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Molecular Cloning of a Novel Crustacean Member of the Aldoketoreductase Superfamily, Differentially Expressed in the Antennal Glands

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Biochemical studies on ecdysteroid metabolism in arthropods suggest that aldoketoreductase enzymes (AKRs) may be involved in this pathway, but very few molecular data are available on these oxidoreductases in invertebrates. Looking for such enzymes in the crayfish *Orconectes limosus*, we have used a PCR strategy with primers deduced from a recent insect 3β-reductase sequence, and from mammalian 5β-reductase sequences. A full-length cDNA, corresponding to a putative AKR, was isolated from crayfish antennal gland. This cDNA contains an open-reading frame of 1008 bp, encoding a predicted protein of 336 amino acids. Northern blots indicated a restricted expression of the transcript in the antennal glands, quite constant during the molting cycle, and *in situ* hybridization demonstrated a strong expression of the transcript in the labyrinth. This is to date the first member of the AKRs superfamily characterized in a crustacean species, and the putative function of the corresponding enzyme is discussed.

Ecdysteroids regulate fundamental events in the life of arthropods such as growth, molting and larval development. At the present time, the metabolic pathway of ecdysteroids, which leads to the production of ecdysone (E), 3-dehydroecdysone (3DE) and/or 25-deoxyecdysone (25dE) is only partially known in arthropods (reviews in Refs. 1 and 2). Biochemical studies allowed the identification of some of the involved enzymes, in arthropod molting glands and peripheral tissues, like cytochrome-P450 (CYPs) or oxidoreductases. Some of these enzymes, especially CYPs, have been structurally characterized in arthropods, e.g., CYP18 from *Drosophila melanogaster* (3), CYP6H1 from *Locusta migratoria* (4), or CYP4C15 from *Orconectes limosus* (5). However, very few molecular data are yet available for invertebrate oxidoreductases. In mammals, these enzymes are divided into three groups: the long-chain alcohol dehydrogenases, which use zinc for catalysis, the short-chain alcohol dehydrogenases (SDRs), which are multimeric non-metallo-oxidoreductases, and the aldoketoreductases (AKRs) (6, 7). An increasing number of enzymes belonging to this third superfamily has been identified by cDNA cloning and there are now more than 40 proteins belonging to this group, with members found in a large variety of organisms (bacteria, yeast, plants, vertebrates). AKRs are monomeric proteins of about 320 amino acids in length, with NADPH-dependent catalytic activity. They metabolize a wide range of substrates like steroids, aldehydes, monosaccharides and polycyclic aromatic hydrocarbons (reviews in Refs. 8 and 9). Several mammalian hydroxysteroid dehydrogenases (HSDs) belong to this superfamily, like the 3α- or 20α-HSD (Review in Ref. 10), whereas 3β-HSD belongs to the SDRs.

Recently, a 3DE-3β-reductase from the cotton leaf worm *Spodoptera littoralis* has been cloned (11) and shown to belong to the AKRs. 3DE is the major ecdysteroid produced by the prothoracic glands of most Lepidopteran species, together with a varying proportion of ecdysone (12). In insects, after its secretion by the prothoracic gland, 3DE is converted into ecdysone (E) by a 3DE-3β-reductase present in the hemolymph and other tissues. 3DE is also the major product of the Y-organs from several crustacean species, as *Orconectes limosus* (13). In this crayfish, *in vitro* studies of 3DE metabolism revealed the same pathway, with a major 3β-reducing activity in the antennal glands (i.e., kidneys) (14). The 5β-reductases (Δ^4-3-ketosteroid-5β-reductases) also belong to the AKR family: they have been characterized and cloned in human (15) and rat (16) and play an important role in bile acid biosynthesis. A 5β-reductase has been demonstrated as an early step of ecdysteroid biosynthetic pathway in the Y-organ of the crab *Carcinus maenas* (17) and also in the crayfish (Blais, pers. comm.).

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Looking for AKRs involved in ecdysteroid metabolism in *O. limosus*, we have used a PCR-based strategy with degenerate primers in an attempt to amplify cDNA fragments from various tissues, i.e., Y-organs for 5β-reductase assays, and antennal glands for 3DE-3β-reductase. We report here the isolation of a full-length cDNA encoding an AKR specifically expressed in the crayfish antennal gland, as observed by Northern blot and *in situ* hybridization. The biological function of the corresponding enzyme is discussed.

MATERIALS AND METHODS

Animals and tissue collection. Crayfish (*Orconectes limosus*) were obtained from the Grand-Lieu Lake, France. They were maintained in running tap water tanks at 10–12°C, under natural photoperiod and fed once a week with cat food pellets. Animals were sacrificed one week following premolt induction by eyestalk ablation (stage of active steroidogenesis), or at different times for the molting stage-related experiment. Molting stages were determined as in (18) by measurement of a gastrolith index (i.e., weight of gastrolith (mg)/cephalothorax length (cm)), and hemolymphatic levels of ecdysteroids were quantified by EIA according to (19). Dissected tissues (antennal glands, Y-organs, epidermis, muscle, hepatopancreas, nervous system, ovaries, and testes) were either immediately frozen in liquid nitrogen and stored at −80°C or directly used for total RNA isolation.

RNA isolation and RT-PCR amplification. Single-stranded cDNAs were synthesized from Y-organ and antennal gland total RNAs (5 μg) extracted with TRIzol reagent (Gibco, BRL) using M-MLV reverse transcriptase (Promega). Two pairs of oligonucleotides (5′-GGSTACCGACACATMGAY-3′ and 5′-AAGTCGAARATMTGGAWGTTYTC-3′; 5′-GATTYITAYYTIRTIGA RDTICC-3′ and 5′-TCRAAIAYYTGIAWRTTYT-3′) were deduced from consensus motifs after alignment of various AKR sequences, among which rat (D17309) and human (Z28339) 5β-reductase and 3DE-3β-reductase from *Spodoptera littoralis* (AJ131966). A cDNA fragment of 723 bp was obtained by RT-PCR but only with the first pair of primers and with RNAs from antennal gland. After 35 cycles (94°C for 30 s, 47°C for 30 s, 72°C for 30 s) followed by a 5 min step at 72°C, the fragment was gel purified (Gene Clean II kit, Bio101, Inc.) and cloned into pCRII-Topo plasmid (Invitrogen). The 5′ region of the cDNA was obtained by 5′RACE (3′–5′ RACE kit, Roche) using two specific primers (5′-CTCTCGCTTGACCTTGCCCT-3′ and 5′-GGAATCACGTAGTTGCCCGAGTT-3′). 3′ RACE amplification was carried out with a sense primer (5′-CGCCTTCTGCCCCCTCGGAG-3′). Sequencing was done by Genomexpress (France).

Northern blots. Equal amounts of total RNA from various tissues (20 μg/lane) were separated on a 1.2% formaldehyde gel and transferred to positively charged nylon membrane. Two PCR-produced DIG-labeled probes were sequentially hybridized to the membrane: (i) a specific probe of 680 bp corresponding to the antennal gland cDNA (sense primer 5′-ACATCGACTTCGCTGCTTAC-3′ and reverse primer 5′-GGATTCGCCGATTTGGGGAT-3′) and (ii) a control probe of 450 bp corresponding to the crayfish ribosomal protein L15 cDNA (rpL15; AF087038; sense primer 5′-GGAAGAAGCAGAGCGATGTC-3′ and reverse primer 5′-CCTTTGTCTGGGAGTAGTGG-3′). Hybridizations were performed overnight at 50°C in high SDS buffer (50 mM sodium phosphate pH 7.0; 50% formamide; 7% SDS; 5 × SSC; 0.1% *N*-lauroylsarcosine; 2% blocking reagent from Roche). After washes (2 × SSC, 0.1% SDS at room temperature followed by 0.5 × SSC, 0.1% SDS at 68°C), the chemiluminescent detection was performed using a DIG luminescent detection kit (Roche).

In situ hybridization. Antennal glands were fixed with Bouin and paraffin-embedded according to standard procedures. Sections (5 μm) were collected on glass slides, dewaxed and hydrated (100–70% ethanol). After washes in PBS, slides were incubated for 10 min in 0.2 M HCl at room temperature (RT), then in 4 × SSC, 1 × Denhardt's for 1 h before dehydration. A DIG-labeled probe was synthesized as described previously, heat denatured at 95°C just before use and added to the hybridization buffer (4 × SSC, 1 × Denhardt's, 50 mg/ml *N*-lauroylsarcosine, 1/10 blocking reagent). Incubation was performed overnight in a moist chamber at 37°C. Slides were then washed at 37°C: 30 min with 4 × SSC, 3 × 20 min with 2 × SSC, 2 × 20 min with 0.5 × SSC and 2 × 20 min with 0.2 × SSC. For detection, slides were incubated during 30 min with a fresh dilution of alkaline phosphatase-conjugated anti-DIG antibody in TBS −3% BSA −0.1% Triton X100, then covered with color buffer (0.45% NBT −0.35% BCIP, 100 mM Tris–HCl pH 9, 100 mM NaCl, 50 mM $MgCl_2$, 1 mM levamisole) and incubated at RT in a moist chamber during 24 h before mounting.

RESULTS AND DISCUSSION

Characterization of an Orconectes limosus reductase (O.l.AKR). The PCR-based strategy allowed us to obtain three overlapping clones of respectively 275, 723, and 348 bp from antennal gland RNA. The compiled nucleotide sequence and the deduced amino acid sequence are shown in Fig. 1. The full-length cDNA of 1170 bp contains an ORF of 1008 bp, if starting numbering at the first ATG. However, the start codon may rather be the second ATG, because the sequence just upstream is very close to the consensus sequence GGCGGC(A/G)CC described by (20), which is usually found before the initiation codon from eukaryotic genes. The 3′UTR is short (162 bp) and a single putative polyadenylation signal is present.

The cDNA gene product was found to be most similar to proteins belonging the AKR superfamily, and was therefore termed O.l.AKR. The predicted protein consists of 336 amino acids, with a calculated molecular mass of 37.6 kDa, which is in good agreement with the already known AKRs size. It shares the common structural features of AKRs, especially the eight (β-strands/α-helices) forming the core of the barrel (Fig. 2), and a strict conservation of amino acid sequence occurs at 11 positions (Gly 22, 45, 164; Asp 50, 112; Lys 84; Pro 119, 186; Asn 167; Gln 190; and Ser 271, with numbering corresponding to rat liver 3α-HSD). To date, seven families of AKRs have been defined, delineation of families occurring at 40% amino acid identity level (8). Here, the predicted protein showed the highest identity to the *Drosophila melanogaster* gene product CG2767 (39.5% amino acid identity), a putative AKR with unknown function, and it also resembles mammalian aldehyde/aldose reductases (32% amino acid identity with hum-ALR and hum-ADR). However, O.l.AKR possesses only 27% of amino acid identity with *S. littoralis* 3DE-3β-reductase, which is closest to aldehyde reductases (42.1% with hum-ALD). Sequence analysis of the predicted protein with the SignalP algorithm (21) revealed no signal peptides: the corresponding enzyme is certainly a non-secretory protein, like other AKRs, but in contrast to *S. littoralis* 3DE-3β-

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                                                                TTATATTCTTCCACCAGGGTG -1
ATGCACAGAGCAGCTGGCGCCATGACGAAAATTCCGACGATTCTTCTCAATTCAGGATCACATATTCCTGTGATGGGG 78
M  H  R  A  A  G  A  M  T  K  I  P  T  I  L  L  N  S  G  S  H  I  P  V  M  G  26
CTGGGTACAGGATCCCTTGGCCGCAATGGCAAAATGTCTGAGGAGGCGGTTACAGCGGTACTGGAAACAGCGCTGGAG 156
L  G  T  G  S  L  G  R  N  G  K  M  S  E  E  A  V  T  A  V  L  E  T  A  L  E  52
TGTGGGTACCGACACTTCGACTCCGCTGCTTACTACGGCAACGAGGCCATCATTGGCCAGGTCCTGCGGCGGTGGATC 234
C  G  Y  R  H  F  D  S  A  A  Y  Y  G  N  E  A  I  I  G  Q  V  L  R  R  W  I  78
TCGGAGGGCAAGGTCAAGCGAGAGGAACTCTTCATCACGACAAAGCTGCCAACTAGAGGTAACCGCGAGAAGGACGTG 312
S  E  G  K  V  K  R  E  E  L  F  I  T  T  K  L  P  T  R  G  N  R  E  K  D  V  104
GCCAGGTTCCTGCAGAAGTCCCTCGACAACCTGCGTCTGCCGTACGTGGACCTCTACCTCGTCCACTATCCCTGTGGC 390
A  R  F  L  Q  K  S  L  D  N  L  R  L  P  Y  V  D  L  Y  L  V  H  Y  P  C  G  130
ATCCTTGATACGGATCGTGAAAGGTTGAGCGCAGATGAAGTTACAGTCGATCCTACGACCGACATTCACGCCATCTGG 468
I  L  D  T  D  R  E  R  L  S  A  D  E  V  T  V  D  P  T  T  D  I  H  A  I  W  156
AGGGCCATGGAGGCACAGCTGGCTGCCGGCAAGACCAAGAACATTGGACTGTCCAACTTCAACGCTGATCAAGTTCAA 546
R  A  M  E  A  Q  L  A  A  G  K  T  K  N  I  G  L  S  N  F  N  A  D  Q  V  Q  182
CGTATAATTAAAGGGTGCCAGGTGAGGCCGGCCGTGCTGCAGGTGGAGGTGCACGTCTACATGCAGCAGGGGGCCCTC 624
R  I  I  K  G  C  Q  V  R  P  A  V  L  Q  V  E  V  H  V  Y  M  Q  Q  G  A  L  208
AGAGCCTTCTGCGCCCAGCACGACATCGTGGTGTGCGCCTTCTGCCCCCTCGGAGGACCCTTCAGGCTCATCAGGAAG 702
R  A  F  C  A  Q  H  D  I  V  V  C  A  F  C  P  L  G  G  P  F  R  L  I  R  K  234
GCTTCCAGACCCGGGGAGGTGAAGCTGCTGCTGGAGGACCCGCAGGTGCTGGACGTGGCCACAGCTCACCACAAGACA 780
A  S  R  P  G  E  V  K  L  L  L  E  D  P  Q  V  L  D  V  A  T  A  H  H  K  T  260
CCGGCGCAGGTCCTCCTTCGGTACCTCCATCAGATCAACGTCATTCCCATCCCCAAGTCGGCGAATCCGACGCGACTG 858
P  A  Q  V  L  L  R  Y  L  H  Q  I  N  V  I  P  I  P  K  S  A  N  P  T  R  L  286
TTTCAGAACACTCAGATTTTTGACTTCGAGTTGACGTCGGCGGAGATGTCGAGGCTCGCCGGTCTGGACCGAGGGCAC 936
F  Q  N  T  Q  I  F  D  F  E  L  T  S  A  E  M  S  R  L  A  G  L  D  R  G  H  312
GAAGGAAGAATCTTTACGGAATTCCGTCGTGGAGACCACACTCATCCAGAGTTCCCTTTCCATCCTCCACACTAGACG 1014
E  G  R  I  F  T  E  F  R  R  G  D  H  T  H  P  E  F  P  F  H  P  P  H  ***  338
GGAAGGCATATGAGAGAGATGAACCTTTCCGTTCCATTGTCCAAACGAGTCTGGAAGCCAAAATGAGATGATGGATCA 1092
TCGAGTCTCTCTTCCATCCTCCGTACCTCCCAGGACGCTTCCAGATGAATTAAATGAAATTGAATTGGATTTAAAAAA 1170
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FIG. 1. Nucleotide and deduced amino acid sequence of O.l.AKR. The start codon (second ATG) is in bold, the Kosak's consensus initiation sequence is underlined, and the putative polyadenylation signal sequence is boxed. This sequence data has been deposited in Genbank under Accession No. AF312369.

reductase, which is synthesized in various peripheral tissues and secreted into the hemolymph (11). Amino acids at positions 54 and 118 vary depending upon the substrate and may have a role in determining steroid versus sugar specificity: residue 54 is generally a leucine or isoleucine in the HSDs and a valine in the ADRs, whereas residue 118 is a tryptophan in ALRs/ADRs and a phenylalanine in HSDs (8, 9). In the O.l.AKR sequence, these amino acids are two tyrosines, that allows no conclusion to be drawn.

Tissue distribution of O.l.AKR and expression during a molt cycle. As shown in Fig. 3A, a single transcript is strongly detected in the antennal gland extract after Northern blot. The size of this transcript, of approximately 1.3 kbp, is consistent with that of the cDNA obtained. For globally similar levels of rpL15 expression, no signal is detected in the other tissues tested, i.e., muscle, epidermis, testicle, and hepatopancreas. This result was confirmed by more sensitive RT-PCR studies, including Y-organ, ovary, and nervous system samples (Fig. 3A): equal amounts of RNA extracted from various tissues were reverse-transcribed and simultaneously amplified with O.l.AKR and rpL15 primers (to obtain fragments of respectively 750 and 500 bp). For similar levels of rpL15 expression, a signal was only observed in antennal glands, and no amplification was seen in other tissues. This restricted expression of O.l.AKR in the antennal gland was not in agree-

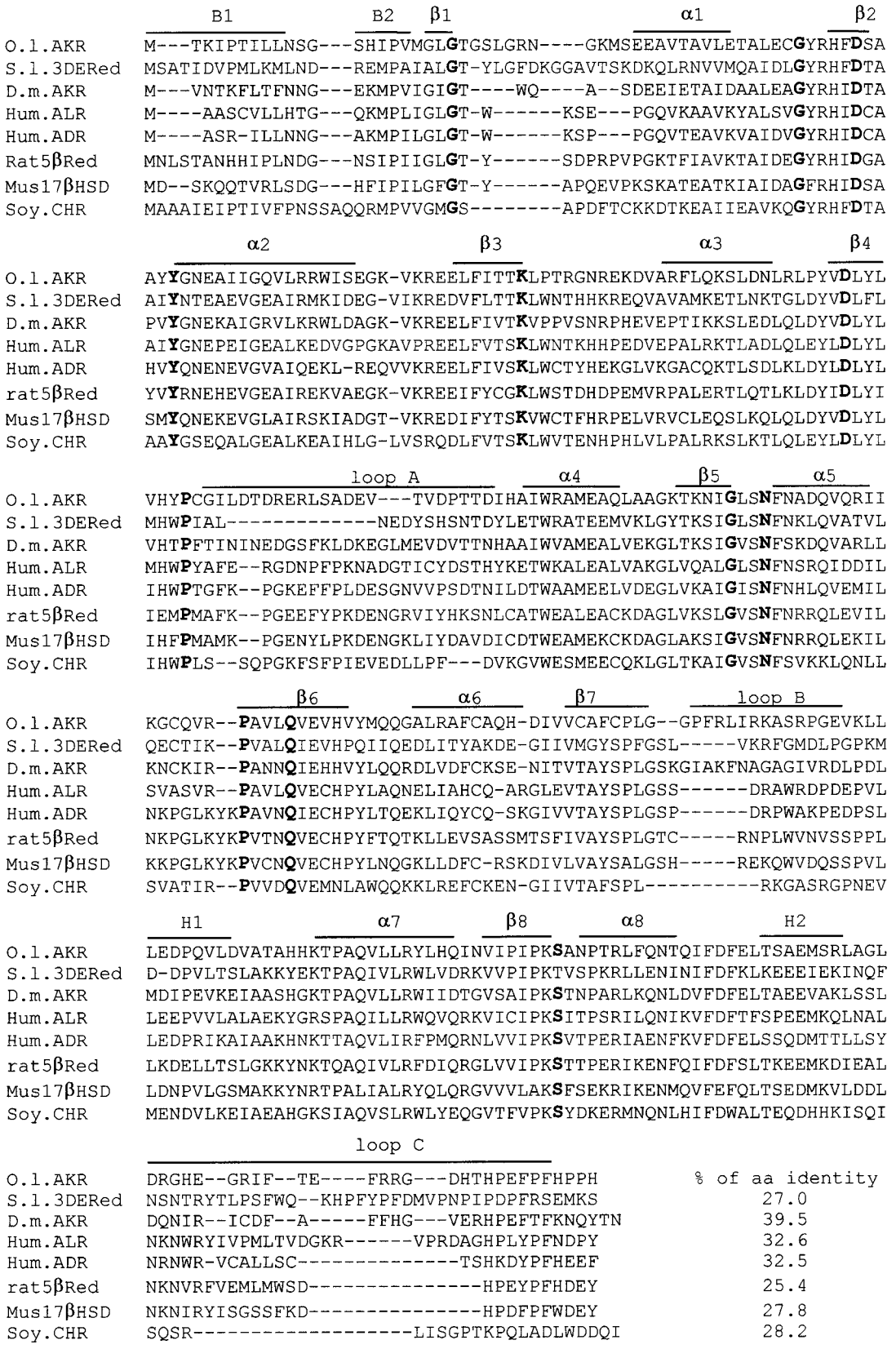

FIG. 2. Alignment of the deduced amino acid sequence of O.l.AKR and some AKRs. The secondary structure with the core $(\alpha/\beta)_8$-barrel structure is noted above the alignment. Invariant residues in all AKR are in bold. S.l.3DE-Red, *Spodoptera littoralis* 3DE-3β-reductase (AJ131966); D.m.AKR, *Drosophila melanogaster* CG2767 gene product (AAF54175.1). Hum.ALD, human liver aldehyde reductase (P14550); Hum.ADR, human placenta aldose reductase (J04795); rat5βRed, rat Δ^4-3-ketosteroid-5βreductase (P31210); Mus 17βHSD, mouse liver 17β-hydroxysteroid dehydrogenase; Soy.CHR, chalcone reductase from glycine (P26690).

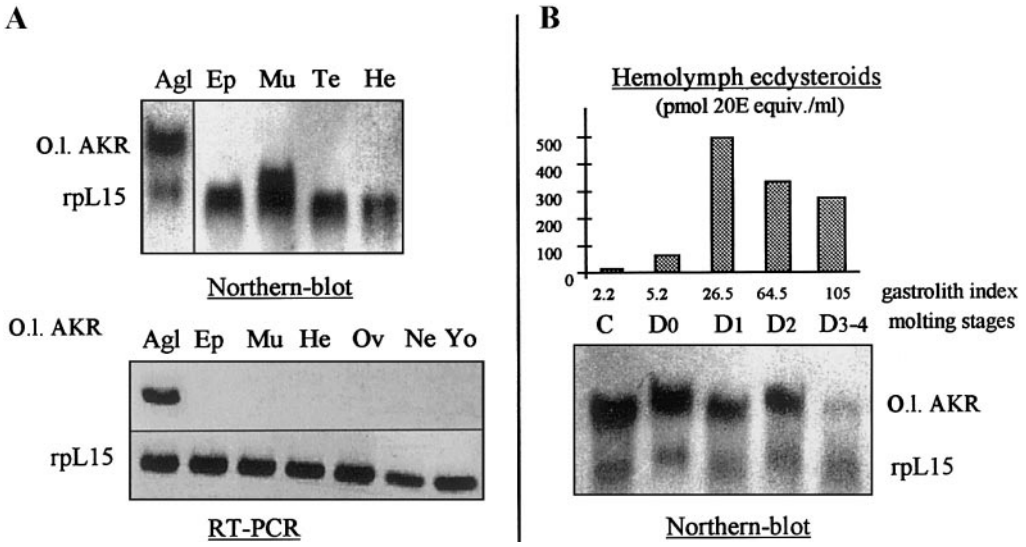

FIG. 3. (A) Analysis of O.l.AKR tissular distribution by Northern blot and RT-PCR. Agl, antennal gland; Ep, epidermis; Mu, muscle; Te, testis; He, hepatopancreas; Ov, ovary; Ne, nervous system; Yo, Y-organ. (B) Determination of the molting stages from the dissected animals and Northern blot analysis of O.l.AKR transcription during a molting cycle.

ment with the hypothesis of a 3DE-3β-reductase activity for this enzyme, since previous studies on 3DE metabolism (14) showed that 3β-reductase activity was maximal in the antennal gland, but also present in nervous tissue, hindgut, hepatopancreas and gonads. Similarly, 3DE metabolism has been studied in other crustacean species, like the crayfish *Procambarus clarkii* (22) or the crabs *Cancer antennarius* (23) and *Menippe mercenaria* (24), and a 3β-reductase activity has been detected in various peripheral tissues, even if the antennal glands were unfortunately not tested in these species.

To precise if there could be a relationship between O.l.AKR expression and ecdysteroid metabolism, we have studied the expression of its transcript during a molting cycle. Antennal gland pairs were dissected from animals, at different times after premolt induction. Hemolymphatic titers of ecdysteroids and gastrolith index were determined to specify the molting stages, and the corresponding RNAs were used for Northern blot experiment. As shown in Fig. 3B, ecdysteroid levels are low in intermolt (C), begin to increase in early premolt (D0), and then exhibit a rapid increase in later D1 premolt stage before to drop in late premolt (D2 to D4). O.l.AKR is strongly expressed both during the ecdysteroid peak (D1) but also when ecdysteroid levels are low, i.e., in C and D0. The signal only decreases in later D3–D4 stages, when the general metabolism of the animal is low. Otherwise, the expression level of the transcript seems quite constant during the molt cycle.

Like the vertebrate kidneys, antennal glands function in maintaining volume and in regulating concentrations of divalent ions, nutrients and other solutes (25). The decapod antennal gland is composed of four parts arranged in series: 1, a coelomosac (cells with podocytic processes) filters hemolymph delivered by the antennal artery; 2, the filtrate passes into a labyrinth, made up of cells having apical brush-border membranes, where absorptive and/or secretory processes take place, especially NaCl and glucose reabsorption (26, 27); 3, the fluid next enters a distal tubule (nephridial tube), specific to fresh-water species, which is thought to function in water absorption and secretion; and 4, lastly, a bladder receives and stores the urine and in some groups, including crabs and crayfishes, is a site of urine modification (28). The pattern of O.l.AKR expression in the antennal gland (*in situ* hybridization) showed a major expression of the transcript in the inner labyrinth (Fig. 4) and also in the nephridial tube cells. As crayfish antennal glands, mammalian kidneys are constantly exposed to osmotic gradients because of the urine-concentrating mechanism. Cells are protected from the osmotic effect of concentrated ions by accumulating various organic osmolytes, especially sorbitol (29). Aldose-reductase (ADR) has been implicated in osmoregulation in vertebrate kidney because it reduces D-glucose to sorbitol. Various ADR genes have been characterized in human (30, 31) and rabbit (32) and shown to be osmotically regulated. ADRs are present in various other tissues, where their physiological role is still unclear, but a renal-specific ADR has been recently characterized in newborn diabetic mice (33). Here, the restricted tissular localization of O.l.AKR and its temporal expression during molting cycle suggest that the corresponding enzyme is probably not involved in ecdysteroid metabolism. Its constitutive expression in the antennal

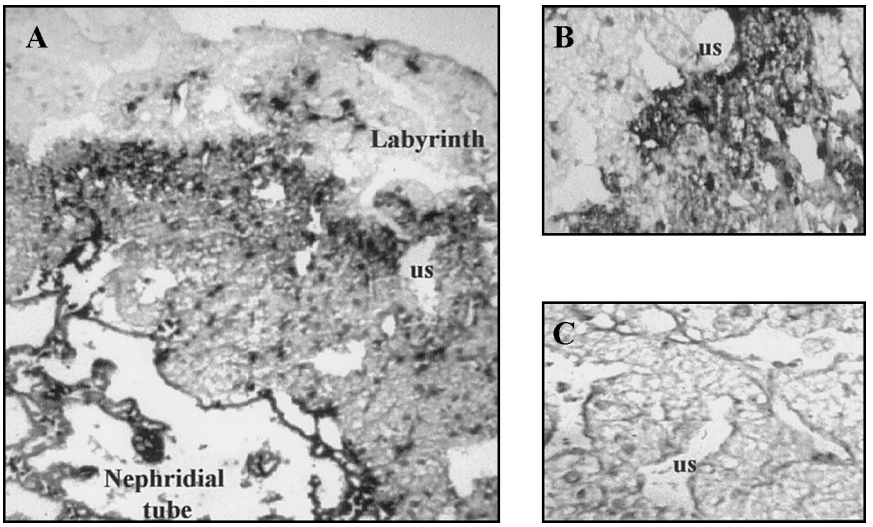

FIG. 4. (A) *In situ* hybridization on section of antennal gland. (B) Detail of the labyrinth part. (C) Negative control (labyrinth). us, urinary space.

glands suggests that O.l.AKR may rather play a role in osmoregulation, and we can hypothesize that the substrate specificity of the corresponding enzyme is rather turned to sugars. Reabsorption of D-glucose in the labyrinth of the lobster *Homarus americanus* was shown to occur by a mechanism similar to that present in the vertebrate kidneys (27), however, no data about sorbitol accumulation in the antennal gland of the freshwater crustaceans are yet available. Only *in vitro* expression of the recombinant protein will allow to study the catalytic properties of the functional enzyme to draw firm conclusions.

This is nevertheless the first member of the AKRs superfamily cloned in a crustacean species, and to date, very few AKRs have been characterized in arthropods. It is interesting to note that, despite the large phylogenetic distance between crustaceans and mammals, the percentages of amino acid identity are relatively high, thus confirming the structural conservation among this superfamily of proteins, which likely evolved from a common ancestor.

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