Identification and characterization of *Mlr1,2*: two mouse homologues of *Mblk-1*, a transcription factor from the honeybee brain[1]

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Abstract **We previously identified the *Mblk-1* gene in the honeybee brain, which encodes a transcription factor containing two DNA binding motifs, termed RHF1 and 2 (Takeuchi et al. (2001) Insect Mol. Biol. 121, 134–140). Here, we identified two mouse *Mblk1* homologues, *Mlr1* and *Mlr2*. Both encode proteins containing a single DNA-binding motif highly conserved with RHF2 and activate transcription mediated by a DNA element recognized by honeybee Mblk-1. *Mlr1* was expressed predominantly in the spermatocytes of the testis, while *Mlr2* was expressed in various tissues other than testis. *Mlr1* transcripts were lost in the testis of *W/W*^{v} mutant mice, suggesting a role in spermatogenesis.**

Key words: Honeybee; Brain; Transcription factor; Spermatocytes; Spermatogenesis; E93

1. Introduction

Transcription factors are key proteins that regulate various biologic processes. The *Mblk-1* gene was originally identified as a gene that is expressed preferentially in the mushroom bodies of the honeybee brain [1]. Mushroom bodies are important regions for learning, memory, and sensory integration in the insect brain [2,3]. Expression of genes involved in neural plasticity is enhanced in the honeybee mushroom bodies [4–6]. Mblk-1 contains several motifs characteristic of transcription factors, including two helix–turn–helix DNA binding motifs termed RHF1 and RHF2, a nuclear localization signal, and glutamine-run motifs [1]. Thus, Mblk-1 is thought to be involved in brain function by regulating transcription of its target genes. Recent in vitro binding site selection and amplification experiments revealed that Mblk-1 binds to a 22-bp double-stranded DNA element 5′-CCCTATCGATCGATCTCTACCT-3′ termed MBE (Mblk-1 binding element). MBE contains a palindromic structure and Mblk-1 protein affords homophilic interaction, suggesting that Mblk-1 functions as a dimer [7].

Mblk-1 has the highest sequence similarity with *Drosophila* CG18389/E93, which is involved in ecdysone-triggered programmed cell death of larval tissues during metamorphosis [8,9], especially in the RHF1 and RHF2 regions [1]. A search of the genome and Expressed Sequence Tag databases revealed that the RHF2 motif is highly conserved among various animals and there are at least two kinds of Mblk-1 homologues in mammals. Thus, isolation and characterization of the Mblk-1 homologues in mice are essential for further study of the gene functions.

Here, we report the identification of both Mblk-1 homologues, termed *Mlr1* (*Mblk1-related protein-1*) and *Mlr2*. Each gene has a distinct expression pattern. *Mlr1* is expressed predominantly in the testis, restricted to the spermatocytes. Furthermore, both gene products recognize the same DNA element, MBE, as honeybee Mblk-1 and are capable of activating transcription.

2. Materials and methods

2.1. Isolation of cDNAs encoding mouse Mblk-1 homologues

Two mouse sequences were retrieved from the mouse genomic trace database by a similarity-search with the honeybee RHF2 amino acid sequence using TBLASTN. Two primer sets for mouse RHF2 were designed from the corresponding genomic traces and two types of cDNA fragments encoding mouse RHF2 were amplified from the mouse brain cDNA library (Stratagene, La Jolla, CA, USA) and used as probes for the following screening. Two cDNA clones were isolated from the oligo(dT)-primed mouse brain cDNA library by plaque hybridization. To isolate cDNA clones corresponding to the N-terminal regions, repeated 5′-rapid amplification of the cDNA end-polymerase chain reaction (PCR) was performed using a Marathon cDNA Amplification kit (Clontech, Palo Alto, CA, USA).

2.2. Northern blot analysis

A multiple tissue Northern blot containing approximately 2 μg of poly(A)+RNA per lane from eight different mouse tissues was purchased from Clontech. cDNA fragments corresponding to the unique region of *Mlr1* and *Mlr2* (+767 to +1276 and +739 to +1218 respectively) were amplified by reverse-transcription PCR (RT-PCR) from mouse brain total RNA and subcloned into the pGEM-T vector (Promega, Madison, WI, USA). ^{32}P-Labeled riboprobes were synthesized using a StripEZ RNA kit (Ambion, TX, USA). All hybridizations and washes were performed as recommended by the manufacturer. After detection, hybridized probes were removed according to the manufacturer's instructions and the same membrane was subjected to a new probe. Human β-actin cDNA (Clontech) was used as the loading control.

2.3. In situ hybridization

In situ hybridization was performed essentially as described previously [10]. Cryosections (10 μm thick) from unfixed frozen testis were

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[1] Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank databases under the accession numbers, AB076078 and AB076079.
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Abbreviations: RT-PCR, reverse transcription-polymerase chain reaction

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collected on silane-coated slides. The sections were fixed in 4% paraformaldehyde in phosphate buffered saline and then hybridized with digoxigenin (DIG)-labeled sense or antisense riboprobes. The DIG-labeled riboprobes were prepared by in vitro transcription using the same constructs used for Northern blot analysis with a DIG RNA labeling mix (Roche, Switzerland). After post-hybridization washes, immunocytochemical detection of DIG-labeled RNA was performed with alkaline phosphatase-conjugated anti-DIG antibody and NBT/BCIP using a DIG nucleic acid detection kit (Roche) according to the manufacturer's instructions.

2.4. RT-PCR analysis of Mlr1

Total cellular RNA was extracted from mouse testis of either ddY or WBB6F1-*W*/*W*^{v} (9 weeks old) mice using TRIzol LS reagent (Gibco BRL). Extracted RNA was treated with RNase-free DNaseI (Gibco BRL) and reverse-transcribed using SuperScriptII (Gibco BRL) with random hexamers. Primers for *Mlr1* were designed from an *Mlr1* unique region, +767 to +796 for sense and +1247 to +1276 for antisense. Primers derived from mouse cytoskeletal actin cDNA (+247 to +276 for sense and +782 to +811 for antisense) were used as a control.

2.5. Luciferase assay

The coding regions of *Mlr1* and *Mlr2* were subcloned into the *Bam*HI and *Spe*I sites of the *actin* 5C expression vector (pPac-PL [11]). A luciferase reporter vector, pGL3-PH-Luc, was constructed by ligating the P-element core promoter and *hsp70* leader into *Bgl*II–*Hind*III of the pGL3-basic vector (Promega). Six tandem copies of MBE or UAS_G (Gal4 upstream activating sequence) [12] were subcloned into the *Sac*I and *Sma*I sites of pGL3-PH-Luc. Transfection experiments were performed essentially as described previously [13,14]. Schneider's Line 2 cells were transfected with a mixture of plasmid DNA (0.5μg) using Cellfectin reagent (Gibco BRL). After 42–44 h, the cells were collected, lysed in the reporter lysis buffer (Promega), and luciferase activity was measured with a luminometer (Lumat LB 9507; BERTHOLD) immediately after addition of the substrate luciferin (Promega). β-Galactosidase activity was measured using *o*-nitrophenyl-β-D-galactopyranoside as substrate, and the values were used to normalize the transfection efficiency. The mixture of plasmid DNA (0.5 μg) consisted of the luciferase reporter vector (50 ng), an *actin* 5C-β-galactosidase reporter vector (50 ng), and 0.4 μg of *Mlr1*, *Mlr2* expression vector or empty pPac-PL.

3. Results

3.1. Isolation of cDNAs encoding mouse Mblk-1 homologues

Searching the database of mouse genomic traces with the RHF2 sequence of honeybee Mblk-1 revealed two kinds of partial genomic sequences. Using these sequences as probes, we screened a mouse brain cDNA library and identified two cDNA clones. The first one was 4981 bp long and encoded a protein, termed Mlr1, consisting of 517 amino acid residues. The other was 2760 bp long and encoded a protein, termed Mlr2, consisting of 433 amino acid residues. In-frame termination codons were located upstream of the putative first methionines in both cDNAs, indicating that these clones contained entire protein coding regions. Both proteins contained a single DNA-binding motif conserved with RHF2 (amino acid positions 428–482 and 336–390, respectively), but not with RHF1 (Fig. 1A). Sequence identity between Mlr1 and Mlr2 was 85% in the RHF2 region, and the overall identity was 27% (Fig. 1B).

RHF2 was thought to form a helix–turn–helix structure, which affords binding to DNA [1,15]. Strict sequence conservation was observed among the mammalian and insect homologues at the third helix, which directly interacts with DNA (Fig. 1B). Furthermore, a novel motif conserved between Mlr1 and Mlr2 was identified (Fig. 1A,C). This motif was not conserved in insect homologues or other genes in the database and was thus called the MM (Mammalian Mlr) motif. A database search revealed that mouse Mlr2 had 98% sequence identity with human KIAA1795 protein, indicating that KIAA1795 is a human orthologue of Mlr2 (Fig. 1A). KIAA1795 was identified in a sequencing project of human cDNAs that encode large proteins [16] and its function remains unknown.

3.2. Distribution of Mlr1,2 transcripts in mouse tissues

The expressions of *Mlr1* and *Mlr2* in various mouse tissues were examined by Northern blot analysis. *Mlr1* was expressed predominantly in the testis and less abundantly in the kidney, liver, and heart; *Mlr2* was expressed in various tissues including the kidney, liver, and heart, but not in the testis (Fig. 2). The expression levels of both genes in the brain are quite low. Thus, in some tissues like heart, liver, and kidney, both *Mlr1* and *Mlr2* were expressed and might function cooperatively or complementarily, while in the testis, only *Mlr1* was expressed strongly, suggesting it has a major role in the testis.

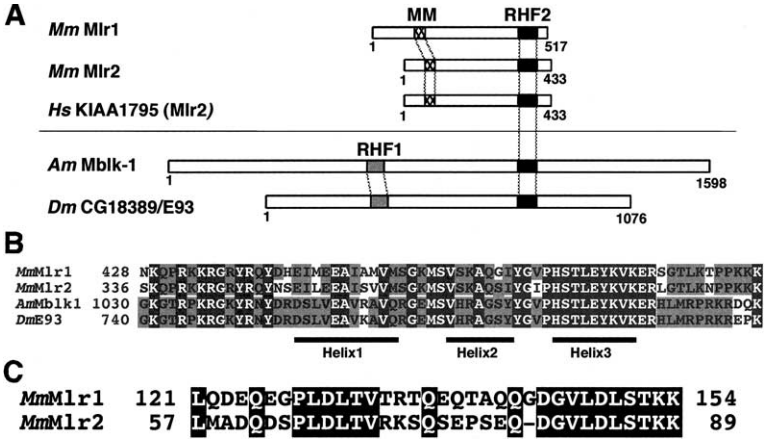

Fig. 1. A: Schematic structure of Mlr1 and Mlr2 from *Mus musculus* (*Mm*) and homologues from other species; *Homo sapiens* (*Hs*), *Apis mellifera* (*Am*), and *Drosophila melanogaster* (*Dm*). Conserved motifs, such as MM, RHF2, and RHF1, are represented by hatched, closed, and shaded boxes, respectively. The numbers indicate the positions of amino acid residues. B,C: Comparison of amino acid sequences of the RHF2 motif (B) or the newly identified MM motif (C). The positions of three helices in RHF2 are indicated by the horizontal bars in B. Amino acids conserved in all or some of the species are shown as inverted or shaded, respectively.

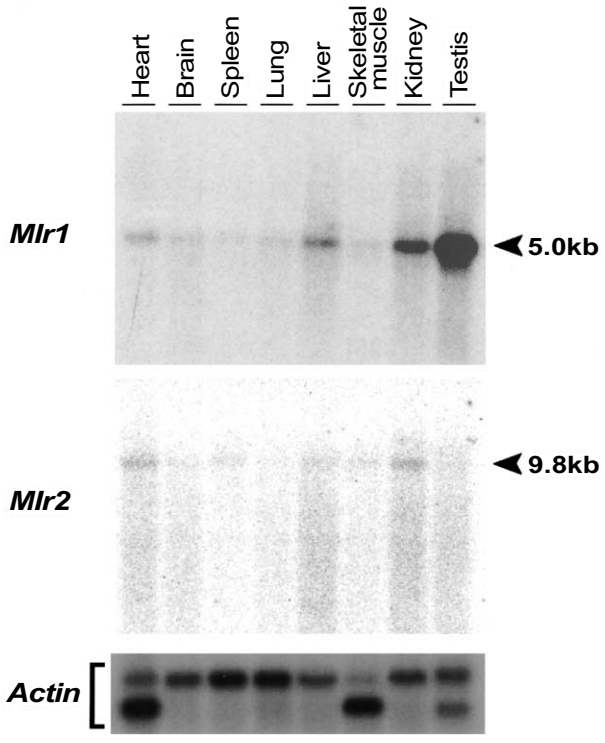

Fig. 2. Northern blot analysis of *Mlr1* and *Mlr2* expression. Poly-(A)+RNA (2 μg) from different mouse tissues probed with +767 to +1276 of *Mlr1* (upper panel) and +739 to +1218 of *Mlr2* cDNA fragments (middle panel) corresponding to the unique region. As a control, the expression of *actin* was examined by probing the same membrane with human β-*actin* gene (lower panel).

3.3. *In situ hybridization analysis of Mlr1 in mouse testis*

Next, in situ hybridization was performed using *Mlr1* cDNA probes and cryosections of adult mouse testis to identify the cell types that express *Mlr1*. In almost all seminiferous tubules, the *Mlr1* probe hybridized to specific stages of germ cells (Fig. 3A,B). Spermatogenesis in the seminiferous tubules can be subdivided into three different phases: spermatogonia (the proliferative phase), spermatocytes (meiotic phase), and spermatids (spermiogenic phase). High *Mlr1* expression levels were observed in large round cells in the seminiferous tubules, which were thought to be spermatocytes based on their morphology (Fig. 3C). No significant expression was detected in the other cells, including spermatogonia and spermatids.

3.4. *Analysis of Mlr1 mRNA in W/W^v mutant mouse testis*

The testis is composed of germ cells as well as somatic cells, including Sertoli and Leydig cells [17,18]. The *W/W^v* mutant mice lack the *c-kit* gene, which encodes a receptor tyrosine kinase required for the differentiation of primordial germ cells [19–21]. Thus, the testis of these mutant mice lack germ cells, but still contain other somatic cells. To confirm that *Mlr1* expression was restricted to the spermatocytes, we performed RT-PCR using testis RNA from *W/W^v* mutant mice with specific primers to *Mlr1*. No signal was detected with the mutant mice RNA compared to the wild type RNA (Fig. 4). In contrast, *actin* mRNA was detected in both mutant and wild-type RNAs. Taken together with the results of in situ hybridization, we conclude that *Mlr1* expression is restricted to the spermatocytes in the testis.

3.5. *Both Mlr1 and Mlr2 recognize the same DNA element as honeybee Mblk-1 and activate transcription*

The RHF2 of both *Mlr1* and *Mlr2* has strict sequence conservation with that of honeybee Mblk-1, especially in its third helix region. Therefore, we hypothesized that both these proteins recognize the same DNA element as the honeybee Mblk-1 and are capable of activating transcription. To test this, we performed a luciferase assay. Six tandem copies of MBE were inserted into the upstream region of the luciferase gene with a core promoter. As a control, 6X UAS_G was used in place of 6XMBE. Both Mlr1 and Mlr2 activated transcription of the luciferase gene driven by the 6X MBE (Fig. 5). In contrast, when 6X UAS_G was used, there was no activation by either Mlr1 or Mlr2. These results indicate that both Mlr1 and Mlr2 recognize the same DNA element as honeybee Mblk-1 and are capable of activating transcription mediated by the MBE.

4. Discussion

In the present study, we identified two novel mouse transcription factors, Mlr1 and Mlr2 that are related to honeybee

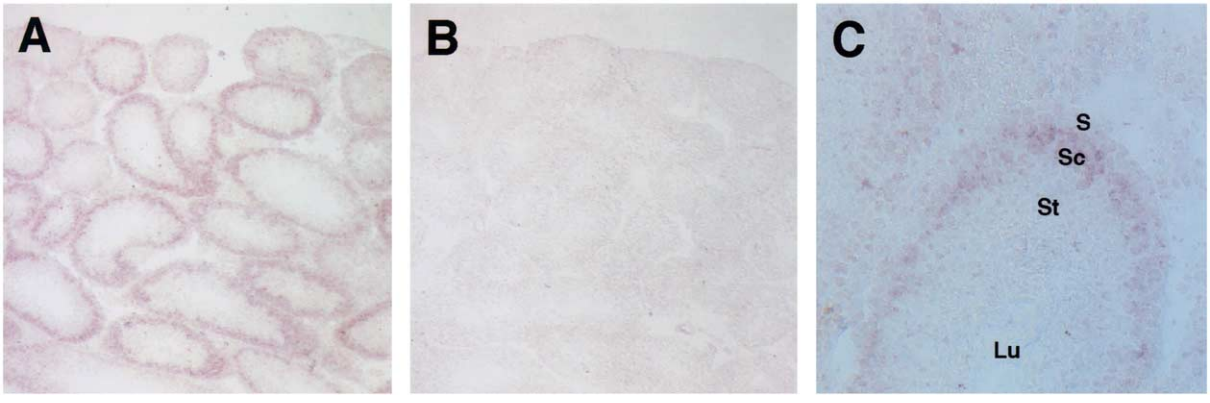

Fig. 3. Localized expression of *Mlr1* mRNA in the testis of adult mouse. Cryosections (10 μm thick) of mouse testis were prepared and treated as described in Section 2. In situ hybridization was performed with DIG-labeled antisense or sense *Mlr1* riboprobes. A: *Mlr1* transcripts were detected in the seminiferous tubule at a specific stage with the antisense probe. In contrast, no hybridization signals were detected in sense probe controls as shown in (B). C: Magnified fields with the antisense probe are shown. The highest signals were detected in spermatocytes. Lu, lumen; Sc, spermatocytes; St, spermatids; S, spermatogonia/Sertoli cells.

Mblk-1. Although both RHF1 and RHF2 are conserved between honeybee Mblk1 and fruit fly E93, mammalian Mlr1 and Mlr2 contain RHF2, but not RHF1. Instead, they have a newly identified MM motif that is specific to mammalian Mlr genes, whose biologic significance remains unknown (Fig. 1).

Mlr1 and *Mlr2* have distinct expression patterns in adult mouse tissues (Fig. 2). Both *Mlr1* and *Mlr2* were expressed in some tissues including the heart, liver, and kidney. It is possible that they function cooperatively or complementarily. As the RHF2 region of honeybee Mblk-1 affords homophilic interaction [7], Mlr1 and Mlr2 might also interact with each other to form a hetero- or homodimer and function cooperatively in those tissues of mice. In contrast, only *Mlr1* is expressed in the testis and its expression is restricted to the spermatocytes where meiosis occurs (Figs. 2–4). These results suggest that Mlr1 is involved in spermatogenesis at the meiotic stage by activating transcription of spermatocyte-specific genes [10,22–24]. The expression of both *Mlr1* and *Mlr2* in the brain is quite low (Fig. 2) and is present in various regions without strict localization (data not shown), suggesting multiple functions of Mblk-1/E93, Mlr-1, and Mlr-2 in insects and mammals.

In contrast to the unique expression patterns of mouse *Mlr1* and *Mlr2* and honeybee *Mblk-1*, the luciferase assay indicated that both Mlr1 and Mlr2 recognize the same DNA element, MBE, as honeybee Mblk-1 (Fig. 5). These results are consistent with the strict sequence conservation of the RHF2 DNA binding motif, especially in the third helix, which interacts directly with DNA [25]. Our results also indicate that both Mlr1 and Mlr2 can activate transcription. Their unique structural characteristics suggest that they are a novel class of mammalian transcription factors. To achieve multiple roles with the same DNA binding activity, they might interact with other proteins to change or specialize their functions or target genes. The MM motif might be a candidate region that is involved in such protein–protein interactions.

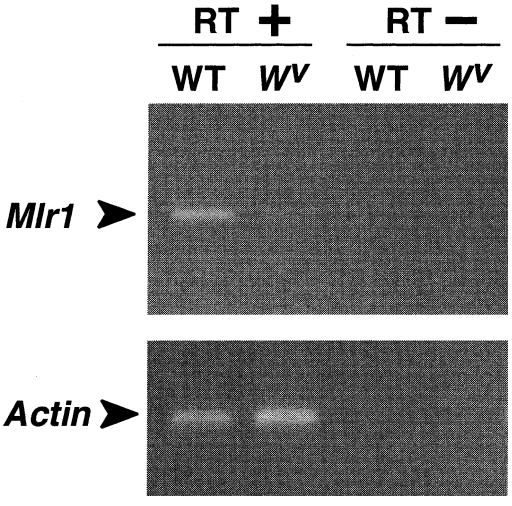

Fig. 4. RT-PCR analysis of *Mlr1* expression in normal and W/W^v mutant mice testis. Total RNA was extracted from the testis of wild-type ddY mice (WT) and germ cell-lacking W/W^v mutant mice (W^v). PCR was performed with the primers specific to *Mlr1* (+767 to +796 for sense, +1247 to +1276 for antisense) and mouse cytoskeletal β-*actin* (+247–267 for sense and +792 to +813 for antisense). RT− indicates the control without RT.

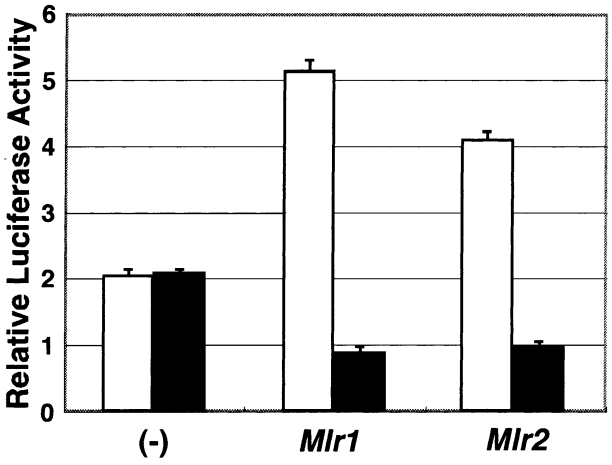

Fig. 5. Both Mlr1 and Mlr2 transactivate a reporter gene driven by MBEs. Expression constructs that contained no insert (−), *Mlr1* or *Mlr2* were co-transfected into cultured cells with a luciferase reporter gene driven by six tandem copies of either MBE (open bars) or control, UAS_G (closed bars) in conjunction with the core promoter. Means of relative luciferase activity from six independent experiments after normalization for activity of a co-transfected internal control, β-galactosidase, are shown. Lines on top of the bars represent standard errors.

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