Specific developmental gene silencing in the honey bee using a homeobox motif

M. Beye*, S. Härtel*, A. Hagen†, M. Hasselmann* and S. W. Omholt†

**Martin-Luther-Universität Halle/Wittenberg, Biozentrum, Institut für Zoologie, Molekulare Ökologie, Halle, Germany;* †*Agricultural University of Norway, Department of Animal Science, AAS, Norway*

Abstract

Manipulating the expression of genes in species that are not currently used as genetic models will provide comparative insights into the evolution of gene functions. However the experimental tools in doing so are limited in species that have not served as models for genetic studies. We have examined the effects of double stranded RNA (dsRNA) in the honey bee, an insect with considerably basic scientific interest. dsRNA derived from a 300 bp stretch of the E30 homeobox motif was injected into honey bee embryos at the anterior pole in the preblastoderm stage. We found that the dsRNA fragment successfully disrupted the protein expression of the target gene throughout the whole embryo. The disruption caused deficient phenotypes similar to known loss of function mutants of *Drosophila engrailed*, whereas embryos injected with nonsense dsRNA showed no abnormalities. We show that the large size of the honey bee egg (D: 0.3 mm, L: 1.6 mm) and the long preblastoderm stage (11–12 h) can be exploited to generate embryos with partial disruption of gene function, which may provide an elegant alternative to classical chimeric analyses. This is the first report of targeted disruption of gene function in the honey bee, and the results prove that the chosen target gene is a functional ortholog to *engrailed* in *Drosophila*.

Keywords: *Apis mellifera*, honey bee, gene silencing, RNA of interference, *engrailed*, development.

Received 22 March 2002; accepted after revision 8 July 2002. Correspondence: Martin Beye, Martin-Luther-Universität Halle/Wittenberg, Biozentrum, Institut für Zoologie, Molekulare Ökologie, Weinberg Weg 22, 06120 Halle, Germany. Tel.: (+ 49)(345)55 21627; fax: (+ 49)(345) 55 27230; e-mail: beye@zoologie.uni-halle.de

Introduction

Understanding the evolutionary dynamics of genes and their functions beyond the 'world' of genetic model organisms will rely on the ability to functionally analyse the genes of interest. The honey bee has not served as a model for genetic studies, but is of considerable basic scientific interest for understanding gene functions in the genetic basis of its complex learning abilities (Menzel & Giurfa, 2001; Fiala *et al.*, 1999), the complex social behaviour (Hunt *et al.*, 1995; Hunt *et al.*, 1998), caste determination (Evans & Wheeler, 2000; Toma *et al.*, 2000) and the haplodiploid mode of sex determination (Beye *et al.*, 1999).

Most genes have been so far isolated by homology cloning in the honey bee. Although these genes are conserved on the nucleotide level, it is not clear whether they also have conserved functions in the honey bee system. Function of genes has been mainly inferred from expression data and *in situ* hybridization experiments, but no direct evidence for the function of the genes is available. Recently it was shown (Fiala *et al.* 1999) that cAMP-dependent protein kinase (PKA) can be down-regulated by the oligonucleotide antisense technique causing changes in long-term memory abilities of bees. The increasing sequence data now going public (e.g. more than 15 000 ESTs (expressed sequence tags) are known so far for the honey bee) and the unique features of the honey bee system necessitates the development of functional genomic tools.

Double stranded RNA interference (RNAi) has been tested so far in a number of organisms and its silencing effect has also been proven, with varying degrees of success, in organisms such as *Tribolium* (Brown *et al.*, 1999), the protozoan *Trypanosoma brucei* (LaCount *et al.*, 2000), hydra (Lohmann *et al.*, 1999), cockroach (*Periplaneta americana*) (Marie *et al.*, 2000), spider *Cupiennius salei* (Schoppmeier & Damen, 2001) and the bug *Oncopeltus fasciatus* (Hughes & Kaufman, 2000). Some reports indicate that RNAi has non-specific effects. In the zebrafish it was found that injection of dsRNA into embryos (Oates *et al.*, 2000; Zhao *et al.*, 2001) resulted in disruption of non-targeted genes. Caution has to be taken with the honey bee system because eggs are large (1.6 mm long) compared to other organisms studied so far and the large size may restrict diffusion of dsRNA throughout the embryo.

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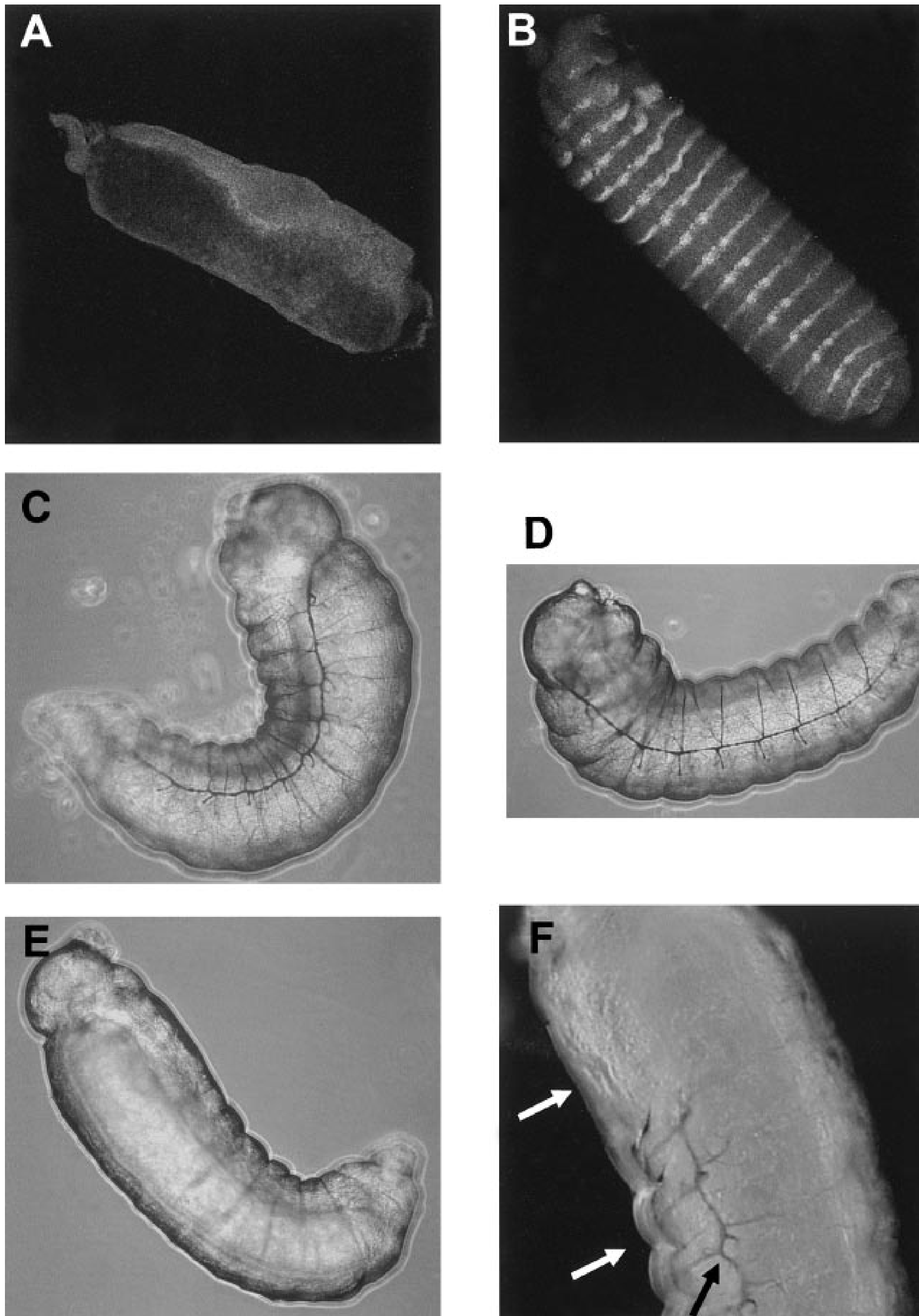

Figure 1. Embryonic *engrailed* antibody staining (58 h old: A, B) and larval phenotypes shortly after hatching (72 h old: C, D, E, F) with and without dsRNA *ben* injection. (A) In *ben* injected 58-h-old embryo no specific stripes were detected although embryonic development before 33 h was normal. Light gray staining of embryos represents unspecific labelling. (B) Uninjected embryos of same age display specific stripes labelling the metameric grooves known to be specific for *engrailed* protein expression. (C) Uninjected hatched larvae showing the full wild-type phenotype. (D) Injected hatched larvae with nonsense dsRNA. The hatched larvae showed a full wild-type phenotype suggesting that injection of dsRNA alone has no effect. (E) Deficient larval phenotype of *ben* RNAi. No tracheal system was detected and metameric units appeared to be fused (see text). (F) *ben* injection resulted in about 12% of all injected embryos showing chimeric development – part deficient and part wild phenotype (see Figure 2). These embryos showed partly segmentation defects (upper white arrow) and partly wild-type phenotype (white arrow below) that correlated with the presence and absence of tracheae (black arrow). The deficient phenotype corresponded to the anterior injection site.

To test the effects of RNAi in honey bee embryos, dsRNA derived from a 300 bp stretch of the E30 homeobox motif showing high similarity to the developmental gene *engrailed* (*en*) of *Drosophila* was injected into honey bee embryos (Walldorf *et al.*, 1989). We refer to this honey bee homologue as *ben.* To document a gene specific silencing effect, assays for *ben* at the level of protein expression and phenotype were performed. We found that the dsRNA fragment successfully disrupted the protein expression of *ben*, resulting in phenotypes similar to known loss of function mutants of *Drosophila engrailed.* This is the first example of specific loss of function genetics in the honey bee.

Results

Injection of dsRNA derived from ben into honey bee embryos indicate high efficiency and specificity of gene silencing

Injection of dsRNA *ben* into preblastoderm honey bee embryos resulted in depleted expression of *engrailed* protein (Fig. 1A). The protein expression was assayed using an antibody specific to the *Drosophila engrailed* protein that adequately detected the protein expression pattern of *engrailed* in control embryos (Fleig, 1990) of the same age as the experimental ones (Fig. 1B). All cells showed loss of expression of *engrailed* protein as compared to the untreated embryos (Fig. 1A,B). This documents that a small amount of dsRNA injected close to the anterior pole at the preblastoderm stage is sufficient to mediate targeted disruption of gene function in the relatively large honey bee embryo (1.6 mm).

Ben deficient embryos show major defects in embryonic development

Old control and injected embryos were examined 33 h, 57 h and 72 h (± 2 h) under phase contrast microscope for morphological and anatomical differences. Experimental and control embryos were put on the same slide for comparison. No differences were detected between untreated and injected embryos (buffer, nonsense, *ben*) at 33 h. Untreated embryos at 72 h inspected by low power microscope showed tracheal branches, all mouth parts and a clear segmentation (Fig. 1C). Embryos injected with nonsense dsRNA or buffer solution only (*n* = 200 and *n* = 73, respectively; Fig. 2) at 72 h showed no differences compared to the untreated 'wild-type' phenotype (nonsense type, Fig. 1D).

Abnormal development was first visible after 57 h in *ben* injected embryos. At the age of 72 h major changes were visible. Over one hundred embryos showing the *ben* deficient phenotype were examined. Mouth parts were poorly formed, tracheal trunks were fragmented and were totally missing in most cases. Metameric units appeared to be fused but the extent of fusion varied between individuals. In

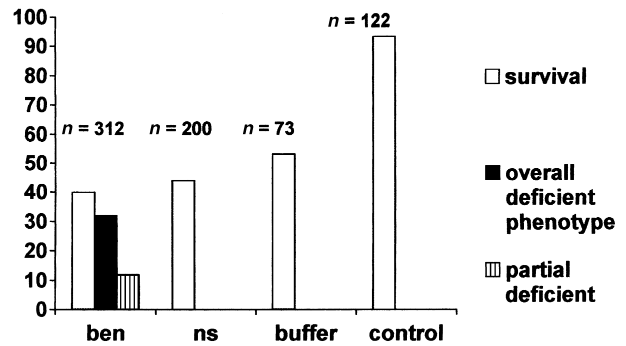

Figure 2. Double-stranded RNA interference (RNAi) in honey bee embryos. Untreated embryos served as control and showed that embryos survived at a rate of 93% with no injection. Embryos (100%) were injected with dsRNA *ben* (*n* = 312), dsRNA nonsense (ns) (*n* = 200) and buffer only (*n* = 73). Injection dramatically reduced survival rate of embryos to 40–53% showing that injection alone had a dramatic effect on survival. Thirty-two per cent of the surviving embryos that received *ben* injection showed deficient phenotypes. Thirty-two per cent of the 40% surviving *ben* injected embryos showed a deficient phenotype resulting in a relative penetrance of 80%. Thirty-seven per cent of those that showed the deficient phenotype were chimeric (see Figure 1F).

some individuals several pairs were partly fused, while in others all bands were totally fused (Fig. 1E). These results demonstrate that *ben* is involved in the basic segmental organization of the insect. In general, embryonic development proceeded until late stages and was arrested in the late germ band stage. Many of these embryos still hatched, but they were developmentally too disrupted to proceed with post embryonic development.

Interestingly, in some individuals only part of the body showed a deficient phenotype, as detected by partial depletion of the tracheal system and partial metameric fusions (Fig. 1F). The upper part of the embryo including the head (site of injection) shows strong segmentation defects with loss of the tracheal system (upper white arrow). The lower part of the embryo (white arrow below) shows the wild-type with normally developed tracheae (black arrow).

This may be caused by incomplete diffusion of the dsRNA from the anterior pole before initiation of the cellular blastoderm stage at 12 h.

Survival rate and penetrance of dsRNA

Embryos were lost just by, for example, handling as demonstrated by the 93% survival rate of uninjected control embryos (control in Fig. 2). Survival rates of 40, 44 and 53% for *ben*, nonsense and buffer, respectively, indicate that the injection by itself has a strong detrimental effect (Fig. 2). The penetrance of RNAi in the surviving *ben* injected embryos was 80% (Fig. 2), demonstrating the strong effect of dsRNA. Thirty-seven per cent of those that showed the deficient phenotype were chimeric (see Fig. 1F).

Discussion

The specific *engrailed* depleted protein expression pattern and the deficient phenotypes demonstrate that the RNAi technique had a specific silencing effect in honey bees. There was a strong functional relationship between the observed 'wild-type' antibody *engrailed* staining pattern and the deficient *ben* phenotype. The antibody raised against Drosophila *engrailed* protein was found to label the honey bee embryonic grooves demarcating metameric units (Fleig, 1990) and to mark parts of embryonic mouth parts, all parts that show a deficient phenotype in the RNAi experiments. The deficient *ben* phenotype resembles the loss of function phenotypes described for *engrailed* (*en*) in *Drosophila* (Gustavson *et al.*, 1996; Kornberg, 2001), e.g. the fusion of metameric units and the poorly formed mouth parts. However, one interesting distinction observed by low power microscopy is the total lack of a tracheal system in the honey bee embryos (Fig. 1C), while in *Drosophila* tracheal trunks develop at least to some extent, generating a fragmented or disrupted tracheal pattern in the embryo (Kornberg, 2001). The data suggest that a 300 bp long dsRNA sequence of a rather common homeobox motif is capable of producing gene-specific deficient embryos in the bee and that *ben* is a functional ortholog to *Drosophila engrailed*.

The relatively large size of the honey bee egg does not limit the targeted disruption of gene function. However, our data show that phenotypic variation occurred between deficient embryos (Fig. 1E). So far we do not understand the nature of partially deficient phenotypes. One explanation is that dsRNA hasn't spread throughout the whole embryo before the cellular blastoderm stage. To operate with an injection window of 1–4 h after egg deposition will be advantageous in future disruption studies. The possibility of making partial knockouts might in some situations become a convenient alternative to the experimentally demanding technique of chimerical analysis for disclosing gene functions.

If the observed specificity is typical for RNAi in honey bees, and considering that our experimental set up allows injection of hundreds of eggs per day throughout the year, the technique might become a very powerful tool for functional genomic research in bees. The need for such a tool has recently become accentuated by the isolation of several behavioural genes (Maleszka, 2000) and the large amount of EST sequence data (http://keck1.biotec.uiuc.edu/bee/honeybee_project.htm) becoming publicly available. The need for this tool is likely to become even greater with the recently announced objective to obtain the full-genome sequence of the honey bee (http://www.nhgri.nih.gov/NEWS/sequencing.html).

Injection of dsRNA into preblastoderm *Drosophila* embryos shows very low penetrance of genes expressed in adult stages (Fire *et al.*, 1998; Kennerdell & Carthew, 1998) (R. W. Carthew, personal communication) emphasizing the potential limitations of the RNAi technique in targeting genes expressed in adult honey bees. Recent results, however, indicate that using expression systems with head to head promoters (LaCount *et al.*, 2000) or inverted repeats (Martinek & Young, 2000) generates a permanent or inducible expression of dsRNA in the cell *in vivo*. A conditional RNAi system for adult honey bees might be established by integrating this strategy with the widely applicable baculovirus system (Oppenheimer *et al.*, 1999). Traditional germ line transformation systems soon may become available in the honey bee (Robinson *et al.*, 2000). However, a somatic approach of gene silencing using RNAi as presented here will help to overcome some basic limitations of the honey bee system, that is the demanding rearing and maintenance conditions of queens and transgenic lines. The queen is the only reproductive individual and has to be maintained in a single colony with thousands of worker bees. In experimental set-ups in which somatic gene silencing approaches are adequate, the large number of eggs that can be obtained from a single queen (up to 1500 eggs per day) will greatly enhance the functional analysis of genes by means of RNAi.

Experimental procedures

Preparation of dsRNA

Primers were designed from sequences of clone E30 (GenBank accession number M29490) showing high similarity to the homeobox containing gene *engrailed* of *Drosophila*. E30 primer sequences were fused with the T7 promoter sequence (underlined) as follows: TAATACGACTCACTATAGGGCGAGCGGACGAGGCG-GGTGAAGC, TAATACGACTCACTATAGGGCGACCTCCCCGTC-CTCGTCAACG. PCR reactions were performed according to standard procedures using cDNA from embryos as template. The resulting fragments were directly sequenced and proved to be 317 bp long. PCR product was purified using the Quiaquick™ PCR purification kit.

Nonsense DNA template was generated from the Q marker (Hunt & Page, 1994) that include no exon sequence. Q marker primer sequences were fused with T7 promoter (underlined) sequence as follows: TAATACGACTCACTATAGGGCGAAGT-GCAGCCAGCTACTGAGAG, TAATACGACTCACTATAGGGCGA-AGTGCAGCCACGTGCCTGAAT. Total DNA from bees was used as template in a standard PCR reaction. RNA was prepared using the Promega RiboMax™ T7 system (Promega, Germany). Sense and antisense strands were transcribed from DNA template in same reaction. RNA was phenol-chloroform extracted and isopropanol precipitated. RNA was resuspended in injection buffer (Fiala *et al.*, 1999) and injected at a concentration of about 4–5 μg/μl.

Provision of eggs and injection of dsRNA

The honey bee colonies providing eggs were kept in a flight room. The eggs were sampled from hives that allow frequent collection of eggs of a defined age with only slight disturbance of the worker bees or the queen (Omholt *et al.*, 1995a,b). The injections of dsRNA were performed with an Oxford micromanipulator (Singer Instruments Co., UK), a microinjector (PLI-100, Medical Systems

Corp., Greenvale, NY), and an ordinary stereomicroscope. The injection pipettes were made from borosilicate capillary tubes (o.d. 1 mm, i.d.: 0.58 mm) (Sutter Instrument Company, Novato, CA), which were pulled by a Brown micropipette puller (Sutter Instrument Company, Novato, CA). The pulled micropipettes were cut with an in-house micropipette cutter. The tips were bevelled to an angle of 15° with a K.T. Brown Type Micropipette Beveller (Sutter Instrument Company, Novato, CA) such that the inner diameter of the pipette tips was 4–6 μm. The injection time was 0.12 s, the injection pressure was 70 kPa, and the balance pressure was 5 kPa. The average amount injected into each embryo was estimated to be 300 pl. Embryos were incubated at 35 °C and 80% RH until inspection.

Phenotype analysis and documentation

Injected eggs were put on tape on object slides covered with a thin layer of paraffin oil 15 h after injection. They were examined under low power microscopy phase contrast 33 h, 57 h, 72 h after egg deposition. Main developmental and apoptotic processes can be seen through the transparent chorion of the honey bee egg.

Immunostaining

Approximately thirty embryos of a control group and of a *ben* injected group were immunostained to look for differences in the *engrailed* protein staining. Only embryos that looked viable (see phenoytpe analysis and documentation) were dechorinated by 45 s exposure to 3.7% $NaOCl_3$ solution, fixed for 1 h in a heptane solution saturated with formaldehyde (obtained from the heptane phase of a stock solution of 1 heptane : 1 formaldehyde) and devitellinized by use of a pair of forceps. The embryos were stored in PBTA solution (1 × PBS, 1% BSA, 0.05% Triton X-100, 0.02% sodium azide). For antibody staining embryos were washed three times in PBST (1 × PBS, 1% BSA, 0.05% Triton X-100) for 1 h at room temperature on a rotator. The primary *engrailed*-antibody (DiNardo *et al.*, 1985) was added to the embryos (6 μg/ml) and incubated overnight at 4 °C. After three washes in PBST (10 min) the embryos were incubated for an additional 3 h with fluorescence labelled secondary antibody (FITC anti-mouse IgG H+L, Immuno Research) in PBST. Secondary antibody was removed by three washes with PBST for 10 min. Embryos were embedded in mounting medium (Cytiflour) and images were taken on a confocal laser scanning microscope (Zeiss).

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