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AKH BIOSYNTHESIS: TRANSCRIPTIONAL AND TRANSLATIONAL CONTROL OF TWO CO-LOCALISED PROHOMONES

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Summary

The neurosecretory cells of the locust corpora cardiaca (CC) express two co-localised transcripts which are translated into the two preprohormones required in adipokinetic hormone I (AKH I) and AKH II biosynthesis. At different stages of postembryonic development, the relative amounts of the two transcripts (AKH I mRNA and AKH II mRNA) change in parallel with the relative rates of synthesis of proAKH I and proAKH II. Differential regulation of transcript expression, however, cannot account for the changes in neuropeptide ratios seen during postembryonic development. Comparison of *in vivo* and *in vitro* translation shows that protein synthesis *in vivo* is biased towards the translation of AKH I mRNA by a factor of about 2.6. This factor appears to be constant during postembryonic development and is required to produce the observed developmental changes in neuropeptide ratios. Both transcriptional and translational mechanisms are therefore necessary to alter neuropeptide ratios in the CC. The mechanisms we describe can account for the developmentally changing pattern of peptide expression. We suggest that regulation of neuropeptide ratios indicates that signalling functions can be attributed to the precise configuration of peptide cocktails.

Introduction

Neurosecretory cells and peptidergic neurones typically produce more than one peptide by the processing of precursor polypeptides which contain the sequences of more than one peptide. In cells producing multiple peptides from a single precursor, the ratios of peptides can be regulated by mechanisms involving segregation of different parts of the precursor into separate subcellular compartments followed by differential degradation

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(Fisher *et al.* 1988; Sossin *et al.* 1990*a*,*b*). Such mechanisms can produce peptides in ratios very different from their representation in the precursor. The fact that peptidergic neurones can configure their peptide cocktails in different ways has led to the idea that peptide ratios may represent an important part of the signal from such cells.

We have studied peptide ratios in neurosecretory cells of the locust (*Schistocerca gregaria*) which produce a set of five peptides (two monomers and three dimers) by the co-expression of two prohormones. Peptide ratios are altered by regulating the relative *in vivo* synthesis rates of the two prohormones, segregation followed by degradation is not involved and peptide ratios can change while remaining in stoichiometric relationship to the precursor structure (Hekimi *et al.* 1989, 1991). Here we examine the mechanisms by which this differential regulation of prohormone biosynthesis is achieved.

In the locust *Schistocerca gregaria*, the paired glandular lobes of the corpora cardiaca (CC) are remarkable structures consisting of a homogeneous population of neurosecretory cells (about 6000 in the adult animal) that synthesize two major neuropeptides called adipokinetic hormone I and II (AKH I and II). AKH I and AKH II are ten and eight amino acids long, respectively (Stone *et al.* 1976; Siegert *et al*. 1985), and are members of an arthropod peptide family that has a wide range of important physiological functions (reviewed by Gäde, 1990). AKH I predominates in the adult CC, where it is co-localised in a ratio of about 4.5:1 with AKH II. They are released into the circulation during flight and stimulate lipid metabolism, enabling locusts to undertake prolonged migratory flights without feeding. The AKHs have also been implicated in the modulation of activity of neurones, skeletal muscle, gut and heart (for reviews, see Orchard, 1987; O'Shea and Rayne, 1992).

Studies on the biosynthesis of AKH I and II showed that they are derived from two very small and structurally simple prohormones called proAKH I (or A-chain) and proAKH II (or B-chain). ProAKH I contains one copy of AKH I, followed by a processing site (Gly-Lys-Arg) and a 28-residue C-terminal sequence called the α-chain (Hekimi *et al.* 1989). ProAKH II contains one copy of AKH II, a processing site (Gly-Arg-Arg) and another 28-residue C-terminal sequence called the β-chain (Hekimi *et al.* 1991). Further characterization of AKH biosynthesis revealed the surprising finding that, prior to processing, the prohormones are arranged into three dimeric precursors called P1, P2 and P3 by the formation of a single disulphide bond between the prohormone subunits. P1 is a proAKH I homodimer (A–A), P2 is a heterodimer (A–B), and P3 is a proAKH II homodimer (B–B). Dimerisation is a prerequisite for *in vivo* precursor processing that will yield five peptides: the two monomeric AKH peptides and three dimeric peptides formed from the α and β sequences of the two prohormones. The dimeric peptides are called the AKH-precursor-related peptides or APRP 1, 2 and 3 (Hekimi *et al.* 1991). As yet, the APRPs have no known functions but they are released with the AKHs (Hekimi and O'Shea, 1989).

In studies undertaken to investigate how the homodimeric precursor P1 is made, the first issue was to determine whether a messenger RNA encoded more than one copy of proAKH I (A-chain), or whether each subunit was independently translated from a small mRNA containing only one copy. Results described by Schulz-Aellen *et al.* (1989) show unambiguously that the CC contains two small mRNAs which are translated *in vitro* into

two very small proteins. The larger of these proteins was identified as preproAKH I. This was further confirmed by DNA sequencing of a clone isolated from a cDNA library, made from CC poly(A)+ RNA, which contains only one copy of proAKH I preceded by a 22 amino acid signal sequence. These results demonstrated that P1 is not made from one larger protein containing two copies of the subunit but from independently translated small protein subunits.

In the present paper, we describe an mRNA encoding preproAKH II and show that it corresponds to the second major product of *in vitro* translation and that it codes for only one copy of an AKH prohormone (proAKH II). This result indicates that AKH biosynthesis involves the production of dimeric precursors from two independently translated and very small preprohormones. The synthesis of the heterodimer P2 (an AKH I and AKH II precursor) therefore requires the translation of two different mRNAs. Previously we showed that the relative synthesis rates of the dimeric precursors P1, 2 and 3, the AKHs and the APRPs change systematically during postembryonic development (Hekimi *et al.* 1991). All of the complex developmental changes in peptide ratios can be explained by the differential regulation of synthesis of the two prohormone subunits of the dimeric precursors. Moreover, our results suggest that the differential regulation of prohormone biosynthesis is driven by both transcriptional and translational mechanisms. A general model accounting for all the postembryonic changes in peptide ratios is presented.

Materials and methods

Animals

Locusts *Schistocerca gregaria* (Forskål) used in this study were reared in our laboratory culture. Both males and females were used since no sex-dependent differences can be measured in the regulation of AKH biosynthesis (S. Hekimi, unpublished results).

Isolation of total and poly(A)+ RNA from corpora cardiaca

One thousand adult CC and 200 fourth- and fifth-instar CC were dissected and immediately frozen in liquid nitrogen. They were stored at $-80^{\circ}C$ until further use. Total cellular RNA was extracted using the guanidinium isothiocyanate–hot phenol method described by Feramisco *et al.* (1982). Several adult CC total RNA preparations were used in this study, yielding approximately 0.56 μg/CC. Total RNA was also obtained from fourth- and fifth-instar CC yielding about 0.25 μg/CC. Poly(A)+ RNA from adult CC was obtained after chromatography on an oligo(dT)-cellulose column. Routinely, 15 μg of poly(A)+ RNA was isolated from 1000 adult CC.

Design of oligonucleotides

The design of the first oligonucleotide probes used in some experiments was based on the amino acid sequence of the A (41 residues) and B (39 residues) subunits of the AKH precursors P1 (Hekimi *et al.* 1989) and P2 (Hekimi *et al.* 1991), which were determined by direct protein sequencing. These probes were used for the screening of the cDNA library and for the hybrid arrest experiments. Their sequences and corresponding protein

residues are as follows. A-chain (proAKH I)-specific oligonucleotides: oligo α2, 5′-GCT GTA GGG GTC TCC GAA GTC CGC AGC GTC-3′ (A-chain 14–23, antisense probe); oligo α3, 5′-GAT GCC GCC GAT TTC GGC GAT CCC TAC TCC TTC CTG TAC CGC CTG ATC-3′ (A-chain 14–29, sense probe). B-chain (proAKH II)-specific oligonucleotides: oligo β1, 5′-GGC CAT GGG GTC AGC GTT GGG GTC GGC GTA-3′ (B-chain 12–21, antisense probe); oligo β2, 5′-TAC GCC GAT CCC AAC GAT GAT CCC ATG GCC TTC CTG TAC AAG CTG ATC-3′ (B-chain 12–27, sense probe).

New oligonucleotides were designed later, on the basis of the cDNA sequences, and used for the Northern analyses presented in this paper. A-chain antisense oligonucleotide probes: oligo AKH I, 5′-GGT GCC CCA GTT GGG GGT GAA GTT GAG CTG-3′ (A-chain 1–10); oligo α3′, 5′-AGA GCA CCC AGA CAT CTT CCT GGC TTC AGC-3′ (A-chain 31–40). B-chain antisense oligonucleotide probes: oligo AKH II, 5′-ACC CCA ACC GGT TGA GAA GTT GAG CTG-3′ (B-chain 1–9); oligo β3, 5′-CGC CAT GGG GTC AGC GTT GGG ATC TGC GTA-3′ (B-chain 12–21).

Construction of cDNA library

The construction of the CC cDNA library in λgt10 has previously been described in detail (Schulz-Aellen *et al.* 1989).

Hybridization screening of CC cDNA library

A library containing about 3×10^5 recombinant phages was screened using standard methods (Benton and Davis, 1977). Oligo β1 was ^{32}P-end-labelled using polynucleotide kinase to a specific activity of about 18 μCi μg^{-1}. Hybridization was done at 50˚C in a 6× SSPE, 5× Denhardt's, 0.1% SDS, 100 μg ml^{-1} salmon sperm DNA for 16h and posthybridization washes were performed with 6× SSPE (60mmol l^{-1} NaH_2PO_4, 90mmol l^{-1} NaCl, 7.5mmol l^{-1} EDTA, pH7.4) at 50˚C followed by with 2× SSPE at 20˚C. The positive recombinant phages were submitted to a second screening under identical hybridization conditions. We here report on the further analysis of one recombinant phage containing a 600 bp insert that hybridized strongly with oligo β1. Southern blot analysis (Southern, 1975) on restriction fragments derived from this insert showed that hybridization with the probe was restricted to a fragment of 270 bp which was transferred into plasmid pUC18 and sequenced.

Sequencing of DNA

Sequencing of DNA was carried out using both the chemical cleavage sequencing method (Maxam and Gilbert, 1980) and the dideoxy chain termination method (Sanger *et al.* 1977). Each nucleotide was read an average of seven times and at least twice in both directions.

In vitro *translation experiments*

Poly(A)+ RNA (0.25 μg) from adult CC was translated in the wheat germ cell-free translational system (Amersham) in the presence of [^{35}S]methionine (1455Cimmol^{-1}, Amersham). After a 2-h incubation at 25˚C, the translated products were analyzed by SDS–PAGE on a 17.5% polyacrylamide gel. The gel was stained with Coomassie Blue,

then treated for 30min with an Enhancer solution (Enlightning, New England Nuclear), dried and exposed under Kodak XAR-5 film. The proteins used on molecular weight standards were from BRL (low range 3–43kDa).

Hybrid arrest of translation

Hybrid arrest was performed as described by Minshull and Hunt (1986). Prior to the addition of mRNA to the translational mix, CC mRNA (0.25 μg) was incubated for 10min at room temperature in 5.5 μl of 0.18mol l^{-1} KCl in the presence of 10 units of RNAsin (Genofit) containing either no oligonucleotide or 31pmol (0.31 μg) of oligo α2 or oligo β1 (antisense oligonucleotides), or 31pmol (0.5 μg) of oligo α3 or β2 (sense oligonucleotides). Each sample was then added to the wheat germ cell-free system and incubated in appropriate conditions. An aliquot of each sample was analyzed by SDS–PAGE (see above).

Labelling of oligonucleotides

Oligonucleotides (0.5 μg) were 5′-end-labelled using 50 μCi of γ-[^{32}P]ATP (6000Ci $mmol^{-1}$, Amersham) by polynucleotide kinase T4 (Biofinex), as described by Sambrook *et al.* (1989). Labelled probes were separated by chromatography on Sep-Pak C_{18} (Waters). Specific activities were 30–50 μCi μg^{-1} oligonucleotide. Northern blot analyses were performed by adding 1.5×10^6–3×10^6 cts min^{-1} of labelled oligonucleotide to 10ml of hybridization buffer.

Northern analysis

Poly(A)$^+$ RNA (0.5 μg) from adult CC and total RNA of adult, fourth and fifth instars CC (5 μg) were denatured in a buffer containing 50% formamide and separated by electrophoresis on 1% agarose–formaldehyde gels according to the procedure described by Sambrook *et al.* (1989). The RNA was then transferred overnight to nitrocellulose membranes. Following the transfer, the damp membranes were irradiated with ultraviolet light to crosslink the RNA, as described by Church and Gilbert (1984). Membranes were then hybridized with ^{32}P-labelled oligonucleotides. Prehybridization (2–4h) and hybridization (16–24h) were done at 42˚C in 6× SSPE, 5× Denhardt's, 1% SDS, 100 μg ml^{-1} of salmon sperm DNA. The filters were washed with 2× SSPE and 0.1 % SDS at successively higher temperature starting at 42˚C up to 60˚C and exposed under Kodak XAR-5 film. The same filter was occasionally hybridized with different probes. In such cases, before hybridization with the next probe, previous probes were washed away by incubating the filter at 85–90˚C for 20min with 1×TE and 0.1% SDS. The filter was exposed overnight to ensure that the label had been removed. Autoradiograms were scanned on a chromoscan 3 (Joyce Loebl) using a 100W tungsten–halogen lamp as light source (400–750nm). To compare mRNA levels, poly(A)+ RNA or total RNA was charged in duplicate on the gel and, after transfer, half of the blot was used for hybridization with the proAKH I mRNA-specific probe (α2) and the other half with the proAKH-II-specific probe (β3).

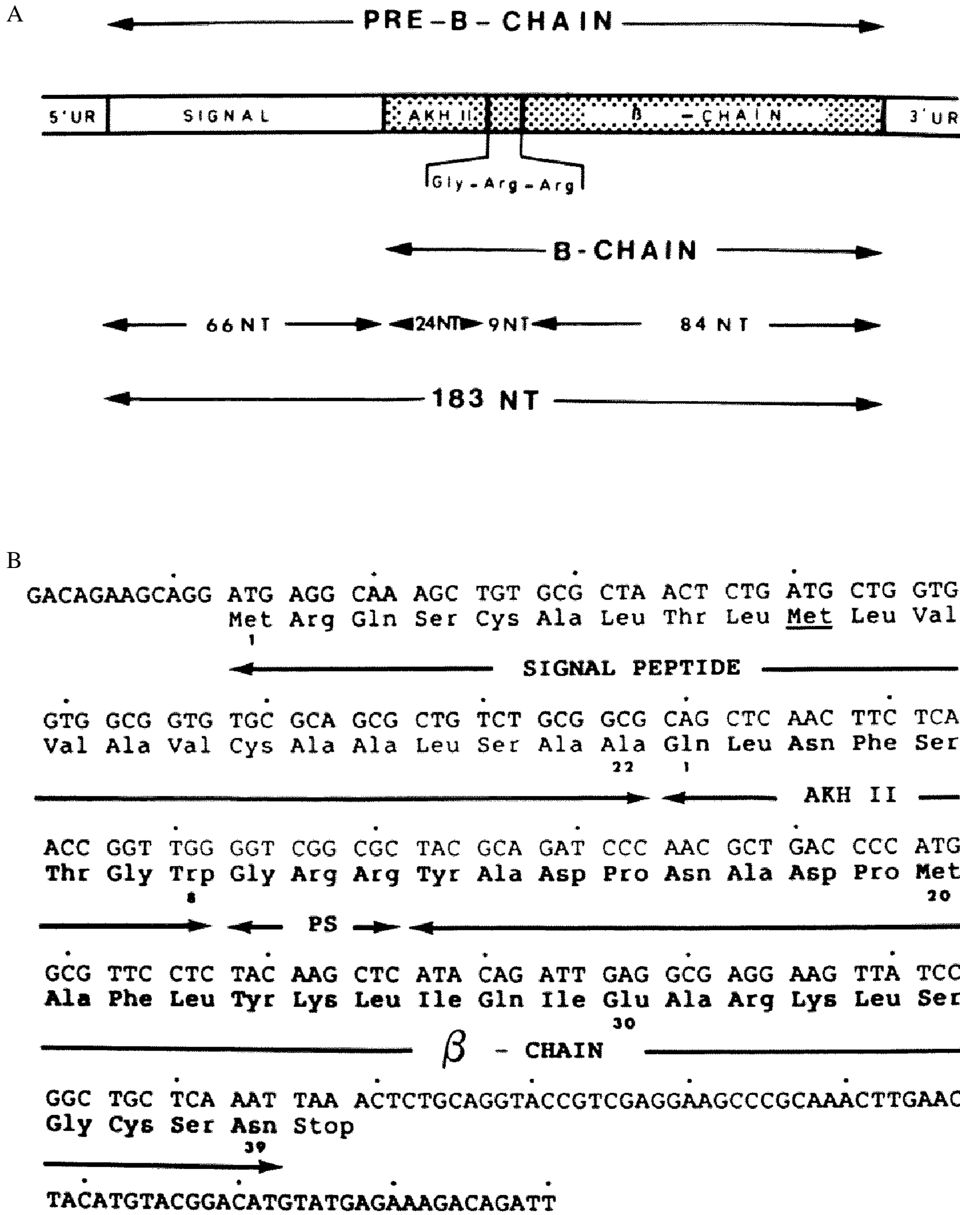

Fig. 1. DNA sequence and deduced amino acid sequence of a positive clone. (A) A schematic representation of the organisation of the preproAKH II or pre-B-chain mRNA. The lengths of segments of mRNA corresponding to functional units of protein are indicated below, together with the total length (183 nucleotides, NT) of the translated region. (B) Nucleotide sequence of the 270 bp fragment of the positive recombinant phage identified in the CC cDNA library is shown alongside a translation giving the proAKH II polypeptide (B-chain) determined by protein sequencing. A deduced signal sequence of 22 amino acids (1–22), or 13 amino acids (10–22) (see text), is followed by the proAKH II sequence (1–39) containing AKH II peptide, the processing site (PS) Gly-Arg-Arg, and the β-chain peptide.

Results

Expression of two prohormones – the subunits of dimeric AKH precursors

Translation of CC poly(A)+ RNA in a wheat germ cell-free system yields two major labelled proteins of small molecular weight estimated by SDS–PAGE to be 6.8 and 5.7kDa (Schulz-Aellen *et al.* 1989). Hybrid arrest experiments demonstrated that the heavier product corresponded to preproAKH I but the identity of the smaller protein remained unresolved because the sequence of the second AKH prohormone was not known.

A CC cDNA library constructed in λgt10 was screened for proAKH II using an oligonucleotide (oligo β1) based on the amino acid sequence of the β-chain of APRP 2. APRP 2 is the dimeric byproduct of processing of P2 (Hekimi *et al.* 1991), a heterodimer of proAKH I and proAKH II. The oligo β1 would therefore be expected to identify clones encoding proAKH II. A positive recombinant phage was characterized by DNA sequencing of the insert and our results show that the insert encodes a 61 amino acid protein, consisting of a 22 amino acid signal sequence, followed by one copy of AKH II, a processing site (Gly-Arg-Arg) and the 28 residue sequence of the β-chain (Fig. 1). This is the expected structure of preproAKH II.

Calculated molecular weights of the preproAKH I and preproAKH II proteins inferred from the cDNA clones are 6.84kDa and 6.64kDa, respectively. Proteins of such similar size cannot be separated under the experimental conditions used for the SDS–PAGE. The two major translation products in the wheat germ system were, however, clearly separated and estimated to be 6.8 and 5.7kDa. The larger is the correct size for the preproAKH I and its translation is specifically blocked by appropriate antisense oligonucleotides (Fig. 2). The smaller protein, however, appears to be too small to be preproAKH II as postulated from cDNA cloning. It was important, therefore, to show that the smaller protein did represent the product of translation of the preproAKH-II-encoding mRNA. Although the protein translated *in vitro* is smaller than the expected weight of preproAKH II inferred from the cDNA clone, a hybrid arrest experiment (Fig. 2) performed using a proAKH-II-specific oligonucleotide, designed to the least homologous portion between the two prohormones, does specifically suppress synthesis of the 5.7kDa protein. Fig. 2 shows a hybrid arrest experiment using both proAKH-I- and proAKH-II-specific oligonucleotides in their sense and antisense forms. Using the proAKH-I (α-chain specific) antisense oligonucleotide, the upper band (6.8kDa) could be specifically suppressed, as expected. The lower band of 5.7kDa is abolished only with the proAKH-II-specific antisense oligonucleotide, which does not block the translation of the upper 6.8kDa protein. Both of the sense oligonucleotides are without effect. The most likely explanation for this is that the smaller protein is indeed preproAKH II, but that the second methionine present in the signal sequence and located eight amino acids downstream from the presumed first methionine (see Fig. 1: second methionine underlined) is used preferentially as the starting codon in the wheat germ cell-free translation system. The calculated molecular weight of a protein starting at this second methionine is 5.63kDa, corresponding to the estimated size of the smaller translational product. We cannot rule out the possibility that the first starting codon is also used *in vitro*. In fact, whereas the

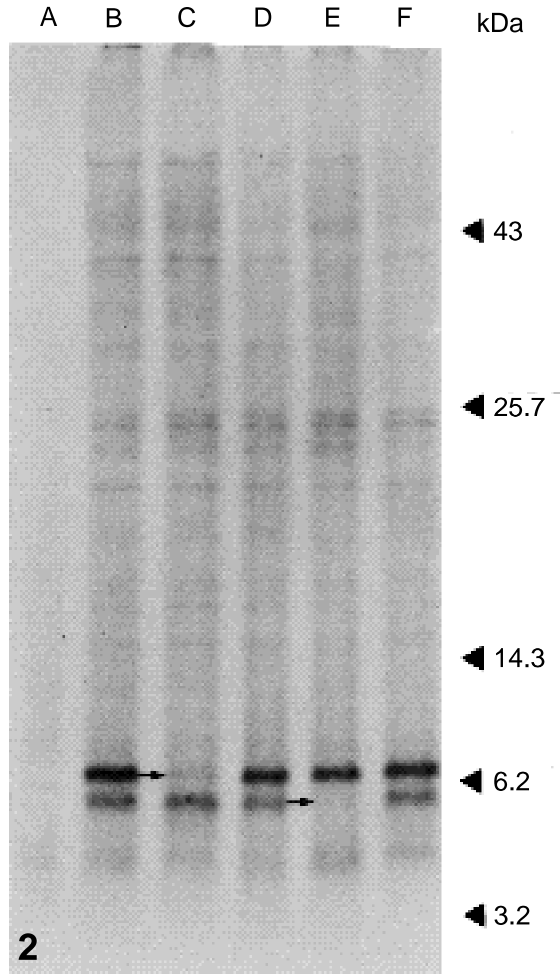

Fig. 2. Hybrid arrest of *in vitro* translation with antisense oligonucleotides. CC mRNA (0.25 μg) was incubated with water (lane B), 31pmol of antisense oligo α2 (lane C), sense oligo α3 (lane D), antisense oligo β1 (lane E) or sense oligo β2 (lane F) before addition of the various components for translation (see Materials and methods). Lane A represents the background level of the wheat germ endogenous synthesis in the absence of exogenous RNA. The arrowheads indicate the migration of molecular weight standards (BRL). The small arrows point to the missing proteins in lanes C and E, whose sizes were estimated from this gel and from other *in vitro* translations to be 6.8kDa and 5.7kDa.

proAKH II antisense oligonucleotide totally abolishes the 5.7kDa protein, prehybridization with the proAKH-I-specific antisense probe does not entirely suppress the 6.8kDa product (Fig. 2). There is usually about 8% of the radioactivity in the 6.8kDa band which cannot be suppressed with the proAKH I antisense oligonucleotide. It is therefore possible that the first methionine is used *in vitro* as the starting codon for preproAKH II but only about 8% of the time, resulting in the translation of some complete preproAKH II, which co-migrates with preproAKH I.

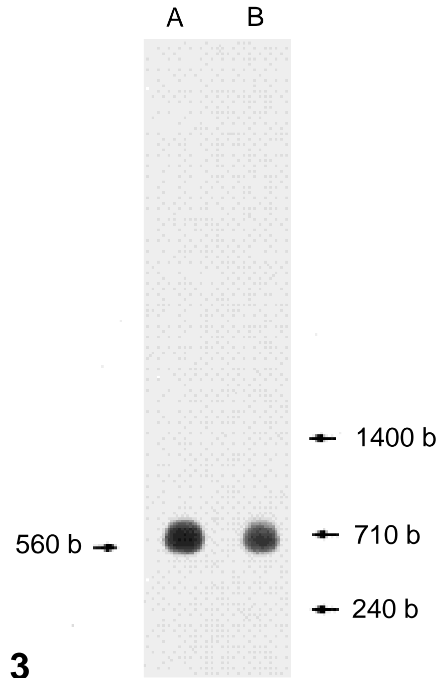

Fig. 3. Northern analysis of adult CC poly(A)+ RNA. Oligo α2 (A) and oligo β3 (B) were ^{32}P-labelled with polynucleotide kinase to matched specific activities and hybridized to poly(A)+ RNA isolated from adult CC (0.5 μg in each lane). The position of migration of haemoglobin RNA (710 bases) and of two fragments of an RNA ladder from BRL (1400 bases and 240 bases) are indicated by arrows, as is the estimated size of the specific mRNA hybridizing with each probe (560 bases).

An estimation of the size of the full-length AKH II mRNA was obtained by Northern blotting. As shown in Fig. 3, the proAKH-II-specific oligonucleotide (oligo β3) hybridizes to a unique band corresponding to an mRNA of about 560 bases, which is very similar to that estimated for the AKH I mRNA (Schulz-Aellen *et al.* 1989).

The sequence identity between preproAKH I and preproAKH II was assessed at the nucleic acid and amino acid levels. Table 1 shows that the sequences encoding the A- and B-chains are more conserved (70% homology) than the signal sequences (59 % homology). There is a high number of single-base mutations leading to the amino acid differences and the prohormones therefore have a higher homology at the nucleic acid level (64.5%) than at the amino acid level (59%).

In summary, the results of the DNA sequencing and *in vitro* translation show that AKH I and II biosynthesis depends on the expression of two different small messenger RNAs. These encode the two subunits of the three dimeric AKH precursors (P1, P2 and P3) identified and characterised by HPLC and direct protein sequencing (Hekimi *et al.* 1989, 1991). How can this arrangement explain the changing ratios of the five peptides generated from these three precursors in the CC?

Table 1. *Comparison of nucleic acid and protein sequences of preproAKH I and preproAKH II in* Schistocerca gregaria

DNA sequences

```
  10  ATGGTGCAGCGGTGCCTGGTGGTAGCCTTGCTGGTGGTGGTGGTGGCGGC  59
      |||  |||  | ||   | |        |||||||||||| ||||   ||
  36  ATGAGGCAAAGCTGTGCGCTAACTCTGATGCTGGTGGTGGCGGTGTGCGC  85

  60  TGCCCTATGCTCGGCGCAGCTCAACTTCACCCCCAACTGGGGCACCGGCA 109
       || || |   ||||||||||||||||| |  ||   |||      ||
  86  AGCGCTGTCTGCGGCGCAGCTCAACTTCTCAACCGGTTGG      GGTC 129

 110  AACGGGACGCTGCGGACTTCGGAGACCCCTACAGCTTCCTCTACCGGCTC 159
        ||  |||| |    |  ||  ||||||      |||||||||  ||||
 130  GGCGCTACGCAGATCCCAACGCTGACCCCATGGCGTTCCTCTACAAGCTC 179

 160  ATACAGGCTGAAGCCAGGAAGATGTCTGGGTGCTCTAATTAG  201
      ||||||  ||| || |||||| |  | || ||||| |||||
 180  ATACAGATTGAGGCGAGGAAGTTAGCCGGCTGCTCAAATTAA  221
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Protein sequences

```
 1 MVQRCLVVALLVVVVAAALCSAQLNFTPNWGTGKRDAADFGDPYSFLYRL  50
   | | |     ||| | |||  |||||   |  | | |    ||  ||| |
 1 MRQSCALTLMLVVAVCAALSAAQLNFSTGW  GRRYADPNADPMAFLYKL  48

51 IQAEARKMSGCSN  63
   || |||| |||||
49 IQIEARKLSGCSN  61
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Top sequence is preproAKH I; lower sequence corresponds to preproAKH II.

	Percentage sequence identity	
	Nucleic acid level	Amino acid level
Signal sequence	59	50
proAKH I/proAKH II	70	64
Overall	64.5	59

Relative transcriptional and translational levels in adult CC

In the adult CC, there is approximately 4.5 times more AKH I than AKH II because the rate of AKH I synthesis is 4.5 times that of AKH II *in vivo* (Hekimi *et al.* 1991). In accordance with this and if transcription were the only mechanism regulating relative AKH expression, one might expect 4.5 times more AKH I mRNA than AKH II mRNA. In our Northern analysis of adult CC poly(A)+ RNA (Fig. 3), using probes that can discriminate between the two mRNAs and labelled to the same specific activity, we have compared the relative amounts of these messages. Densitometrical measures performed on this Northern blot and on several independent poly(A)+ RNA preparations showed that the mean ratio of proAKH I mRNA to proAKH II mRNA is about 1.7:1 (mean 1.65±0.2, S.D.). Thus, in the adult the mRNA ratio is far from the expected 4.5:1 indicated by *in vivo* protein synthesis.

The discrepancy between the 4.5:1 ratio of AKH I:AKH II synthesis and the 1.7:1 ratio of the mRNAs could be explained if the AKH I mRNA were more efficiently translated than the AKH II mRNA by a factor of about 2.6. Evaluation of mRNA translatability was made in the *in vitro* wheat germ translation system. Densitometrical analyses of the two

proteins synthesized *in vitro* are consistent with the results of Northern analyses, giving a mean ratio of 1.8:1 (preproAKH I to preproAKH II), i.e. similar to the ratio of the mRNAs. This indicates that both mRNAs are translated *in vitro* with equal efficiency, producing an amount of protein directly related to the relative mRNA levels. We know, however, that during translation *in vivo* there is relatively more proAKH I produced. This indicates that the relative translatability of the two mRNAs *in vivo* is not equal, the AKH I mRNA being more efficiently translated by a factor calculated from the mean relative levels of the two mRNAs and the relative levels of AKH I and AKH II synthesis. These calculations suggest that *in vivo* AKH I mRNA is translated about 2.6 times more efficiently than AKH II mRNA and indicate the presence in the CC, but not in the wheat germ system, of a factor or factors which differentially regulates translation of the two mRNAs. We cannot, of course, distinguish between the relative upregulation of AKH I mRNA or the downregulation of AKH II mRNA. Nevertheless, the bias towards proAKH I translation is a significant factor which determines ultimate peptide ratios and this view is further supported by developmental results described below, which show that levels of the two transcripts change while the translational bias remains constant.

Developmental changes in relative transcript and translation levels

The adult AKH I:AKH II ratio is achieved following systematic changes in the relative rates of precursor biosynthesis during postembryonic development, progressively to favour AKH I synthesis (Hekimi *et al.* 1991). There are in the locust five larval stages or instars prior to adulthood. In the first stage (first instar), the ratio of AKH I to AKH II is about 1:1, it reaches 2.5:1 in the fourth instar, 3:1 in the fifth instar and thereafter increases to the adult ratio of 4.5:1, sometimes reaching 5:1 in older adult animals. In order to assess the contribution of transcript levels to these changes we have determined the relative amounts of AKH I and AKH II mRNAs present at different stages of development. Owing to the minute amount of tissue available from the CC of larval animals, experiments were performed on total RNA preparations using the specific antisense oligonucleotides described above. Furthermore, it proved impractical to perform Northern analysis on RNA from stages of development earlier than the fourth instar. As the most pronounced changes in relative AKH I and AKH II synthesis occur between the fourth and fifth instars and the adult, these stages provide the best opportunity to detect correlated changes in relative mRNA levels.

Comparisons were made, using Northern analysis and densitometry, between amounts of AKH I mRNA and AKH II mRNA present in the same quantity of total RNA extracted from fourth and fifth instars and adult (Fig. 4). Levels of both mRNAs increase but they do not increase at the same rate. In the fourth instar, the two messengers are present in approximately equal amounts, with slightly more AKH II mRNA. By the fifth instar AKH I mRNA predominates, giving a ratio of 1.14:1 (AKH I mRNA:AKH II mRNA), and this difference increases further to the adult ratio of 1.7:1 (Fig. 5). Note that the adult ratio obtained from a total RNA preparation is almost precisely that obtained by analysis of polyA+ RNA (see above and Fig. 3). Fig. 5 also shows that changes in relative transcript levels parallel the changes in the AKH ratios over this period of development and suggests that differential transcriptional regulation can

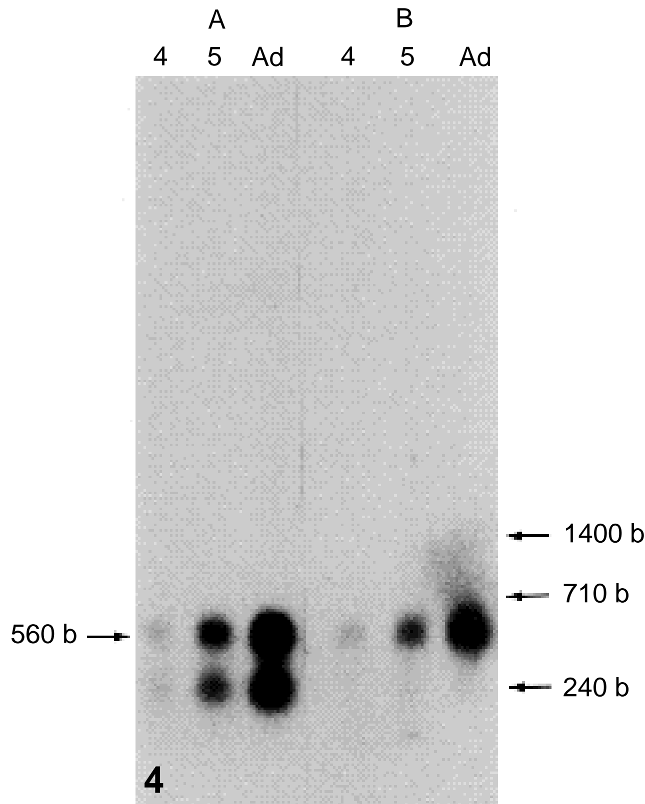

Fig. 4. Northern analysis of AKH I and AKH II mRNAs during development. Total RNA (5 μg) extracted from fourth instars (4), fifth instars (5) and adult (Ad) CCs was analyzed by Northern blotting. Hybridization was performed with ^{32}P-labelled oligo α2 (A) for AKH I mRNA and oligo β3 (B) for AKH II mRNA. Three marker RNAs of known length (1400, 710, 240 bases) are shown on the right side of the figure. The estimated size of the messenger RNA hybridizing with oligo α2 and oligo β3 is indicated on the left. Notice the presence of a small RNA revealed by oligo α2 but not oligo β3.

account for the trend of the changing peptide ratios. Notice that the abrupt change in the transcript ratios between the fifth instar and the adult is accounted for by a reduction in the rate of increase in AKH II mRNA rather than by an upregulation of AKH I transcript expression. This feature was also noted for developmental changes in AKH I and II biosynthesis (Hekimi *et al.* 1991).

While differential control of transcript levels clearly operates during postembryonic development, it alone cannot explain the differences in the levels of AKH I and AKH II biosynthesis. For example, as described above for the adult, the AKH I:AKH II translational ratio is 4.5:1 *in vivo,* whereas the corresponding transcript ratio is only 1.7:1. It was argued from the translation ratio *in vitro* that this difference indicated an *in vivo* translational bias of about 2.6 favouring proAKH I production. This factor appears to be preserved in fourth and fifth instars also, as indicated in Fig. 6, in which the mRNA and

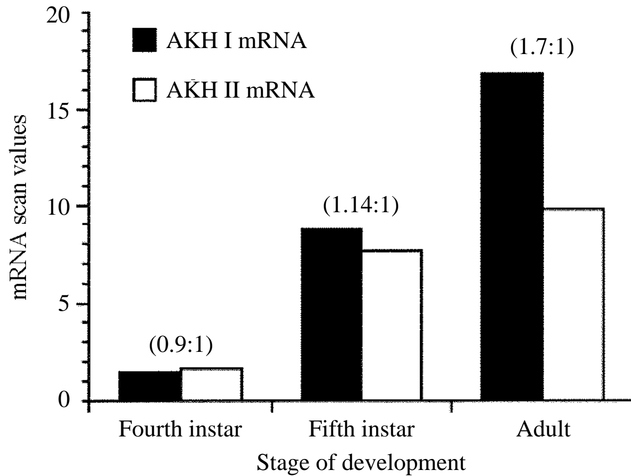

Fig. 5. Relative changes in transcript levels during development. By scanning Northern analyses of the type shown in Fig. 4, relative levels of AKH I and AKH II mRNA were compared. Labelled oligonucleotides that distinguish between the two were used to reveal the developmental shift towards AKH I mRNA late in development. The scan ratios are indicated (I:II) above each pair of histograms.

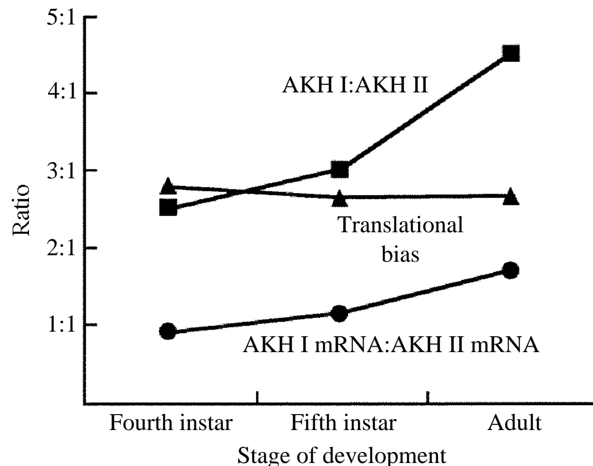

Fig. 6. Comparison of peptide and mRNA ratios. Changes in peptide (squares) and mRNA (circles) ratios are parallel, but differ by a factor (triangles, translational bias) which varies little over the measured range of postembryonic development from the fourth larval stage to the adult.

peptide ratios are compared. At the fourth instar, the peptide ratio is 2.77 times higher than the corresponding mRNA ratio, at the fifth instar 2.63 times and in the adult 2.64 times. It appears, therefore, that the developmental changes in peptide ratio can be explained by a combination of the differential regulation of transcript levels played

against a background of a consistent difference in the relative translatability of the two mRNAs *in vivo*.

In all the experiments performed on several different total RNA preparations, a second band was observed when α-chain oligonucleotides were used for hybridization. This small RNA of about 250 bases is present in instar and adult total RNA (Fig. 4) but not in poly(A)+ RNA preparations. It hybridizes neither with the β-chain-specific probe nor with probes corresponding to the AKH I and AKH II sequences. A probe complementary to the proAKH I mRNA, encoding the C-terminal of the α-chain (oligo α3′), hybridizes with the same stringency as the N-terminal α-chain-specific oligonucleotide (α2) (result not shown). This suggests that this small RNA may encode the complete α-chain of proAKH I but not AKH itself. The amount of this RNA compared with that of the proAKH I mRNA was estimated by scanning the autoradiograms. In five different preparations, the radioactivity associated with the smaller RNA was 60–70% of that of the proAKH I mRNA. Although there is no clue at the moment as to the primary structure and possible role of such RNA, it seems to be related to the α-chain only.

Discussion

Two small mRNAs and prohormones form three dimeric precursors

Our experiments show that two different mRNA species encode two prohormones that subsequently dimerise to form the three precursors of AKH I and II. These mRNAs are homologous in arrangement and coding sequence and are of similar size, around 560 bases. They both have one open-reading frame encoding equal-length but different hydrophobic signal peptides followed by the hormone sequence (AKH I or AKH II), a glycine residue for carboxy-terminal amidation, a dibasic processing site (Lys-Arg or Arg-Arg) and a C-terminal chain of 28 residues (α or β). By blocking their translation *in vitro* with specific oligonucleotide probes we have shown that the AKH I mRNA yields a preproAKH I protein of 6.8kDA and the AKH II mRNA yields a protein of 5.7kDA. The smaller *in vitro* protein corresponds to preproAKH II but appears to have a signal peptide nine amino acids shorter than inferred from cDNA sequencing. This may be due to the preferential use of a second starting codon located within the leader sequence, although the first AUG seems to lie in a more optimal context for initiation when the sequences flanking both AUGs are compared with the optimal nine-nucleotide consensus sequence, CC<u>A/G</u>CC**AUG<u>G</u>**, determined by Kozak (1986) for initiation in eukaryotic mRNAs. The underlined positions -3 and -4 are particularly important to ensure a high efficacy of translation initiation. Inspection of the sequence surrounding the first starting codon (GCAGGATGA) shows a better match of five nucleotides out of nine with position -3 conserved, whereas the sequence around the second codon (CTCTGATGC) shows a poor match of only four nucleotides out of nine, with those in positions -3 and -4 not conserved. Secondary structures of mRNA are involved in facilitating recognition of initiator codons (Kozak, 1990, 1991) and several small inverted repeats have been noticed within the cDNA sequenced, but the possible ‘hairpin’ structures that may result from these would seem to favour the first starting codon even more. Whether this second starting codon is preferentially used *in vivo* is unknown, but if it were, one may argue that

the 13-residue signal peptide that would result from translation initiated at the second AUG may not be long enough for a protein to be translated into the endoplasmic reticulum for subsequent secretion. However, the analysis of von Heijne on the minimal requirements for a signal peptide (von Heijne, 1985) indicates that 13 residues would be the minimum length. Furthermore, the (-3,-1) role for the determination of the cleavage site of the signal peptide (von Heijne, 1984*a*) is followed here with suitable residues in these positions (serine and alanine respectively) or preproAKH II. The net charge of the signal peptide has been shown to influence both the level of translation and the efficiency of transport: a positively charged N-terminal sequence of the peptide appears to be advantageous (Vlasuk *et al.* 1983; von Heijne, 1984*b*). In this respect, a signal peptide of 22 residues would be favoured because it presents a net charge, according to principles established by von Heijne (1984*b*), of +2, whereas the shorter form of 13 residues bears no charge. A net charge of zero has been shown to affect synthesis of a prokaryotic proprotein, but not its secretion (Vlasuk *et al.* 1983). Therefore, if the second AUG codon is indeed used *in vivo*, then this could be the factor, or one of the factors, limiting translation of the proAKH II mRNA.

Homologies between the prohormones and mRNAs were determined in *S. gregaria* and compared with the sequences obtained by Noyes and Schaffer (1990) in another locust species, *Schistocerca nitans*. The overall identities at the nucleic acid level are higher between preproAKHs of the two locust species (97.4% for preproAKH I and 96.7% for preproAKH II) than between preproAKH I and preproAKH II in the same animal (64.5%, Table 1). The same is observed at the amino acid level, where preproAKH I and II share an overall identity of 96.8% and 93.4%, respectively, between the two species, whereas in *S. gregaria* the two preproAKHs only have 59% homology. The ancestral proAKH gene must therefore have duplicated and diverged before phylogenetic divergence of the two locust species.

Cells that express the AKH peptides contain all three forms of precursors P1, P2 and P3 generated by the dimerisation of two co-localized prohormones. Prohormone dimerisation appears to be a random process because it results in the production of binomial distributions of the dimeric precursors (Hekimi *et al.* 1991). The relative amounts of proAKH I and proAKH II determine the shape of the binomial distribution of precursors and, following enzymatic processing, the ratios of the peptides. For example, a 5:1 ratio of proAKH I:proAKH II produces a 25:10:1 binomial for P1:P2:P3 and, following processing, a 5:1 AKH 1:AKH II ratio (because there is one AKH copy per prohormone) and a 25:10:1 distribution of the dimeric APRPs. An explanation of the shifting peptide ratios therefore resides in the mechanisms that regulate the differential production of proAKH I and proAKH II.

Developmental regulation of AKH I and AKH II synthesis

Fig. 7 represents the ideal situation where the proAKH I and proAKH II are produced in a ratio of 5:1, resulting in a 5:1 ratio of AKH I and II (approximately the expected adult ratio). To achieve this ratio, we have assigned a 2:1 ratio of transcripts and a bias of 2.5 towards the transcription of proAKH I over proAKH II. These figures are based on our experimental findings, but the adult transcript ratio has been rounded up and the

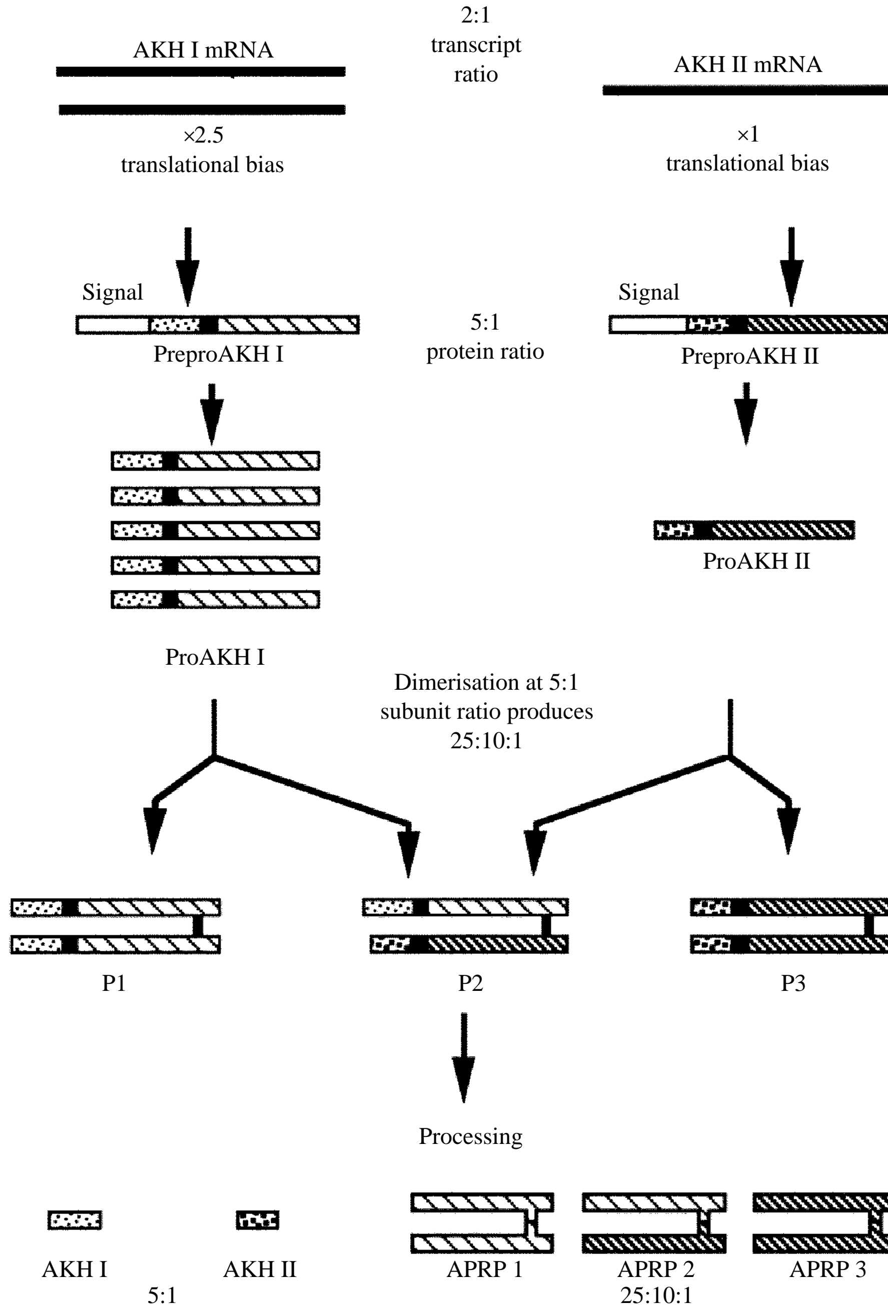

Fig. 7. Model for peptide ratio regulation. For explanation see Discussion.

translational bias rounded down to 2.5. These changes simplify the binomial calculations, producing whole numbers, and result in peptide ratios (AKH and APRP) close to those normally achieved in the adult CC.

Although Fig. 7 approximates the adult situation, it can be simply adapted to generate correct peptide ratios at any stage of development. For example, in the fourth instar the transcript ratio is (simplified) 1:1. Application of the transcriptional bias of 2.5 produces a 2.5:1 ratio of the prohormones, which generates the binomial distribution 6.25:5:1 for the dimeric precursors (P1:P2:P3). The binomial distribution is obtained by expanding $(2.5x+y)^2$, where x is proAKH I and y is proAKH II. Following processing, the expected 2.5:1 AKH I:AKH II ratio and the above binomial distribution of the APRPs characteristic of the fourth instar are produced.

During postembryonic development, the absolute levels of both mRNAs increase as a proportion of total RNA in the CC (Fig. 4). Throughout postembryonic development there is also an increase in the number of AKH-producing neurosecretory cells (S. Kirshenbaum and M. O'Shea, in preparation) and this increase in cell number probably accounts for the absolute increase in mRNA levels. The change in the relative levels of the two mRNAs, however, indicates transcriptional differential regulation of AKH I and AKH II genes as development progresses.

Why AKH I and II peptide ratios change systematically during postembryonic development is unknown, but the most rapid changes occur late in development when the animal's wings start to differentiate. The change may, therefore, be related to the transition between peptide function in the larval stages and in the adult, when flight behaviour first appears, suggesting that the peptide ratios must be configured appropriately for their adult functions.

Dimerisation between different subunits encoded by distinct mRNAs is clearly a process that considerably extends the diversity of the final proteins and can lead to mature products with totally different activities, or even opposite activities, as in the case of the mammalian activin/inhibin gonadal dimeric peptides (Vale *et al.* 1990). The AKH precursors are formed from two identical or different subunits that each carry a neurohormone with similar biological activity, i.e. AKH I and II, and a 28-residue chain, α or β. Physiological functions for the dimeric APRPs, generated by the enzymatic processing of these precursors, remain to be uncovered. One may be tempted to draw an analogy with the dimeric activin/inhibin family. These peptides have opposite effects on synthesis and secretion of FSH (follicle-stimulating hormone) from the anterior pituitary (Vale *et al.* 1990). In a similar manner, enzymatic processing of the AKH precursors would furnish the flying insect with two hormones (AKHs) to control fuel metabolism. The APRPs might exert different functions on identical targets or similar functions on distinct targets, possibly in relation to the complex physiology of flight behaviour. Interestingly, a potential dibasic processing site is observed in the sequence of the α- and β-chains (Arg-Lys at positions 21–22 of both 28-residue chains). Cleavage at these sites does not occur within the neurosecretory cells of the CC (Hekimi *et al.* 1991). It is possible that these sites are not accessible for endoproteolysis because they are in the vicinity of the cysteine residue that forms the disulphide bridge or they might be cleaved at target sites. We are now looking forward to elucidating the physiological functions of the dimeric APRPs.

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