

Honeybee (*Apis mellifera* L.) *mrjp* gene family: computational analysis of putative promoters and genomic structure of *mrjp1*, the gene coding for the most abundant protein of larval food

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Abstract

Mrjp1 gene belongs to the honeybee *mrjp* gene family encoding the major royal jelly proteins (MRJPs), secreted by nurse bees into the royal jelly. In this study, we have isolated the genomic clone containing the entire *mrjp1* gene and determined its sequence. The *mrjp1* gene sequence spans over 3038 bp and contains six exons separated by five introns. Seven mismatches between the *mrjp1* gene sequence and two previously independently published cDNA sequences were found, but these differences do not lead to any change in the deduced amino acid sequence of MRJP1. With the aid of inverse polymerase chain reaction we obtained sequences flanking the 5' ends of other *mrjp* genes (*mrjp2*, *mrjp3*, *mrjp4* and *mrjp5*). Putative promoters were predicted upstream of all *mrjp* genes (including *mrjp1*). The predicted promoters contain the TATA motif (TATATATT), highly conserved both in sequence and position. Ultraspiracle (USP) transcription factor (TF) binding sites in putative promoter regions and clusters of dead ringer TF binding sites upstream of these promoters were predicted computationally. We propose that USP, as a juvenile hormone (JH) binding TF, might possibly act as a mediator of *mrjp* expression in response to JH. *Mrjp1*'s genomic locus is predicted to encode an antisense transcript, partially overlapping with five *mrjp1* exons and entirely overlapping with the putative promoter and predicted transcriptional start point of *mrjp1*. This finding may shed light on the mechanisms of regulation of *mrjps* expression. Southern blot analysis of genomic DNA revealed that all so far known members of *mrjp* gene family (*mrjp1*, *mrjp2*, *mrjp3*, *mrjp4* and *mrjp5*) are present as single-copy genes per haploid honeybee genome. Although MRJPs and the yellow protein of *Drosophila melanogaster* share a certain degree of similarity in aa sequence and although it has been shown that they share a common evolutionary origin, neither structural similarities in the gene organization, nor significant similarities between intron sequences of *mrjp1* gene and fourteen yellow-like genes of *D. melanogaster* were found.

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Abbreviations: aa, amino acid(s); ARID, A/T rich interaction domain; bp, base pair(s); cDNA, DNA complementary to RNA; DIG, digoxigenin; dNTP, deoxynucleoside triphosphate; Dri, dead ringer; EGFP, enhanced green fluorescent protein; HG, hypopharyngeal glands; JH, juvenile hormone; kb, kilobase(s); MRJP, major royal jelly protein; mRNA, messenger RNA; nt, nucleotide(s); ORF, open reading frame; PCR, polymerase chain reaction; RJ, royal jelly; TF, transcription factor; Usp, ultraspiracle; UTR, untranslated region; UV, ultraviolet.

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1. Introduction

A characteristic feature of honeybee (*Apis mellifera* L.) society is the phenomenon of caste determination named insect polyphenisms. Young female larvae fed on royal jelly (RJ) develop into queen bees while genetically identical female larvae fed on worker jelly (nutritionally less valuable than RJ) develop into worker bees. This is a case of insect polyphenisms regulated by differential nourishment (for reviews see: Beetsma, 1979; Evans and Wheeler, 2001). RJ is a secretion product of the cephalic glands of nurse bees and serves as a food for bee larvae. It is a mixture of

substances, which determine the development of a honeybee queen. Hypopharyngeal glands (HG) of nurse bees were found to be the major synthesizing and secretory organ of RJ (Hanes and Šimúth, 1992).

A substantial part of RJ is made of proteins, which form about 50% of the dry mass of RJ (Šimúth, 2001). Major proteins account for 82–90% of total RJ proteins and belong to one MRJP protein family (Schmitzová et al., 1998). The *mrjp* gene family, encoding at least five closely related MRJPs, has been described at the cDNA level (Klaudiny et al., 1994a; Ohashi et al., 1997; Schmitzová et al., 1998; Albert et al., 1999a). The closest similarity of MRJPs found is to yellow protein of *Drosophila melanogaster*. MRJPs share a common evolutionary origin with the yellow protein of *D. melanogaster* (Albert et al., 1999a). Although MRJPs are structurally closely related to each other, the expression profiles of *mrjp* genes are not identical. The mRNA of MRJP1 is present in higher amounts in the HG of both nurses and foragers, while MRJP2, MRJP3 and MRJP4 are synthesized predominantly in the HG of nurses (Klaudiny et al., 1994b; Kubo et al., 1996; Ohashi et al., 1997). The level of *mrjp4* expression in HG is very low compared to the expression of the other *mrjps* (Klaudiny et al., 1994b; Kubo et al., 1996; Ohashi et al., 1997). Moreover, mRNA of MRJP1 was found in Kenyon cells of the mushroom bodies of worker bee's brain (Kucharski et al., 1998). Recently it was found that *mrjp2* is expressed in the brain of foragers (Kucharski and Maleszka, 2002). The MRJP1 is present also in pollen pellet, pollen bread and honey (Šimúth, unpublished results). From the physiological point of view, the stimulatory effect on proliferation of primary cultured rat hepatocytes caused by RJ protein with a signal sequence identical to MRJP1 (Kamakura et al., 2001) is interesting. These findings indicate that MRJPs have polyfunctional properties in the honeybee colony and could participate in control of development as regulatory factors.

The age-dependent division of labor (role change), the phenomenon named age polyethism (Winston, 1987) has been observed. The age dependent changes in the expression profile of worker bees' HG (Kubo et al., 1996; Ohashi et al., 1997) as well as a correlation between the age of worker bees and the juvenile hormone (JH) titer in their haemolymph (Huang et al., 1991, 1994) were observed. This leads to the hypothesis that JH may be involved in the reprogramming of the HG function from production of MRJPs (in nurses) to the synthesis of sugar processing enzymes (in foragers) (Ohashi et al., 1997; Bozic and Woodring, 2000). These facts suggest that the expression of the *mrjp* genes is precisely regulated to be highly tissue specific and time restricted. The study of *mrjp* genes expression and regulation will help us to understand the differential expression of these homologous genes and their role in the ontogenesis of social insect honeybee. These genes serve as unique models for further study of molecular mechanisms of the age polyethism of the honeybee.

The characterization of honeybee genes on the genomic

level is in its infancy. To date, no regulatory mechanism of gene expression in the honeybee genome has been described. In order to fill this gap in the field of honeybee genomics and to understand molecular mechanisms regulating different processes in honeybee development, both at individual and social levels, we started with the characterization of honeybee genes and the analysis of their regulatory regions. In our work we present the genomic structure of the gene coding for MRJP1, the most abundant protein of RJ with a unique self-assembly property (Šimúth, 2001), in silico analysis of its genomic sequence as well as description of putative promoter regions of five known *mrjp* genes.

2. Materials and methods

2.1. Biological samples

Female worker honeybees (*Apis mellifera* L.) were obtained from the Institute of Apicultural Research, Liptovská Hrádok, Slovak Republic.

2.2. Honeybee genomic library screening

The honeybee genomic cosmid library No. 72 (Beye et al., 1998), obtained from the Resource Center/Primary Database of the German Human Genome Project (Berlin, Germany), was screened in order to obtain the clone containing the gene coding for MRJP1. The probe, a 1300 bp long cDNA coding for MRJP1 (without signal peptide sequence), was digoxigenin (DIG) labeled by PCR amplification out of the pQE32-MRJP1 construct (Júdová et al., 1998) in reaction mixture containing 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM MgCl₂, 0.001% w/v gelatin, 200 μM of dATP, dCTP, dGTP, 190 μM dTTP, 10 μM DIG-dUTP (Boehringer Mannheim, Germany) and 1 unit of *AmpliTaq* DNA polymerase (Perkin Elmer, USA) using following primers: forward 5'-CAT TCT TCG AGG AGA GTC TTT AAA-3', reverse 5'-GGA TTG AAA TTT TGA AAG GTG TTC G-3'. The initial denaturation step of 30 s at 95°C was followed by 30 cycles of 15 s at 95°C, 40 s at 59°C and 3 min at 72°C. The last cycle was followed by a 5 min extension step at 72°C. Hybridization was carried out under the following conditions: one set (four filters) of the genomic library was pre-hybridized for 2 h at 65°C in Church buffer (0.25 M sodium phosphate pH 7.2, 5% sodium dodecylsulfate and 1.25 mM EDTA). A total of 300 ng of the DIG-labeled probe was denatured for 10 min at 100°C, diluted with 10 ml Church buffer pre-warmed to 65°C and sprayed directly onto the filters, 2.5 ml per filter. The hybridization was carried out overnight at 65°C. Afterwards, the filters were washed for 15 min at room temperature followed by 20 min at 65°C with washing buffer containing 0.2 M sodium phosphate pH 7.2 and 0.1% sodium dodecylsulfate. After background blocking in

1 × PBS buffer containing 5% skimmed milk (Merck, Germany) for 45 min at room temperature, alkaline phosphatase linked antibody against digoxigenin was sprayed onto filters and incubated for 1 h at room temperature. Subsequently, the filters were washed in 1 × PBS buffer twice for 30 min at room temperature followed by washing with 0.1 M Tris/1 mM MgCl₂ pH 9.5 twice for 10 min at room temperature. One mM Attophos (JBL Scientific, USA) in 10 ml 0.1 M Tris/1 mM MgCl₂ pH 9.5 buffer was sprayed on the filters and incubated at 37°C for 4 h. Fluorescent signals were detected by CCD camera (Photometrics, USA).

2.3. Shotgun library construction

Cosmid DNA of MPMGc72F01195Q3 clone, obtained after honeybee genomic library screening, was prepared as described (Sambrook et al., 1989). A shotgun library was generated from the cosmid DNA as described (Radelof et al., 1998). The size range for subcloned fragments was 1.5–2.0 kb. The bacterial cultures of shotgun clones were grown in 384-well microtitre plates (Genetix, UK) at 37°C overnight. PCR amplification of the shotgun clones was carried out in 384-well microtitre plates (Perkin Elmer, USA) in reaction mixture containing 50 mM KCl, 15 mM Tris–HCl (pH 8.5), 35 mM Tris-Base, 1.5 mM MgCl₂, 0.1% Tween 20, 0.015 mM Cresol Red, 200 μM dNTPs, 10 pmol of M13 forward and 10 pmol M13 reverse PCR primer, 1 U *Taq* DNA polymerase. The microtitre plates were sealed using the Microseal 'A' Film (MJ Research, USA). PCR amplification of the shotgun library was performed for 30 cycles consisting of 15 s at 94°C, 3 min at 65°C in 384-well PCR machines (MJ Research, USA). High-density filter arrays of the derived PCR products were generated robotically as previously described (Meier-Ewert et al., 1998). All together 2 300 shotgun clones were spotted onto Hybond N+ nylon membrane (Amersham, UK) as doublets to avoid ambiguity. cDNA of MRJP1 (PCR amplified as described in Section 2.2, without DIG-labeling) was spotted as guide dots for later image analysis.

2.4. Shotgun library analysis

The shotgun library arrayed on nylon membranes was hybridized with 50 ng of MRJP1 cDNA (PCR amplified as described in Section 2.2, without DIG-labeling), radioactively labeled in a random hexamer priming reaction using (α-³³P) dCTP (Amersham International, UK) and Klenow polymerase (New England Biolabs, USA). The hybridization was performed overnight at 65°C in hybridization bottles containing 10 ml Church buffer in a hybridization oven (Appligene, Germany), with a probe concentration of 5 ng/ml. Filters were washed with Church buffer twice for 20 min at 65°C. Hybridization signals were detected by phosphor storage auto-radiography. The hybridization data were analyzed by Visual Grid, Version

2.01 (GPC Biotech AG, Germany). Clones, which gave positive signals, were sequenced. In order to extend the sequence data into the non-coding upstream region of the *mrjp1* gene, the shotgun library was additionally probed with a PCR product derived from a positive clone matching close to the 5' end of the preliminary consensus sequence. In the end a total of 90 shotgun clones were sequenced.

2.5. Shotgun clones DNA sequencing and sequences assembly

Sequencing reactions of shotgun library clones were carried out using fluorescent dye-terminator sequencing kit (Perkin Elmer, USA) with standard M13 primers (both forward and reverse). Specific primers were designed for primer walking on certain clones. ABI PRISM 377 DNA Sequencers and ABI PRISM 3700 DNA Analyzers were used for separation and detection of sequencing reaction products. Data were processed using ABI's sequence analysis software (Perkin Elmer Applied Biosystems, USA), v.3.2 (for ABI PRISM 377 DNA Sequencers) or v.3.6 (for ABI PRISM 3700 DNA Analyzers). To compile and analyze the obtained sequencing data the GCG package Version 10.0 software was used. A total of 140 shotgun clones sequences were assembled into the contig using the Gap4 program (GCG-STADEN). A 7084 bp long consensus sequence, covered entirely by sequence readings in both sense and antisense directions, was obtained. In order to deduce the intron/exon structure of the *mrjp1* gene, the BLAST program (Altschul et al., 1990) was used to compare the sequence of MRJP1's cDNA (Ohashi et al., 1997; Schmitzová et al., 1998) with the obtained consensus sequence.

2.6. Genomic DNA preparation

Honeybee (*Apis mellifera* L.) genomic DNA was isolated from deep frozen female bee workers (whole body) according to (Beye and Raeder, 1993). Isolated genomic DNA was dissolved in 10 mM Tris–Cl (pH 8.0) buffer.

2.7. Southern blot analysis of honeybee genomic DNA

Ten μg of honeybee genomic DNA was digested overnight with restriction enzymes, recognizing 6-bp sites: *Bgl*II, *Eco*RI, *Hind*III, *Pst*I and *Spe*I (New England BioLabs, USA). Digested DNA was phenol-chloroform-isoamyl alcohol extracted, dissolved in 1 × TE buffer and the resulting DNA fragments (5 μg of digested genomic DNA) were separated through 0.8% agarose gel in 1 × TAE buffer (0.04 M Tris-acetate, 1 mM EDTA). DNA was denatured and transferred from the gel onto Hybond N+ nylon membrane (Amersham, UK) by capillary transfer in 10 × SSC buffer (150 mM sodium-citrate, 1.5 M NaCl) overnight as described (Southern, 1975). Afterwards the membrane was washed for 5 min in 6 × SSC (90 mM

sodium-citrate, 0.9 M NaCl), dried and the transferred DNA was UV-crosslinked with the membrane. The Southern blot membrane was hybridized in a hybridization oven (Appligene, Germany) with the following probes: (i) probe for *mrjp1*, a 1300 bp long cDNA of MRJP1 was PCR amplified out of the pQE32-MRJP1 construct (Júdová et al., 1998) with the primers described in Section 2.2; and (ii) probes for *mrjp2*, *mrjp3*, *mrjp4* and *mrjp5* were PCR amplified from honeybee genomic DNA (100 ng DNA/reaction) with primers designed to match non-conserved regions of particular cDNAs corresponding to *mrjp* genes: *mrjp2* probe, 290 bp long (forward: 5'-TTT CGT AGG AAG CAA CAA ACC TG-3', reverse: 5'-GCA ATT TTG AAA AAC GAC CAA CTT A-3'), *mrjp3* probe, 400 bp long – the primers flanking the sequence of the repetitive region of *mrjp3* were used (Albert et al., 1999b), *mrjp4* probe, 850 bp long (forward: 5'-CGT TTT CGA CAG TCA ATT AAC C-3', reverse: 5'-TTT AGC ACG TTC TGG TTT CTG-3'), *mrjp5* probe, 400 bp long (forward: 5'-GAT GCA TCT CCC ACA ATC CA-3', reverse: 5'-GTA TCC ATC CTA TCC ACC CT-3'). The PCR conditions were as follows: 15 pmol of each primer, 75 mM Tris–Cl (pH 8.8), 20 mM ammonium sulfate, 1.5 mM magnesium chloride, 0.01% Tween 20, 1.3 M betaine, 300 µM each dNTP. To reduce possible sequence errors a *Taq-Pfu* DNA polymerase mixture (15:1 units) was used. The initial denaturation step at 95°C for 5 min was followed by 35 cycles of 30 s at 93°C, 30 s at 54°C and 1 min at 68°C. The last cycle was followed by an extension step for 10 min at 72°C. The probes were purified from the agarose gel using QIAEX II kit (Qiagen, Germany). 100 ng of each probe was radioactively labeled with (α -³²P) dCTP (Amersham International, UK) as described in Section 2.4. The hybridization was performed as described in Section 2.4, with the probe concentrations 10 ng/ml Church buffer. Hybridization signals were detected by phosphor storage auto-radiography.

As a control for hybridization and probe specificity a dot blot membrane was used in each hybridization experiment. Fifty pg of each PCR amplified probe was applied to onto Hybond N+ nylon membrane (Amersham, UK) and processed as described above for the Southern blot membrane. For each probe, hybridization of the dot blot membrane was carried out simultaneously with the Southern blot membrane in the same hybridization bottle.

2.8. Inverse PCR

One µg of genomic DNA isolated from female worker bees (Section 2.6) was partially digested with three different 4-bp recognizing restriction enzymes in parallel: *Msp*I, *Mse*I and *Mbo*I (New England Biolabs, USA). Digested DNA was phenol-chloroform-isoamyl alcohol extracted (Sambrook et al., 1989) and dissolved in 50 µl of 10 mM Tris–Cl (pH 8.0) buffer. The obtained fragments were circularized by self-ligation in series of 6 dilutions. The

reaction was performed with 80 U T4 DNA ligase (New England BioLabs, USA) in 20 µl reaction mixture containing 5% polyethyleneglycol at 16°C overnight. Circularised DNA fragments were ethanol precipitated (Sambrook et al., 1989) and dissolved in 20 µl of 10 mM Tris–Cl (pH 8.0) buffer. Five µl of circularized DNA was taken as a template for the first round of inverse PCR amplification with primers (forward: 5'-GGT GTA CCT TCT TCT TTG AAC GTG G-3' and reverse: 5'-CGC CAG ATT GAA TCG CAG CTT GTC TTC-3') designed to anneal to the most conserved sequences of 5-prime ends of all five MRJPs' cDNAs in order to amplify all the possible genomic DNA fragments flanking the 5-prime end of all *mrjp* genes. The PCR was carried out in a 50 µl mixture containing 15 pmol of each primer, 75 mM Tris–Cl (pH 8.8), 20 mM ammonium sulfate, 2.5 mM magnesium chloride, 0.01% Tween 20, 1.3 M betaine, 400 µM of each dNTP. *Taq-Pfu* DNA polymerases mixture (15:1 units) was used. The cycling conditions were as follows: an initial denaturation for 5 min at 95°C was followed by 35 cycles of 1 min at 93°C, 30 s at 54°C and 5 min at 68°C. The last cycle was followed by the extension step for 10 min at 72°C. One µl of PCR mixture from the first round of PCR was used for second round PCR with the following nested primers specific to particular *mrjp* genes (for GenBank Accession Nos. of cDNAs coding for *mrjp* genes see Section 2.10):

mrjp2-forward: 5'-GGT AAA ACT GGT AAG GGT GGA CGA C-3',
mrjp2-reverse: 5'-TCG CTA CCG AAG TCA TAA TCA AAA TAC-3',
mrjp3-forward: 5'-GGT AAC TAA TAA AAA GGG CAA AGG TGG AC-3',
mrjp3-reverse: 5'-ATT TGC AGA TTT TCT TTG ATG ATT CAC AG-3',
mrjp4-forward: 5'-GGT ATC TGA CAA AAC TGG CAA CGG-3',
mrjp4-reverse: 5'-GAA TTT TCT CGA ACA ACG CCA CCT C-3',
mrjp5-forward: 5'-ATA TCT GAG AAA ATT GGC AAC GGT GG-3',
mrjp5-reverse: 5'-CAA ACG ACA GTT TCA AGA GAA CGC AG-3'.

Nested PCR was carried out under the same conditions as the first round PCR except that a higher annealing temperature (62°C) and a lower number of cycles (30) were used. The PCR products were purified from the agarose gel using QIAEX II kit (Qiagen, Germany) and sequenced by GATC company (Constance, Germany).

2.9. Putative *mrjp1* promoter constructs and reporter gene assay

An enhanced green fluorescent protein (EGFP) reporter vector pIE1-SV-EGFP for the high-level expression in

insect cell lines (Huynh and Zieler, 1999) was used in these studies. The AcNPV promoter of this vector was cut out with restriction endonucleases *Bgl*II (cutting site in position 1201) and *Spe*I (cutting site in position 97) (New England BioLabs, USA) and replaced by the PCR fragment of putative regulatory region of *mrjp1* gene. The PCR of the fragments was done as described in Section 2.8 with 30 s annealing step at 50°C and 3 min elongation at 68°C. The following primers, with overhang sequences bearing *Bgl*II, respectively *Spe*I recognition site (shown in bold letters), were used:

1-forw-*Spe*I: 5'-GGACTAGT GGT TAA ACG TAT AAC CAC GAA C-3',
 1223-forw-*Spe*I: 5'-GGACTAGT TAG CGG ACG GTT TTG CCA-3',
 2175-forw-*Spe*I: 5'-GGACTCGT AAT TAA ATA AAC ATA ATA TTT TTC AAT AT-3',
 2275-forw-*Spe*I: 5'-GGACTCGT GAA TTA TAT GAT GAA GCG AGA CG-3',
 2637-rev-*Bgl*II: 5'-GAAGATCT AAT AAA ATA TTT CTT ATA GGA AAT ATT AC-3',
 2674-rev-*Bgl*II: 5'-GAAGATCT TTT TCT ACA AAA TAT TTC GTC TTG-3',
 2865-rev-*Bgl*II: 5'-GAAGATCT TTA TTC TTG TAG TCG TAT TCG CC-3',
 3032-rev-*Bgl*II: 5'-GAAGATCT CTC AGC ATG GTG ACA AAA ATC TTA TC-3'.

A total of seven different fragments were PCR amplified using the following primer combinations (the numbers in primer names reflect the sequence position in 7084 bp *mrjp1* genomic locus (Figs. 1A,B)): 1223-forw-*Spe*I and 3032-rev-*Bgl*II (1810 bp fragment), 1223-forw-*Spe*I and 2865-rev-*Bgl*II (1643 bp fragment), 1-forw-*Spe*I and 2637-rev-*Bgl*II (2637 bp fragment), 1-forw-*Spe*I and 2674-rev-*Bgl*II (2674 bp fragment), 2275-forw-*Spe*I and 2637-rev-*Bgl*II (363 bp fragment), 2175-forw-*Spe*I and 2637-rev-*Bgl*II (463 bp fragment), 2175-forw-*Spe*I and 2674-rev-*Bgl*II (500 bp fragment). The fragments contain either a short region 500 bp upstream of the translational start point or different regions, up to 2.7 kb in length upstream of the translational start point, with or without the first exon and first intron of *mrjp1*. The fragments were, after *Bgl*II and *Spe*I restriction, cloned into *Bgl*II – *Spe*I digested pIE1-SV-EGFP vector. All seven *mrjp1*-pIE1-SV-EGFP constructs were verified by sequencing before use. The S2 insect cells (Invitrogen, USA) were cultured in 6-well plates with 3 ml of DES (Invitrogen, USA) supplemented with 10% heat-inactivated fetal bovine serum (Biochrom KG, Germany), 50 units/ml penicillin and 50 µg/ml streptomycin at 24°C until they reached 60–70% confluence. The cells were transiently transfected with 20 µg of DNA (pIE1-SV-EGFP as a control, respectively seven different *mrjp1*-pIE1-SV-EGFP constructs) by the Ca-phosphate co-precipitation technique (Gorman et al., 1984). Forty-eight

hours after transfection cells were washed with 1 × PBS buffer. The fluorescent signal of expressed EGFP in S2 cells was detected by fluorescent mikroskop (Axioplan 2, ZEISS, Germany).

2.10. GenBank Accession Numbers

Genomic sequence of *mrjp1* gene locus: AF388203; genomic sequence flanking the 5' end of *mrjp2* gene: AY078399; genomic sequence flanking the 5' end of *mrjp3* gene: AY078400; genomic sequence flanking the 5' end of *mrjp4* gene: AY078401; genomic sequence flanking the 5' end of *mrjp5* gene: AY078402; cDNA of MRJP1: D79207 (Ohashi et al., 1997) and AF000633 (Schmitzová et al., 1998); cDNA of MRJP2: AF000632 (Schmitzová et al., 1998); cDNA of MRJP3: Z26318 (Klaudiny et al., 1994a; Albert et al., 1996); cDNA of MRJP4: Z26319 (Klaudiny et al., 1994a); cDNA of MRJP5: AF004842 (Albert et al., 1999a).

2.11. Software used in DNA sequence analysis

GAP4, MZEF, MEME – in GCG program package (Wisconsin Package Version 10.2, Genetics Computer Group (GCG), Madison, USA); CHIPS – in EMBOSS program package (Rice et al., 2000); BLAST: <http://www.ncbi.nlm.nih.gov/BLAST/> (Altschul et al., 1990); MATCH (Transfac database 5.4): <http://www.biobase.de/> (Wingender et al., 2000); Neural Network Promoter Prediction (NNPP2.1): http://www.fruitfly.org/seq_tools/promoter.html (Rees and Eeckman, 1995).

3. Results

3.1. DNA sequence of *mrjp1*'s genomic locus and its structure

In order to analyze the *mrjp1* gene, a honeybee genomic library was screened with a DIG-labeled cDNA of MRJP1 as a probe. One positive cosmid, MPMGc72F01195Q3, was identified and purified. A shotgun library from this clone was prepared and clones containing the *mrjp1* gene fragments were identified by hybridization with radioactively labeled (³³P) cDNA of MRJP1. After sequencing of selected positive shotgun clones, sequence reads were assembled by the Gap4 program into a single contig of 7084 bp in length (GenBank Accession no. AF388203). This genomic sequence is entirely composed of reliable sequence readings from both DNA strands. The average sequence coverage of this genomic locus is 8.5. Fig. 1A shows the intron-exon organization of *mrjp1* gene on the sense strand, obtained by comparison of the 7084 bp *mrjp1* genomic locus with the cDNA coding for MRJP1 (Ohashi et al., 1997). The coding region of the *mrjp1* gene spans over 3038 bp. Six exons were found in the coding region and all

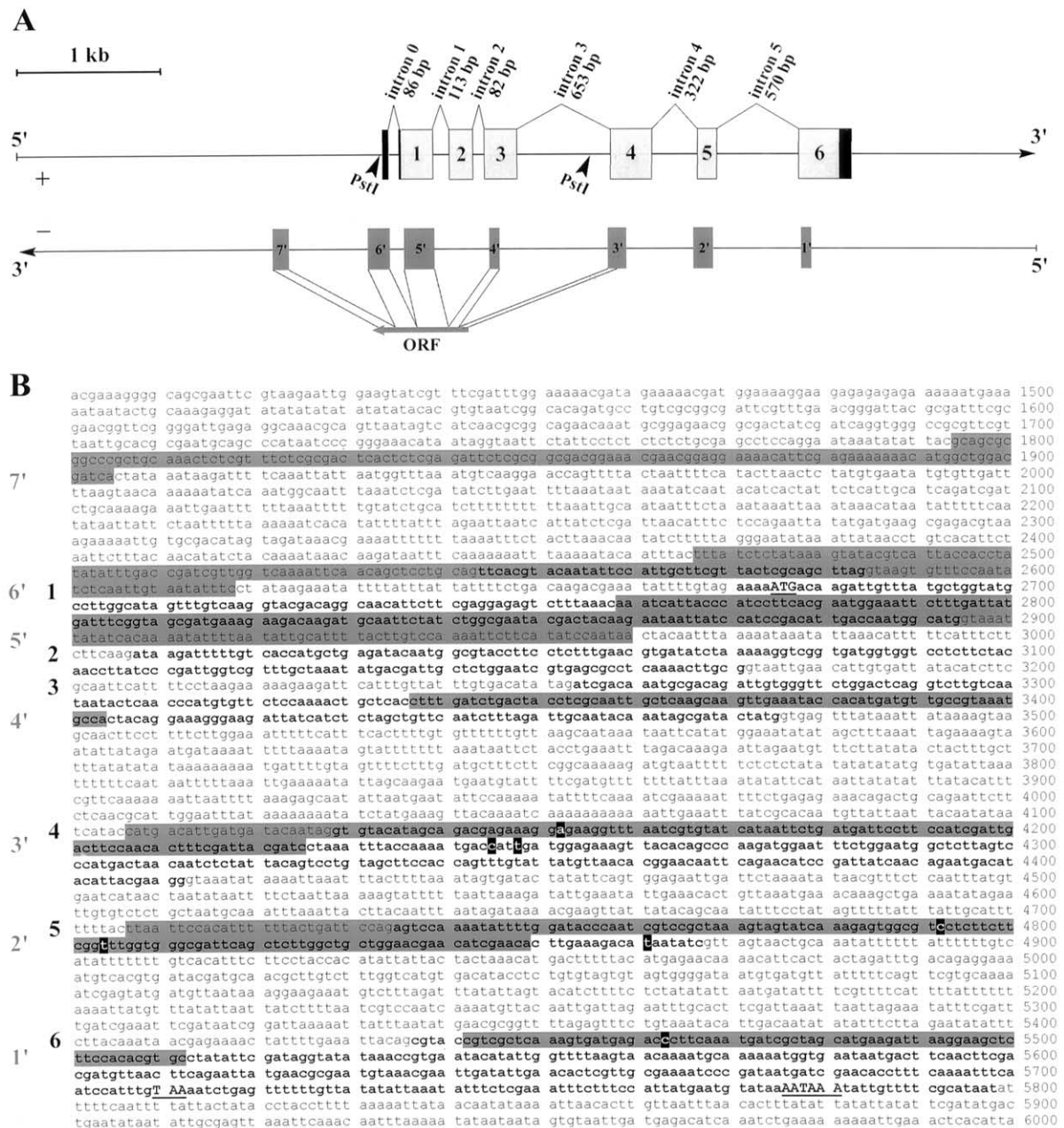


Fig. 1. Genomic organization of *mrjp1* gene's locus. (A) The sense (+) DNA strand: the exon-intron structure of *mrjp1*: light-gray numbered boxes (1–6) indicate exons while the black boxes indicate untranslated regions transcribed into mRNA of MRJP1. Arrows show *PstI* restriction sites. The antisense (–) DNA strand: dark-grey numbered boxes (1'–7') indicate putative exons predicted by MZEF program. Four antisense exons (4', 5', 6' and 7') together with part of the 3' antisense exon show ORF of 576 nt in length. (B) Part of the 7084 bp honeybee genomic locus sequence bearing the *mrjp1* gene (GenBank Accession no. AF388203). The exons of *mrjp1* are shown in black bold letters. The start codon ATG (2675–2677), the stop codon TAA (5710–5712) and the polyadenylation signal AATAAA (5776–5781) are shown in bold black underlined uppercase letters. Mismatches are indicated by white letters in black boxes. Putative predicted antisense exons are shaded in gray. The numbers on the left indicate the corresponding *mrjp1* exons (black) or putative antisense exons (gray).

intron-exon boundaries follow the gt-ag rule (Fig. 1B). The continuous ORF encodes the MRJP1 protein, which is 432 aa in length. The ORF was investigated with the CHIPS program (Rice et al., 2000) for codon usage statistic. The codon usage ratio 50.9 (out of 61 corresponding to all the possible codon usages) indicates low codon bias of *mrjp1*'s coding region.

The sequence comparison analysis revealed seven mismatches between the genomic DNA sequence of the *mrjp1* gene and the corresponding cDNA sequence, available from the public databases and determined by two independent groups (Ohashi et al., 1997; Schmitzová et al., 1998) (Fig. 1B). Nevertheless, these mismatches do not lead to any changes in the aa sequence of MRJP1. With

the aid of the MZEF program, seven exons were predicted on the antisense (–) DNA strand of the genomic sequence (Figs. 1A,B; Table 1) coding for a putative RNA transcript of 857 bp in length. Interestingly, five of these antisense exons (1'–5') partially overlap with five *mrjp1* exons (1, 3, 4, 5 and 6) (Figs. 1A,B). Antisense exon 6' overlaps with the 5' UTR of *mrjp1* gene (Figs. 1A,B). The last four antisense exons (4', 5', 6' and 7') together with part of the antisense exon 3' contain an ORF of 576 nt in length, starting at the position 4158 nt of 7084 bp genomic locus (Fig. 1A). The derived putative 192 aa antisense protein would have its first methionine at position 44. The Initiation codon ATG of the antisense transcript, corresponding to this methionine, together with G in position at –3 from this ATG, is within a context that conforms well to the Kozak consensus sequence for initiation of translation by eukaryotic ribosomes. Thirty-eight aa (positions 97–134 of this putative protein) show a 36% identity and 64% similarity (BLAST) with a *Caenorhabditis elegans* chemoreceptor (GenBank Accession No. NM_062033). Nevertheless, neither a frame stop codon, nor a polyadenylation signal were found on the 3' end of the potential antisense transcript.

3.2. Southern analysis of honeybee genomic DNA

To determine the copy numbers of *mrjp1* gene and other known members of the *mrjp* gene family (*mrjp2*, *mrjp3*, *mrjp4*, *mrjp5*) in the honeybee genome, Southern analysis of the genomic DNA was performed using the whole body genomic DNA of worker bee digested separately with 6 bp recognizing restriction endonucleases: *Bgl*II, *Eco*RI, *Hind*III *Pst*I or *Spe*I. The blot was probed with the following radioactively-labeled (^{32} P) probes: cDNA encoding MRJP1 or a part of genomic DNA corresponding to the unique non-conserved parts of *mrjp2*, *mrjp3*, *mrjp4* and *mrjp5*, genes belonging to one *mrjp* gene family. The specificity of particular probes is shown in Fig. 2B. There are two strong bands observed on the membrane in the lane corresponding to *Pst*I restriction after probing with *mrjp1* probe (Fig. 2A). The smaller, 1.4 kb, band is consistent with the predicted 1450 bp fragment containing the first three exons of the

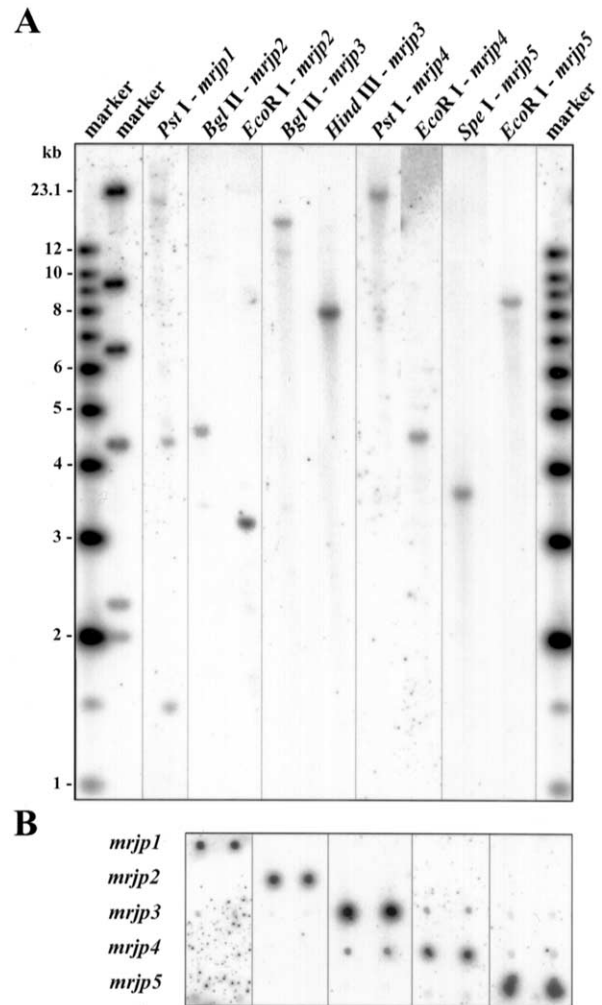


Fig. 2. Southern analysis of honeybee genomic DNA. (A) Five μ g/lane of digested genomic DNA (using five different restriction endonucleases: *Bgl*II, *Eco*RI, *Hind*III, *Pst*I and *Spe*I) was loaded on gel. Southern blot membrane was probed with probes specific to the genes of the honeybee *mrjp* gene family: *mrjp1*, *mrjp2*, *mrjp3*, *mrjp4* and *mrjp5*. (B) Fifty pg of each probe was applied in duplicates to the dot blot membrane, which was used in each hybridization experiment together with Southern blot membrane to confirm specificity of the hybridized probe.

mrjp1 gene according to the restriction map of the *mrjp1* gene locus (Fig. 1A). The second, 4.6 kb, band corresponds obviously to the fragment containing exons 4, 5 and 6 of *mrjp1* gene, spanning to the 3' non-coding region (Fig. 1A). The weak band of high molecular size reflects probably incomplete digestion of the honeybee genomic DNA with *Pst*I. The hybridization patterns of the four *mrjp2*–5 probes show one discrete band in each case (Fig. 2A). These results indicate that *mrjp* genes are present as single copy genes per haploid honeybee genome.

3.3. Transcription factors and regulatory regions mapping on putative *mrjp* promoters

In order to obtain the unknown DNA sequences immediately upstream of the 5' UTR of the *mrjp* genes

Table 1
Predicted exons on the antisense strand of 7084 bp honeybee genomic locus (containing *mrjp1* gene) with the MZEF program

Putative anti-exons	Score	Length (bp)	Position related to the sense strand (bp)
1'	0.975	71	5512–5442
2'	0.988	143	4849–4707
3'	0.979	119	4225–4107
4'	0.817	68	3404–3337
5'	0.865	202	2960–2759
6'	0.926	152	2618–2467
7'	0.945	112	1905–1794

Table 2

Putative promoters predicted by NNPP2.1 tool on upstream sequences flanking the 5' end of five *mrjp* genes^a

Gene related to the analysed sequence ^b	Length of the 5' end flanking sequence ^c	Position of promoter	Score	Promoter sequence ^d
<i>mrjp1</i>	2543	2490–2540	0.86	ATTACCACCTATATATTGACCGATCGTTGGTCAAAATTC A ACAGCTCCT
<i>mrjp2</i>	744	675–725	0.81	CATTACCACCTATATATTGACCAACCGTTAGTCAAAATTC A ACAGTTCTT
<i>mrjp3</i>	625	556–606	0.51	CATTACAACCTATATATTGACCAACAGTTAGTCAAAATTC T GCAGTTTG
<i>mrjp4</i>	613	551–601	1.00	TTATCGTATATATATATTGACCGAGCATTCACTCAAAATTC C TGCAGTTT
<i>mrjp5</i>	709	650–700	0.95	CATTATCGCCTATATATTGACCGACCATTAGTCAAAATTC A ACAAATCT

^a Neural network promoter prediction (see Section 2.11).^b For GenBank Accession Nos. of cDNAs coding for MRJPs see Section 2.10.^c For GenBank Accession Nos. of these genomic sequences see Section 2.10.^d Predicted transcriptional start points are indicated in bold type, predicted TATA box sequences are underlined.

and to compare putative regulatory regions among all known *mrjp* genes, the inverse PCR approach on honeybee genomic DNA was applied. A total of 744 bp upstream of *mrjp2*, 625 bp upstream of *mrjp3*, 613 bp upstream of *mrjp4* and 709 bp upstream of *mrjp5* sequences were determined (for GenBank Accession Nos. see Section 2.10).

The Neural Network Promoter Prediction (NNPP2.1) tool (see Section 2.11) was used in order to find putative promoter regions in these putative regulatory sequences of *mrjp* genes. The consensus sequence of the TATA boxes found is in all cases highly conserved (TATA-TATT) and is located 30–31 bp upstream from the transcriptional start point predicted by the NNPP2.1 prediction tool (Table 2). A putative regulatory region of *mrjp1* gene contains the CAAT box sequence (CCAAT), not present in the other four cases (Fig. 3).

Several transcription factor (TF) binding sites were mapped within *mrjp* putative regulatory sequences, according to the Transfac database using the MATCH program (Wingender et al., 2000). Insect matrices of TF binding sites were selected for the analysis. TF binding sites for dead ringer (Dri) and ultraspiracle (USP) were detected in all the *mrjps*' putative regulatory sequences (Fig. 3). A high density of binding sites for Dri (binding site core sequence: GATTA or AATTA) in all five putative regulatory sequences can be observed. For comparison, the whole coding region of *mrjp1* gene

spanning over 3038 bp shows only two binding sites for Dri TF under the chosen parameters (results not shown). Although the pattern of Dri binding sites in these five sequences differs from gene to gene, the closest Dri binding site, related to the coding regions of the genes, appears to be at a similar distance from the 5' UTR for all *mrjp* genes (Fig. 3). The binding site for a second potentially interesting TF, USP (binding site core sequence: GGTCA), is located close to the 5' UTR region of all the *mrjp* genes, immediately downstream of the predicted TATA box sequence, in the region of predicted *mrjp* promoters. The *mrjp1* related sequence contains probably two USP binding sites, the other four *mrjp2*–5 related sequences contain one USP binding site (Fig. 3). The chosen parameters for TF binding sites mapping were as follows: the core similarity cut off was 1.00 for both TFs, the matrix similarity cut off for Dri was 0.97, and the matrix similarity cut off for USP was 0.74. Distribution of the predicted regulatory elements found in the putative regulatory sequences of *mrjp* genes is shown in Fig. 3.

The presence of multiple binding sites for TFs in 5' non-coding regions of *mrjp* gene family and the similarity of their distribution pattern prompted us to test these putative regulatory regions functionally. Unlike the positive control of pIE1-SV-EGFP containing AcNPV insect promoter, none of the seven *mrjp1*-pIE1-SV-EGFP promoter constructs gave a positive fluorescent signal of EGFP to

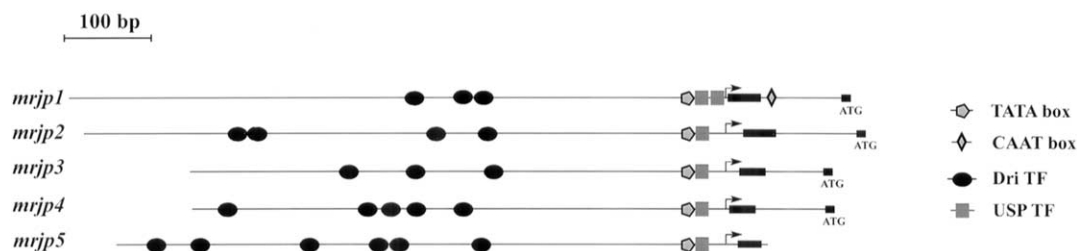


Fig. 3. Scheme of putative transcription factor binding sites mapped on upstream (putative regulatory) sequences of members of the *mrjp* gene family (for GenBank Accession Nos. see Section 2.10). ATG start codons for four of them are shown. The arrow indicates NNPP2.1 predicted transcriptional start point. Black solid boxes represent 5' UTRs transcribed into cDNAs of MRJPs. dead ringer (Dri) and ultraspiracle (Usp) TF binding sites were mapped using MATCH program in the Transfac database.

indicate promoter activity in S2 insect cell line (data not shown).

4. Discussion

The obtained genomic sequence of honeybee *mrjp1* gene differs from the MRJP1 cDNA sequences found in the public database (Ohashi et al., 1997; Schmitzová et al., 1998) in seven nucleotides. None of these seven mismatches changes the derived aa sequence of MRJP1 compared to the aa sequence derived from the MRJP1 cDNA sequences. It is worth mentioning that these MRJP1 cDNA sequences were characterized by two different and geographically distant research groups, and are identical on the nucleotide level. The conclusion from these results would be that the MRJP1 protein is very conservative in its structure and that any mutations in its gene, causing changes in aa sequence, seem to be unfavourable. Contradicting this is the finding of Kucharski et al., who also sequenced a cDNA of MRJP1, that the translated protein sequence differs at aa position 9 (cysteine instead of arginine) (Kucharski et al., 1998).

The closest relative to the honeybee MRJP protein family so far known is yellow protein of *D. melanogaster*. The family of 14 yellow-like proteins was found in the annotated genomic sequence of *D. melanogaster* (Drapeau, 2001). They share 27–30% identities and 46–48% similarities on the protein level with MRJP1 (BLAST, data not shown). We compared the genomic structures of the *mrjp1* gene and the yellow-like gene family. *Drosophila*'s yellow-like genes differ in structure from one another, containing 0–3 introns and spanning from 1207 bp (yellow-g2) to 5595 bp (yellow-e). The genomic organization of *mrjp1* (six exons and five introns and coding region spanning over 3038 bp (Fig. 1A)) differs significantly from the yellow-like genes of *Drosophila*. A search for sequence similarities between *mrjp1* and yellow-like genes on the DNA level using the BLAST program and for possible conserved motifs between these genes with the MEME program (GCG program package) was done. Although yellow protein of *D. melanogaster* and honeybee MRJPs share a common evolutionary origin (Albert et al., 1999a) and similarity described above, no similarity in gene structure, nor similarities in intron sequences between honeybee *mrjp1* gene and any of the *Drosophila* yellow-like gene family were found.

The finding of a potential gene encoded on the reverse DNA strand of *mrjp1* locus, so called antisense transcript, opens a new point of view to the yet unknown mechanism of *mrjp1* expression and its regulation. There are many eukaryotic genes described at the moment, coding for antisense transcripts, originating from the same locus as the sense transcripts (*cis*-encoded). They overlap each other at least partially. Naturally occurring antisense transcripts may regulate the expression of their complementary sense transcripts at any step from transcription to translation. Evidence for nuclear regulation of gene expression usually

comes from antisense transcripts that are non-polyadenylated and consequently not transported to the cytoplasm. Antisense transcripts may therefore regulate sense expression in the nucleus either at the level of transcription, processing, nucleo-cytoplasmic transport or degradation. They are generally believed to down-regulate the sense gene expression. However, up-regulation has also been proposed, by impairing normal degradation of the sense transcript by masking at least part of the 3' UTR (for review see Vanhee-Brossollet and Vaquero, 1998). The mechanism of possible up-regulation of *mrjp1* by antisense transcript can be excluded from further consideration, since the predicted antisense transcript does not span over the 3' UTR region (Figs. 1A,B). Since we have not been able to locate a polyadenylation signal on the predicted antisense transcript, it can be either located outside of the sequenced region or the possible *mrjp1* antisense transcript may be non-polyadenylated and may moderate the level of expression of the *mrjp1* gene within the nucleus. Another presumption for possible *mrjp1* gene regulation comes from Ohashi et al. (1997). They suggest the hypothesis, based on northern and immunoblot analysis of nurse-bee hypopharyngeal glands, that expression of the *mrjp1* gene might be regulated on the translational level. Further experimental work has to be done to confirm or reject the hypothesis of *mrjp1* regulation by its antisense transcript.

Because of the spatially and temporally similar expression pattern of *mrjp1* gene and other four known *mrjp* genes, similarities in regulation and regulatory regions of the *mrjp* gene family are expected to be present. Regulatory regions of eukaryotic genes are mostly located in immediate proximity to the transcriptional start point. We compared the putative regulatory sequences of *mrjp1*, *mrjp2*, *mrjp3*, *mrjp4* and *mrjp5*. Indeed, TF mapping analysis of immediate upstream 5' non-coding sequences of the *mrjp* genes localized putative regulatory elements common for all five *mrjp* genes (Fig. 3). Especially intriguing are the patterns of putative binding sites for Dri and USP TFs found in 5' non-coding regions of *mrjp* genes. Dri is a member of a family of DNA-binding proteins that share an A/T rich interaction domain (ARID). ARID-containing proteins are responsible for both positive and negative transcriptional regulation and are likely to be involved in the modification of chromatin structure. They are also known to play regulatory roles during development (Kortschak et al., 2000). USP is a member of a family of nuclear hormone receptors, a family of ligand-modulated transcription factors that regulate cell differentiation and development as well as homeostasis and reproduction (Sergaves, 1991). It was reported, that two active JH isoforms (JH acid and JH methylester), found in *D. melanogaster*, bind specifically to *Drosophila* USP TF (Jones and Sharp, 1997). This binding induces conformational changes and homo-oligomerization of USP. This may confer different activities to USP influencing transcription. Based on these results it was proposed that JH is a

physiological regulator of USP action (Jones and Sharp, 1997). There was found to be a correlation between JH titer in honeybee hemolymph, HG size and task performance of honeybee worker (Huang et al., 1991, 1994). The USP is a candidate to test for a role as the connecting element to the JH response of HG in honeybee.

The negative result in functional *mrjp1* promoter assay in S2 insect cell line derived from *Drosophila* supports the idea of a highly organ and physiological stage specific regulation mechanism of *mrjp1* expression in HG of honeybee workers. Most probably there is a strong repression of *mrjp1* expression in the absence of activating element(s), occurring naturally in the environment of honeybee HG.

To find out what the molecular mechanisms of *mrjp* genes expression regulation exactly are remains to be determined. Recent approaches to evaluation of differential gene expression during honeybee caste determination (Evans and Wheeler, 2000) and during behavioral development (Kucharski and Maleszka, 2002) by using microarrays showed acceleration in this field as well as requirement of study of these regulational processes. The presented results will be useful for the identification of mechanisms and pathways that translate the hormonal signals into developmental responses. Up to now, there are no stable cell lines derived from honeybee available, which would provide a homologous system for investigation of honeybee gene regulation. The honeybee genome sequence is unknown. Our negative results in promoter-reporter assay showed also the necessity to develop molecular genetic tools for honeybee, which would drive the research in the field of molecular genetics of this social insect forward and would enable us to prove the suggested hypothesis. The prepared seven putative promoter constructs (*mrjp1*- pIE1-SV-EGFP) will be valuable tools for testing of honeybee cell cultures derived from different organs and tissues during development of honeybee and honeybee casts.

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