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Transcriptional profiling reveals multifunctional roles for transferrin in the honeybee, *Apis mellifera*

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Abstract

Transferrins belong to a family of iron-binding proteins that have been implicated in innate immunity and in vitellogenesis in insects. Here we have sequenced and characterized a full-length cDNA encoding a putative iron-binding transferrin (AmTRF) in the honeybee. AmTRF shows high level of sequence identity with transferrins in both vertebrates and insects (26-46%) suggesting that the primary function of the predicted 712 amino acid protein is binding and transporting of iron. AmTRF is expressed ubiquitously, but particularly high levels of its mRNA are found in the central brain and in the compound eye. Using northern blotting and a microarray based approach we have examined the levels of AmTRF mRNA by expression profiling under a wide range of conditions including developmental stages, septic injury and juvenile hormone treatment. Increased expression of AmTRF is seen during early pupal stages, in the brain of mature foragers and in the abdomen of virgin queens, whereas treatment with juvenile hormone leads to a decrease of AmTRF levels in the abdomen. We show that a transcriptional response of transferrin to septic injury with *E. coli* is relatively moderate as compared to a dramatic up-regulation of an antibacterial polypeptide, Hymenoptaecin, under similar conditions. We conclude that major fluctuations of AmTRF mRNA in time and space are consistent with context-dependent functional significance and suggest broader multifunctional roles for transferrin in insects.

Keywords: iron homeostasis, innate immunity, microarray, Hymenoptaecin, vitellogenesis, gene expression, context-dependent protein function

Abbreviation:

AmTRF	*Apis mellifera* transferrin
EST	expressed sequence tag
Cy-dUTP	amino-propargyl-2'-deoxyuridine triphosphate coupled to cyanine fluorescent dye (Cy3 or Cy5).
JH	juvenile hormone

Introduction

Iron is one of the essential elements required by all organisms, but it is also a potent toxin because of its ability to produce free radicals in the presence of oxygen (Crichton *et al*., 2002, Nichol *et al*., 2002). In some tissues, such as brain or retina, where anti-oxidative defences are relatively low and oxygen consumption is very high, iron accumulation in specific regions is associated with a number of neurodegenerative diseases (Crichton *et al*., 2002). Hence, organisms must balance their nutritional requirements with the necessity to control this potential toxic property. In animals, iron-binding and transporting proteins provide an important way to minimise the reactivity of iron towards oxygen in addition to their facilitating role in iron metabolism. One class of iron-binding proteins that have attracted much attention in recent years belongs to a highly conserved family of transferrins. These proteins are well characterised in vertebrates, but in recent years there has been some progress in studies on their relatives in insects (Nichol *et al*., 2002). Despite a high level of sequence conservation of transferrins from different lineages there are significant differences in their biochemical properties as well as in their involvements in cellular functions. In vertebrates, transferrins are glycoproteins of approximately 80 kDa with two ferric-binding lobes, most likely resulting from a duplication of an ancient gene encoding a 40 kDa protein (Jamroz *et al*., 1993, Nichol *et al*., 2002). Most insect transferrins bind only one ferric ion because their C-terminal lobes have no iron-binding capacity. One notable exception is transferrin in *Balberus discoidalis* that has been found to contain two functional iron-binding sites (Jamroz *et al*., 1993).

In addition to binding iron, mammalian transferrin has been shown to act as a growth factor and as a regulator of gene expression at the transcriptional level (Raivich *et al*., 1991, Espinosa-Jeffrey

et al., 2002). A correlation between myelination and transferrin synthesis and secretion in oligodendrocytes has also been demonstrated. In vertebrates, transferrin transports iron in blood, and many cells can access transferrin-bound iron by way of the transferrin receptor pathway. At present it is not known whether a similar pathway exists in insects. The lack of a gene encoding a protein similar to the vertebrate transferrin receptor in *Drosophila* was taken as evidence that insects may use a different transport mechanism or a different receptor (Nichol *et al*., 2002). Clearly, this issue needs to be re-examined as more insect genomes become available for comparative analyses.

Transcriptional profiling is a powerful way to determine not only the functional state of the cell, but also to provide insights into the underlying biology and to identify hitherto unknown genes as well as new functions for previously described genes. As part of our ongoing study on molecular mechanisms underlying complex biological processes in the honeybee, *Apis mellifera*, we have found that a transcript encoding a putative transferrin was differentially expressed under a range of conditions including adult development, physical insults and following drug treatment (Kucharski and Maleszka, 2002). This finding prompted us to examine the spatio-temporal expression pattern of this transcript, designated AmTRF1, in more detail to gain a better understanding of its biological significance in the honeybee. On the basis of recent data Nichol *et al*. (2002) considered the following roles for transferrin in insects: as an iron binding protein, an antibiotic agent, a vitellogenic protein, and a protein repressible by juvenile hormone. Here, we show that the levels of transferrin mRNA in the honeybee are responsive to an even wider range of developmental and physiological conditions suggesting that the functional roles of transferrin are context-dependent and can only be viewed as part of a complex genetic network that is likely to include other related molecules and signalling factors.

Materials and Methods

Sample collection

Foraging honeybee workers were captured near the hive entrance and snap-frozen in liquid nitrogen. To ensure that fully matured workers were harvested, only those that carried pollen or nectar were selected. We estimate their age to vary from 20-35 days. To obtain newly emerged honeybees a single brood frame was removed from the hive and incubated at 32° C (80% humidity). Individual insects were collected within 1-5 min after emergence and snap-frozen in liquid nitrogen. All dissections were done under permanent cooling (dry ice or liquid nitrogen). Pupae of different ages were collected on the basis of eye coloration. We estimate their approximate age to be 10-12 days (white eyes), 13-15 days (pink eyes) and 16-19 days (red eyes) respectively.

Tissue dissection

For all dissections the honeybees were snap-frozen in liquid nitrogen. Preparation of brain tissue was carried out under permanent liquid nitrogen cooling in the following manner. A drop of Tissue Tek O.C.T. Compound (Miles Scientific) was placed on a brass block protruding from a liquid nitrogen bath. While the drop was cooling, a bee head was removed from storage and placed immediately in the drop of Compound dorsal side uppermost. The head was held in position until the Compound had frozen around it. Following removal of the head capsule, the pigmented eyes were sliced away and stored separately. A transverse incision was made through the antenno-glomerular tract and the brain was prised gently out. To obtain individual compartments the brains were placed in a small plastic dish and separated under a dissection microscope into three regions: the mushroom bodies, the antennal lobes and the optic lobes. Dissections were performed on a brass block submerged in LN and brains were never allowed to thaw. Both thoraces and abdomens were separated from frozen bees using a pair of tweezers and a scalpel, whereas antennae and legs, which typically disintegrate from the bodies, where picked up from the bottom of the LN jar.

Juvenile hormone and caffeine treatment

Emerging adult insects were treated with juvenile hormone (JH) (typically within one hour after emergence) by applying 1 µl of JH (1.25% JH-III from Sigma (www.sigmaaldrich.com) in dimethyl formamide) on the thorax, transferred to a small cage containing a tube of honey and incubated at 32° C until desired age. To obtain caffeine-treated bees, a colony of 30 newly born individuals in a small cage was fed for 3 days with honey containing 10 mM caffeine. A similar colony, but fed only with pure honey was used as control.

Light exposure

Newly emerged bees were collected in a dark room illuminated with red light and transferred to a small cage containing a tube of honey (~50 individuals per cage). One cage was left in a dark incubator and the other one was placed between two light boxes and exposed to light for 24-48 hrs.

Septic injury

One microliter of either bacterial or yeast cells (10 x concentrated overnight cultures in sterile bee Ringer) were injected into the thorax of ice-chilled 3-4-day old bees using a 25 µl Hamilton syringe attached to a dispenser. We used *E. coli* strain XL1-Blue from Stratagen (www.stratagene.com) and *S. cerevisiae* strain Dip2 (Skelly and Maleszka, 1991). Aseptic injuries were done by either injecting 1 µl of sterile Ringer or by making a small incision in the thorax with a micro-scalpel. All bees were snap frozen in liquid nitrogen 6 hrs after treatment.

Array preparation

We employed spotted arrays containing either 2,500 or 9,000 cDNAs. The 9000-cDNA arrays were purchased from the University of Illinois at Urbana-Champaign. Their design is described elsewhere (Whitfield *et al.*, 2002). The construction of the 2500-cDNA array as well as labelling and hybridisation were performed according to protocols established by Brown with minor modifications (http://cmgm.stanford.edu/pbrown). We used a standard, unannotated and non-normalised honeybee brain cDNA library made in lambda ZAP that was kindly provided by G. Robinson, Urbana-Champaign. Arrays were prepared by printing 4608 samples, in triplicates, on poly-lysine coated glass slides (Menzel) using a robotic device from Genetic Microsystems (model

418) (http://www.geneticmicro.com). These samples correspond to approximately 2500 unique cDNAs (Kucharski and Maleszka, 2002).

RNA extraction, hybridisation and data analysis

Total RNA was extracted from frozen tissues as described previously (Kucharski *et al*., 2000). Labelled probes were prepared by incorporating Cy3 and Cy5 dUTP (Amersham, www.apbiotech.com) during a reverse transcription of total RNA (SuperScript II, Life Technologies, www.synovislife.com). Hybridisation was conducted for 4-6 hrs at 62° C in a small volume (60-120 µl) of ExpressHyb buffer (Clontech, www.clontech.com) under a plastic coverslip. Following a washing step, slides were scanned with the Affymetrix 428 scanner and analysed using Affymetrix Pathways software v. 1.0 (www.affymetrix.com) and Microsoft Excel spreadsheets.

Bioinformatics

Database searches were performed at the National Center for Biotechnology Information (NCBI) using the BLAST server (www.ncbi.nlm.nih.gov). Additional searches were conducted at the Keck Center for Comparative and Functional Genomics, University of Illinois at Urbana-Champaign (titan.biotec.uiuc.edu/bee/honeybee_project.htm) and at the TAGC University of Marseille (http://tagc.univ-mrs.fr). The presence of the signal peptide and the most likely cleavage site were predicted with SignalP VI.I (Nielsen *et al*., 1997).

Northern blot analysis

Total RNA was isolated using Trizol reagent from Gibco BRL (www.lifetech.com) followed by mRNA purification on Oligo $(dt)_{25}$ magnetic beads from Dynal (www.dynal.no). RNA samples were denatured by mixing with an equal volume of formamide, containing 0.05% bromophenol blue and 0.01% SybrGreen II, incubated at 90° C for 5-7 min and immediately chilled on ice. Electrophoresis was performed in small horizontal tanks (Hoeffer HE33) using 1.5% agarose gels submerged in TBE buffer (50 mM Tris-borate, 1 mM EDTA, pH 8. 2) at 20V/cm. Alternatively, the glyoxal based system (Ambion, www.ambion.com) was used for RNA separation. See the manufacturer's instruction manual (Ambion, catalogue #1946) for details. Following electrophoretic resolution the gels were quantified with the Vistra FluoroImager (www.amershambiosciences.com) and then soaked in 1M ammonium acetate, 0.02 M NaOH and blotted onto Hybond N+ nylon membranes (Amersham, www.amershambiosciences.com) by capillary transfer. RNA was cross-linked to the membrane by UV irradiation and after a brief wash in 2x SSC the blots were pre-hybridised for 5-30 min. Hybridisation was carried out either at 68° C (ExpressHyb solution, Clontech, www.clontech.com), or at 42° C (UltraHyb buffer, Ambion) for 16 hrs using P^{32}-labelled probes (RediPrime kit, Amersham). Blots were washed 3-4 times in 2x SSC, 0.1% SDS at 50° C and exposed to a phosphorstorage screen (Molecular Dynamics, www.moleculardynamics.com) without drying. Computer generated images (Molecular Dynamics, Phosphor-Imager 400S) of individual gels were analysed using ImageQuant (Amersham) software. Optical density measures for a given transcript were normalised against the corresponding optical density for loaded RNA. The expression levels were calculated and shown relatively to the highest hybridisation signal that was set as 100.

Results

AmTRF encodes a highly conserved transferrin

In our previous study we have used a spotted cDNA microarray representing 2,500 transcripts in the honeybee brain to identify genes that are differentially expressed during adult development and as a result of caffeine treatment (Kucharski and Maleszka, 2002). Coupled with northern blot verification this approach revealed thirty-seven genes showing at least 2-fold change under our experimental conditions. One of these genes, represented by bEST92, was found to be up-regulated in the abdomen of mature worker bees. Subsequent sequencing and bioinformatics analyses revealed that bEST92 encodes a conserved transferrin. Here we present the molecular characterization and transcriptional profiles of the honeybee transferrin, designated AmTRF. AmTRF is transcribed as a single message of approximately 2400 base pairs that spans an open reading frame of 2136 nucleotides. This open reading frame encodes a 712 amino acid polypeptide with a predicted molecular weight of 78.6 kDa and a most likely cleavage site between positions 26 and 27 (IAA-QD) indicating a secreted protein (Fig. 1). AmTRF shares the highest sequence identity (45%) with two insect transferrins in the cockroach *B. discoidalis* and in the termite *Mastotermes darwiniensis* (Fig. 1). Like other transferrins in both insects and vertebrates the honeybee protein appears to be a product of intragenic duplication and contains relatively large number (25) of conserved cysteine residues. The level of amino

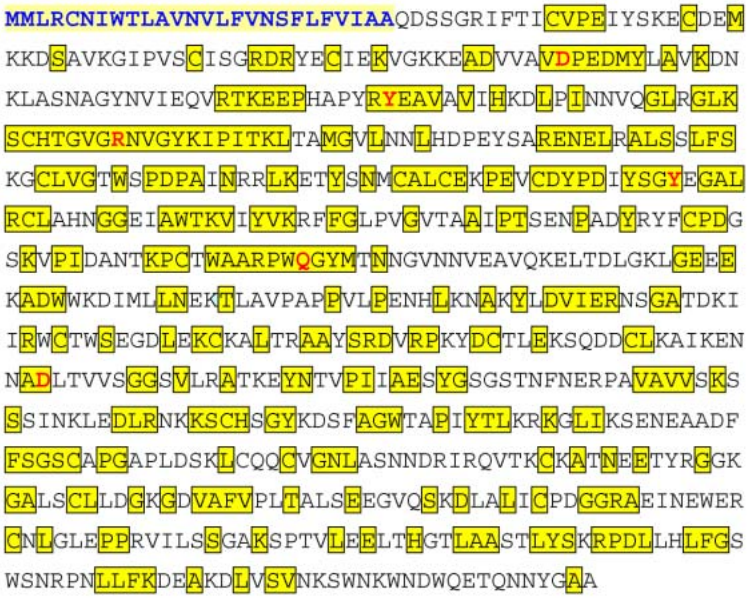

Figure 1. Predicted amino acid sequence of the honeybee transferrin AmTRF. Residues conserved in the honeybee protein (*Apis mellifera)* and in two insect transferrins from the cockroach (*Balberus discoidalis*) and the termite (*Mastotermes darwiniensis*) are boxed and shaded in yellow. The leader peptide is typed in blue and the conserved amino acids implicated in iron binding are typed in red. GenBank accession No. *Am*: AY217097, *Bd*: AAA27820, *Md*: AAN03488.

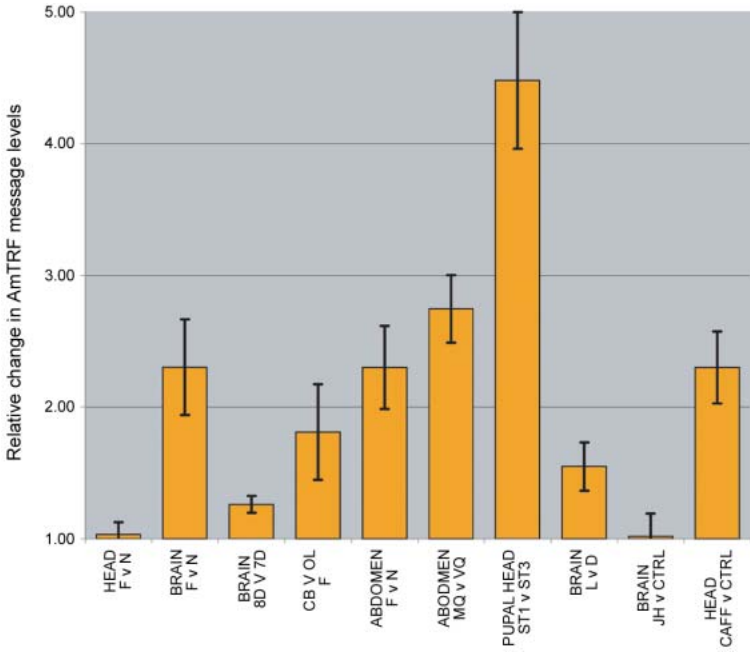

Figure 2. Microarray analysis of AmTRF expression under various conditions. Fold changes in the level of AmTRF message are shown for the following experiments: forager (F) head versus nurse (N) head, F brain v N brain, 8-day old brain v 7-day old brain, central brain (CB) v optic lobes (OL), F abdomen v N abdomen, mature queen (MQ) v virgin queen (VQ), early pupae head (ST1) v late pupae head (ST3), brains of light-exposed bees (L) v dark-kept (D) bees, brains of juvenile hormone treated bees (JH) v controls (CTRL), heads of caffeine and honey-fed bees (CAFF) v honey-fed bees (CTRL). Error bars represent standard deviations from 8 independent samples. Nurses refer to 1-day old bees, foragers to 20-30-days old bees.

acid positional identity between the N-terminal and C-terminal halves in AmTRF is 22%. The residues implicated in iron binding in insects (Jamroz *et al*., 1993, Thompson *et al.,* 2003) are conserved only in the N-terminal half of AmTRF suggesting that its C-terminal lobe has no iron-binding capacity (Fig. 1).

AmTRF is differentially expressed in time and space

Transcriptional responses of AmTRF were investigated by means of both northern blots and microarray hybridizations using RNA populations representing major body parts, brain compartments, developmental stages, caffeine- or juvenile hormone-treated bees, and bees exposed to light. Spotted cDNA microarrays can be used to profile the expression of thousands of gene targets in a single experiment. However, they also can be used to evaluate changes in the expression pattern of a single gene by extracting data points from a series of microarray-based physiological and developmental studies. We used fluorescently labelled total RNAs to interrogate spotted arrays containing either 2,500 or 9,000 cDNAs. These arrays were developed in our lab (Kucharski and Maleszka, 2002) and by Whitfield *et al*. (2002) respectively. Figure 2 shows fold-changes in AmTRF levels measured across a series of experiments.

The most profound difference (4.5 fold decrease) was found between the heads of late and early pupae (Fig. 2, pupal heads ST3 v ST1). This dramatic down-regulation of AmTRF expression in late pupae coincides with cellular reorganization of the insect nervous system during metamorphosis that involves both scrapping and recycling of the larval neurons to make way for new adult neurons (Levine, 1987). In our study, late and early pupal heads represent newly rewired adult brain and larval nervous system respectively. A two-fold increase was detected in the brains of older foragers as compared to brains of newly born individuals (in agreement with the northern blot analysis shown in Figure 3) and in the abdomens of mature worker bees and newly born individuals (Fig. 2, brain F v N, abdomen F v N). A similar increase in the level

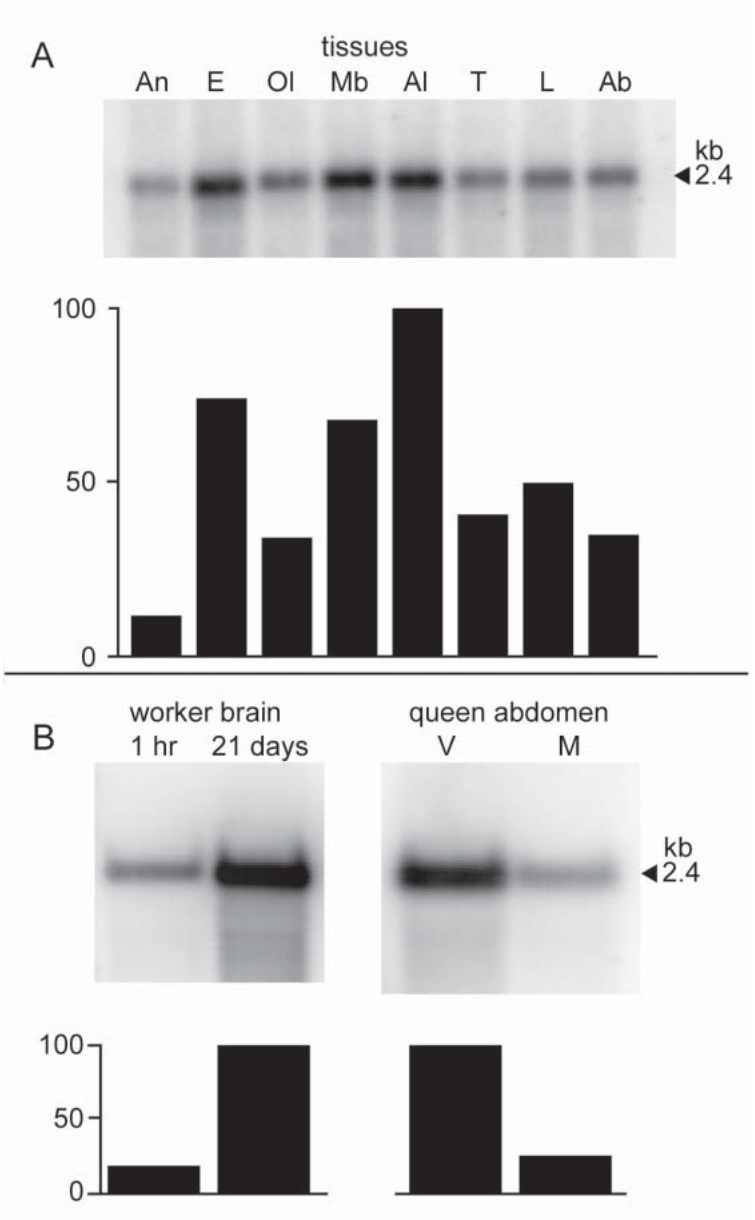

Figure 3. A. Northern blot (top panel) showing the expression of AmTRF in various tissues: antennae (An), compound eyes (E), optic lobes (Ol), mushroom bodies (Mb), antennal lobe (Al), thorax (T), legs (L) and abdomen (Ab). Equal amounts of poly(A)$^+$RNA (~2 µg) were loaded per lane. B. Northern blot showing the expression of AmTRF in brains of newly emerged individuals (1 hr) and experienced foragers (21 days) and in the queen abdomen (V-virgin, M-mature, egg-laying). Lower panels in A and B show the relative expression levels calculated using ImageQuant software as described in materials and methods.

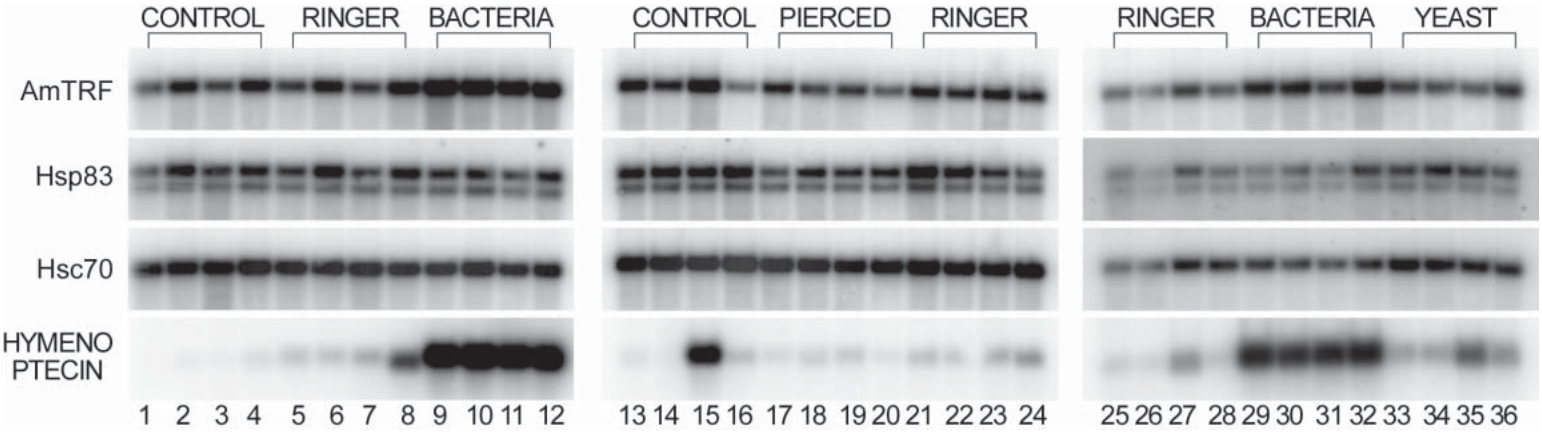

Figure 4. Transcriptional responses of AmTRF and Hymenopteacin to injury. Northern blot analyses were performed on total RNAs extracted from individual heads. Each lane (No. 1- 36) represents total RNA from the same head hybridised with four different probes: AmTRF, Hsp83, Hsc70, and Hymenopteacin. Bees were injected either with Ringer or with a microbial suspension (yeast or *E. coli*), control refers to untreated bees. The second band hybridising with the Hsp83 probe represents either a differentially spliced transcript or a closely related member of this family. Image Quant software was used to evaluate relative levels of each transcript. GenBank Acc. No. AmTRF, AY217097; Hsc70, BI946410; Hsp83, BI946435; Hymenoptaecin, U15956.

of AmTRF is seen in central brain neuropils that include the mushroom bodies as compared to optic lobes (Fig. 2, CB v OP, forager). It is important to note that differential expression of AmTRF in brains of newly emerged bees and mature forages cannot be detected by comparing the levels of AmTRF mRNA in the entire heads. As shown in Figure 2 transferrin expression in the head does not show any change between the young and older bees (Head F v N). This finding emphasises the need for dissecting well-defined tissues or localised cellular compartments as a critical step in transcriptional evaluation of certain messages. AmTRF is up-regulated in the abdomen of a mature forager and in the abdomen of a virgin queen. A more than two-fold increase is also seen in heads of caffeine-fed bees. A moderate increase (1.5x) is detectable in brains of young bees exposed for 24 hrs to light. No differences are seen in brains of juvenile hormone-treated and untreated individuals and in brains of 7- and 8-day old caged bees. The 7-day and 8-day old individuals represent two distinct developmental stages of adult maturation that can be distinguished by both behavioral and pharmacological approaches (Guez *et al*., 2001, 2003).

To examine in more detail the spatio-temporal pattern of AmTRF expression in the honeybee and to verify our microarray-based results we performed a series of northern blot analyses using various tissues and developmental stages. Although in most cases the differential expression uncovered by microarrays is reproducible in northern blots (Taniguchi *et al*., 2001, Kucharski and Maleszka, 2002), it is worthwhile to complement the microarray-based screens with northern analyses of individual genes to either confirm quantitative differences, or to rule out the existence of multiple transcripts from the same gene. AmTRF is expressed at relatively high levels in all examined tissues including the legs and antennae (Fig. 3, panel A). However, the levels of the corresponding mRNA are quite variable with the highest expression found in the brain (mushroom bodies, optic lobes and antennal lobes) and in the compound eye (Fig. 3, panel A). This figure also illustrates the profound developmental differences in the level of AmTRF message in the brains of newly emerged bees and experienced foragers and in the abdomens of virgin and mature queens (Fig. 3, panel B). The northern blot detected differences are somewhat greater than those seen on microarrays, ~5-fold increase in the mature brain and a 4-fold increase in the abdomen of a virgin queen.

AmTRF is up-regulated upon bacterial, but not yeast infection

To determine if AmTRF is involved in antimicrobial defence we examined its transcriptional responses following injections with either bacterial (*E. coli*) or yeast cultures (*S. cerevisiae*). Injections of bee Ringer as well as piercing with an empty needle were employed as "aseptic" treatments. Untreated bees were included as controls for baseline expression. For comparison, we contrasted the transcriptional profiles of AmTRF with those of Hymenoptaecin, a peptide implicated in bacterial defence in honeybees (Casteels *et al*., 1993). In addition, we measured the levels of mRNAs encoding two members of the heat shock protein family, Hsc70 and Hsp83. Like transferrin, these genes have been identified in our microarray screen as differentially expressed during development (Kucharski and Maleszka, 2002). Heat shock proteins perform multiple functions such as signal transduction and developmental regulation and also are excellent indicators of stress responses (Pirkkala *et al*., 2001). We used RNAs extracted from single insects to ensure that inter-individual differences were detected. Figure 4 shows hybridisation patterns of these four genes in 36 individuals. As expected, the levels of Hymenoptaecin mRNA, which are either very low or undetectable in control bees, were dramatically induced (>100 fold) in those individuals that were injected with *E.coli*, but not in those injected with yeast (bees No. 9-12 and 29-32). This finding confirms that the role of Hymenoptaecin in the honeybee is antibacterial defence. By comparison, the transcriptional response of AmTRF to bacterial infection appears to be relatively restrained with approximately 45-50% increase in the level of AmTRF mRNA following injections with *E. coli*. Because the increased expression of AmTRF is seen only in those samples that also have high levels of Hymenoptaecin

we interpret this effect as infection-induced. No significant up-regulation of the AmTRF message was detectable in yeast-injected bees. It is noteworthy that the increased levels of both transcripts AmTRF and Hymenoptaecin are observed in some untreated individuals, for example in bee No. 15, suggesting a prior infection with bacteria in the hive. Further, a moderate increase in the level of Hymenoptaecin observed in bees numbered 8, 27 (Ringer injected) and 35 and 36 (yeast injected) may be interpreted as a secondary bacterial infection associated with the experimental procedure.

The expression patterns of both Hsc70 and Hsp83 indicate some variability in the tested group of bees (Fig. 4). However, we found no clear correlation of this variability with the expression level of Hymenoptaecin and thus with microbial infection (Fig. 4). On the other hand, some degree of co-regulation between the expression of AmTRF and both heat shock genes is evident. For example, in individuals numbered 2, 13 and 27, an increased level of AmTRF is accompanied by higher levels of both heat shock genes (Fig. 4). It is reasonable to assume that many steps involved in this experimental procedure, such as handling and cooling, are stressful to the insects and may lead to the activation of molecular machinery responsible for stress recognition. Genes encoding heat shock proteins are expected to be involved in early stages of stress responses and as our results suggest, transferrin may also be part of this process.

AmTRF expression is responsive to juvenile hormone

To explore further possible functional roles of AmTRF in the honeybee we have examined the effects of juvenile hormone (JH) on the expression of this gene in female worker bees. Suppression of transferrin in the fat body by JH has been reported in a cockroach (Jamroz *et al*., 1993) and in a bean bug, *Riptortus clavatus* (Hirai *et al*., 2000). Given the importance of JH in insect development and reproduction and the proposed involvement of transferrin in vitellogenesis (Nichol *et al.*, 2002) we were curious to examine the effects of JH-treatment on transferrin expression in worker honeybees that are essentially sterile and in normal colonies do not lay eggs. In contrast to some other insects, juvenile hormone titers increase in maturing worker bees leading to suppression of their vitellogenin (yolk protein) synthesis. We measured the levels of AmTRF mRNA in both heads and abdomens of 1-day old workers treated with JH shortly after emergence. A decreased expression of AmTRF (~50%) was found in the abdomens of treated bees as compared to control individuals (Fig. 5). By contrast, the levels of AmTRF mRNA were not significantly different in the heads of control and JH-treated bees (Fig. 5). Also, as mentioned before, no JH-induced change in AmTRF expression was detected in the brain by using the microarray-based profiling (Fig. 2).

Because JH has an effect on maturation of the adult honeybee (Maleszka and Helliwell, 2001, and references therein) the observed alteration in AmTRF expression in the abdomen could result from JH-accelerated developmental processes rather than from the specific transcriptional control. Hence, we also examined the expression pattern of a gene called SPARC that encodes a protein belonging to the cell adhesion family. This gene is developmentally regulated in the honeybee and the level of the corresponding message decreases with age (Kucharski and Maleszka, 2000). As shown in Figure 5 the expression of SPARC in both heads and abdomens is not significantly affected by JH treatment suggesting that the observed JH-induced down-regulation of AmTRF reflects a specific hormonal modulation of the level of this transcript.

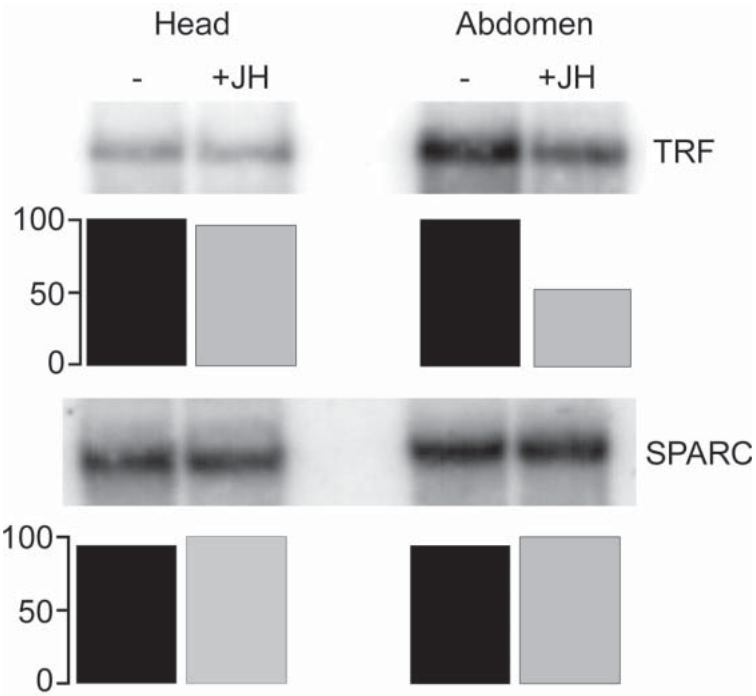

Figure 5. Northern blot analysis of AmTRF expression in heads and abdomens of juvenile hormone-treated honeybees. Newly emerged individuals were treated with JH as described in materials and methods and snap frozen 24 hrs later. For comparison, the expression patterns of SPARC, a member of the cell adhesion molecules family (Kucharski and Maleszka, 2002) is also shown. The relative intensity of hybridisation signals was calculated using ImageQuant software as described in materials and methods.

Discussion

We have characterized a putative iron-binding transferrin in the honeybee. The predicted protein is highly conserved and shares 45% identical residues with two transferrins in the cockroach *B. discoidalis* and in the termite *M. darwiniensis*. It is noteworthy, however, that the cockroach and the termite transferrins are more closely related to each other (76% identity) then to AmTRF raising the possibility that the honeybee genome may encode another related protein that is a true ortholog of these two transferrins. This important question awaits the publication of the honeybee genome which is in progress.

The highest level of AmTRF message is found in the central brain neuropils and in the pigmented eye. By analogy to mammals, these tissues are likely to have low anti-oxidative defences (Crichton *et al*., 2002). Thus, transferrin may play a role in the honeybee central nervous system as a component of a protection mechanism against reactive oxygen intermediates. It has been shown that the vertebrate retina consumes more oxygen per unit weight than any other tissues and the consequent generation of these intermediates is thought to

underlie several retinal diseases in mammals. Light is particularly important in ocular injury mediated by reactive oxygen intermediates (Lu *et al*., 2002). In this context it is noteworthy that the exposure of newly emerged bees to constant light for 24 hrs leads to a 50% up-regulation of AmTRF message (Fig. 2). The high levels of AmTFR in the brain and in the compound eye of mature adults could also be related to a potential role of transferrin bound iron in flight and directional sensing.

A major drop in the level of AmTRF is observed in pupae during the transition from the larval to adult nervous system. The destruction of larval neurons is followed by massive biosynthesis that creates adult nervous system. Our study reveals a dynamic expression of AmTRF during this complex process that has been shown to consist of postembryonic neurogenesis, programmed cell death and modifications of persistent neurons (Levine, 1987). One possibility is that lower levels of transferrin make more iron molecules available for reactions that are promoted by transition-metal ions. Iron is required for generating di-oxygen species (O_2) that are critical for many biological processes such as biosynthesis of DNA, serotonin, fatty acids and other bio-molecules (Kovacs, 2003). Iron is also an essential cofactor of a number of key enzymes needed in energy metabolism. Interestingly, the levels of AmTRF message increase quite dramatically in the adult brain suggesting that transferrin may play contrasting roles in the larval insect nervous system and in the adult brain.

In addition to being part of anti-oxidative defences transferrin may act as a growth factor that can regulate other proteins even at the transcriptional level. In mammals, transferrin is a ubiquitous growth factor that plays a critical role in cellular iron uptake, growth and proliferation (Raivich *et al*., 1991). For example, rat transferrin selectively regulates MBP (myelin basic protein) at the transcriptional level and together with IGF-1 (insulin-like growth factor) increases both the maturation and myelinogenic properties of oligodendrocytes (Espinosa-Jeffrey *et al*., 2002). A link between transferrin and the expression of neuronal cytoskeletal proteins has also been demonstrated in the mouse (Marta *et al*., 2002).

Although the extent to which the properties of mammalian transferrin are relevant to insects is not clear at present, both the high levels of AmTRF message in the adult brain and major fluctuations during developmental stages suggest that transferrin in the bee is an important protein with multiple functions in the nervous system. The changes in AmTRF expression under some conditions, such as light exposure, are more subtle than during development. However, the biological implications of such minor changes for specific processes may be as profound as more dramatic changes under different conditions. For finely balanced processes even a small alteration in gene expression may cause major perturbations.

Insect transferrin has been considered an infection-inducible gene (Yoshiga *et al*., 1997, Thompson *et al*., 2003) and has been implicated in innate immunity to fight microbial infections by sequestering iron. We report here that AmTRF is up-regulated following an injection with *E.coli*, but not with yeast. Yet, the level of AmTRF induction is relatively low comparing to that of Hymenoptaecin, which is highly inducible under the same conditions. Hymenoptaecin inhibits viability of both Gram-negative and Gram-positive bacteria, including several human pathogens (Casteels *et al*., 1993). This may suggest that transferrin may only be loosely linked to a pathway involved in defence against Gram-negative bacteria. Recent studies in *Drosophila* have shown that innate immunity involves distinct signalling pathways that control a subset of targets activated by microbial agents (Boutros *et al*., 2002). Transferrin in *Drosophila* is primarily dependent on *Toll* pathway signalling that is induced during infection with Gram-positive bacteria. A close link between the control of tissue repair and antimicrobial processes has also been found (Boutros *et al*., 2002). Thus, it is not unexpected that transferrin is up-regulated in bees injected with sterile Ringer or pierced with an empty needle. The fact that high levels of Hymenoptaecin have been found in some control (untreated) individuals suggests that these bees had been naturally infected in the hive.

In accord with two previous reports (Jamroz et al., 1993, Hirai et al., 2000) we show that treatment with juvenile hormone results in suppression of transferrin expression in the honeybee. This effect is best seen in the abdomen of worker bees and may illustrate the fact that during adult maturation increased titres of JH correlate with suppression of vitellogenin in worker bees that in normal colonies are sterile and do not lay eggs. Interestingly, AmTRF levels are high in the virgin queen and decrease quite dramatically in the mature egg-laying queen. One possibility is that in a mature queen iron is associated with other proteins, such as ferritins, or alternatively, its transport is no longer required. However, further experiments with antibodies against AmTRF are important to determine if there is a temporal correlation between the levels of the AmTRF message and the encoded protein.

Our results clearly show that transcriptional regulation plays a crucial role in modulating the levels of AmTRF under diverse physiological and developmental conditions. AmTRF is ubiquitously expressed suggesting that it may play a more general role in diverse pathways, and that additional mechanisms may further modulate its activity. A general conclusion that emerges from the above analyses is that function makes no sense in the absence of context; iron-binding capacity of transferrin has quite different phenotypic implications in the eye or brain as compared to immune responses or developmental processes. The biological significance of a particular protein will depend on when and where its message is expressed. It has been reported that in higher Metazoa, each gene or protein is estimated on average to interact with four to eight other genes (Arnone *et al*., 1997), and to be involved in 10 biological functions (Miklos and Rubin, 1996). Such multi-functional roles of proteins are increasingly being found to be a rule rather than an exception (Miklos and Maleszka, 2001). A good example is lactate dehydrogenase that can act both as a dehydrogenase and an eye lens structural protein, depending on its context (Bork, 2000). The functional significance of transferrin can only be viewed as part of a highly interconnected cellular signalling network that includes many other components, in particular related molecules such as ferritins and other metalo-transferases, but also receptors, signalling factors and mitochondrial proteins.

As the trend accelerates from single gene analyses to global changes in gene expression patterns we are likely to uncover novel pathways in which transferrin in the honeybee and other insects plays a role. In particular, the relationship between transferrin(s) and iron regulatory proteins that permit iron-responsive translational control of protein synthesis via binding to iron responsive elements

found in the 3'-regions of mRNAs deserves more attention.

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