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SHORT COMMUNICATION

ACTIN GENE EXPRESSION DURING MUSCLE GROWTH IN *CARCINUS MAENAS*

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As in other crustaceans, growth in *Carcinus maenas* (L.) is closely associated with the moult cycle because of the restrictions imposed by the calcified exoskeleton. The greatest increase in body mass occurs after ecdysis when the old exoskeleton is shed and rates of water uptake are elevated with a consequent increase in body volume (Drach, 1939; Mykles, 1980; Hartnoll, 1982). Growth of the walking leg extensor muscles in crustaceans such as *Homarus americanus* and *Carcinus maenas* is achieved both by the addition of sarcomeres to lengthen the muscle fibres and by increasing the number of myofibrils to add to their diameter (Govind, 1982; El Haj *et al.* 1984; El Haj and Houlihan, 1987). Addition of sarcomeric proteins to the fibres is relatively rapid, being restricted to the few days when ecdysis and the immediate postecdysial stages occur, while increases in fibre diameter may occur over the longer intermoult periods (Houlihan and El Haj, 1985; El Haj and Houlihan, 1987). The factors regulating muscle growth in crustaceans have not been identified and the stages of the moult cycle where regulation occurs have not been defined.

The hormones recognized as having important roles in the control of the metabolic and physiological changes associated with moulting in crustaceans are the ecdysteroids 20-hydroxyecdysone and ponasterone A. Ecdysteroids have a direct influence on protein biosynthesis in the hypodermis and hepatopancreas of several species of freshwater crayfish (Gorell and Gilbert, 1969, 1971; McWhinnie and Mohrherr, 1970; McWhinnie *et al.* 1972) and in the land crab *Gecarcinus lateralis* (Paulson and Skinner, 1991), with an indication that regulation of gene expression occurs in the hypodermis during transition from the intermoult to the premoult stage (Traub *et al.* 1987). Muscle tissue may also be influenced by ecdysteroids, as an increase in haemolymph ecdysteroid titre by a factor of 10 between premoult stages D_{1-2} and D_{3-4} in *Carcinus maenas* (Lachaise *et al.* 1976) coincides with a sixfold increase in the rate of protein synthesis in the extensor muscles (El Haj and Houlihan, 1987). A similar response is found in the chelae muscles of *Gecarcinus lateralis,* where incorporation of radioactive amino acids

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into protein is at a maximum during premoult stages D_0–D_2 (Skinner, 1965) when haemolymph ecdysteroid titres are elevated (Soumoff and Skinner, 1980). Studies on the mode of action of steroid hormones at the cellular and molecular level indicate that similar pathways are involved in both insects and vertebrates (Peronnet *et al.* 1986), but little information is available on the mode of action of crustacean ecdysteroids. In insects, ecdysteroids regulate the activity of specific genes by inducing gene transcription as a primary response (e.g. Karlson, 1974; Lepesant and Richards, 1989) and ecdysteroid response regions on specific genes have been identified (Segraves and Richards, 1990). The mechanisms of steroidal regulation may be the same in all arthropods as crustacean ecdysteroids are very similar in chemical structure to those found in insects (Gilbert *et al.* 1977) and receptors specific to ecdysteroids have been identified in the cytoplasm and nucleus of the cells of the hypodermis in several crustaceans including *Carcinus maenas* (Spindler *et al.* 1980, 1984).

The purpose of this study was to investigate actin synthesis in the walking leg muscles of *Carcinus maenas* by identifying changes in actin gene expression at different stages of the moult cycle and by initiating studies into the possible role of ecdysteroids in the regulation of this process. Actin is a major structural component of crustacean muscles (Mykles and Skinner, 1985), forming the thin filaments of the sarcomeres. This protein is well-conserved throughout the animal kingdom so that there is a high degree of homology between the actin genes of animals as diverse as mammals, insects and echinoderms (Engel *et al.* 1982; Vaudekerckhoe and Weber, 1984). Consequently, the possibility of utilizing a heterologous mammalian cDNA probe to skeletal α-actin, in order to follow changes in actin gene expression in crustacean muscle, was investigated. Rates of total RNA synthesis *in vitro* were determined in isolated leg muscles exposed to different concentrations of the ecdysteroid 20-hydroxyecdysone, in an initial attempt to establish a role for ecdysteroids in the initiation of protein synthesis in crustacean muscle over the moult cycle. Although ponasterone A is the predominant ecdysteroid in premoult *Carcinus maenas* (Lachaise *et al.* 1986, 1989), it is not commercially available and, therefore, 20-hydroxyecdysone was used in this study since it is relatively easy to obtain and the biological activity of the two hormones is very similar.

For *in vivo* experiments adult male crabs (30–80 g, N=29), caught from the wild along the south coast of Devon, were separated into intermoult (C_4: N=9), premoult (D_{2-4}: N=9) and early postmoult (A, B: N=11) stages according to the criteria devised by Drach and Tchernigovtzeff (1967). They were frozen on site and returned to Birmingham for storage at −70°C. The extensor and flexor muscles were dissected from the merus segments of the third and fourth walking legs of each crab and pooled into 2 g portions within each stage for the extraction of total RNA using a hot phenol method (Scherrer and Darnell, 1962). RNA samples were subjected to Northern blot analysis (Fourney *et al.* 1988; Dabre, 1988) using a mammalian cDNA probe for α-actin. This probe is a *Pst*I–*Pst*I insert cloned in plasmid pAM91 and is 1150 base pairs in length, coding for approxi-

mately 90 % of the α-actin mRNA and with approximately 200 nucleotides of 3′ noncoding sequence (Minty *et al.* 1981). The *Pst*I–*Pst*I insert (donated by Margaret Buckingham) was labelled with α[-^{32}P]dCTP (Amersham) by multiprime labelling (Amersham kit no. RPN 1600Y) using the 'Klenow' fragment of DNA polymerase I (Feinberg and Vogelstein, 1983). Using this method, 100 ng of the cDNA insert was labelled to a specific activity of 1.8×10^9 disints min^{-1} μg^{-1}. The Northern blots were prehybridized for 4 h and hybridized for 18 h in 50 % formamide at 42 °C (Anderson and Young, 1988). The washed filters were exposed to X-ray film (Hyperfilm-Hp Amersham) in the presence of intensifying screens for 4–16 h at −70 °C.

For *in vitro* determination of total RNA synthesis rates, 17 adult male intermoult crabs (70–80 g) collected from the south coast of Devon were taken to Birmingham where they were held in recirculated, aerated sea water (35 ‰, 15 °C) and fed on a diet of fish or ox heart for 2–8 weeks. For each crab, three walking legs were removed by autotomy (only third and fourth walking legs were used) and the flexor muscles of the merus segment were dissected free as described by El Haj and Houlihan (1987) to expose the extensor muscle (mean wet mass=117±11 mg, N=51) within the remaining exoskeleton of the carpopodite. These muscle preparations were kept viable after dissection, in Medium M199 (Flow Laboratories) modified from Mattson and Spaziani (1986) by adding appropriate salts to adjust the ionic concentration to that characteristic of *Carcinus maenas* haemolymph in 70 % sea water and by adding $NaHCO_3$ to a final concentration of 10 mmol l^{-1} to buffer the pH of the medium to 7.8 during incubation in an atmosphere of 0.5 % CO_2. The muscle preparations were immediately placed into a culture chamber maintained at 15 °C and supplied from a mixing pump (Wöstoff, Bochum) with a gas mixture of humid air containing 0.5 % CO_2. After preincubation for 6 h, fresh medium containing the hormone 20-hydroxyecdysone (Sigma Chemical Co. Ltd) was added and incubation continued for a further 14 h. Two hormone concentrations were used, 10^{-7} mol l^{-1} and 10^{-6} mol l^{-1}, which correspond to the mean values of 20-hydroxyecdysone measured by radioimmunoassay in the haemolymph of *Carcinus maenas* during premoult (10^{-6} mol l^{-1}) and postmoult (10^{-7} mol l^{-1}) by Lachaise *et al.* (1976). The three muscle preparations taken from each crab were treated differently, with one muscle preparation exposed to each hormone level and the third incubated in hormone-free medium as a control. This resulted in three muscle preparations from the same crab in three different incubation media. After 12 h of hormone treatment, [5,6-^{3}H]uridine at 5 μCi ml^{-1} (Amersham 44 Ci $mmol^{-1}$) was added to the media with cold uridine to a final concentration of 0.3 mmol l^{-1}. [^{3}H]uridine incorporation was terminated after 2 h by snap-freezing the extensor muscles in liquid nitrogen. Total RNA was extracted from the muscle samples as described previously. RNA specific activity was determined by counting subsamples of dissolved RNA in Triton Z-100/xylene-based scintillation fluid with a Packard Tricarb Scintillation System (460C) using an internal standard and a counting efficiency of 15 %. Rates of RNA synthesis at each hormone concentration are represented as means ±S.E.M.

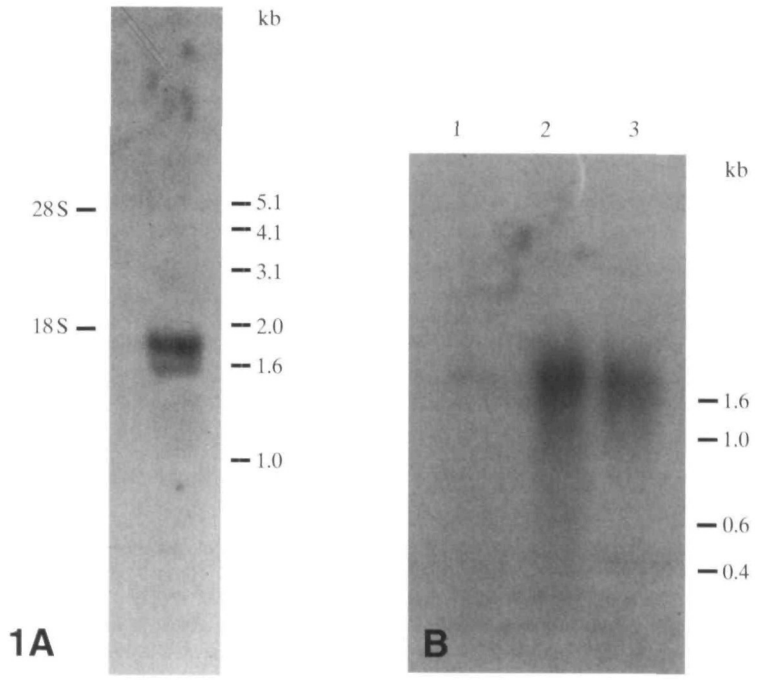

Fig. 1. Autoradiographs of Northern blots: (A) total RNA (22 μg) from the walking leg muscles of intermoult *Carcinus maenas* and (B) total RNA (10 μg) from leg muscles of intermoult (lane 1), premoult (lane 2) and postmoult (lane 3) crabs. In A the Northern blot was hybridized with 10 ng of the labelled cDNA probe to mouse skeletal α-actin and washed in 4× standard saline citrate (SSC)/0.1 % SDS (5 min at room temperature) and in 2×SSC/0.1 % SDS (60 min at 60 °C). 1×SSC is 150 mmol l^{-1} sodium chloride, 15 mmol l^{-1} trisodium citrate, pH 7.4. In B the Northern blot was hybridized with 30 ng of the labelled actin cDNA probe and washed in 4×SSC/0.1 % SDS (45 min at room temperature) and in 2×SSC/0.1 % SDS (30 min at 60 °C). Numbers on the right of the autoradiographs correspond to RNA markers in kilobases.

(N=17) in Fig. 2. The significance of apparent differences between the mean values was tested using Student's t-test at the 95 % level of confidence (P<0.05).

The result of Northern blot analysis using the cDNA insert of pAM91 on total RNA extracted from the leg muscles of intermoult, premoult and early postmoult crabs is shown in Fig. 1. Minty *et al.* (1981) showed that mouse skeletal muscle α-actin and nonmuscle actin mRNAs have lengths of 1.65 and 2.1 kb, respectively. In this study the cDNA hybridized to crustacean actin mRNAs in two bands of 1.6 and 1.8 kb (Fig. 1A), showing that a heterologous probe can be used to identify actin mRNA species from crustacean muscle. The degree of hybridization varied, with higher levels of actin mRNAs in premoult and postmoult muscles (Fig. 1B).

The rate of incorporation of [^{3}H]uridine into the RNA of isolated extensor muscles measured *in vitro* over the last 12–14 h of incubation was higher in those muscles exposed to 20-hydroxyecdysone than in the corresponding control muscles (Fig. 2). RNA synthesis rates, expressed as disints per min per μg total RNA per

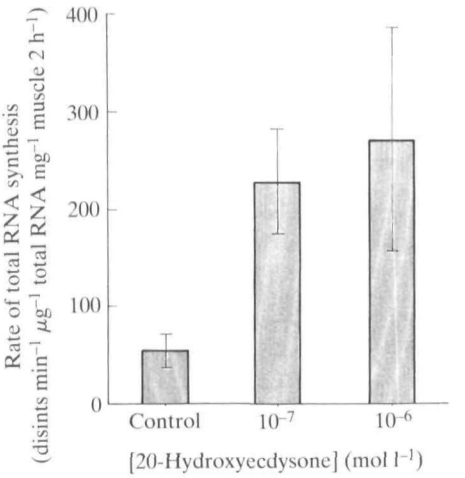

Fig. 2. *In vitro* rates of total RNA synthesis in isolated extensor muscles (117±11 mg, N=51) of intermoult crabs incubated for 14 h in culture media containing 20-hydroxyecdysone at either 10^{-7} or 10^{-6} mol l^{-1}. Control leg preparations were incubated in hormone-free media. Rates of RNA synthesis were determined by the addition of [^{3}H]uridine (5 μCi ml^{-1}) and cold uridine (0.3 mmol l^{-1}) to the culture media for the last 2 h of incubation. Values are given as means±S.E.M. (N=17).

mg muscle per 2 h, were 4.2 times higher than control levels, in muscles treated with 10^{-7} mol l^{-1} 20-hydroxyecdysone and 5.0 times higher in muscles treated with 10^{-6} mol l^{-1}. These increased rates of synthesis were significantly different from control rates (P<0.05) but were statistically similar to each other (P>0.05).

Use of the heterologous cDNA probe to mouse skeletal α-actin has identified crab actin mRNAs of 1.6 and 1.8 kb. The exact identity of the transcripts is unknown but they could correspond to mRNAs for nonmuscle as well as muscle isoforms of actin as this cDNA probe has been shown to hybridize to β and γ isoforms in a myogenic mouse cell line (Minty *et al.* 1981). It is also possible that multiple isoforms of muscle actins encoded by one or more of a closely related family of genes are being identified, a situation similar to that in mammals (Engel *et al.* 1982).

Northern blot analysis of total RNA extracted from *in vivo* muscle samples with skeletal α-actin cDNA shows that actin mRNA transcripts increase in abundance in the premoult and postmoult stages. This increase in actin mRNA levels corresponds to the increase in rates of protein synthesis and lengthening of muscle fibres observed in *Carcinus maenas* extensor muscles by Houlihan and El Haj (1985) and El Haj and Houlihan (1987) over the moult cycle. The premoult increase in actin mRNA levels (Fig. 1B) coincides with an increase in haemolymph ecdysteroid titre from 0 to 3×10^{-7} mol l^{-1} in intermoult to 10^{-7} to 2×10^{-6} mol l^{-1} in premoult *Carcinus maenas* (Lachaise *et al.* 1976). However, actin mRNA levels remained elevated in postmoult muscles, when circulating ecdysteroid titres have fallen back to intermoult levels (Lachaise *et al.* 1976), suggesting that factors other

than hormonal regulation are involved. These elevated postmoult levels could be due to an extended cellular half-life for actin mRNAs, transcribed during the premoult peak in haemolymph ecdysteroid titre. Alternatively, mechanical stimulation during post-ecdysial expansion could result in the transcription of actin mRNAs, as experimental manipulation of muscle fibres from intermoult *Carcinus maenas* resulted in a 10% increase in muscle length in response to continuous stretch (Houlihan and El Haj, 1985). Addition of 10^{-7} and 10^{-6} mol l^{-1} 20-hydroxyecdysone to the culture medium of the *in vitro* leg preparations increased the rate of total RNA synthesis in the extensor muscles, but further studies are needed to characterize these changes before the possible influence of ecdysteroids on actin synthesis can be established.

Low levels of actin mRNAs were detectable in *Carcinus maenas* muscle during intermoult (Fig. 1A). These were considerably lower than in the pre- and postmoult stages and larger quantities of total RNA from intermoult muscles were required to achieve hybridization. These relatively low levels of actin transcription in intermoult muscles may indicate a continuous turnover of muscle proteins, as is found in mammals and other invertebrates. Alternatively, the low levels of actin mRNAs may be associated with intermoult increases in muscle fibre width, which have been measured histologically in both *Carcinus maenas* (El Haj and Houlihan, 1987) and *Homarus americanus* (El Haj *et al.* 1984).

The close homology between the actin genes of diverse species has enabled this study to investigate changes in mRNA levels for a specific myofibrillar protein in decapod crustaceans, as a first step towards understanding the molecular mechanisms regulating muscle growth over the moult cycle. Hybridization of total RNA from *Carcinus maenas* leg muscles to the mammalian cDNA for α-actin has demonstrated higher levels of actin mRNAs in the pre- and postmoult stages of the moult cycle. The factors responsible for regulating these differences in actin mRNA levels still need to be elucidated but increased rates of transcription could be involved, influenced by both hormonal and mechanical factors.

Recently, crustacean cDNAs for muscle actins have been isolated from *Artemia salina* (Macias and Sastre, 1990) and current work in our laboratory is successfully developing an actin cDNA for lobster (*Homarus gammarus)* muscle (P. Harrison and A. J. El Haj, personal communication). Use of these crustacean probes for actin will enable a more specific characterization of changes in the levels of actin mRNAs over the moult cycle and further studies of the specific role of ecdysteroids in muscle gene transcription are planned, using the more homologous cDNAs in dot blot analysis.

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