Cloning of a shrimp (*Metapenaeus ensis*) cDNA encoding a nuclear receptor superfamily member: an insect homologue of E75 gene

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Abstract Degenerate primers were derived from the amino acid sequence in the DNA binding domain of the *Drosophila* ecdysone receptor (DmEcR). Several partial cDNAs were amplified from the shrimp epidermis by reverse transcription polymerase chain reaction (RT-PCR). One of these fragments shows the highest amino acid sequence homology to the insect ecdysone inducible gene E75. This partial cDNA was used as a probe to screen the swimming leg cDNA library of the shrimp, *Metapenaeus ensis*. A 3.6 kb cDNA clone was obtained. The longest open reading frame of this cDNA consists of 606 amino acids and its deduced amino acid sequence has all five domains typical of a nuclear receptor. The putative polyadenylation signal is located at about 400 bp 3′ to the stop signal. The deduced amino acid sequence of this cDNA shows the highest identity to that of the E75A reported in *Manduca sexta*, *Galleria melonella*, *Drosophila melanogaster*, and *Choristoneura fumiferana*. Based on the amino acid sequence comparison, the shrimp nuclear receptor is considered the insect homologue of E75A. Northern blot analysis shows that the shrimp E75 is expressed in the epidermis, eyestalk and the nerve cord of the pre-molt shrimp. Moreover, E75 transcripts can be detected in the epidermal tissues of early pre-molt shrimp by in situ hybridization. To determine whether the shrimp could also express other E75s like the insects, 5′ end RACE and RT-PCR were performed on epidermal cDNA of a single shrimp. Subcloning and DNA sequence determination of the PCR products confirmed the presence of two other forms of E75 (tentatively called E75C and E75D) in shrimp. By RT-PCR, different levels of E75 expression can be detected in the epidermis, nerve cord and the eyestalk of early pre-molt shrimp. In addition to the different levels of expression of the shrimp E75s in the epidermis, the pattern of their expression is also different during the molting cycle. This is the first report on the cloning of a shrimp nuclear receptor superfamily member.

Key words: Shrimp; Nuclear receptor; E75; Ecdysone inducible gene

1. Introduction

Crustaceans and insects share a number of hormones that regulate their important physiology. For example, molting is stimulated by an increase of ecdysteroid titer during the early pre-molt stage. Ecdysteroid stimulates the detachment of the epidermis from the endo-cuticle and triggers a series of events leading to the formation of a new cuticle [1]. This change is also accompanied by a change in different enzymatic activities corresponding to the degradation of the old cuticle and the formation of a new cuticle. It has been suggested that ecdysone stimulates the molting event through a coordination of changes in gene transcription via its receptor [2]. In insects, ecdysone induction of gene expression has been the focus of research for many years [3]. Studies of ecdysone action have been initiated from the puffing of the salivary gland polytene chromosome [4,5]. In this model, ecdysone binds to its receptor and turns on the expression of the ecdysone responsive genes. This results in the formation of early puffs in the polytene chromosome [2]. Most of these ecdysone responsive genes encode steroid receptor protein and function as transcription factors. Although the initial hormonal event of both insect and crustacean molting is similar, there is no information on the role of ecdysteroid and its receptor in the regulation of gene expression in crustaceans. Moreover, crustaceans and insects appear to regulate the synthesis and production of ecdysteroid by a different mechanism. For example, the synthesis of ecdysone is inhibited by the molt-inhibiting hormone in crustaceans [6]. However, in insects, the brain releases the prothoracicotropic hormone (PTTH) which stimulates the production of ecdysone by the prothoracic gland [7]. It is possible that the following events after the initial increase of ecdysteroid titer and the following molecular events in molting may be different. To advance our understanding of the regulatory role of ecdysone in crustacean molting, we attempted to isolate ecdysteroid and ecdysone inducible gene(s) in shrimp. Because of the lack of shrimp-specific probes, we attempted to isolate steroid hormone receptor in shrimp by a combination of RT-PCR cloning and cDNA library screening approaches.

2. Materials and methods

2.1. Animals

Shrimp were purchased from a local seafood market and acclimated at 25°C in a 12 h light and 12 h dark photoperiod. The molt stages of these shrimp were identified by pleopod setogenesis [8]. Briefly, a small piece of pleopod (swimming leg) was cut and setal development was observed under the microscope. Shrimp at different molt stages were used for total RNA extraction as described below.

2.2. Preparation of total RNA and RT-PCR

Shrimp tissues were dissected with a pair of sharp scissors, rinsed in 70% ethanol and quickly homogenized in a glass homogenizer for total RNA using the guanidine isothiocyanate method [9]. The quality of total RNA was monitored by running on a 1.2% denatured RNA gel. Intact RNA (1 μg) was used for first strand cDNA synthesis by the reverse transcription reaction. Reverse transcription (RT) was performed in a final concentration of 1×transcription buffer (50 mM Tris-HCl, 8 mM $MgCl_2$, 30 mM KCl, 2 mM each of dNTP, 10 mM DTT), 2 pmol of oligo dT17 primer, and 1 unit of reverse transcriptase (Promega, USA). The reaction mixture was incubated at 42°C for

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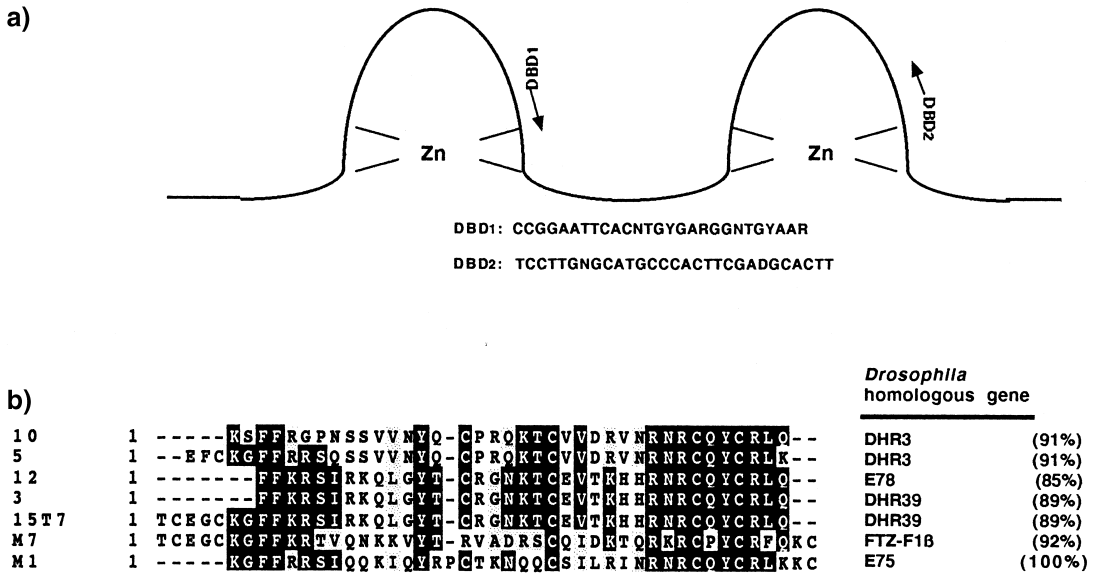

Fig. 1. RT-PCR cloning the DNA binding domain of shrimp nuclear receptor epidermis. a: Location and sequence of degenerated primers (DBD1 and DBD2) on the zinc finger region. b: Comparison of the predicted amino acid sequence of the shrimp nuclear receptors resulting fromRT-PCR. The amino acid sequences of the shrimp putative nuclear receptors are named as 10, 5, 12, 3, 15T7, M7 and M1 on the left. The corresponding *Drosophila* nuclear receptor homologues are indicated on the right. The amino acid identities (%) are shown in parentheses.

2 h and later used as template for PCR. For RT-PCR, we designed forward (DBD1) and reverse (DBD2) primers based on the known sequence of the ecdysone receptor [10] (DBD1: CCGGAATTC-ACNTGYGARGGNTGYAAR and DBD2: TCCTTGNGCATGC-CCACTTCGADGCACTT). The final PCR mix (30 μl) consisted of 10 mM Tris-HCl, pH 8.0, 1.5 mM $MgCl_2$, 50 mM KCl, 0.5 pmol primer and 2.5 μl reaction mix from the reverse transcription as described above. The PCR conditions were 35 cycles of denaturation at 95°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. At the end of the last cycle, the reaction mix was further incubated for 10 min at 72°C for the completion of DNA synthesis. PCR products were analyzed on a 1.5–2.0% agarose gel. Target DNA fragments of expected sizes (approximately 140 bp) were excised from the gel and subcloned into a pBluescript cloning vector (Stratagene, USA). DNA sequencing determination was performed using a T7 DNA polymerase sequencing kit from Pharmacia (USA) based on the dideoxy chain termination method [11]. DNA and amino acid sequence derived from the cDNAs were compared and analyzed with the GenBank database.

2.3. cDNA library construction and screening

Poly(A)$^+$ RNA was purified using the polyT tract (Promega, USA) mRNA synthesis kit. Using 1–2 μg of poly(A)$^+$ RNA, an eyestalk cDNA library was constructed in the vector lambda Zap-II according to the instruction of the manufacturer (Stratagene, USA). Phage particles were transferred on duplicated circular nylon membrane (Hybond, Amersham, USA). Hybridization of replica filters was performed at 65°C overnight in a hybridization buffer (0.5 M sodium phosphate, 1% SDS, 2 mM EDTA, 1×Denhardt's solution and 100 μg/ml denatured salmon sperm DNA without formamide). The probe used in the screening was derived from the cDNA of the initial PCR clone (134 bp). Filters were washed at a final concentration of 0.5×SSPE and 0.1% SDS at 65°C twice for 30 min. After the third round of screening, potential positive phage plaques were purified and the recombinant pBK-CMV phagemids were rescued from the bacteriophage clones by in vivo excision according to the instruction of the manufacturer (Stratagene, USA). DNA sequence determination on both strands was performed using a T7 DNA polymerase and the dideoxynucleotide chain termination method.

2.4. RT-PCR cloning of E75C and E75D forms

Epidermal total RNA from the pre-molt stage of one shrimp was extracted for total RNA. Ten micrograms of the RNA were used in the RT reaction using a gene-specific reverse primer (LBD2). The 5′ end of the partial 5′ end cDNA was linked to an adapter. PCR was performed using an adapter-linker primer. A second round of nested PCR was performed using either the forward primers 75A, 75C or 75D or the common reverse primer 75R1.

2.4.1. In situ hybridization. Pleopod were fixed in 4% paraformaldehyde, 2.5% NaCl, 50 mM sodium phosphate (pH 7.5) and embedded in parafilm. Sections of 6 μm thick were mounted on subbed slides and the parafilm was removed by soaking the slide in xylene, hydrated in decreasing concentrations of ethanol, and digested with 1 μg proteinase K (5 μg/ml) at 37°C. Samples were hybridized at 42°C in a hybridization buffer containing formamide and digoxigenin labeled non-radioactive E75 probe. Washes consisted of 2×SSC, 0.1% SDS twice at 60°C for 30 min and 0.1×SSC, 0.1% SDS twice at 60°C for 30 min. Detection of the signal was performed according the instruction of the non-radioactive labeling kit (Boehringer-Mannheim, Germany).

Table 1
Comparison of the predicted amino acid sequences of the shrimp and insect E75 homologues

Region	Length (amino acids)						Identity (%)				
	MeE75	DmE75	MsE75	CfE75	GmE75	HsEar-1	DmE75	MsE75	CfE75	GmE75	HsEar-1
A/B	31	244	41	46	46	102	9	21	30	37	11
C	67	67	56	67	67	67	100	92	100	100	77
D	67	67	68	68	67	67	34	35	35	35	19
E	213	207	206	206	206	105	54	54	54	54	52
F	228	652	314	302	325	244	14	23	23	23	13
Overall	606	1151	685	689	711	579	21	41	44	44	22

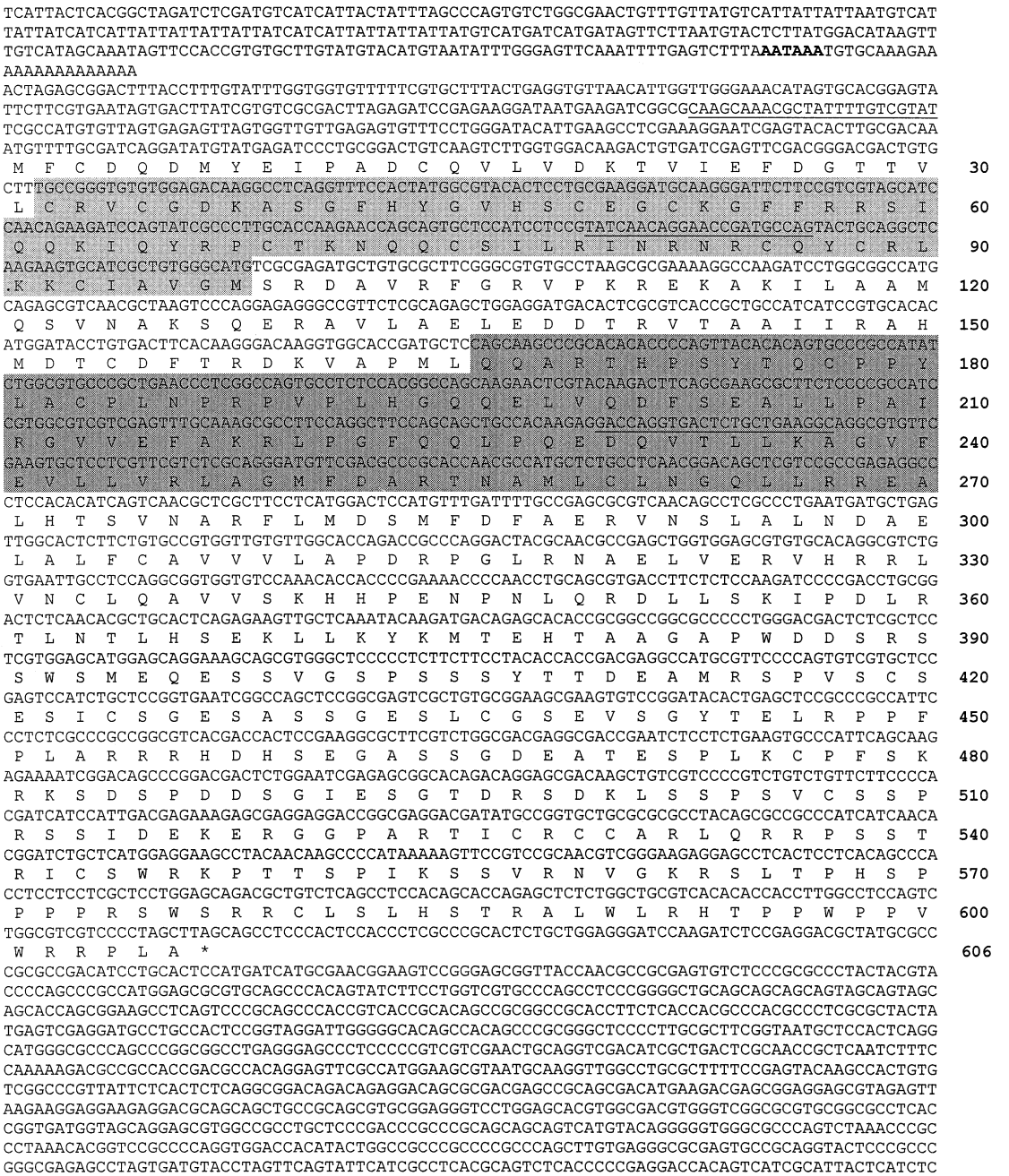

Fig. 2. Nucleotide and predicted amino acid sequence of the shrimp MeE75A. The putative DNA binding domain is shown in the gray box and the ligand binding domain is shown in the darker box. Numbers on the right indicate amino acid positions with the cDNA. The putative poly(A) signal is in bold letters. The primers used in RT-PCR (E75A and E75R1) are underlined.

3. Results

3.1. Isolation and characterization of shrimp cDNA encoding E75

RT-PCR amplification of the shrimp swimming leg cDNA and subsequent cloning of target fragments revealed that some of these fragments encoded proteins that were homologous to the DNA binding domain of nuclear receptors (Fig. 1). The deduced amino acid sequence of M1 showed 100% sequence identity to the insect ecdysone inducible gene, E75A. The shrimp partial cDNA was then used as a probe to screen 150 000 plaques of the shrimp pleopod (swimming leg) cDNA library. After the third round of library screening, a positive clone of approximately 3.6 kb was isolated. This cDNA revealed a 1818 bp open reading frame (ORF) encoding 606 amino acids and consisted of a polyadenylated signal only 9 bp 5′ of the poly(A) tail (Fig. 2). The deduced amino acid sequence of the cDNA revealed a high level of similarity with that of nuclear receptor superfamily members. Like other nuclear receptors, the deduced amino acid sequence of the

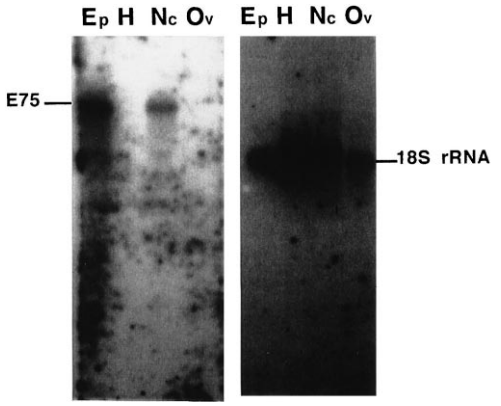

Fig. 3. Northern blot detection of the shrimp E75. Twenty micrograms of total RNA were used for Northern blot analysis. The blot was hybridized by a probe derived from the DBD and LBD region DBD of the E75 cDNA (left panel). The right panel shows the Northern blot analysis of the same nylon membrane with a shrimp 18S rRNA probe to show the integrity of the RNA samples. Lanes are: Ep (epidermis), Hp (hepatopancreas), Nc (nerve cord) and Ov (ovary).

shrimp cDNA could be divided into five domains (A/B, C, D, E, and F). A/B represents the variable N-terminal end of the protein, region C represents the DNA binding domain, region D is the variable linker region joining the DBD and LBD while region E is the ligand binding domain. Comparison of the shrimp E75 with the *Drosophila* DmE75 [12], *Choristoneura* CfE75 [13] and *Galleria* GmE75 [14] showed an overall 100% identity in the DBD region (Table 1). Another region that is conserved among members of the nuclear receptor superfamily is the E region. It is required for trans-activation function [15]. The ligand binding domain consists of 206–216 amino acids and is separated by a linker of 67–69 amino acids. The LBD of the shrimp E75 showed an overall 54% amino acid identity with that of other insects. When MeE75 was compared to the vertebrate steroid receptor genes (Table 1), it was most similar to the human ear-1 [16].

3.2. Expression of shrimp E75 in shrimp

MeE75 was expressed in the epidermis and nerve cord of the pre-molt shrimp (Fig. 3). By in situ hybridization, shrimp E75 was detected in the epidermis of the early pre-molt (stage Do) shrimp but it was not detected in the muscle (Fig. 4). To determine whether the shrimp also expressed other E75s like the insect, RT-PCR and 5′ end RACE were performed using cDNA from the swimming leg of one pre-molt shrimp. A gene-specific reverse primer E75R1 derived from the LBD of the MeE75A and a non-specific primer from the 5′ end RACE kit (Clontech, USA) was used in the 5′ end RACE. The resulting PCR products were analyzed by Southern blot analysis using an internal probe containing the sequence for the DBD. The results (not shown) indicated that three DNAs of various sizes were amplified. DNA sequence determination was performed and confirmed the presence of two other E75s in the shrimp (Fig. 5b). Based on our initial PCR cloning of the shrimp genomic DNA with E75R and these three primers, an intron (approximately 300–400 bp) was detected in the first zinc finger of all three forms (data not shown). PCR amplification of mRNA containing the E75s with primers flanking the DBD resulted in smaller fragments when compared to PCR amplification with genomic DNA as a template. To study the expression of these three forms in the epidermis during the molting cycle, RT-PCR was performed and three different primers (75A, 75C and 75D) were designed (Fig. 5). From the result of the RT-PCR, E75A appeared to be expressed continuously throughout the molting cycle. E75C was expressed most abundantly. Two peaks appeared for the expression of E75C. The first peak occurred during the transition from the inter-molt to the pre-molt stage. The second peak appeared in the later pre-molt stage. The pattern of expression for E75, however, was similar to that of the E75A (Fig. 6).

4. Discussion

Since the DNA binding domain and the P-box of the shrimp E75 (EGCKG) is 100% identical to that of other E75s, the MeE75 may also compete with ecdysone for binding in the response element [14]. The E region of the MeE75 is less homologous to the LBD of insects. Among insects, the LBD shares over 97% amino acid identity. Since this region is where the ligand will bind, the diversity of sequence similarity suggested that a different ligand may bind to the LBD of shrimp. However, there are specific regions in the LBD which are conserved among these E75s. Based on the high degree of amino acid identity in both region C and region E, the shrimp cDNA may be considered an insect homologue of E75. Since there is no information on the ligand for the shrimp E75, it

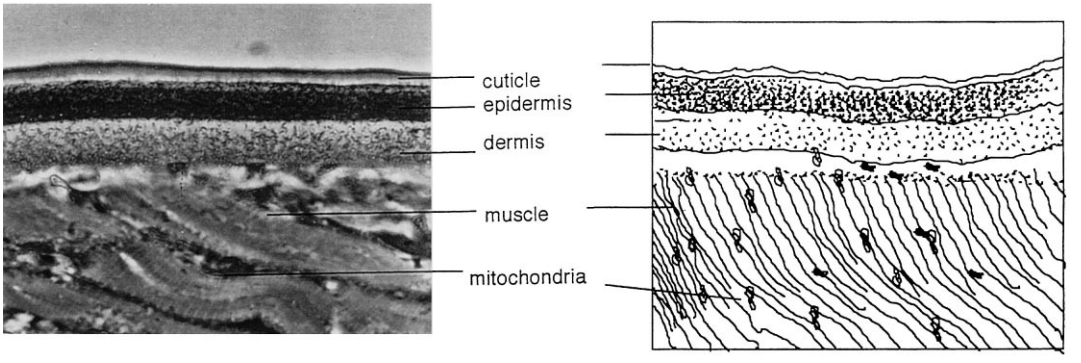

Fig. 4. Localization of E75 in the epidermis of shrimp. a: In situ hybridization to demonstrate expression of the shrimp E75 in the early pre-molt stage (Do). The darker color indicates the localization of the MeE75 messages in epidermal tissue. No hybridization signals were observed in the cuticle and the muscle. b: Schematic drawing of the corresponding epidermal and dermal regions of the shrimp integument.

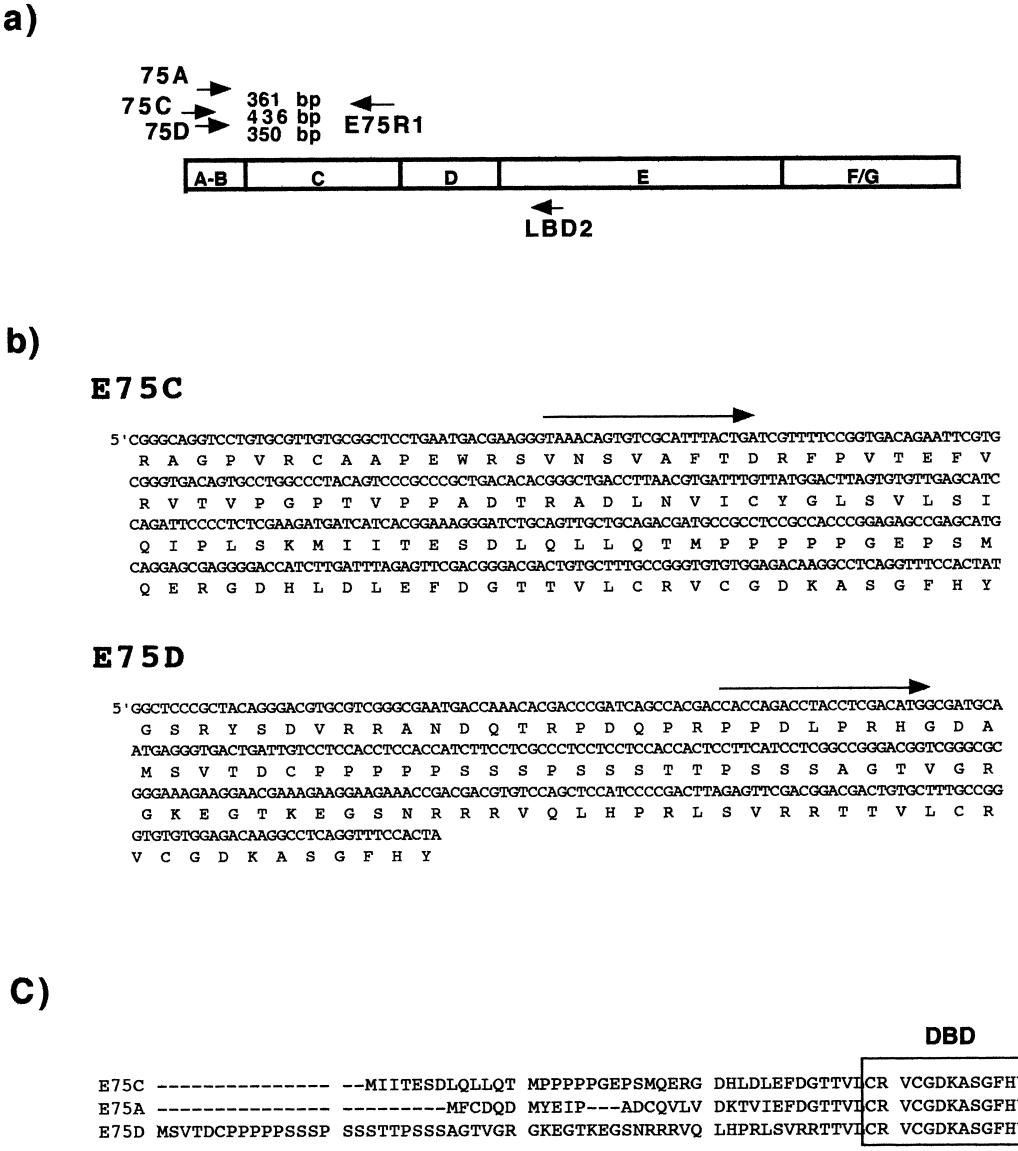

Fig. 5. RT-PCR cloning of other E75s in shrimp. a: Sketch of the MeE75 cDNA and the relative positions of the primers (75C, 75D, E75R1) used in the RT-PCR and 5′ end RACE (LBD2). b: Nucleotide sequence and amino acid sequence of the E75C and E75D generated from the cloning of 5′ end RACE. The locations of primers (E75C and E75D) used in RT-PCR are indicated by arrows. c: Comparison of the N-terminal end of the shrimp E75s.

remains classified under the category of orphan steroid receptors.

Although the level of their expression may be different, other forms of E75s have been identified in shrimp. Like the insect, E75 is widely distributed in shrimp. In the abdominal tissue of *Choristoneura fumiferana*, RNA for CfE75 is most abundant at the time of the ecdysteroid peaks during molting. In the waxworm, *G. melonella*, two peaks of GmE75A RNA were detected. Each of these corresponded to the time of ecdysteroid rise. Thus, the pattern of expression for the shrimp E75C is similar to that of the waxworm. In *Drosophila* and *Manduca*, the E75 gene consists of two overlapping transcription units [12,17]. E75B results from the alternative splicing of the E75 gene. This receptor consists of only one zinc finger and may be responsible for competing with other forms of E75 for regulation of certain genes. However, a similar cDNA in shrimp has not been detected.

This is the first report on the cloning of a nuclear receptor superfamily member in shrimp. There are only a few reports on the cloning of nuclear receptors in decapod crustaceans. For example, a lobster nuclear receptor homologous to the insect MHR3 and two nuclear receptors homologous to the insect ecdysone receptor and ultrasprical (USP) have been reported [18,19]. In the crab and lobster, the DBD and the LBD of these receptors show only 91–93% amino acid identity to the respective domains of the insects. However, the DBD and LBD of the shrimp MeE75 show a much higher amino acid sequence identity to that of the insect counterpart. As in insects, MeE75 can be detected in the epidermis. The high degree of amino acid identity (100%) in the DBD region of the shrimp E75 with the insect suggests that it may bind to the same recognition sequence as the E75 of the insects. On the other hand, because of the lower degree of amino acid identity in the LBD, the shrimp E75 may not need a ligand for its

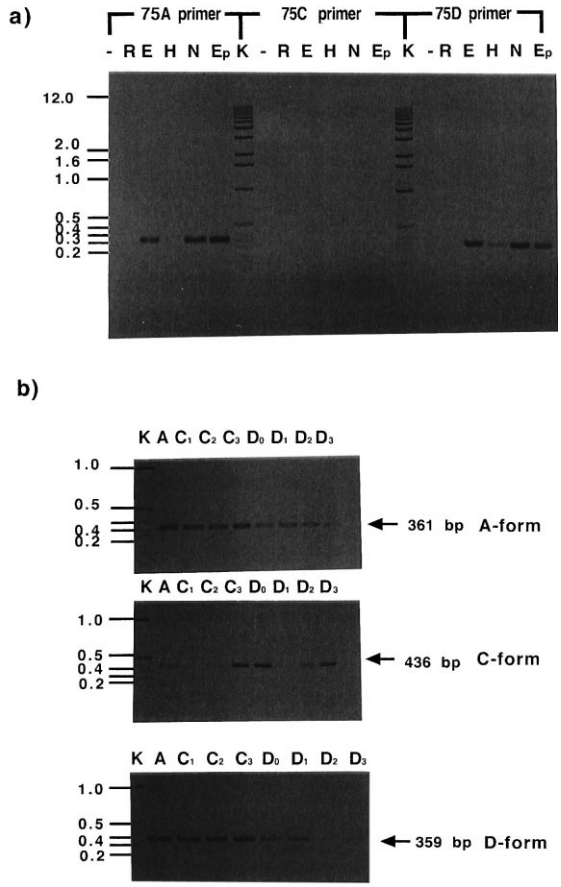

Fig. 6. Southern blot analysis of RT-PCR products to detect the expression of MeE75. a: detection of E75A, E75C and E75D in various tissues. Lanes are: −: negative control without RT; R: no template (cDNA) control; E: eyestalk; H: hepatopancreas; N: nerve cord, Ep: epidermis with some muscle. b: Expression of E75s in the epidermis (with muscles) of an individual shrimp during the molting cycle (stage A–D3). K: 1 kb DNA marker labeled with [γ-^{32}P]ATP with a kinase. The probe used derived from the DBD domain of the cDNA.

proper functioning. However, we cannot exclude the possibility that the LBD may be bound by a ligand similar to that of the insect. In summary, using a combination of RT-PCR and library screening, a cDNA clone from the swimming leg of shrimp has been isolated. This cDNA is a potential shrimp nuclear receptor superfamily member homologous to the insect E75. We have also identified several partial cDNAs encoding other potential nuclear receptor superfamily members. Some of these nuclear receptors are also homologous to other ecdysone inducible genes, i.e. DH39 [20], FTZ-F1 [21], E78 and DHR3 as discovered in insects. The isolation of several shrimp cDNAs homologous to insect suggests that crustaceans and insects may share a similar regulatory mechanism for the control of molting at the molecular level.

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