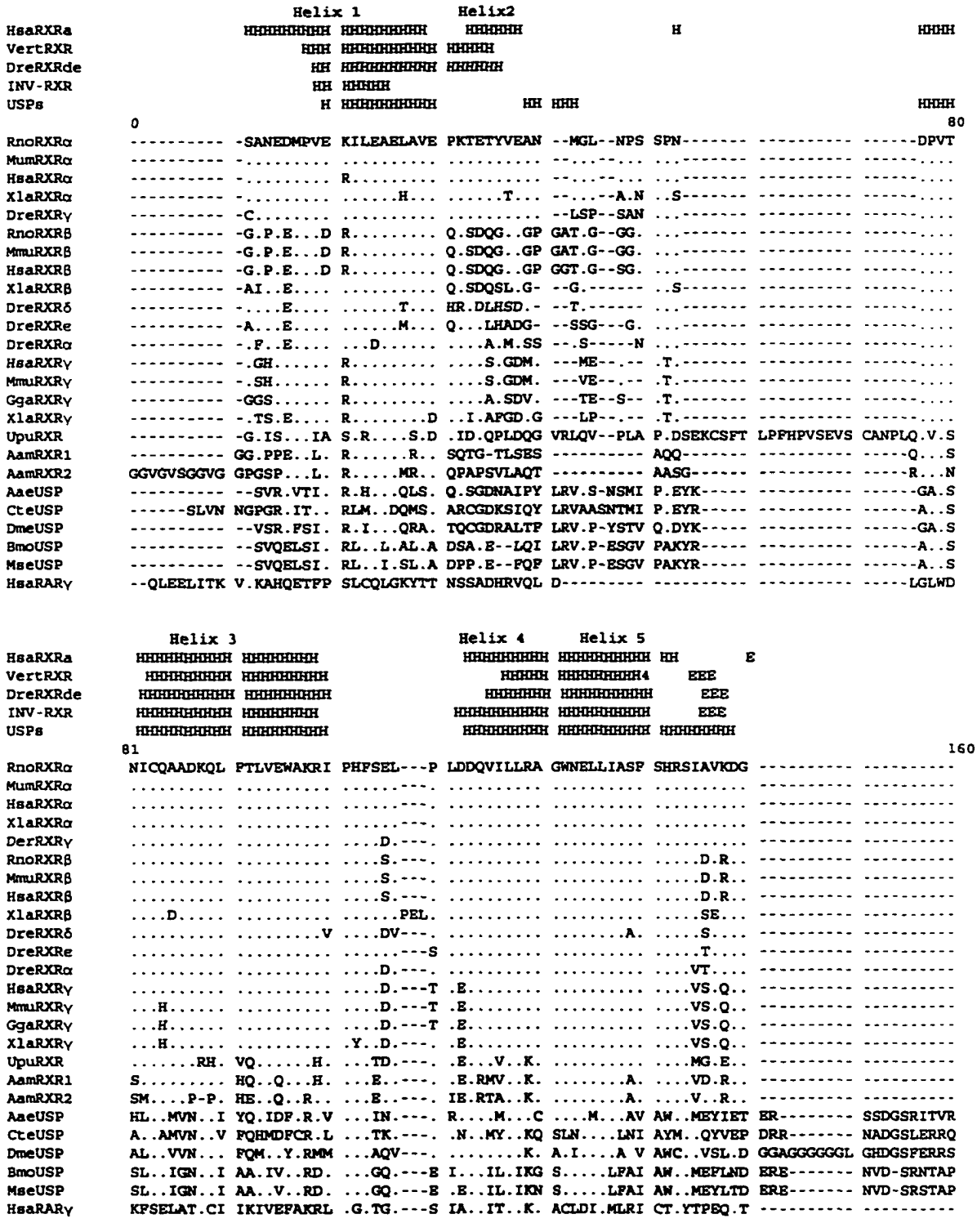


FIG. 3


Helix 1
Helix2
HsaRXRa
VertRXR
DreRXRde
INV-RXR
USPs
RnoRXRα
MumRXRα
HsaRXRα
XlaRXRα
DreRXRγ
RnoRXRβ
MmuRXRβ
HsaRXRβ
XlaRXRβ
DreRXRδ
DreRXRe
DreRXRα
HsaRXRγ
MmuRXRγ
GgaRXRγ
XlaRXRγ
UpuRXR
AamRXR1
AamRXR2
AaeUSP
CteUSP
DmeUSP
BmoUSP
MseUSP
HsaRARγ
Helix 3
Helix 4
Helix 5
HsaRXRa
VertRXR
DreRXRde
INV-RXR
USPs
RnoRXRα
MumRXRα
HsaRXRα
XlaRXRα
DerRXRγ
RnoRXRβ
MmuRXRβ
HsaRXRβ
XlaRXRβ
DreRXRδ
DreRXRe
DreRXRα
HsaRXRγ
MmuRXRγ
GgaRXRγ
XlaRXRγ
UpuRXR
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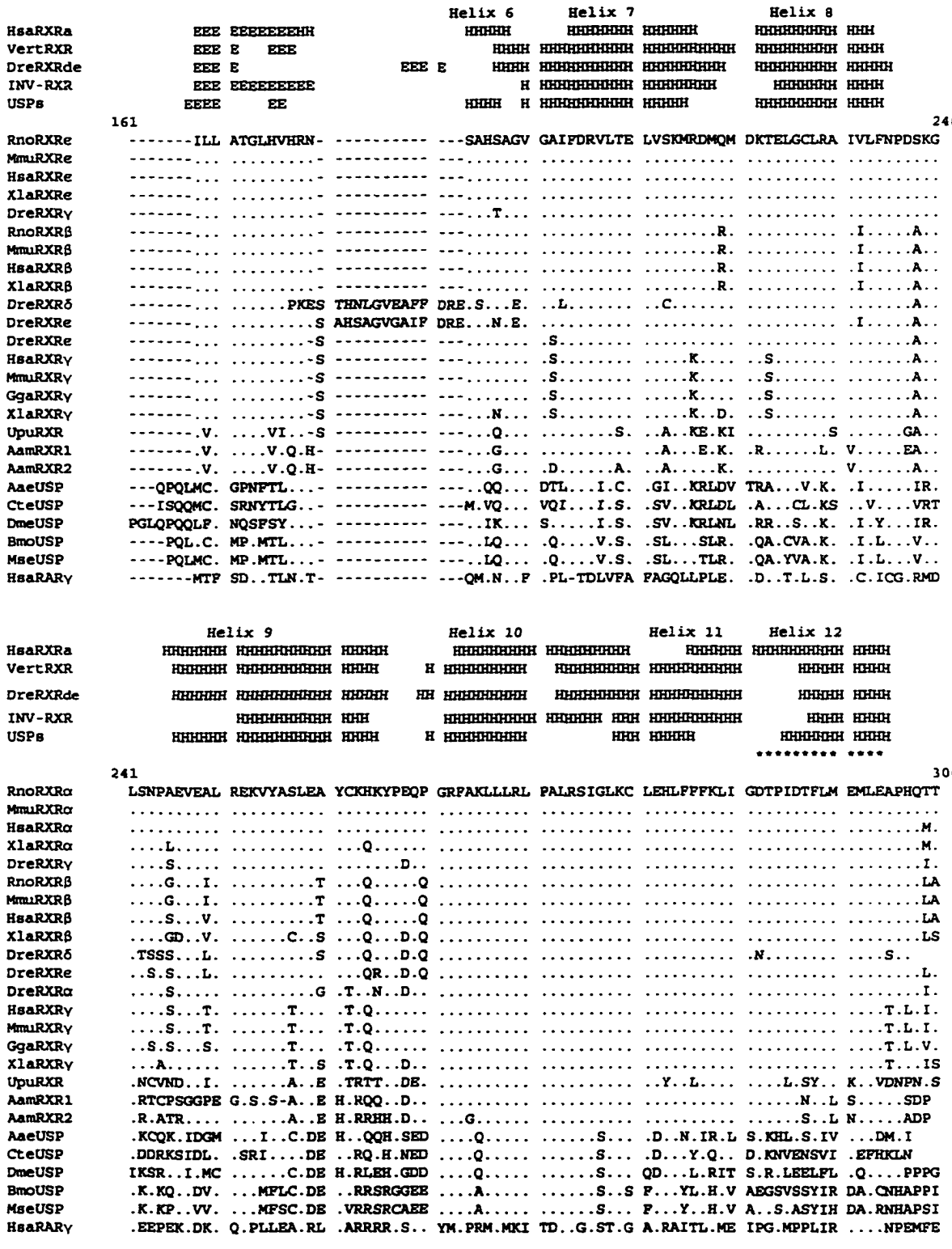
AamRXR2
AaeUSP
CteUSP
DmeUSP
BmoUSP
MseUSP
HsaRARγ

FIG. 4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Helix 7
Helix 8
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VertRXR
DreRXRde
INV-RXR
USPs
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240
RnoRXRe
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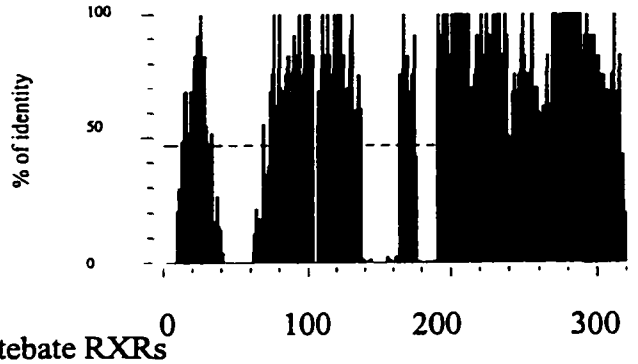
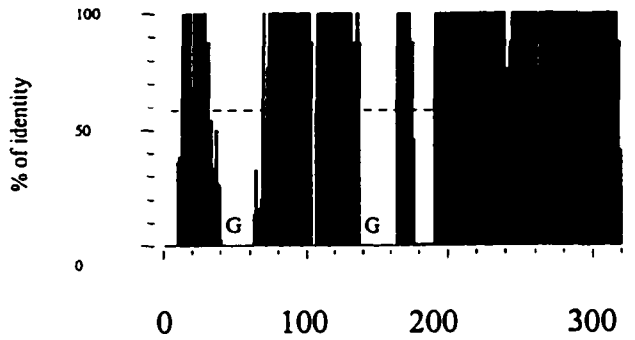
HsaRXRe
XlaRXRe
DreRXRγ
RnoRXRβ
MmuRXRβ
HsaRXRβ
XlaRXRβ
DreRXRδ
DreRXRe
DreRXRe
HsaRXRγ
MmuRXRγ
GgaRXRγ
XlaRXRγ
UpuRXR
AamRXR1
AamRXR2
AaeUSP
CteUSP
DmeUSP
BmoUSP
MseUSP
HsaRARγ
Helix 9
Helix 10
Helix 11
Helix 12
HsaRXRa
VertRXR
DreRXRde
INV-RXR
USPs
241
300
RnoRXRα
MmuRXRα
HsaRXRα
XlaRXRα
DreRXRγ
RnoRXRβ
MmuRXRβ
HsaRXRβ
XlaRXRβ
DreRXRδ
DreRXRe
DreRXRα
HsaRXRγ
MmuRXRγ
GgaRXRγ
XlaRXRγ
UpuRXR
AamRXR1
AamRXR2
AaeUSP
CteUSP
DmeUSP
BmoUSP
MseUSP
HsaRARγ

FIG. 4 (con't)

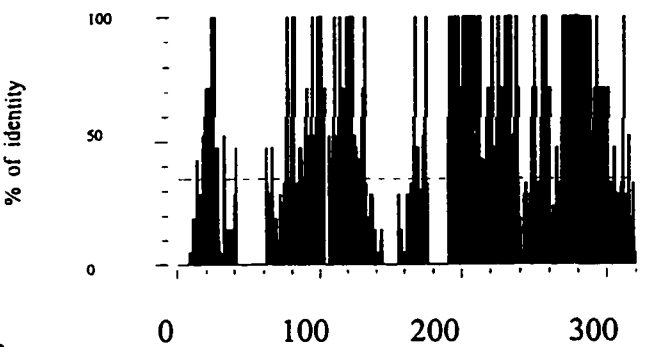
(a) All RXRs



(b) Vertebate RXRs



(c) All invertebrate RXRs



(d) USPs

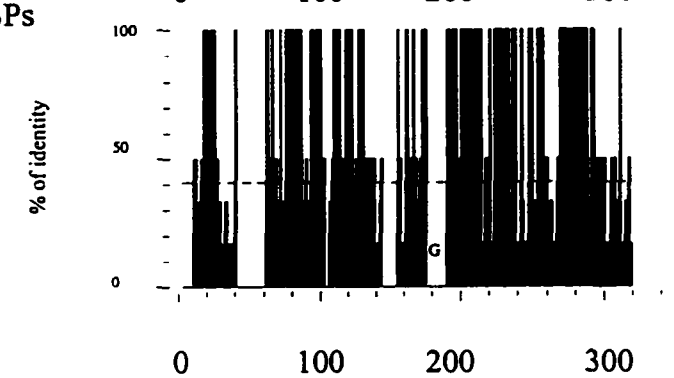


FIG. 5

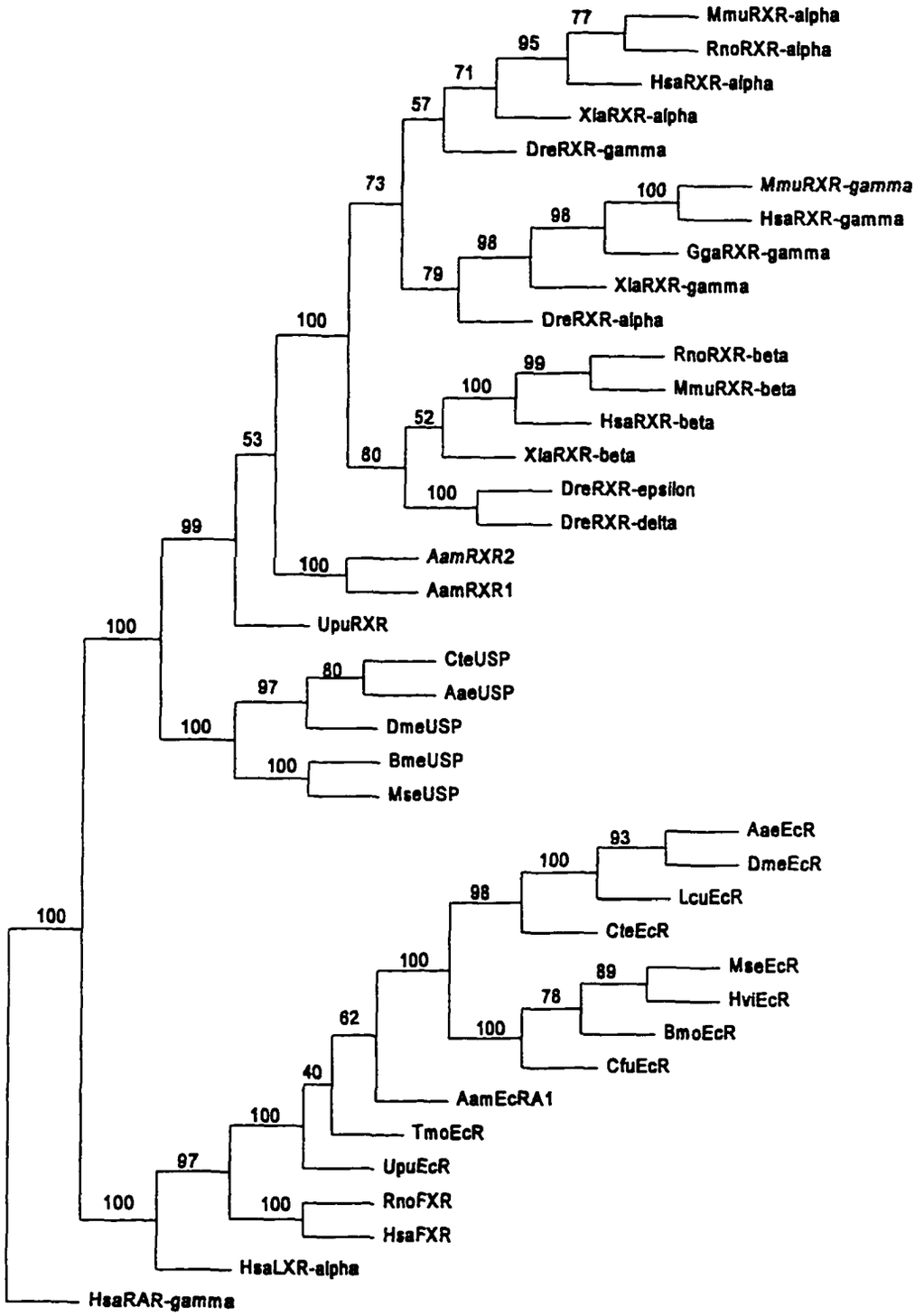
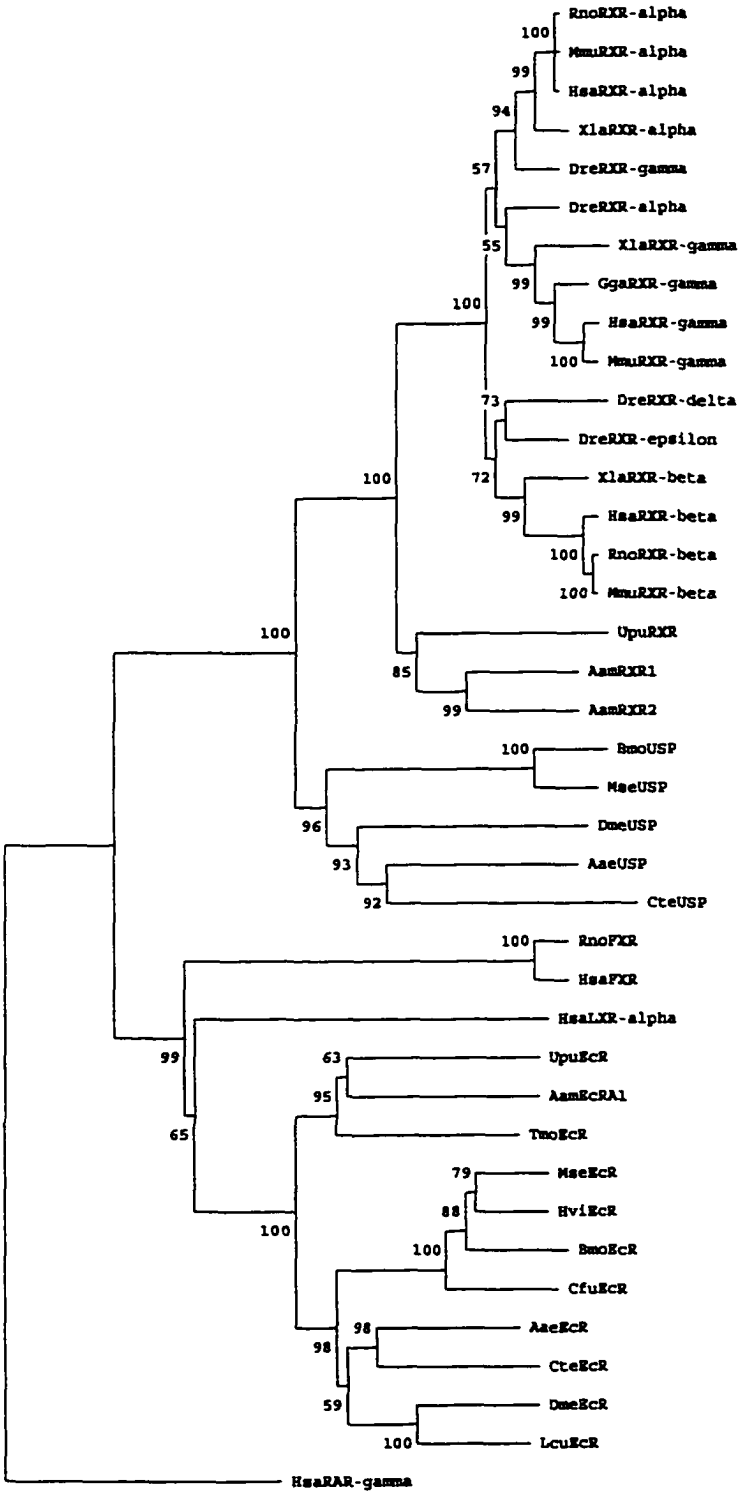

MmuRXR-alpha
RnoRXR-alpha
HsaRXR-alpha
XlaRXR-alpha
DreRXR-gamma
MmuRXR-gamma
HsaRXR-gamma
GgaRXR-gamma
XlaRXR-gamma
DreRXR-alpha
RnoRXR-beta
MmuRXR-beta
HsaRXR-beta
XlaRXR-beta
DreRXR-epsilon
DreRXR-delta
AamRXR2
AamRXR1
UpuRXR
CteUSP
AaeUSP
DmeUSP
BmeUSP
MseUSP
AaeEcR
DmeEcR
LcuEcR
CteEcR
MseEcR
HviEcR
BmoEcR
CfuEcR
AamEcRA1
TmoEcR
UpuEcR
RnoFXR
HsaFXR
HsaLXR-alpha
HsaRAR-gamma

FIG. 6a



Scale: each – is approximately equal to the distance of 0.008076

FIG. 6b

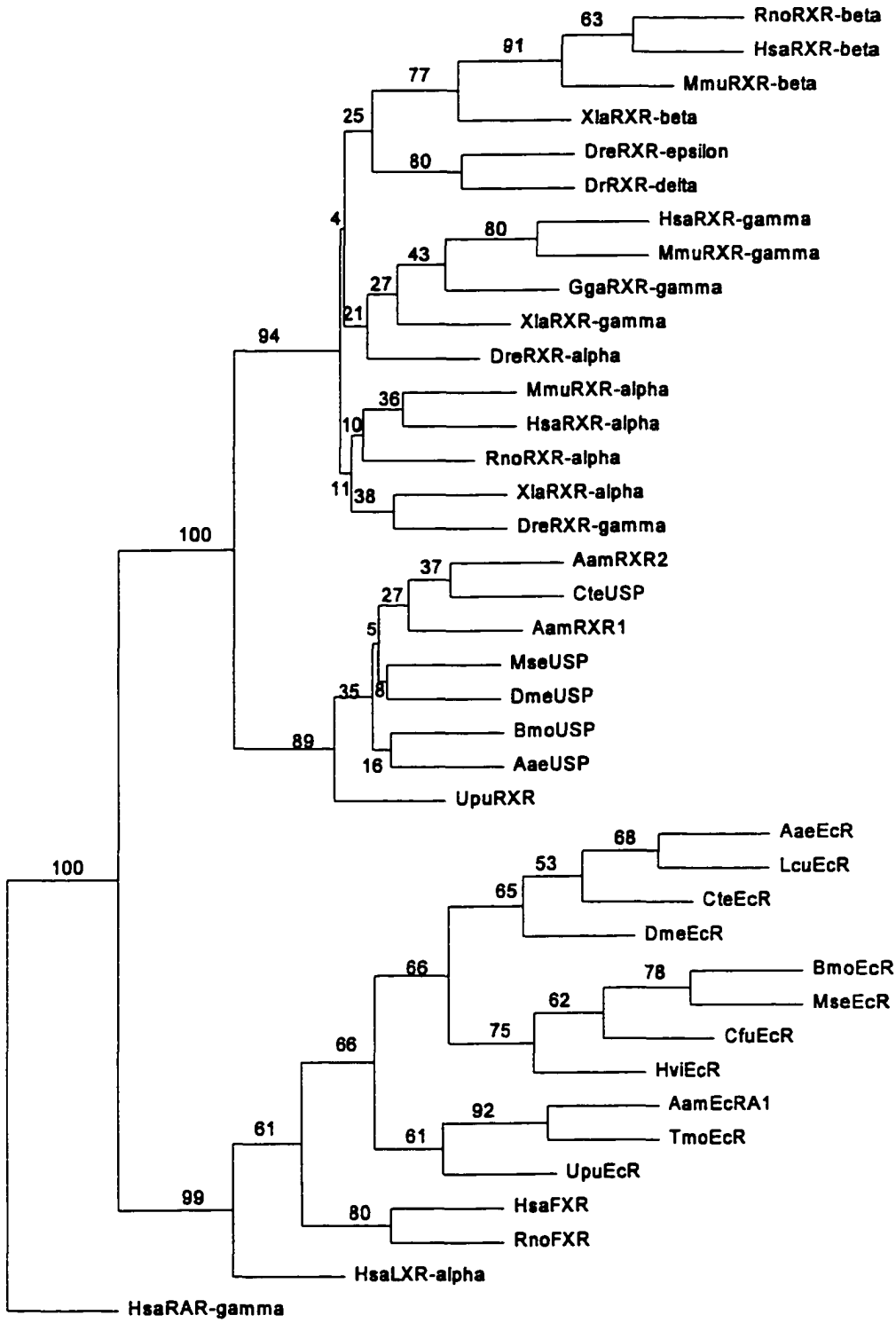
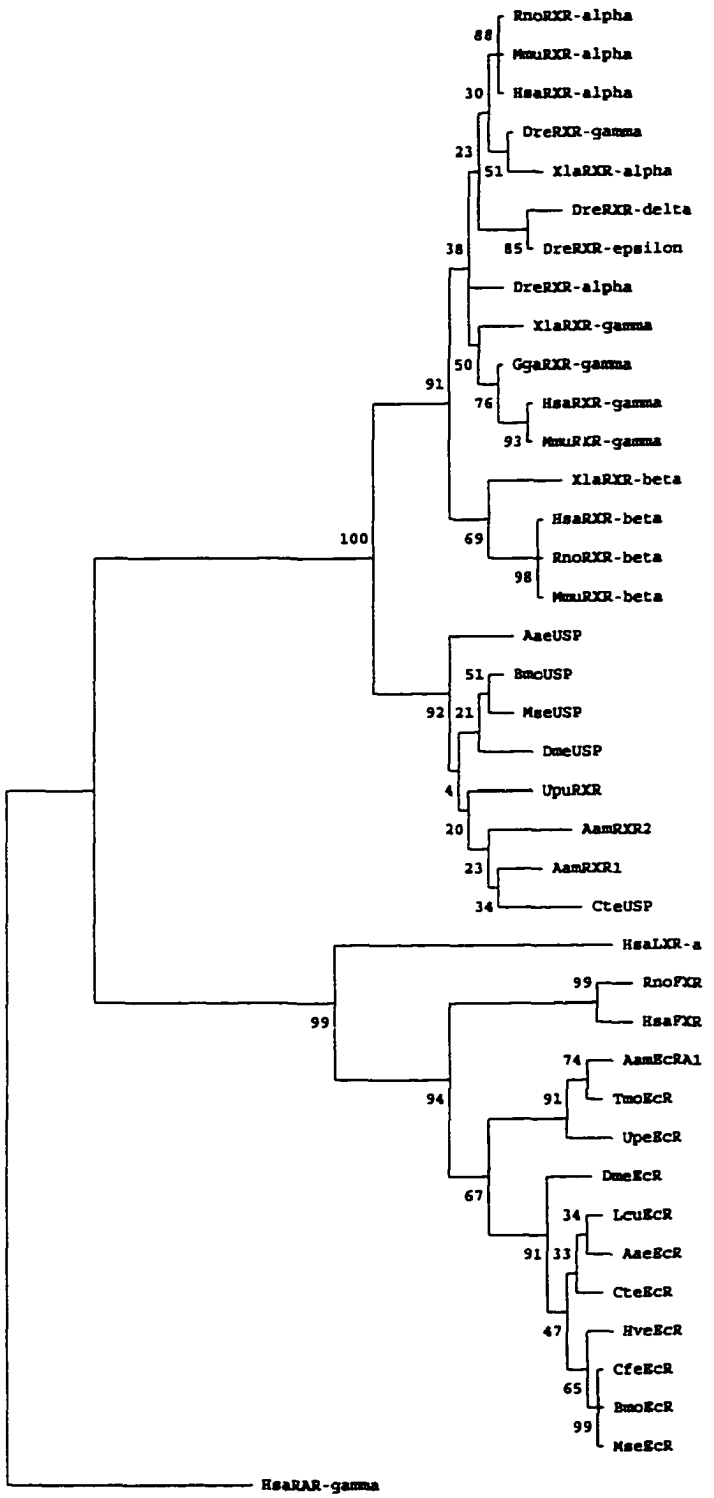

RnoRXR-beta
HsaRXR-beta
MmuRXR-beta
XlaRXR-beta
DreRXR-epsilon
DrRXR-delta
HsaRXR-gamma
MmuRXR-gamma
GgaRXR-gamma
XlaRXR-gamma
DreRXR-alpha
MmuRXR-alpha
HsaRXR-alpha
RnoRXR-alpha
XlaRXR-alpha
DreRXR-gamma
AamRXR2
CteUSP
AamRXR1
MseUSP
DmeUSP
BmoUSP
AaeUSP
UpuRXR
AaeEcR
LcuEcR
CteEcR
DmeEcR
BmoEcR
MseEcR
CfuEcR
HviEcR
AamEcRA1
TmoEcR
UpuEcR
HsaFXR
RnoFXR
HsaLXR-alpha
HsaRAR-gamma

FIG. 7a



Scale: each – is approximately equal to the distance of 0.005042

FIG. 7b

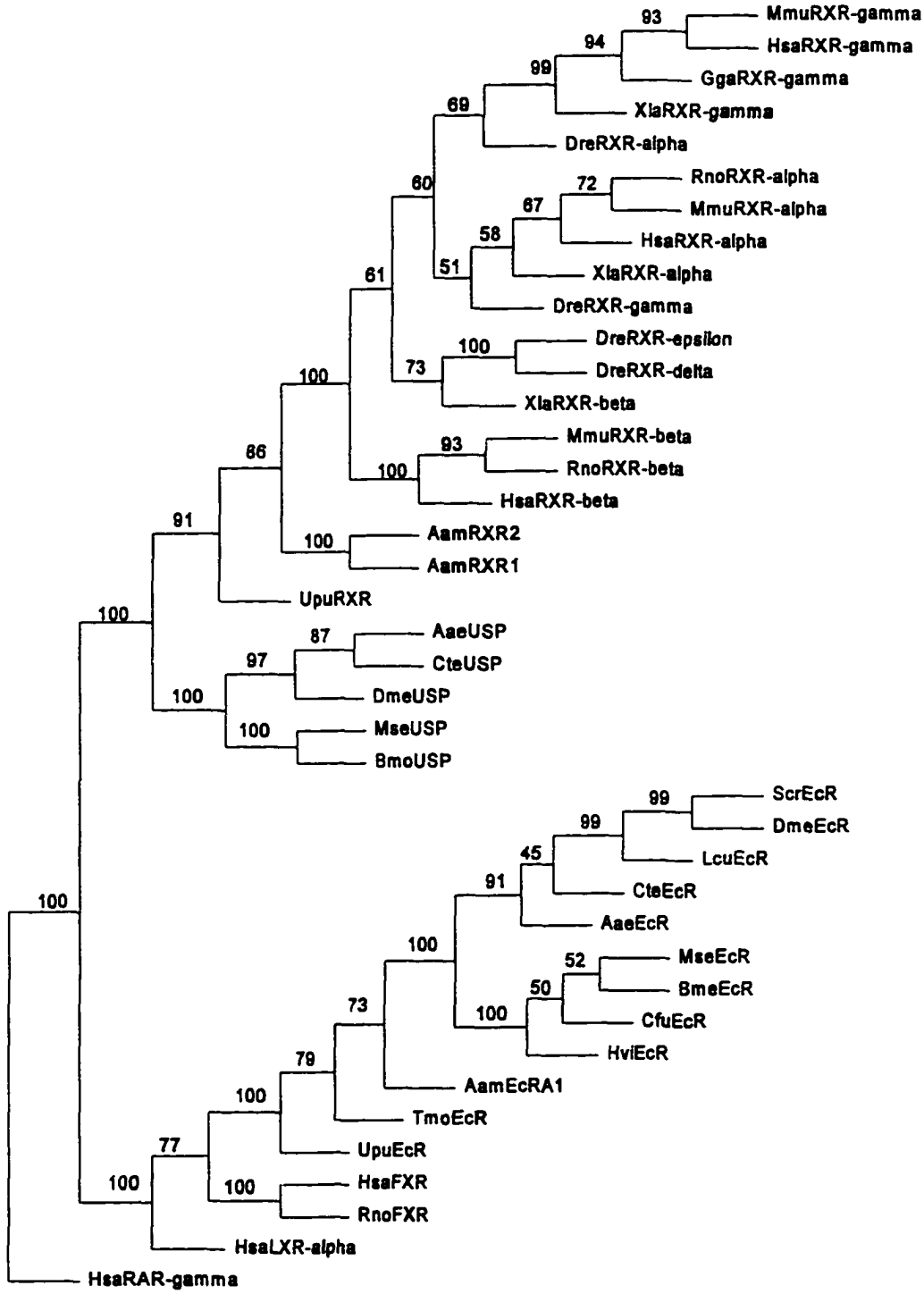
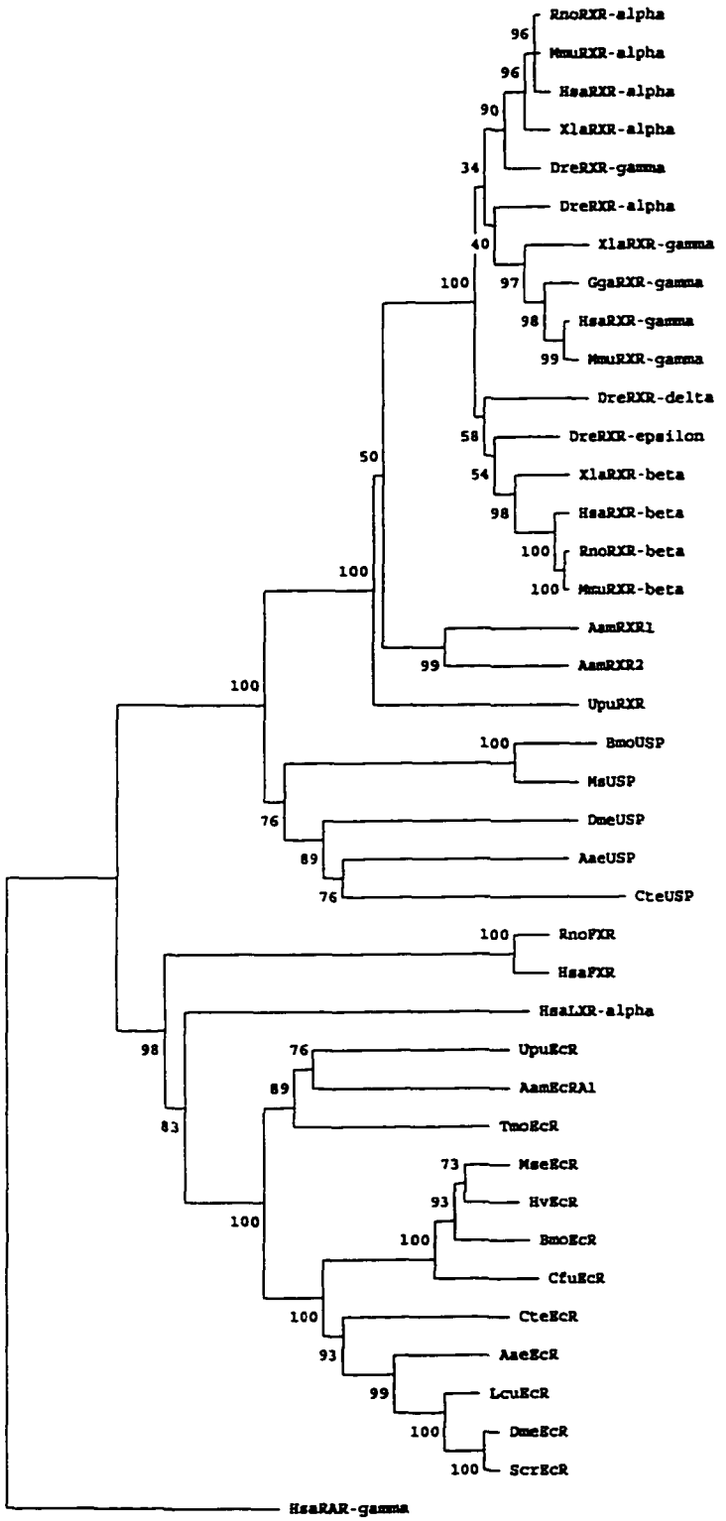

MmuRXR-gamma
HsaRXR-gamma
GgaRXR-gamma
XlaRXR-gamma
DreRXR-alpha
RnoRXR-alpha
MmuRXR-alpha
HsaRXR-alpha
XlaRXR-alpha
DreRXR-gamma
DreRXR-epsilon
DreRXR-delta
XlaRXR-beta
MmuRXR-beta
RnoRXR-beta
HsaRXR-beta
AamRXR2
AamRXR1
UpuRXR
AaeUSP
CteUSP
DmeUSP
MseUSP
BmoUSP
ScrEcR
DmeEcR
LcuEcR
CteEcR
AaeEcR
MseEcR
BmeEcR
CfuEcR
HviEcR
AamEcRA1
TmoEcR
UpuEcR
HsaFXR
RnoFXR
HsaLXR-alpha
HsaRAR-gamma

FIG. 8a



Scale: each – is approximately equal to the distance of 0.008991

FIG. 8b

(a) Alignment

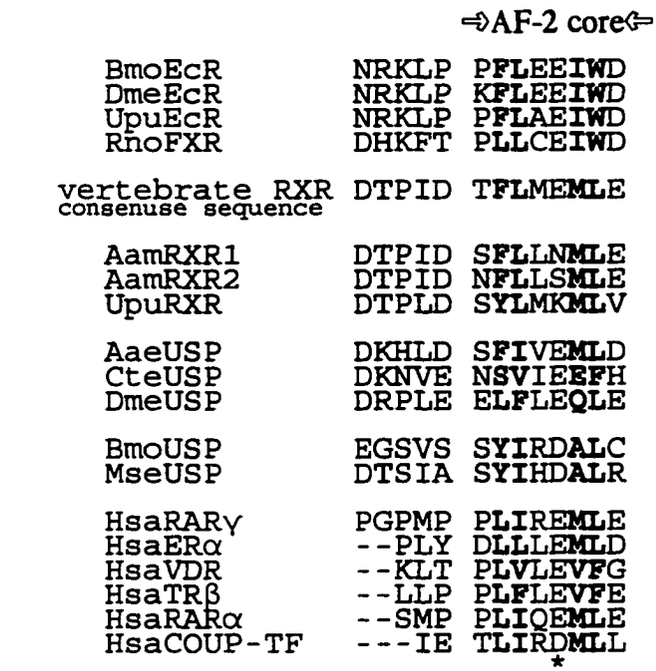


FIG. 9

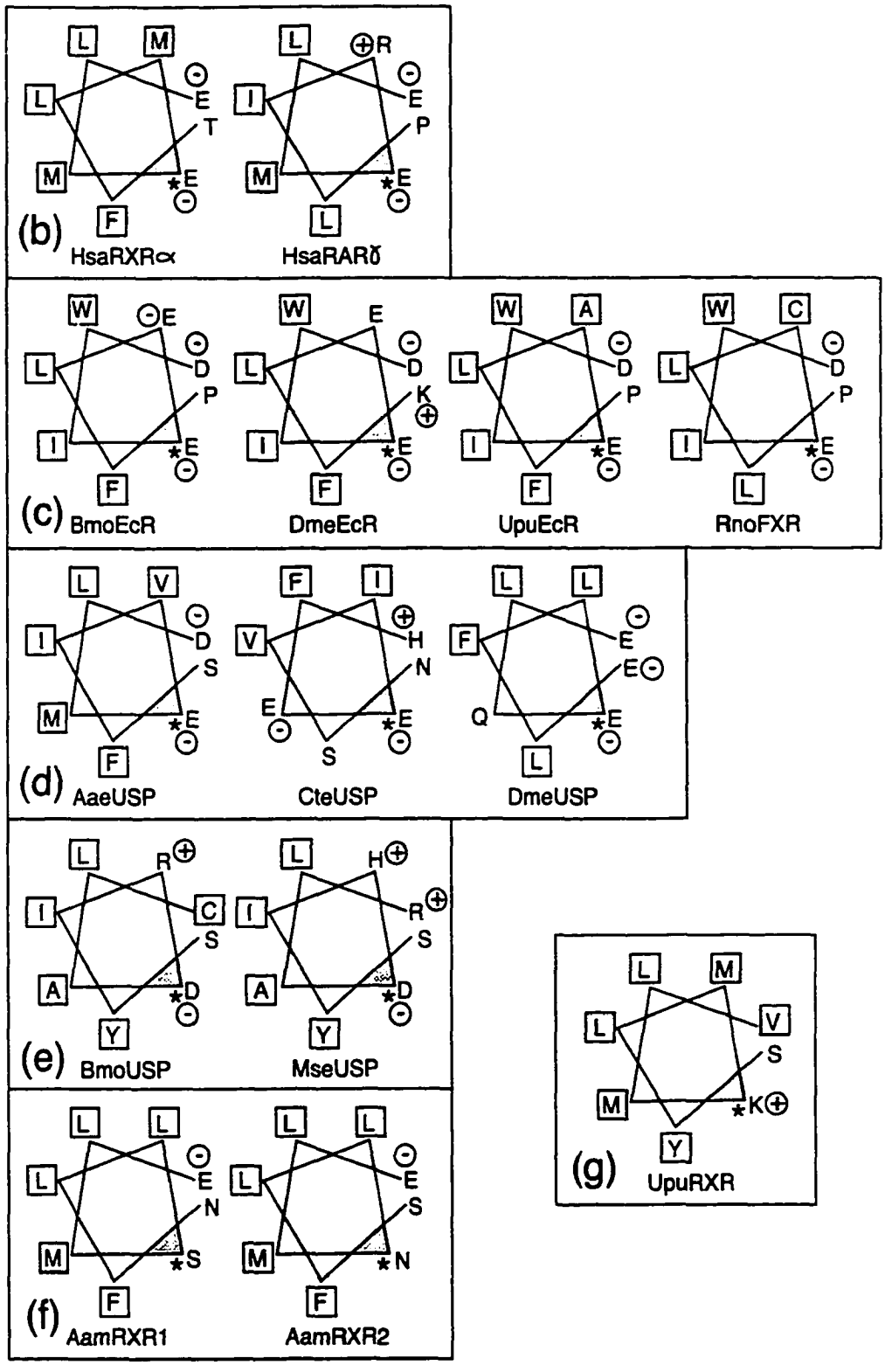

(b)
HsaRXRα
HsaRARδ
(c)
BmoEcR
DmeEcR
UpuEcR
RnoFXR
(d)
AaeUSP
CteUSP
DmeUSP
(e)
BmoUSP
MseUSP
(f)
AamRXR1
AamRXR2
(g)
UpuRXR

FIG. 9 (con't)

GENERAL CONCLUSIONS

Molting and limb regeneration in crustaceans have been studied for a century. Basic hormonal mechanisms underlying these two processes have been understood for decades although the details are still awaiting to be explored. In the future, molecular tools should enable us to investigate the mechanism of these two processes at the molecular level. Cloning of the *Uca* ecdysteroid receptor (*UpuEcR*) and *Uca* retinoid-X receptor (*UpuRXR*) gene homologs from *Uca pugilator* should be helpful for future investigation.

In this dissertation, I report the characterization of these two crustacean receptor gene homologs. With the oligo-dT primed cDNA library constructed by the poly(A)$^+$ RNA from proecdysial limb buds, *Uca* homologs of *EcR* and *RXR* genes have been identified. The deduced amino acid sequence of UpuEcR is similar to insect EcRs in both the DNA binding (DBD) and ligand binding (LBD) domains, suggesting that this crustacean receptor should have properties similar to the insect EcRs. Interestingly, the deduced amino acid sequence of UpuRXR gives a similar story as chelicerate RXRs. The DBD of UpuRXR shares greatest identity with insect USPs, insect homologs of vertebrate RXRs, whereas the LBD is similar to the vertebrate RXRs. These findings are supported by the phylogenetic analysis. UpuRXR may have similar DNA binding properties similar to insect USPs because of a high level of amino acid conservation in the DBD. Since the amino acid sequences of the helices and beta-turn, which form the putative ligand binding pocket in UpuRXR, are similar to those in vertebrate RXRs, UpuRXR may be able to bind *9-cis* retinoic acid (RA) as most vertebrate RXRs, instead of juvenile hormone as in *Drosophila* USP (DmeUSP). The substitution of

the critical acidic amino acids in the ligand-dependent activation subdomain, AF-2 subdomain, is consistent with the hypothesis that the crustacean RXR has a different transactivation mechanism. Further studies using bacteria-expressed protein may shed more light on these speculations.

Cloning of these two genes also provides tools to investigate the expression of *UpuEcR* and *UpuRXR* transcripts in crustacean tissues. Findings in insects show that the dimerization of EcR and USP are important for both DNA-binding and ecdysteroid-binding. As insects, EcR must bind to USP to be a functional receptor to mediate the actions of ecdysteroids. Transcripts of these two receptors are found in regenerating limb buds, gills, eyestalks, hypodermis, hepatopancreas, muscle of non-regenerating walking legs and large cheliped, suggesting that these are ecdysteroid target tissues. The simultaneous expression of *UpuEcR* and *UpuRXR* in these tissues also supports the possibility that heterodimerization of these two receptors occurs *in vivo*.

The physiological events in target tissues may be associated with different ranges of the circulating ecdysteroid titers during the molt cycle and limb regeneration. Thus, changes of total circulating ecdysteroid titers could regulate the rate of physiological events related to molting or limb regeneration, and could ensure the appropriate temporal and spatial coordination of the different events. Transcript levels of *UpuEcR* exhibit marked variation between tissues at any given time during the molt cycle. These results support the hypothesis that various tissues have different potential to respond to ecdysteroids. Although there is no correlation between high levels of *UpuEcR* transcripts in some tissues and total ecdysteroid titers, the expression pattern of *UpuEcR* is correlated with some physiological processes in

these tissues during the molt cycle. Moreover, *UpuEcR* expression in tissues from multiply autotomized crabs differs from the expression patterns in tissues from singly autotomized crabs.

In insects, specific isoforms of EcRs and USPs are expressed differentially in larval and adult tissues during larval development. At least four active ecdysteroid metabolites are found in crustacean hemolymph. It is possible that UpuEcR and/or UpuRXR may have isoforms that mediate different events during the molt cycle and limb regeneration and that interact with different ecdysteroids. Results from the early western blot studies using anti-DmeEcR antibody and restriction digestion studies on the cDNA clones suggest that UpuEcR may have isoforms. Our present RNA probes are produced from the sequences encoding a common region of the receptor genes. By using these RNA probes, my results do not detect the variation of any isoform transcripts. Isolation of the cDNA clones encoding UpuEcR and/or UpuRXR isoforms may enable us to investigate the expression of these isoforms temporally and spatially during the molt cycle and limb regeneration. In addition, it may be useful to determine the relationship between the expression of these isoforms and the various ecdysteroids.

Early blastemal differentiation is important for crustacean limb regeneration. Exposure to *all-trans* retinoic acid (RA) in this stage often disrupts normal limb regeneration. Other studies show that low ecdysteroid titers are necessary for the mitotic activity during blastemal differentiation. Preliminary binding studies in our laboratory show that nuclear extracts from blastemas have both ecdysteroid- and retinoic acid-binding abilities. *UpuEcR* and *UpuRXR* mRNAs are found in this early stage and transcript levels

increase four days after autotomy. In addition, immersion of the crabs in sea water with *all-trans* RA elevates the transcript levels of *UpuRXR* and alters the pattern of ecdysteroids. These results suggest that UpuEcR and UpuRXR should play an important role in early blastemal differentiation. In the future, molecular tools, such as anti-UpuEcR and anti-UpuRXR antibodies, will be useful to identify how the ecdysteroids and their receptors participate at this critical moment.

Following blastemal differentiation, crustacean limb regeneration can be divided into two different growth processes, basal growth and proecdysial growth. Basal growth is an epimorphic growth, whereas proecdysial growth is a hypertrophic one. These two growth processes occur during periods of different ecdysteroid titers in the blood. Basal growth, as blastemal differentiation, occurs when total ecdysteroid titers are low and when levels of 25-deoxyecdysone and Ponasterone A are higher relative to other ecdysteroids. As proecdysial growth begins, there is a switch in the predominant ecdysteroids from 25-deoxyecdysone and Ponasterone A to ecdysone and 20-OH ecdysone. The completion of proecdysial growth requires high total ecdysteroid titers, and Ponasterone A is the single most predominant steroid of the total circulating ecdysteroids.

Contralateral removal of half of the limb buds during basal growth in *U. pugilator* reduces the growth rate of the primarily regenerating limb buds and extends the molt cycle. Unlike a previous study in the land crab, *Gecarcinus lateralis*, removal of half of the limb buds in *U. pugilator* does not lower the titers of total circulating ecdysteroids immediately but delays the late proecdysial peak of total ecdysteroids. These results suggest that the reduction of growth rate in the primarily regenerating limb buds is not caused by the drop

of total ecdysteroid titers and these results also support the assumption that the basal growth of secondarily regenerating limb buds requires low levels of total circulating ecdysteroids. The transcript levels of *UpuEcR* and *UpuRXR* in the secondarily regenerating limb buds are high when the proecdysial growth rate of the secondarily regenerating limb buds is high. These results are similar to the previous results that during the limb regeneration of *U. pugilator*, the transcript levels of *UpuEcR* and *UpuRXR* in limb buds are high when the growth rate of proecdysial limb buds is high. The receptor transcript levels in limb buds may have a close relationship with the growth rate of proecdysial limb buds. These results raise a possibility that a certain EcR isoform may be synthesized specifically for fast proecdysial growth.

Results from this study do not explain the cause of the reduction of growth rate in the primarily regenerating limb buds after removal of contralateral limb buds. Together with the results of a recent report in *G. lateralis*, I speculate that other factor(s), besides ecdysteroids, may be involved in the regulation of crustacean limb regeneration. Also, the reduction of growth rate may be a result of a downregulation of specific *UpuEcR* isoforms necessary for proecdysial growth. Multiple autotomy is well known to accelerate the molting events in crustacean but the underlying mechanism is still unclear. The identification of these putative factors may be helpful to understand the mechanism of multiple autotomy.

EcRs have been found only in arthropods and the functions of EcR rely on its dimerization with RXR (or USP in insects). The evolution of these two receptors should be closely related to arthropod evolution. In addition, EcR and USP are the only invertebrate nuclear receptors that have been shown to possess ligand binding ability. Ligand binding

ability of the steroid hormone receptor /nuclear receptor superfamily has been proposed to have been acquired after the vertebrates separated from the invertebrates during evolution. It is unknown how the ligand binding ability of EcRs and USPs have evolved. Results of structural analysis suggest that the vertebrate EcR homologs, FXR and LXR, may have different ligand binding ability from that of arthropod EcRs. This supports other studies that FXR binds to farnesoids and the LXR binds oxysterols. There is no evidence that FXR and LXR bind ecdysteroids. Phylogenetic analysis indicates that FXRs and LXR are only distantly related to EcR. Thus, the ecdysteroid-binding ability of EcR may have been evolved after the evolutionary separation of the invertebrates and the vertebrates.

Results of both structural and phylogenetic analyses show that the LDBs of insect USPs are different from other RXRs, including chelicerate and crustacean RXRs although the DBDs of all invertebrate RXRs are closely related. These results suggest that all invertebrate RXRs may have evolved after the evolutionary separation of the invertebrates and the vertebrates. At that time, this invertebrate RXR ancestor might have been an unliganded receptor and the vertebrate RXRs might have acquired *9-cis* retinoic acid-binding ability later during further vertebrate evolution. The recent report about the binding of DmeUSP to insect juvenile hormone and the results of this study suggest that during arthropod evolution, arthropod RXRs may have acquired a different ligand binding ability and acquired a functional ligand-dependent transactivation subdomain, AF-2 subdomain.

The results from this evolutionary study indicate that the functions and properties of UpuEcR should be similar to those of insect EcRs. Since the LBDs of UpuRXR is similar to vertebrate RXRs, instead of insect USPs, UpuRXR may bind to *9-cis* retinoid acid, or

other metabolites of retinoic acid. The substitution of critical acidic amino acids in the AF-2 subdomain suggests that UpuRXR may have a different ligand binding transactivation. These speculations remain to be confirmed by future analysis using the bacteria-expressed UpuEcR and UpuRXR proteins.

APPENDIX

The ecdysone receptor (EcR) during proecdysis in the fiddler crab, *Uca pugilator*

Abstract

During the proecdysial period of the crustacean molt cycle, ecdysteroid levels in the hemolymph fluctuate significantly. Ecdysteroids coordinate many physiological events which are important for ecdysis (molting). One of the physiological events that occur during proecdysis is the final stage of regeneration of lost limbs. During proecdysis, regenerating limb buds undergo hypertrophic growth and prepare for molting. During late proecdysis, apolysis occurs and frees the old exoskeleton from the hypodermis. Following the separation from the exoskeleton, a new cuticle is secreted underneath the old one. Both proecdysial regeneration, apolysis, and cuticle secretion are influenced by ecdysteroids. Using a heterologous monoclonal antibody to *Drosophila* ecdysone receptor (EcR) (GGD 11.6, gift of Dr. David S. Hogness lab, Stanford University), we have conducted Western blot experiments designed to examine the temporal expression of cross-reacting proteins in hypodermis and limb buds during proecdysis of the fiddler crab *Uca pugilator*. In *Drosophila*, this antibody binds specifically to a 104 amino acid region containing the DNA-binding domain of the EcR protein. The *Uca* EcR DNA-binding domain has greater than 90% amino acid identity to *Drosophila* EcR. This suggests that this antibody should cross-react with *Uca* EcR. A complex pattern of immunoreactive proteins is seen in *Uca* tissues

during proecdysis. At least three bands cross-react with the monoclonal anti-*Drosophila* EcR antibody. These bands appear in hypodermis, limb buds, and muscle from nonregenerating walking legs removed during proecdysis but their patterns appear to be different, raising the possibility that *Uca*, like *Drosophila*, contains multiple EcR isoforms.

Introduction

During proecdysis of the crustacean molt cycle, ecdysteroid levels in the hemolymph fluctuate significantly (Hopkins, 1992). The ratio of individual ecdysteroids also changes significantly (Hopkins, 1992). During early proecdysis (D_0), total ecdysteroids are low in hemolymph and 20-OH ecdysone is the major ecdysteroid. During this time, regenerating limb buds undergo hypertrophic growth. In late proecdysis (D_{1-4}), the concentration of total ecdysteroids increases and Ponasterone A becomes a dominant ecdysteroid. During D_{1-4}, apolysis occurs and a new cuticle is secreted by the hypodermis. The hypertrophic growth of limb buds and the secretion of a new cuticle by hypodermis are controlled to a certain extent by ecdysteroids.

The DNA-binding domain of the *Uca* EcR protein is highly conserved between *Uca* and *Drosophila* (Durica and Hopkins, 1996). Thus , we decided to use heterologous anti-EcR monoclonal antibodies, which bind specifically to the DNA-binding domain of *Drosophila* EcR, for a preliminary examination of the temporal expression of EcR in *Uca* tissues. Our goal in these experiments was to determine whether the appearance of protein(s) immunoreactive to the EcR antibody is tissue- or molt stage-specific in *Uca*.

Materials and Method

Animals

Uca pugilator were purchased from Gulf Specimen Company, Florida, USA. As soon as the crabs were acclimated to laboratory conditions, each crab had 6 limbs

autotomized (multiple autotomy). After the limb buds emerged, the length of the third right limb bud was measured. The length of the bud was divided by the width of the carapace and expressed as an R-value (Bliss, 1956). The molt stage and rate of growth of the crabs were determined by their R-values and circulating levels of ecdysteroids (n= >4) were determined by radioimmunoassay (RIA):

R-value	Rate of Growth	Apolysis	Molt stage	Average ecdysteroid level
8-12	45+	No	Early D_0	30.1(±2.86)pg/ul
13-17	45+	No	Late D_0	33.1(±4.37)pg/ul
> 18	> 20	No	Early D_{1-4}	33.3(±3.95)pg/ul
> 18	< 20	Yes	Late D_{1-4}	57.7(±9.50)pg/ul

Western blot analysis

Proteins were extracted from tissues at different molt stages using EcR 40 buffer or cracking buffer (Koelle et al., 1991). The amount of protein was quantified by Bradford and Lowry methods. The samples were electrophoresed in polyacrylamide gels using the procedures of Laemmli (1970). The gels were then electroblotted onto nitrocellulose paper using Towbin buffer. The protein blots were probed with murine anti-*Drosophila* EcR antibodies (Gifts of Dr. D. S. Hogness, Stanford University), followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Bands were visualized with enhanced chemiluminescence reagents (Amersham), according to the specifications of the manufacturer.

Results

Preliminary tests of the monoclonal antibodies

Four anti-*Drosophila* EcR monoclonal antibodies were examined: AD 4.4, JG 6.2, EEC 11.1 and GGD 11.6 (Gifts of Dr. D. S. Hogness, Stanford University) (Talbot et al., 1993). AD 4.4 is directed against the N-terminal of EcR B1 protein, which has a molecular weight of about 105 kd (Talbot et al., 1993). The other three antibodies bind specifically to the DNA-binding domain of *Drosophila* EcRs. After testing these antibodies against the protein extracts from pupae of *Drosophila* Canton-S flies, GGD 11.6, as well as AD 4.4, showed a band at 105 kd (Fig. 1). Although JG 6.2 and EEC 11.1 were shown to bind to the same epitope as GGD 11.6 by Talbot et al. (1993), they did not show a strong 105 kd band in the protein extracts from pupa (Fig. 1). GGD 11.6 was selected for examination of EcR in *Uca* tissues because it showed a 105 kd EcR band in *Drosophila* and, most importantly, this antibody was raised against the highly conserved DNA-binding domain of EcR.

In addition, a protein with molecular weight around 70 kd was found in pupal extracts. It always cross-reacted with the three monoclonal antibodies directed against the DNA-binding domain (Fig. 1). Talbot et al. (1993) report a <65 kd protein in pupal tissue which non-specifically cross-reacts with the AD 4.4 monoclonal antibody.

Western blot analysis of EcR in *Uca* tissues

Three crustacean tissues were examined: hypodermis, proecdysial limb buds and muscle from nonregenerating limbs. These tissues were removed from the crabs during early proecdysis (D_0) and late proecdysis (D_{1-4}).

During stage D_0, a protein band at approximately 70-80 kd from *Uca* hypodermis cross-reacted with GGD 11.6 (Fig. 2). This protein band appeared to be more intense in samples undergoing apolysis (lanes 5-8) and where the hemolymph ecdysteriod titer was found to be rising (lane 2). The presence of this protein band is consistent with early binding studies of crustacean EcR in which the molecular weight of EcR in crayfish intermolt hypodermis was shown to be 70 kd (Londershausen and Spindler, 1981; Londershausen et al., 1982). Another band with an approximate molecular weight of 120 kd appeared at late D_{1-4}. This band was also found in samples of limb bud as well as muscle from unregenerating limbs (Fig. 3 and 4). Interestingly, this 120 kd band was also seen in the early Do sample where the ecdysteroid titer was found to be rising (Fig. 2, lane 3).

Western blots of proecdysial limb bud extracts showed a number of bands which cross-reacted with GGD 11.6 (Fig. 3). As in hypodermis, the major cross-reacting bands were observed at approximately 70 kd and 120 kd. In later stages, (late Do to D1-4), numerous other cross-reacting bands were observed, the most prominent occurring at 105 kd and >200 kd.

The pattern of proteins from limb muscle which cross-reacted with GGD 11.6 appeared to be relatively uniform during proecdysis (Fig. 4). In addition to the 70 and 120 kd cross-reacting proteins described above, a diffuse band at approximately 105 kd was consistently observed. There were no major changes in the relative intensities of these protein bands during proecdysis.

Summary

Using an antibody directed against the highly conserved DNA binding domain of the *Drosophila* EcR, a number of different cross-reacting proteins can be identified in Western blots of *Uca* tissues. The molecular weights of the most consistent bands were 70, 105, and 120 kd. These forms seem to be distributed differently in different tissues, consistent with the hypothesis that they represent different isoforms.

In hypodermis, a 70-80 kd protein cross-reacted with the murine anti- *Drosophila* EcR monoclonal antibody GGD 11.6 during all of proecdysis. Another protein band at 120 kd appeared in the samples in which apolysis was observed or where the ecdysteroid titer was found to be rising.

Immunoreactive bands at approximately 70, 105 and 120 kd were observed in limb bud tissue samples examined. A protein band at 120 kd appeared consistently during all of proecdysis, whereas a protein band at 105 kd appeared only late in D_0 and D_{1-4}.

Results of muscle from nonregenerating limbs showed three bands at 70, 105 and 120 kd. There were no qualitative differences (i.e. no major pattern shifts) in the relative intensities of these bands during proecdysis.

Discussion

A 70-80 kd immunoreactive protein which appears in hypodermis during proecdysis is consistent with earlier molecular weight determinations of crustacean intermolt hypodermal EcRs based on binding studies (Londershausen and Spindler, 1981; Londershausen et al., 1982). These results suggest that this 70 kd protein may be a major

EcR in hypodermis, and by extension, other tissues.

Immunoreactive proteins with molecular weights of 105 and 120 kd appear consistently in limb buds and muscle. The protein bands at 105 and 120 kd appear to increase in intensity in limb buds late in D_0 and D_{1-4}. This occurs at the same time as circulating levels of ecdysteroids begin to rise prior to ecdysis. Thus the accumulation of these immunoreactive proteins correlates with the switch from D_0 to D_{1-4} in limb buds. The 120 kd protein is also found in greater amounts in hypodermis after apolysis. Since ecdysteroid titers increase from about 25 pg/µl during D_0 to over 100 pg/µl during D_{1-4} these data suggest that the appearance of the 120 kd protein may be dependent on ecdysteroid titers in hypodermis. A protein sample containing the 120 kd band, staged at D_0 by growth measurements but shown to have an elevated ecdysteroid titer by RIA, supports this hypothesis.

In nonregenerating muscle, however, the bands at 105 and 120 kd are relatively constant throughout proecdysis. The immunoreactive 105 and 120 kd proteins may therefore subserve different functions in limb buds and muscle.

A complex pattern of proteins immunoreactive with an EcR antibody is seen in *Uca pugilator* tissues during proecdysis. Such a pattern is reminiscent of the distinctive pattern of tissue expression of isoforms of EcR reported for *Drosophila* at metamorphosis (Robinow et al., 1993). We have recently cloned a portion of the *Uca* EcR receptor and are in the process of developing homologous immunological probes which will clarify the relationships between the various proteins identified in this study and their distributions during crab growth and development.

Literature cited

Bliss, D. E. (1956) Neurosecretion and the control of growth in a decapod crustacean. *In* "Bertil Hanstrom. Zoological papers in honour of his sixty-fifth birthday" (K. G. Wingstrand, Eds.), pp. 56-75. Zoological Institute, Lund.

Durica, D. S. and P. M. Hopkins (1995) Expression of the genes encoding the ecdysteroid and retinoid receptors in regenerating limb tissues from the fiddler crab, *Uca pugilator*, Gene 171:237-241.

Hopkins, P. M. (1986) Ecdysteroid titers and y-organ activity during late anecdysis and proecdysis in the fiddler crab, *Uca pugilator*. Gen. Comp. Endocrinol. 63: 362-373.

Hopkins, P. M. (1992) Hormonal control of the molt cycle in the fiddler crab *Uca pugilator*. Amer. Zool. 32: 450-458.

Koelle, M. R., W. S. Talbot, W. A. Segraves, M. T. Bender, P. Cherbas, and D. S. Hogness (1991) The *Drosophila* EcR gene encodes an ecdysone receptor, a new member of the steroid receptor superfamily. Cell 67: 59-77.

Laemmli, U. K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. Nature 227: 680-685.

Londershausen, M. and K.-D. Spindler (1981) Characterization of cytoplasmic ecdysteroid receptors in the hypodermis of the crayfish, *Orconectes limosus*. Mol. Cell. Endocrinol. 24: 253-265.

Londershausen, M., P. Kuppert and K.-D. Spindler (1982) Ecdysteroid receptors: a comparison of cytoplasmic and nuclear receptors from crayfish hypodermis. Hoppe-Seyler's Z. Physiol. Chem. 363: 797-802.

Robinow, S., W. S. Talbot, D. S. Hogness, and J. W. Truman (1993) Programmed cell death in the *Drosophila* CNS is ecdysone-regulated and coupled with a specific ecdysone receptor isoform. Development 119: 1251-1259.

Talbot, W. S., E. A. Swyryd, and D. S. Hogness (1993) *Drosophila* tissues with different metamorphic responses to ecdysone express different ecdysone receptor isoforms. Cell 73: 1323-1337.

FIG. 1 Western blot analysis of protein extracts from *Drosophila* pupae. Proteins from *Drosophila* pupae were electrophoresed, blotted, and probed with murine anti-*Drosophila* EcR monoclonal antibodies AD 4.4 (lane 1), GGD 11.6 (lane 2), JG 6.2 (lane 3) and EEC 11.1 (lane 4), followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Then the bands were visualized with enhanced chemiluminescence reagents (Amersham).

FIG. 2 Western blot analysis of protein extracts from hypodermis in *Uca pugilator*. Proteins from hypodermis during early D_0 (lane 1-4) and late D_{1-4} (lane 5-8) were electrophoresed, blotted, and probed with anti-*Drosophila* EcR monoclonal antibodies GGD 11.6, followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Then the bands were visualized with enhanced chemiluminescence reagents (Amersham). Partial apolysis was observed in the samples at lane 5 and 6, while complete apolysis was observed in the samples at lane 7 and 8. Lane 9 was protein extract of *Drosophila* pupae.

FIG. 3 Western blot analysis of protein extracts from limb buds of *Uca pugilator*. Proteins from limb buds during early D_0 (lane 1-4), late D_0 (lane 5-8), early D_{1-4} (lane 9-12) and late D_{1-4} (lane 13-16) were electrophoresed, blotted, and probed with anti-*Drosophila* EcR monoclonal antibodies GGD 11.6, followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Then the bands were visualized with enhanced

chemiluminescence reagents (Amersham). Lane 17 was protein extract of *Drosophila* pupae.

Figure 4 Western blot analysis of protein extracts from muscle of nonregenerating legs of *Uca pugilator*. Proteins from muscle during early D_0 (lane 1-4), late D_0 (lane 5-8), early D_{1-4} (lane 9-12) and late D_{1-4} (lane 13-16) were electrophoresed, blotted, and probed with anti-*Drosophila* EcR monoclonal antibodies GGD 11.6, followed by a horseradish peroxidase-conjugated secondary antibody (Bio-Rad). Then the bands were visualized with enhanced chemiluminescence reagents (Amersham). Lane 17 was protein extract of *Drosophila* pupae.

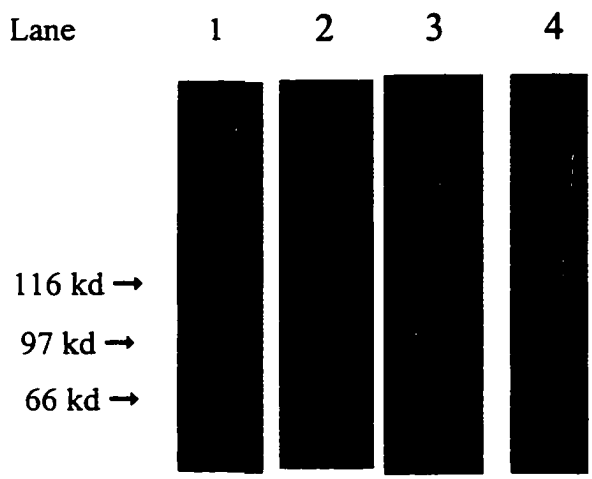


FIG. 1

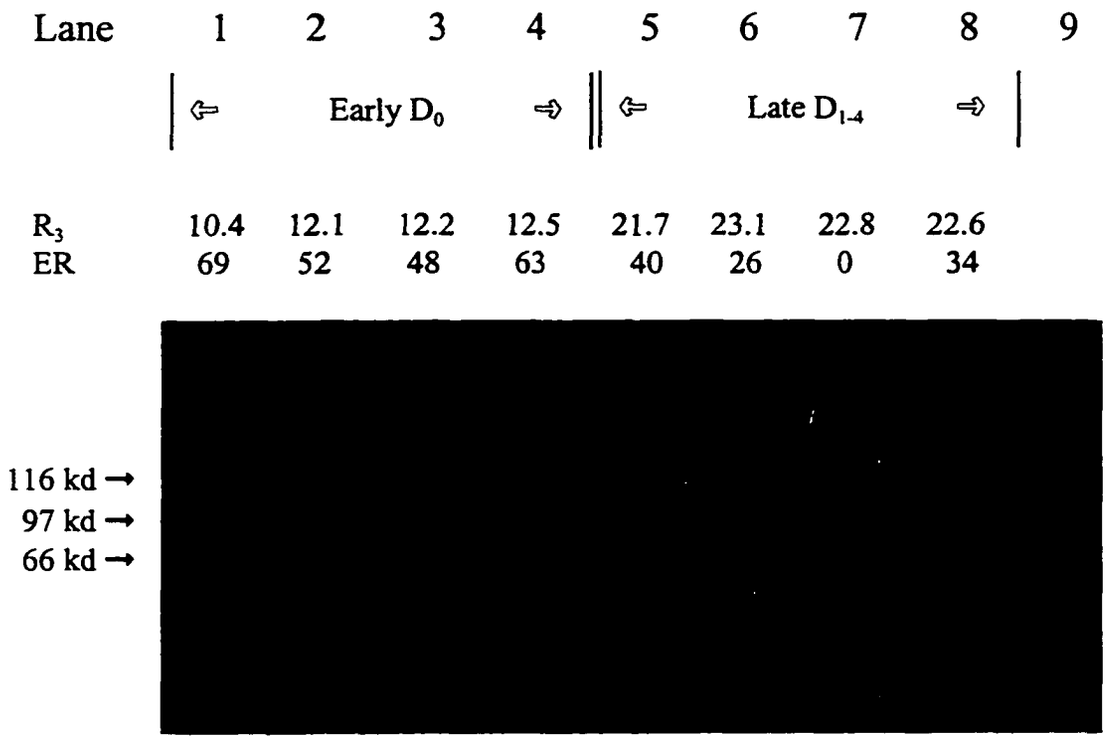

Lane 1 2 3 4 5 6 7 8 9
Early D_0
Late D_{1-4}
R_3 10.4 12.1 12.2 12.5 21.7 23.1 22.8 22.6
ER 69 52 48 63 40 26 0 34
116 kd
97 kd
66 kd

FIG. 2

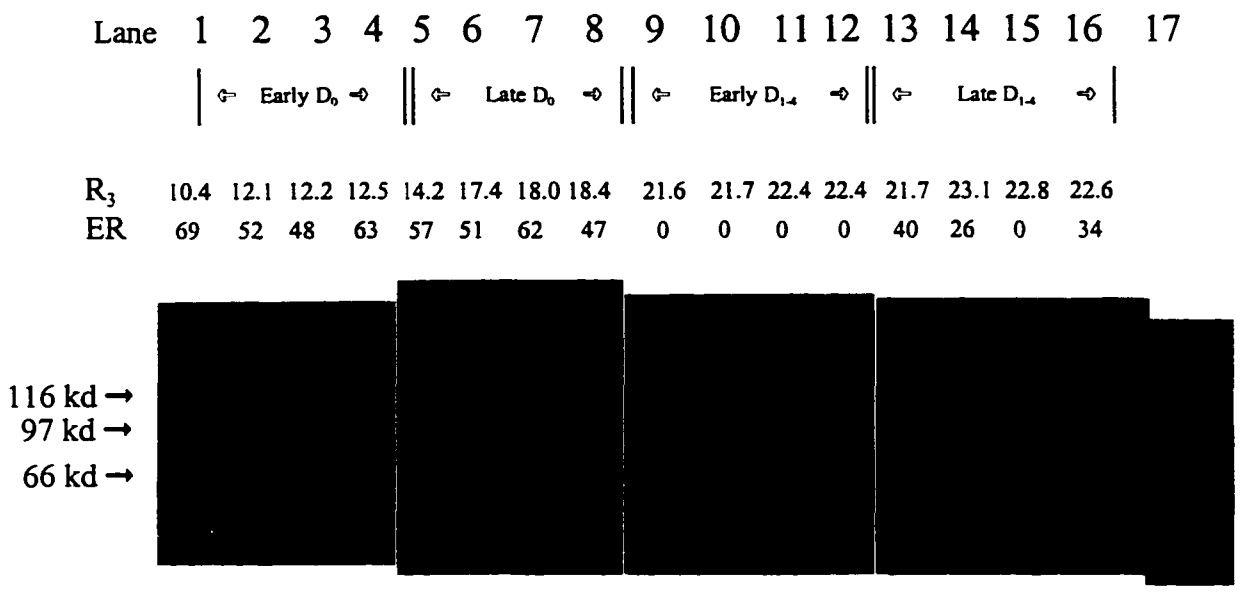

Lane 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
Early D_0
Late D_0
Early D_{1-4}
Late D_{1-4}
R_3 10.4 12.1 12.2 12.5 14.2 17.4 18.0 18.4 21.6 21.7 22.4 22.4 21.7 23.1 22.8 22.6
ER 69 52 48 63 57 51 62 47 0 0 0 0 40 26 0 34
116 kd
97 kd
66 kd

FIG. 3

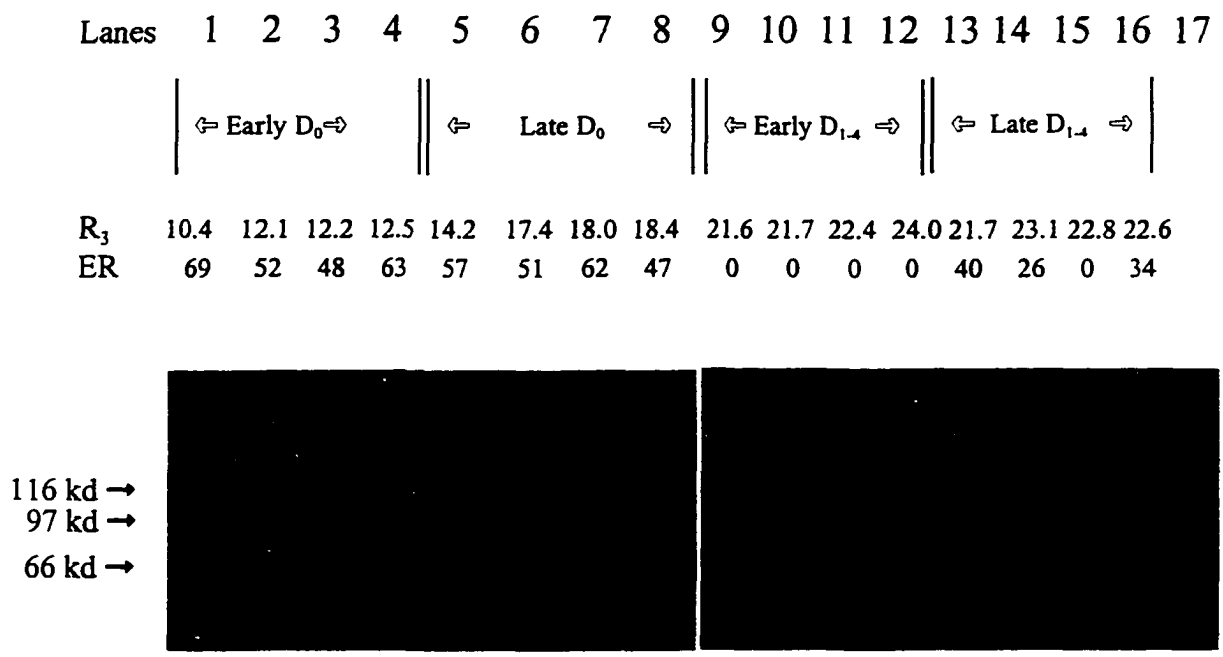
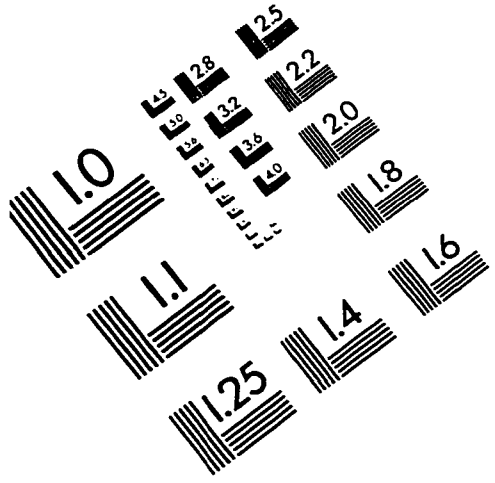
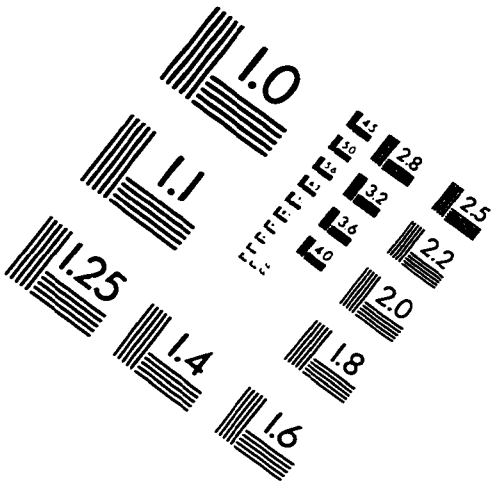
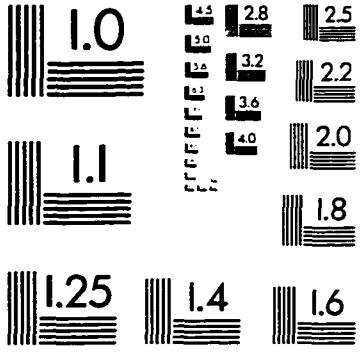

Lanes 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
⇦ Early D0⇨
⇦ Late D0 ⇨
⇦ Early D1-4 ⇨
⇦ Late D1-4 ⇨
R3 10.4 12.1 12.2 12.5 14.2 17.4 18.0 18.4 21.6 21.7 22.4 24.0 21.7 23.1 22.8 22.6
ER 69 52 48 63 57 51 62 47 0 0 0 0 40 26 0 34
116 kd →
97 kd →
66 kd →

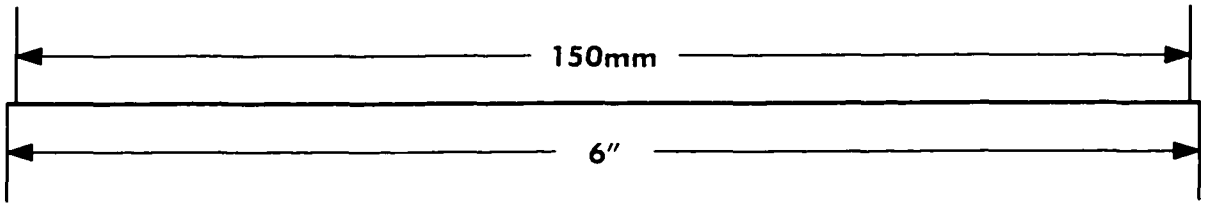
FIG. 4

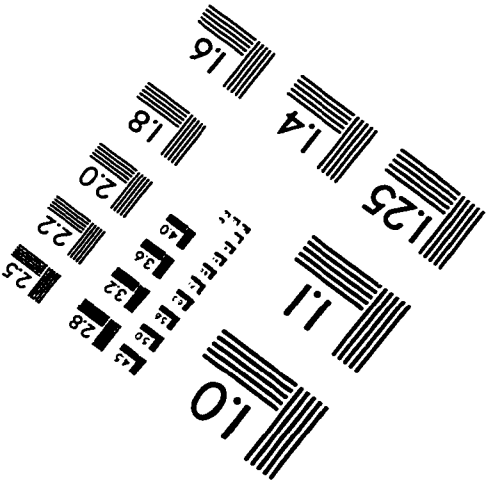
IMAGE EVALUATION
TEST TARGET (QA-3)

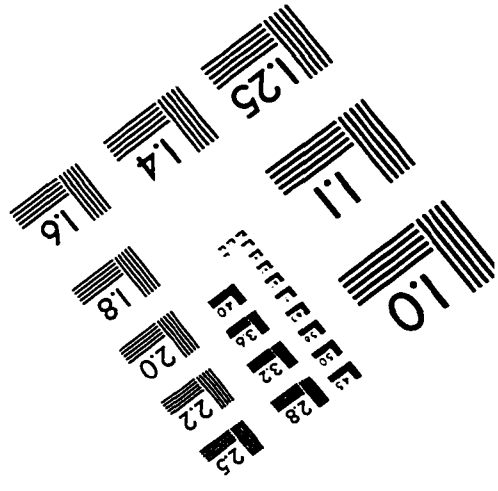












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