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DIFFERENTIAL EXPRESSION OF MITOCHONDRIAL GENES BETWEEN QUEENS AND WORKERS DURING CASTE DETERMINATION IN THE HONEYBEE *APIS MELLIFERA*

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Summary

The nourishment received by female honeybee larvae determines their differentiation into queens or workers. In this study, we report the first molecular analysis of differences that occur between queens and workers during the caste-determination process. RNA-differential display experiments identified a clone that encodes for a gene that is homologous to the nuclear-encoded mitochondrial translation initiation factor (AmIF-2_{mt}). Semi-quantitative analysis by reverse transcriptase/polymerase chain reaction (RT-PCR) throughout honeybee development detected a higher level of expression of this gene in queen larvae than in worker larvae. Analysis of two other genes encoding mitochondrial proteins, cytochrome oxidase subunit 1 (COX-1; mitochondrial-encoded) and cytochrome *c* (cyt *c*; nuclear-encoded) also showed differential expression of these two genes between queens and workers. In particular, the cyt *c* transcript is more abundant in queen larvae and throughout the metamorphosis of the queen. These results indicate that the higher respiratory rate previously documented in queen larvae is accomplished through a higher level of expression of both nuclear- and mitochondrial-encoded genes for mitochondrial proteins.

Key words: honeybee, *Apis mellifera*, caste determination, mitochondrial gene, gene regulation.

Introduction

A particular feature of social insects is the phenomenon of caste determination which, since the eighteenth century, has been investigated by numerous researchers using, initially, the honeybee as a model (for reviews, see Weaver, 1966; Bettsma, 1979; Brian, 1979). Perez (1889) was the first to establish that the development of honeybee larvae to queen or worker is not due to genetic differences, but is regulated by the differential nourishment that the female larvae receive from the nurse bees. Queen larvae are nourished with royal jelly, a mixture of hypopharyngeal and mandibular gland secretions. In contrast, worker larvae are nourished with worker jelly formed at first as a hypopharyngeal gland secretion by the nurse bees and later, this diluted with honey and pollen (Jung-Hoffman, 1966).

Worker and royal jelly thus differ in chemical composition (Rembold, 1965). One of the most important differences between these types of food is the sugar concentration: royal jelly contains approximately 12 % sugar (wet mass) while worker jelly contains only 4 % sugar (Shuel and Dixon, 1959). In addition, nourishment quantity is also drastically different between queens and workers, since nurse bees visit a queen larva 1600 times compared with 150 times for worker larvae (Lindaver, 1952). Therefore, the nutrition that the queen and worker larvae receive is quantitatively and qualitatively different.

In general, three major consequences result from the differential nutrition between worker and queen larvae. First, at the early fifth instar (approximately 96 h of larval development), the worker larva is larger than the queen larva (Asencot and Lensky, 1976); however, by the end of the fifth day (approximately 120 h of larval development), the queen larva is approximately 60 % heavier (Wang, 1965). Second, the queen larva has a higher metabolic rate, reflected in a higher rate of oxygen uptake starting at mid third instar (approximately 50 h of larval development) and reaching a maximum in the mid fourth instar (72 h) (Shuel and Dixon, 1968). Third, there are important differences in the levels of juvenile hormone between the two castes. It is known that juvenile hormone secretions of the corpora allata depend on the quality and quantity of the food ingested by the larvae (Bettsma, 1979; Wirtz and Bettsma, 1972). Juvenile hormone levels increase in queen larvae during the third to fifth instars, reaching a peak at early stages of the fifth instar, when they are 15 times higher than in worker larvae (Lensky et al., 1978; Rembold, 1987; Rachinsky et al., 1990).

Several physiological and biochemical approaches have been documented in the study of caste determination in the honeybee. However, no molecular strategies have been used in analyses

of this fascinating developmental problem. In this work, we report the first studies that describe some of the molecular differences that occur during the process of caste determination between queen and worker larvae in *Apis mellifera*. Using RNA-differential display (RNA-DD), we have isolated a cDNA that encodes the *A. mellifera* homologue of the mitochondrial translation initiation factor (AmIF-2_{mt}). This transcript is significantly more abundant in queen larvae than in worker larvae throughout the fourth- and fifth-instar larval stages, but no significant differences are found during prepupal and pupal development. A similar set of expression analyses was performed with two other mitochondrial genes, cytochrome oxidase 1 (COX-1) and cytochrome *c* (cyt *c*); in both cases, differences in transcript levels were found between queens and workers, in particular for the nuclear-encoded cyt *c* gene. Our results indicate that, during the differentiation of queen bees, there is an increase in the activity of nuclear- and mitochondrial-encoded genes that play roles in different mitochondrial functions. The possible role of juvenile hormone in coordinating the respiratory status of the honeybee larva with its nutritional state, and the possible influence of larval size on the onset of the genetic-caste-specific program are discussed.

Materials and methods

Honeybees

Larvae, pupae and adults were collected from *Apis mellifera ligustica* colonies. The mass and age of the queen larvae were similar to those reported by Wang (1965). However, the mass of worker larvae was lower than the reported values, probably because of the age of the cells used in our study. In the case of larvae collected after cell sealing, the age (using as reference the eclosion and cell sealing time) and morphology of the individuals were considered. The age of pupae was estimated according to eye pigmentation levels, but in the case of queens, pupae of less than 225 mg were excluded.

RNA display

Total RNA was isolated from worker and queen larvae at 4.5 days of development using the method of Chirgwin et al. (1979). RNA was treated with 10 units of RNAse-free DNAase (Boehringer Mannheim) for 30 min. RNA-differential display (RNA-DD) used for the amplification of cDNAs was performed with random decamers and an oligo-(dT) primer with two random bases at the 3′ end (dT11 MNs). The display was performed as reported by Liang and Pardee (1992). Synthetic oligonucleotides used in the amplification reactions were purchased from Operon Technologies Inc.

RACE

Owing to the small size of the polymerase chain reaction fragments obtained during the RNA display experiments, we used a rapid amplification of cDNA ends modification (RACE) technology with 5′-oligo dT-NN primers (M. Corona and M. Zurita, unpublished results) for the 5′ extension of the cDNAs obtained using RNA-DD (Frohman et al., 1988).

cDNA library construction and screening

Poly(A)$^+$ RNA (5 μg) from queen larvae at 4.5 days of development was used for the construction of a cDNA library in the λZAP vector. This library was prepared using the ZAP-cDNA synthesis kit and the Gigapack II gold packing extract following the recommendations of the supplier (Stratagene). Approximately 6×10^6 independent recombinants were obtained, and the library was then amplified to give 1.8×10^9 plaque-forming units ml^{-1}. To screen the library, a 600 base pair (bp) cDNA RACE-amplified fragment from clone 1 was used as a probe in approximately 2×10^5 plaque-forming units. Hybridization was performed following the protocol supplied by Stratagene. From this screening, two independent positive clones were isolated and characterized in further detail.

DNA manipulations and sequence analysis

All cloning procedures and Southern hybridizations were performed according to the methods of Sambrook et al. (1989). Nucleotide sequencing was performed using the thermo Sequenase kit (Amersham Life Science). DNA and protein sequence analyses were performed using the Gap and Pileup programs from the Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison WI, USA.

RT-PCR

Poly(A)$^+$ RNA was purified using an oligo-dT-cellulose column (Boehringer Mannheim) following the protocols of Sambrook et al. (1989). The first-strand reaction was made using 200 ng of poly(A)$^+$ RNA with 1× first-strand buffer (50 mmol l^{-1} Tris-HCl, 75 mmol l^{-1} KCl, 3 mmol l^{-1} $MgCl_2$), 0.01 mmol l^{-1} dithiothreitol (DTT), 200 units of M-MLV reverse transcriptase (Gibco-BRL), 0.5 mmol l^{-1} dNTPs, 2 mmol l^{-1} oligo$(dT)_{15}$, 20 units of RNAase inhibitor (Boehringer Mannheim) in a final volume of 20 μl. The mixture was initially incubated at 65 °C for 5 min, before DTT and the enzyme were added. The mixture was then incubated for 1 h at 38 °C. A sample of the first-strand reaction (1 μl) was used for the PCR reaction in 1× *Taq* PCR buffer (0.1 mmol l^{-1} Tris-HCl, 1.5 mmol l^{-1} $MgCl_2$, 50 mmol l^{-1} KCl, pH 8.3), 150 mmol l^{-1} dNTPs, 0.5 mmol l^{-1} of each specific oligonucleotide and 1.5 units of *Taq* polymerase (Boehringer Mannheim). PCR reactions were carried out in a Perkin Elmer 2400. The reactions were incubated at 94 °C for 3 min and at 65 °C for 5 min before *Taq* polymerase was added. The mixtures were then incubated at 72 °C for 1 min for one cycle. Specific oligonucleotides were used in each reaction, and their sequences and PCR conditions are as follows.

Nuclear-encoded cytoplasmic elongation factor 1a (EF-1α) (Walldorf and Hovemann, 1990). Control product length: 348 bp, oligonucleotide sequence, direct primer (D): 5′-AAGATCGGTGGTATCGGTACTG-3′ (sequence position, 763–784); reverse primer (R): 5′-GCAAGCGATAT-GAGCGGTGTG-3′ (sequence position, 1329–1309). PCR conditions 94 °C for 30 s, 50 °C for 40 s, 72 °C for 30–60 s; 30 cycles. *Nuclear-encoded mitochondrial transition initiation factor* (this study). Product length: 396 bp; (D): 5′-

ATCTTGAAGGTTTAGTGGAAGG-3′ (sequence position, 1028–1049); (R): 5′-CTTTTTATTTTTAACATTTTCTT-3′ (sequence position, 1424–1402). PCR conditions: 94 °C for 30 s, 50 °C for 40 s, 72 °C for 30 s; 30 cycles. *Nuclear-encoded cytochrome c (cyt c)*. Product length: 275 bp; (D): 5′-CARA-ARTGYGCNCARTGYCAYACN-3′ (sequence position, 49–72); (R): 5′-TTNGANGCYTGYTCRATRTANGCDAT-3′ (sequence position, 323–298); Y=T or C; R=A or G; and D=A, C or T. PCR conditions: 94 °C for 30 s, 55 °C for 40 s, 72 °C for 30 s; five cycles. *Mitochondrial-encoded cytochrome oxidase subunit I (COX-1)*. Product length: 1044 bp, the oligo primers were identical to those reported by (Hall and Smith, 1991); (D): 5′-TTAAGATCCCCAGGATCATG-3′ (sequence position, 115–134); (R): 5′-TGCAAATACTGCACCTATTG-3′ (sequence position, 1158–1139). PCR conditions: 94 °C for 30 s, 50 °C for 40 s, 72 °C for 60 s; 30 cycles. All RT-PCR products from both ethidium-bromide-stained gels and Southern blot hybridizations of the RT-PCR products were quantified by densitometry using the NIH image (1.60 b7) scanning system, and graphs were produced using KaleidaGraph 3.0. Error bars given in the figures correspond to a confidence interval of 99 % calculated by the program.

Dot blot hybridizations

Total DNA (nuclear and mitochondrial) was isolated from different larval and pupal stages using the Nucleon ST kit for soft tissue (Amersham Life Science). DNA concentration was determined by spectroscopy and visualized in agarose gels. A sample (1 μg) of this total DNA was applied to a Hybond N^+ nylon membrane (Amersham Life Science) using a Minifold (Schleicher and Schuell) apparatus. To determine the relative number of nuclear genomes, the EF-1α cDNA fragment (348 bp) was used as a probe. This probe was removed by boiling the membrane in 0.5 % SDS for 30 min, and the same membrane was then rehybridized with a COX-1 1044 bp fragment for the detection of mitochondrial genomes. Membranes were prehybridized at 40 °C for 4–8 h in 50 % formamide, 5× SSC (1× SSC is 0.15 mol l^{-1} sodium chloride, 0.015 mol l^{-1} sodium citrate), 5× Denhardt's solution (1× Denhardt's solution is 0.02 % bovine serum albumin, 0.02 % polyvinylpyrrolidone, 0.02 % dextran sulphate), 0.5 % sodium pyrophosphate plus 100 μg ml^{-1} calf thymus DNA. Filters were then hybridized for 8 h at 40 °C in this solution plus 0.1 % SDS. Successive washes were performed in 0.2× SSC, 0.2 % SDS at 50 °C and 60 °C, each for 20 min. Filters were used for autoradiography using X-AR Kodak films with intensifying screens at −70 °C for 3 h in the case of COX-1 and for 24 h in the case of EF-1α. The dot blot hybridizations of three independent experiments were quantified by densitometry using the NIH image (1.60 b7) system.

Results

Identification of cDNAs expressed differentially in queen and worker larvae by RNA-differential display

Fourth-instar honeybee worker larval (2.5–3.5 days old) development is reversible, in the sense that, if at this point these larvae are fed with royal jelly, they can develop queen characters (Woike, 1971). After the fifth instar, the caste-determination process is no longer reversible. Therefore, larvae of the mid fifth instar (approximately 4.5 days of larval development) that had been fed throughout larval development with royal jelly (future queens) and larvae fed with worker jelly (future workers) were collected. Total RNA was purified from both types of larvae and used in typical RNA-differential display experiments (for details, see Materials and methods). Several cDNA fragments present only in the queen larvae were identified in sequencing gels. These fragments were gel-purified, reamplified, cloned and sequenced (data not shown). One of these clones (clone 1) is analyzed in further detail in this work.

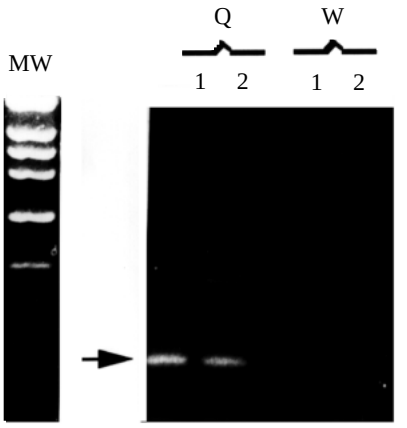

Fig. 1. Reverse transcriptase/polymerase chain reaction (RT-PCR) of the RNA-differential display of clone 1 between queen and worker larvae at 4.5 days of larval development (fourth instar). Lanes 1 and 2 show different cDNA reactions. Q represents queen larvae, and W indicates worker larvae. MW denotes molecular mass markers. The arrow indicates the PCR product of the expected size that is present in the RNA preparation from queen larvae (for details, see the text).

To confirm that clone 1 is more abundant in queen larvae, specific oligonucleotide primers were used for RT-PCR experiments with the original total RNA from queen and worker larvae. Fig. 1 shows a cDNA fragment of the expected molecular size (100 bp indicated in the figure with an arrow) amplified in the RNA from queen larvae. No signal was detected in the worker preparation. This result confirms that clone 1 cDNA is more abundant in total RNA preparations from queen larvae than in those prepared from worker larvae at this developmental stage, and a detailed analysis of its expression throughout larval development was therefore performed.

As the clone 1 fragment isolated by RNA display was too short to be easily used for molecular procedures, the 250 bp fragment was extended at the 5′ end using a variant of the RACE technique (see Materials and methods). Using this procedure, a fragment of 600 bp was obtained (data not show) and used as a probe in library screening (see below).

Clone 1 encodes for the Apis mellifera *mitochondrial translation initiation factor*

To obtain information about the nature of the product

encoded by clone 1, a cDNA library from 4.5-day-old queen larva RNA was prepared (see Materials and methods). This library was screened using the clone 1 RACE-extended product as probe. Two different cDNA clones were identified. One of these clones was nearly full-length and was analyzed further. Sequence analysis of this clone identified an open reading frame on 2158 bases in the correct orientation; although no initiation codon was found at its 5′ end, subsequent analyses indicated that this clone lacks only the first eight amino acid residues of the complete gene product. A computer search to identity previously reported homologous sequences showed that the conceptual open reading frame has a substantial degree of identity with mitochondrial translation initiation factor 2 (IF-2_{mt}) from mammals (Ma and Spremulli, 1995; Ma et al., 1995) and yeast (Vambutas et al., 1991) and with bacterial translation initiation factor 2 (Sacerdot et al., 1984; Friedrich et al., 1988) (Fig. 2). The best identities in overall protein sequence found were with two previously reported animal mitochondrial initiation factors: 41.34 % identity with the bovine sequence and 40.86 % with human IF-2_{mt}. On the basis of the high degree of homology between clone 1 and IF-2_{mt}, but not with the cytosolic IF-2 (data not shown), we may conclude that clone 1 encodes the *Apis mellifera* homologue of the IF-2_{mt} (AmIF-2_{mt}). The gene for AmIF-2_{mt} has two particular features; it contains a high A/T content along its coding region (72.3 %), even compared with other IF-2_{mt} homologues (64.2 % in yeast, 59.1 % in bacteria, 58.7 % in humans and 57.7 % in the bovine sequence). Second, the last five amino acid residues at the carboxyl terminal region are 100 % conserved among the animal IFms, even at the nucleotide sequence level (TGGGATCCAGGATT). Interestingly, this sequence contains seven bases that are identical, but in inverse orientation, to a single thyroid hormone response element (Sap et al., 1989).

AmIF-2_{mt} mRNA levels are differentially expressed in queen and worker larvae

It is known that, during caste determination, an increase in the rate of oxygen consumption takes place in queen larvae (Shuel and Dixon, 1968). The fact that one of the cDNAs identified in our RNA-differential display assays encodes honeybee IF-2_{mt}, a fundamental product for the translation of mitochondrial-encoded mRNAs, suggests that the higher mitochondrial activity in the queen larvae requires an increase in the whole set of mitochondrial products. These products can be encoded either in the nucleus or in the mitochondria themselves. To study AmIF-2_{mt} mRNA levels during critical queen *versus* worker developmental stages in more detail, we performed a semiquantitative RT-PCR analysis (Harting and Wiesner, 1997). Total RNA samples from queens and workers of fourth-instar and fifth-instar larvae, carefully classified by age and mass, were used as templates (see Materials and methods). This classification is fundamental since previous work has demonstrated that a significant increase in size and mass in larvae of both castes takes place in each instar (Wang, 1965), which could have an effect on the general metabolism of the organism. Identical amounts of poly(A)$^+$ RNA from queen and worker larvae were used for the RT-PCRs. Because few gene sequences have been reported in the honeybee so far, we used the *A. mellifera* cytoplasmic translation elongation factor (EF-1α) as an internal control. Although it is known that EF-1α RNA levels change during the development of insects such as *Drosophila melanogaster* (Hovemann et al., 1988) and in honeybees during metamorphosis (see below), these changes are identical in queens and workers. These results indicate that EF-1α responds to the developmental program in both castes, but not to the caste-determination process; EF-1α can therefore be used as a control in these experiments.

The differences between the two castes in the amount of RT-PCR product were quantified using a densitometric analysis in at least three independent experiments (both ethidium-bromide-staining and Southern blotting of the RT-PCR products), and the mean ratio between the experimental RT-PCR product and the RT-PCR control was then plotted. Examples of the RT-PCR gel and the densitometric analysis are presented in the Fig. 3. These results shows that the expression of AmIF-2_{mt} from the early fourth instar is significantly greater in queen larvae than in worker larvae at each time point analyzed. The most dramatic difference in AmIF-2_{mt} RNA expression level between the castes is observed in the last 18 h before cell sealing (108 h), where it is approximately 30 times higher in queen larvae. This result explains why we found differential expression of this transcript with the RNA-differential display performed using RNA from this stage of development.

The abundance of AmIF-2_{mt} RNA in the future queens and workers was also analyzed in larvae after cell sealing, in prepupa and in different pupal stages and adults. The criteria for classification of the organisms in this experiment after cell sealing in the case of larvae were developmental timing in hours and the morphological differences between the pupal stages. In contrast to our observations in fourth- and fifth-instar larvae, there are no significant differences in amounts of AmIF-2_{mt} RNA between queens and workers during these stages (Fig. 4A,B). AmIF-2_{mt} RNA levels are constant in comparison with the control in the last larval stages, prepupae and adults in both queens and workers. As expected, EF-1α RNA levels, used as an internal control, showed similar variations during different developmental stages but not between castes. Higher levels of EF-1α mRNA are accumulated during metamorphosis (Fig. 4).

Differential expression in queens and workers of other nuclear- and mitochondrial-encoded genes involved in mitochondrial function

The fact that the AmIF-2_{mt} transcript is more abundant in fourth- and fifth-instar larvae in the queen than in the workers raises the question of whether this is a general phenomenon for other nuclear- or mitochondrial-encoded genes involved in mitochondrial function. To test this possibility, we quantified the mRNA levels of the mitochondrial-encoded COX-1 gene (cytochrome oxidase subunit 1) and of the nuclear-encoded

cytochrome *c* (cyt *c*) gene in both queen and worker castes. For COX-1 gene amplification, oligonucleotides were synthesized on the basis of the previously reported set which amplifies a 1044 bp fragment (Hall and Smith, 1991). Although the *A. mellifera* cyt *c* sequence has yet not been reported, this is a highly conserved gene, and synthetic oligonucleotide

Fig. 2. Comparison of the conceptual amino acid sequence of the *Apis mellifera* mitochondrial translation initiation factor (AmIF-2_{mt}) and the reported amino acid sequences of mitochondrial translation initiation factors from other organisms. Amino acid residues conserved in all the organisms are indicated in black and with asterisk; residues conserved only between bacteria are indicated in green; residues present in at least two organisms are indicated in pink. The AmIF-2_{mt} sequence is indicated in light blue (cyan). Sf is *Streptococcus faecium* (Accession no. M36878); Bs is *Bacillus stearothermophilus* (Accession no. X04399); Bt is the bovine sequence (Accession no. L37835); Hs is *Homo sapiens* (Accession no. L34600); Am is *Apis mellifera* (Accession no. It200974); Sc is *Saccharomyces cereviseae* (Accession no. X58379).

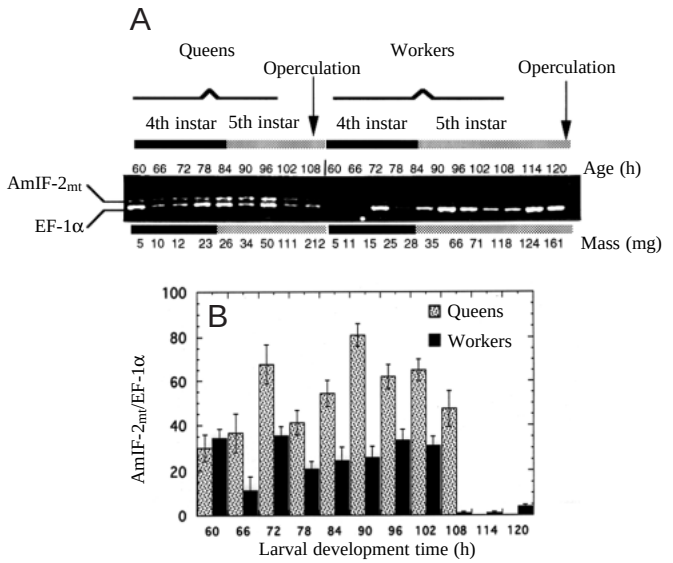

Fig. 3. Semi-quantitative reverse transcriptase/polymerase chain reaction (RT-PCR) of the *Apis mellifera* mitochondrial translation initiation factor (AmIF-2_{mt}) RNA between queen and worker larvae during the caste-determination process. Poly(A)$^+$ RNA from different developmental stages of the fourth and fifth larval instars from queens and workers was purified and subject to a semi-quantitative RT-PCR assay (see Materials and methods). The larvae were classified by both the development time (h) and mass of each specimen (mg) as indicated. EF-1α is the cytoplasmic elongation factor used as an internal control. An example of an agarose gel showing the RT-PCR products is shown in (A). (B) Quantification of the RT-PCR products; the mean of three independent experiments in which the ratio of the experimental PCR/control PCR was plotted against developmental time. The fourth- and fifth-instar developmental stages and the time of operculation are indicated in A. Values are means ±99 % confidence intervals

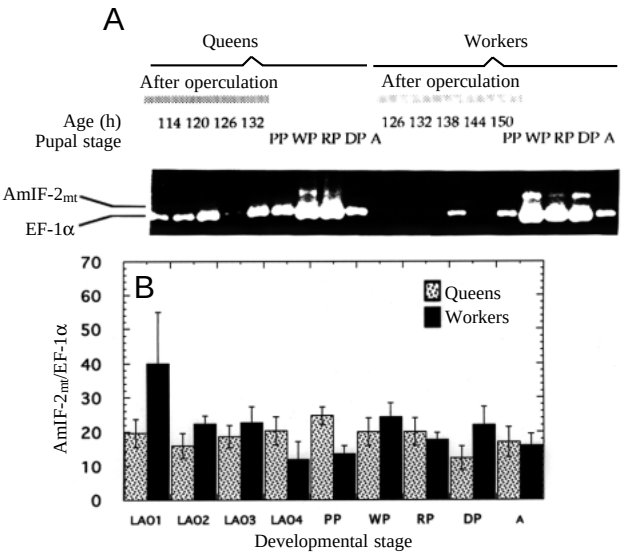

Fig. 4. Semi-quantitative reverse transcriptase/polymerase chain reaction (RT-PCR) analysis of the *Apis mellifera* mitochondrial translation initiation factor (AmIF-2_{mt}) between queens and workers after operculation (cell sealing) and during metamorphosis. (A) An agarose gel with the RT-PCR products; (B) quantification of the PCR products as described in Fig. 3. Again, the different larval stages in queens and workers after operculation were classified by age (h). Since the larval age is different between queens and worker after operculation, these were classified into four equivalent developmental stages indicated as LAO1–LAO4 (*L*arvae *A*fter *O*perculation). PP, prepupa; WP, white-eye pupa; RP, red-eye pupa; DP, dark-eye pupa; A, adult (for details, see the text). Values are means ±99 % confidence intervals for three independent experiments.

primers were designed on the basis of the reported amino acid sequence of the honeybee protein (data not shown; Inoue et al., 1985). RT-PCR amplifications were performed using the same RNA samples as for the AmIF-2_{mt} transcript analyses and quantified by densitometry. The COX-1 transcript is very abundant in queens at all time points analyzed (Fig. 5A). In contrast to what was observed with the AmIF-2_{mt} transcript, COX-1 mRNA levels in worker larvae were also high, although lower than in queen larvae. During the last hours of worker larval development, just before operculation, COX-1 mRNA levels decreased significantly (Fig. 5A). After operculation and during the different pupal stages, COX-1 mRNA levels were almost identical in queens and workers (Fig. 5B). Again, we can observe that the EF-1α RNA levels used as an internal control are more abundant during metamorphosis, as in the previous analyses, probably as consequence of the high degree of cell proliferation.

Cytochrome *c* mRNA levels were very low in both queen and worker larvae. Nevertheless, as in the case of AmIF-2_{mt}, clear differences between queen and worker larvae were detected. In queen larvae, cyt *c* RNA was detected after 35 cycles of PCR amplification at levels comparable to COX-1 RNA detected after only 30 cycles (Fig. 5C). In worker larvae samples treated in the same way, the cyt *c* transcript could be detected only by Southern blot hybridizations of the RT-PCR products (data not shown). Densitometric analyses showed that the lowest concentration of cyt *c* RNA in worker larvae is detected in the same development stage as for the AmIF-2_{mt} and COX-1 transcripts, during the last 18 h before cell sealing. For instance, at 4.5 days (108 h of larval development), cyt *c* RNA levels are approximately 100 times higher in queen larvae than in workers of the same age. In contrast to AmIF-2_{mt}, cyt *c* mRNA levels after cell sealing and during metamorphosis were much higher in the queens than in the workers (Fig. 5D). These results indicates either that cyt *c* transcription is maintained at the same rate in queens during caste determination and metamorphosis or that cyt *c* mRNA is more stable than AmIF-2_{mt} mRNA.

No significant differences in the mitochondrial copy number occurred between queens and workers

The differences in the amounts of AmIF-2_{mt}, COX-1 and cyt *c* transcripts between queen and worker larvae could result from a higher copy number of the mitochondrial genome

resulting from greater mitochondrial biogenesis in queen larvae compared with worker larvae. We compared the number of mitochondrial and nuclear genomes in queen and worker individuals at different developmental stages. Dot blot hybridization of total purified DNA from different queen and worker larval stages was performed. This method compares the relative mitochondrial copy number per cell in each specific developmental stage, since each nuclear genome can represent one cell (Forsburg and Guarente, 1989). This dot blot was sequentially hybridized with nuclear (EF-1α) and mitochondrial (COX-1) DNA probes (Fig. 6). After densitometric quantification of signals from three independent

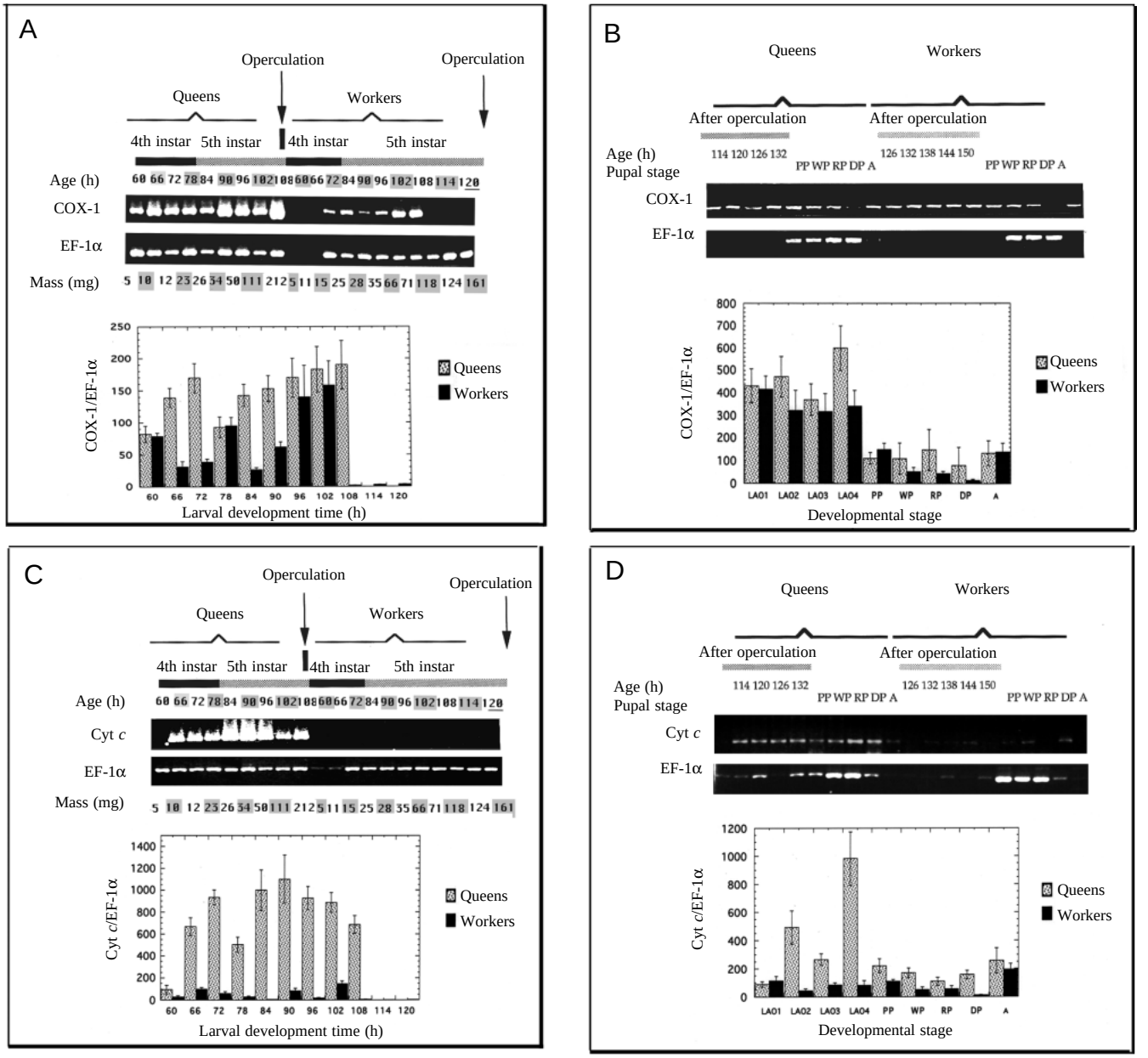

Fig. 5. Semi-quantitative reverse transcriptase/polymerase chain reaction (RT-PCR) of the cytochrome oxidase subunit 1 (COX-1) and cytochrome *c* (cyt *c*) transcript levels during larval development at the time of caste determination and after operculation and during metamorphosis. (A) COX-1 during larval development; (B) COX-1 after operculation and during metamorphosis; (C) cyt *c* during larval development; (D) cyt *c* after operculation and during metamorphosis. In each case, the quantification of the RT-PCR products is represented as in Figs 3 and 4. The same cDNAs used for the analyses of the transcript levels of the AmIF-2_{mt} RNA were used to amplify PCR fragments of COX-1 and cyt *c* in queens and workers. The variables used for classification are indicated in the figure, age (h) and mass (mg) for the larval stages before and after operculation, and the colour of the eye for pupae; PP, prepupa; WP, white-eye pupa. Larvae after operculation were classified into four equivalent developmental stages indicated as LAO1–LAO4 (*L*arvae *A*fter *O*perculation) pupae; RP, red-eye pupa; DP, dark eye for prepupal and pupal stages; A, adult. ET-1α is the cytoplasmic translation elongation factor used as internal control. Values are means ±90 % confidence intervals for three independent experiments

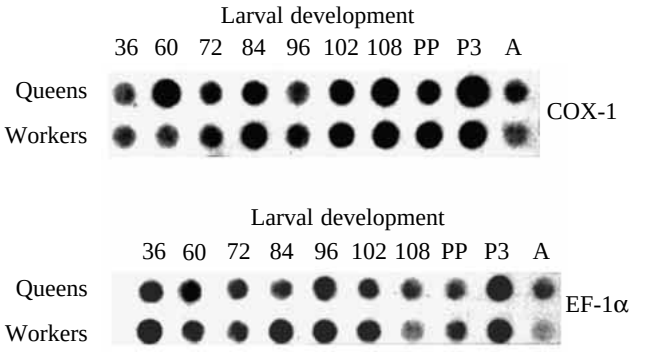

Fig. 6. Comparison of the number of mitochondrial and nuclear genomes between queens and workers. Cytochrome *c* (cyt *c*) was used as a probe for mitochondrial DNA, and the cytoplasmic elongation factor (EF-1α) was used as probe for nuclear DNA. Total DNA (1 μg from each stage) was dotted and hybridized sequentially against these two probes (for details, see Materials and methods). The DNA from different larval stages at the moment of caste determination is indicated by the age (36–108 h); PP, prepupae; P3, pupae with dark eyes (late pupae); A, adult. No significant differences between queens and workers in the number of mitochondrial genomes in relation to genomic DNA were observed in three independent densitometric experiments (data not show).

experiments with both nuclear and mitochondrial probes (data not shown), we found that there were no significant changes in the mitochondrial/nuclear genome ratio between queens and workers, indicating that the differences in transcript levels may be related to a higher transcriptional activity of mitochondrial products in the queens and not to the biogenesis of more mitochondria in this caste.

Discussion

For the first time, we have demonstrated differences in gene expression between queens and workers during the process of caste determination in *Apis mellifera*. The genes analyzed in this study can now be used as molecular markers for a more specific characterization of the gene regulation events in response to signal molecules, such as the juvenile hormone involved in this differentiation process.

Differences between queens and workers in the expression of genes that encode for mitochondrial proteins

Since the amount and quality of food received by the queen larvae is particularly rich, it is reasonable to think that the general metabolism of these individuals might be more active than in the worker larvae. In other words, more ATP is required to fulfil the metabolic demands of the accelerated growth of the queen larvae. This ATP may be produced either by increasing the mitochondrial activity per organelle or by increasing the number of mitochondria in queen individuals.

Higher mitochondrial activity can be achieved among other possibilities by increasing mitochondrial protein synthesis. In animal mitochondria, the molar concentration of all the mitochondrial mRNAs exceeds by far the concentration of ribosomes, a situation that is different from that in the cytosolic compartment (Garstka et al., 1994; Harting and Wiesner, 1997). Since the rate of translation initiation depends on mRNA and ribosome concentrations (Xia, 1996), the translation initiation factor may be a key regulator for mitochondria protein synthesis. The fact that we found more AmIF-2_{mt} mRNA in queen than in worker larvae suggests that mitochondrial translation might be increased in queen larvae, resulting in enhanced mitochondrial activity.

In animals, it has been reported that most mitochondrially encoded genes are regulated at the post-transcriptional level (Gillhan et al., 1994); however, in the case of the honeybee during the caste-determination process (fourth- and fifth-instar larvae), higher levels of COX-1 mRNA were detected in the queen larvae (Fig. 5). Although COX-1 mRNA levels are high in both castes, levels are approximately 1.5-fold greater in queens than in workers. The only exception is seen during the late fifth instar of the worker larval stage, in which COX-1 mRNA levels are very low (detectable only by hybridization of the RT-PCR product; Fig. 5). This stage coincides with a decrease in juvenile hormone levels just before cell sealing. Interestingly, at this point, only the worker larvae, but not the queen larvae, undergo a reduced rate of growth, and this phenomenon may have some effect on COX-1 transcript levels, either by reducing transcription of the COX-1 gene or by reducing the stability of the mRNA. Although we have analyzed the expression levels of only one mitochondrial-encoded gene, we believe that a similar situation will be found for other mitochondrial-encoded gene transcripts because the animal mitochondrial genes encoding polypeptides are transcribed as a polycistronic RNA (Ojala et al., 1981; Montoya et al., 1983).

The nuclear-encoded cyt *c* gene analyzed in this work showed higher levels of RNA expression in the queen, even during metamorphosis and in the adult stages. The mRNA levels for this gene and for the AmIF-2_{mt} gene suggest that, in queens, there is a higher level of expression of the nuclear-encoded genes for mitochondrial proteins. The changes observed in the mitochondrial protein genes between queens and workers are not only a response to the rapid development of the queen, since they are detected from the fourth instar, in which the development of worker larvae is faster than that of queen larvae.

The accumulation of mitochondrial transcripts might indicate that, during the queen-determination process, the developing larvae require either more active mitochondria or a higher rate of biogenesis of these organelles. Our experiments shows that the ratio between mitochondrial and nuclear genomes is the same during the caste-determination process in both queen and worker larvae, suggesting that mitochondrial biogenesis is not greater in queens during caste determination. The accumulation of nuclear transcripts for mitochondrial products that we have observed may be an indication that the strategy used by *A. mellifera* to deal with a large input of energy during the feeding of the queen larva may be to increase the larval respiratory capacity, thus increasing

the activity of their mitochondria more than increasing organelle biogenesis. The lower levels of nuclear-encoded and mitochondrial-encoded transcripts for mitochondrial proteins detected in worker larvae may be related to a slower larval development during the later stages of the fifth instar.

Juvenile hormone and queen determination

In mammals, it has been demonstrated that nuclear genes that encode some mitochondrial proteins are activated by the action of nuclear receptors, in particular by the thyroid hormone receptor (Demonacos et al., 1996; Wiesner et al., 1992). In honeybees, juvenile hormone secretion is stimulated by food ingestion, and the high concentration of juvenile hormone in the queen larvae also correlates with the time of highest respiratory rate in the queen. Juvenile hormone may therefore coordinate the nutritive state of honeybee larva with its respiratory status by activating the metabolic machinery that allows the conversion of food into chemical energy (Rachinsky et al., 1990). Furthermore, in other insects, juvenile hormone has been implicated in the transfer and utilization of stored products to generate the energy for larval growth (L'Hélias, 1970; Locke, 1980) and to direct the induction of the increase in respiration rate (L'Hélias, 1970; Asencot and Lensky, 1976).

It is possible that, in honeybees, juvenile hormone could be implicated in the transcription of the AmIF-2_{mt} gene and of other mitochondrial- and nuclear-encoded genes. It has been reported in mammals that the application of thyroid hormone increases the accumulation of mitochondrial-encoded transcripts, suggesting that this hormone may act directly or indirectly in the transcription of mitochondrial genes (Mutvei et al., 1989). A similar situation could occur in honeybee mitochondria during the caste-determination process when juvenile hormone is present. In future experiments, it will be interesting to determine whether the application of juvenile hormone to worker larvae results in an increase in the levels of AmIF-2_{mt}, cyt *c* and COX-1 transcript levels.

Size, caste determination and metamorphosis in social insects

In many insects, it has been proposed that larval size is an important factor inducing the onset of metamorphosis (Safranek and Williams, 1984). In social insects, metamorphosis is linked to the determination of the morphological characters of the caste. In several hymenopteran species, physiological differences and dimorphism in body size are achieved by the quantity fed and by an extension of the feeding period during the larval stage (Brian, 1979). It has been suggested that, at least in some *Bombus* species, it is larval size that determines pupation and caste (Plowright and Pendrel, 1977). It seems, however, that this is not the case for *A. mellifera*. Although there is a correlation between the size of the queen, pupation and the development of queen characters (Asencot and Lensky, 1976; Dietz and Lambreton, 1970; Woike, 1971), size is not a determinant in queen pupation and development. This conclusion is based on experiments in which the external application of juvenile hormone to a worker larva accelerated the onset of metamorphosis and the development of queen characters independently of the size of the larva (Wirtz and Bettsma, 1972). It is possible that under natural conditions the increase in larval size of the queen larva during development is the result of an important adaptive strategy for the success of the queen in the colony.

The observations reported in this work on the expression of genes that encode mitochondrial proteins suggest that these genes could also be good molecular markers in a search for common factors in the queen-determination processes in other social insects. Experiments similar to those reported here could be performed with ants and wasps to verify whether there is a general difference between the queen and the rest of the colony in different species.

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