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DNA-Binding Properties of Mblk-1, a Putative Transcription Factor from the Honeybee

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We previously identified a gene, *Mblk-1*, that encodes a putative transcription factor with two DNA-binding motifs expressed preferentially in the honeybee brain [H. Takeuchi *et al., Insect Mol. Biol.* 10, 487–494 (2001)]. In the present study, we identified its preferred binding sequence as 5′-CCCTATCGATCGATCTCTACCT-3′ and characterized its DNA-binding properties using truncated Mblk-1 mutants. An electrophoretic mobility shift assay revealed that the full-length Mblk-1 binds to the sequence with high affinity, whereas each truncated DNA-binding motif of Mblk-1 binds with much lower affinities. An *in vitro* pull-down assay indicated that each DNA-binding motif affords homodimeric bindings, suggesting that Mblk-1 functions as a dimer.

Key Words: **honeybee; brain; mushroom body; Mblk-1; transcription factor; DNA binding; E93.**

The honeybee *Apis mellifera* L. is a social insect, and various exquisite communications are performed by colony members to maintain colony activity. For example, workers provide other workers with information related to both the distance and the direction of a food source using dance language (1, 2). Very little is known, however, about the molecular basis of their highly advanced behavior.

Mushroom bodies (MBs) are important for learning, memory, and sensory integration in the insect brain (3, 4). The honeybee MBs are characteristically well developed compared with those of other insects. In the honeybee, each MB contains two calyces composed of two morphologically distinct intrinsic neurons, termed large- and small-type Kenyon cells (5–7). On the other hand, in *Drosophila melanogaster* there is only one calyx and the neurons are morphologically indistinct (8).

In the search for genes expressed preferentially in the MBs using the differential display method, we recently identified a gene, termed *Mblk-1*, that is expressed preferentially in the large-type Kenyon cells of the honeybee MBs, and demonstrated that it encodes a novel protein composed of 1598 amino acid residues (9). A data base search revealed that the highest sequence similarity was between this protein and the *D. melanogaster* CG18389/*E93* nuclear protein, which is required for ecdysone-triggered programmed cell death of larval tissues (10, 11), suggesting that E93 is a *Drosophila* homologue of Mblk-1 (9). The overall sequence similarity is 22%; however, two putative DNA-binding motifs, termed RHF 1 and 2, have higher sequence similarities (44% and 98%, respectively). Both proteins share a nuclear localization signal and Gln-runs (9). These findings suggest that Mblk-1 functions as a transcription factor in the MB neuronal circuits and has an important role in sensory integration and memory of the honeybee. Furthermore, RHF 2 has significant sequence similarities with proteins encoded by genes from various animal species, suggesting that the function of the protein is conserved among the animal kingdom (9). The characteristics of the *Mblk-1* protein, however, have not been analyzed.

To clarify the role of *Mblk-1* as a transcription factor, we performed the binding site selection method and demonstrated that Mblk-1 binds preferentially to a sequence containing a palindromic motif. An *in vitro* pull-down assay suggested that Mblk-1 functions as a dimer.

MATERIALS AND METHODS

Expression and purification of GST-Mblk-1 fusion proteins. The full-length *Mblk-1* cDNA was obtained by ligating six *Mblk-1* subclones isolated previously, and subcloning them into pGEX4T-3 (Pharmacia) with a His6 tag at the C-terminus. For the truncated

Abbreviations used: MB, mushroom body; BSS, binding site selection; EMSA, electrophoretic mobility shift assay; MBE, Mblk-1 binding element; UAS_G, Gal4 upstream activating sequence; MCS, multiple cloning site; GST, glutathione *S*-transferase; SDS, sodium dodecyl sulfate.

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Mblk-1 mutants containing RHF 1 or 2 [Mblk-1(384-808) or Mblk-1 (776-1207)], corresponding cDNA regions (+1400 to +2671 and +2576 to +3871, respectively) were amplified by PCR and subcloned into pET-22b (Novagen), whose multiple cloning site (MCS) (*Nde*I-*Xho*I) had been replaced by His6-MCS-glutathione *S*-transferase (GST). Each GST-fusion protein was produced in *Escherichia coli* BL21 (DE3) and purified using glutathione–Sepharose 4B (Pharmacia).

Binding site selection (BSS) method. The BSS method was performed essentially as described previously (12). Briefly, random oligonucleotides of 5′-CGGCTGAGATCAGTCTAGATCT$(N)_{20}$GGATCCGAGACTGAGCGTCGTC-3′ (N is any nucleotide) were converted into double-stranded (ds) DNA by *Taq* polymerase. Approximately 200 ng of partially purified GST-Mblk-1 fusion protein was spotted onto 1 cm^2 of nitrocellulose membrane (Schuleicher & Schuell) and blocked with binding solution (25 mM Hepes-NaOH, pH 7.9, containing 40 mM KCl, 3 mM $MgCl_2$, and 1 mM dithiothreitol) containing 0.5% skim milk. The membrane was then incubated with 200 μl of binding solution containing 10 pmol of the ds-oligonucleotide mixture for 2 h at 4°C. After the membranes were washed three times with binding solution containing 0.25% skim milk, the bound ds-oligonucleotides were dissociated by incubating with 200 μl of 1 M KCl for 10 min at 4°C and amplified by PCR. The PCR mixture consisted of 10 μl of the dissociated ds-oligonucleotide solution, 10 μl of 10×PCR buffer (100 mM Tris-HCl, pH 8.3 containing 15 mM $MgCl_2$), 8 μl of 2.5 mM dNTPs, 100 pmol of each forward and reverse primer, and 5 U of *Taq* polymerase in a final volume of 100 μl. After 20 cycles of 95°C for 50 s, 64°C for 50 s, and 72°C for 1 min, 5 μl of the amplified ds-oligonucleotide solution was used directly for the next round of filter binding selection. After six rounds of selection, the resulting ds-oligonucleotides were digested with *Xba*I and *Bam*HI, subcloned into pBluescript SK, and transformed into *E. coli* DH5α. A total of 46 clones, including inserts, were randomly selected and sequenced.

Electrophoretic mobility shift assay (EMSA). The reaction mixture (20 μl) consisted of 10 mM Hepes-NaOH, pH 7.6, containing 10% glycerol, 50 mM KCl, 0.1 mM EDTA, 20 fmol ^{32}P end-labeled MBE (Mblk-1-binding element) oligonucleotide, 12 ng partially purified GST-Mblk-1 fusion proteins, 300 ng purified GST-Mblk-1 (384-808), or GST-Mblk-1 (7765-1207), 0 to 50 ng poly(dI-dC), and 0 to 1 pmol unlabeled MBE, UAS_G (Gal4 upstream activating sequence) (13) or mutant MBE oligonucleotides (M1–M6) as competitors. The reaction mixture was kept on ice for 30 min and run on a 5% nondenaturing polyacrylamide gel. The gel was then fixed, dried, and subjected to autoradiography. Radioactivity was measured by scanning the autoradiogram with a Bioimaging analyzer (Fuji BAS-2500). The apparent dissociation constant (K_{eq}) values were determined according to the equation K_{eq} = [free protein][free DNA]/[protein-DNA complex] (14).

In vitro pull-down assay. ^{35}S-Met-labeled GST-Mblk-1(384-808) or GST-Mblk-1(776-1207) were prepared using TNT wheat germ *in vitro* transcription-translation system (Promega). The lysates containing *in vitro* translation products were mixed with 10 μl of glutathione beads bound with GST, GST-Mblk-1(384-808), or GST-Mblk-1(776-1207) in 200 μl of 10 mM PBS (phosphate buffered saline; phosphate buffer, pH 7.4, containing 100 mM NaCl, 5 mM KCl) and incubated at 4°C for 12 h. After the beads were washed five times with 1 ml PBS containing 0.1% Triton X-100, the bound material was dissolved in sodium dodecyl sulfate (SDS) sample buffer (150 mM Tris-HCl buffer, pH 6.8, containing 1.2% SDS, 30% glycerol, and 15% 2-mercaptoethanol) and then subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography.

RESULTS

Identification of the Binding Sequence for Mblk-1 by the BSS Method

To identify the binding sequence of Mblk-1, the BSS method was employed using a partially purified GST-full length-Mblk-1 fusion protein. When the ds-oligonucleotides, recovered after six rounds of selection, were subcloned and sequenced, 19 of 46 independent clones had identical sequences, 5′-CCCTATCGATC-GATCTCTACCT-3′, in arbitrary positions and two other clones had sequences that differed from the above clones by only one base (Fig. 1). These 21 clones contained a palindromic motif, ATCGATCGAT, indicating that Mblk-1 binds preferentially to this motif. Although the remaining 25 clones had sequences with

1	TCTAGATCT	CCCTATCGATCGATCTCTGCCT	GGATCC
2	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
3	TCTAGATCT	CCCTATCGATCGATCTCTGCCT	GGATCC
4	TCTAGATCT	CCTGCGGTGCGGGCCGGCTT	GGATCC
5	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
6	TCTAGATCT	CCTGCGGTGCGGGCCGGCTT	GGATCC
7	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
8	TCTAGATCT	CCAACTTGGCGTATACA	GGATCC
9	TCTAGATCT	ATANACGCAGGGGCCAAACG	GGATCC
10	TCTAGATCT	ATCAGGGGCATGTTTGTTA	GGATCC
11	TCTAGATCT	CGGGGGGGAGGCCGGCTACG	GGATCC
12	TCTAGATCT	CCTGCGGTGCGGGCCGGCTT	GGATCC
13	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
14	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
15	TCTAGATCT	CTTTCACAAATCTGAACCCC	GGATCC
16	TCTAGATCT	AGGCTGGTTCTAGTTTTCG	GGATCC
17	TCTAGATCT	GGGGTCGGTGTTCTTCATAA	GGATCC
18	TCTAGATCT	CCCGCAGCTCACGGTCGCAT	GGATCC
19	TCTAGATCT	CTGCTGCGAGTCAGAGCCCTAG	GGATCC
20	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
21	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
22	TCTAGATCT	CTGCTGCGAGTCAGAGCCCTAG	GGATCC
23	TCTAGATCT	CTTTCACAAATCTGAACCCC	GGATCC
24	TCTAGATCT	CCCGCAGCTCACGGTCGCAT	GGATCC
25	TCTAGATCT	CTGCTGCGAGTCAGAGCCCTAG	GGATCC
26	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
27	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
28	TCTAGATCT	CCAACTTGGCGTATACA	GGATCC
29	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
30	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
31	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
32	TCTAGATCT	TCGGCGTGCTTTGTGCTCT	GGATCC
33	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
34	TCTAGATCT	AGGCTGGTTCTAGTTTTGG	GGATCC
35	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
36	TCTAGATCT	CCCGCAGCTCACGGTCGCAT	GGATCC
37	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
38	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
39	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
40	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
41	TCTAGATCT	CAGCCCGACGACGCTCAGTCTC	GGATCC
42	TCTAGATCT	CAGCCCGACGACGCTCAGTCTC	GGATCC
43	TCTAGATCT	CCCTATCGATCGATCTCTACCT	GGATCC
44	TCTAGATCT	TTAGTTCTCCGTGGTCTGTT	GGATCC
45	TCTAGATCT	ACCCAGAAGTGATGGTTGGCA	GGATCC
46	TCTAGATCT	GCTGCGAGTCAGAGCCTAG	GGATCC

A	0	0	0	0	21	0	0	0	21	0	0	0	21	0	0	0	0	0	19	0	0	0
T	0	0	0	21	0	21	0	0	0	21	0	0	0	21	0	21	0	21	0	0	0	21
C	21	21	21	0	0	0	21	0	0	0	21	0	0	0	0	0	21	0	0	21	21	0
G	0	0	0	0	0	0	0	21	0	0	0	21	0	0	21	0	0	0	2	0	0	0
	C	C	C	T	A	T	C	G	A	T	C	G	A	T	C	T	C	T	A	C	C	T

FIG. 1. DNA sequences obtained using the BSS method. The oligonucleotides recovered after six rounds of selection were subcloned and sequenced. The selected sequences are aligned with respect to the restriction sites. The table below summarizes the frequency with which each nucleotide is represented in the 22 positions of the 21 almost identical clones. The bottom sequence represents the consensus MBE sequence. The palindromic motif is shown by arrows.

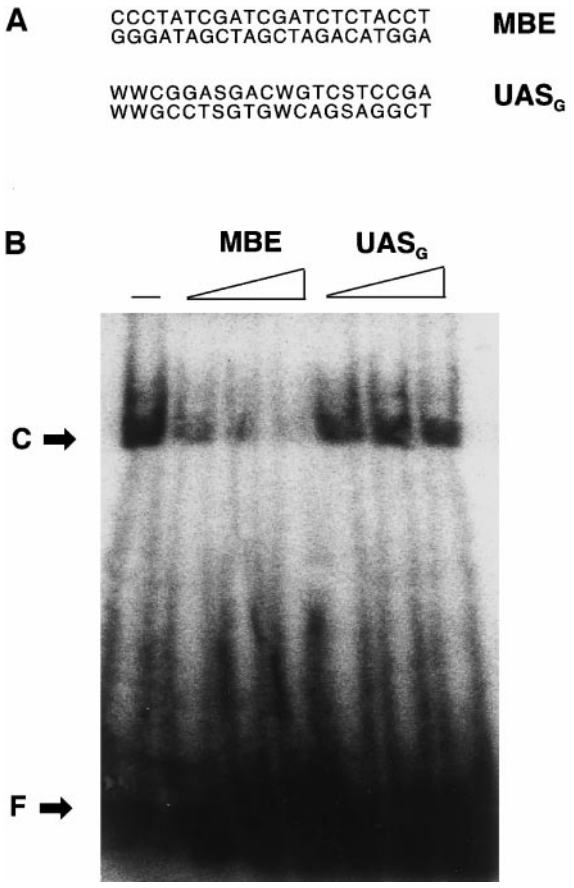

FIG. 2. Analysis of the MBE-binding activity of Mblk-1. (A) Sequences of ds-oligonucleotides used in EMSA. (B) Partially purified recombinant GST-full length Mblk-1 fusion protein was incubated with ^{32}P-labeled MBE probe in the presence or absence of non-labeled MBE or UAS$_G$ and subjected to EMSA. The triangles indicate that 10-, 25-, 50-fold excess of competitor was added to each sample from left to right. C and F indicate positions of protein/DNA complex and free probe, respectively.

no significant similarity to this motif, many of them (more than 30 clones), including the above sequence, contained CT-rich sequences in their 5′ and 3′ ends. In particular, the 5′ ends of these clones tended to start with a C-rich sequence, suggesting that Mblk-1 might also recognize the flanking nucleotides as well as the central palindromic motif.

MBE Represents High-Affinity Target Sequence Recognized by Mblk-1

Next, we used EMSA to examine whether the recombinant Mblk-1 specifically binds to the above identical sequence 5′-CCCTATCATCGATCTCTACCT-3′, which we termed MBE. When the recombinant GST-full-length Mblk-1 fusion protein (12 ng) was mixed with ^{32}P end-labeled ds-MBE (20 pmol, Fig. 2A), a single DNA–protein complex was detected (Fig. 2B). Addition of increasing amounts (10- to 50-fold excess) of unlabeled MBE probe resulted in a gradual diminution of the complex formation. In contrast, addition of the unlabeled non-specific oligonucleotide, UAS$_G$, failed to compete for complex formation even at a molar ratio of as high as 50-fold of labeled MBE, clearly indicating that the binding is specific.

Mblk-1 Recognizes the Entire MBE Sequence

To identify important regions in MBE for recognition by Mblk-1, competition experiments were performed with a series of mutant oligonucleotides, each with base a substitution at four or eight positions. In all, six mutant oligonucleotides (M1–M6) were created (Fig. 3B). When these mutant oligonucleotides were added at 40-fold molar excess over the probe, the decrease in the intensity of the band for the DNA–protein complex was much smaller than when MBE was used as a competitor and similar to that when UAS$_G$ was used. There were no significant differences between the results for any of the six (M1–M6) oligonucleotides (Fig.

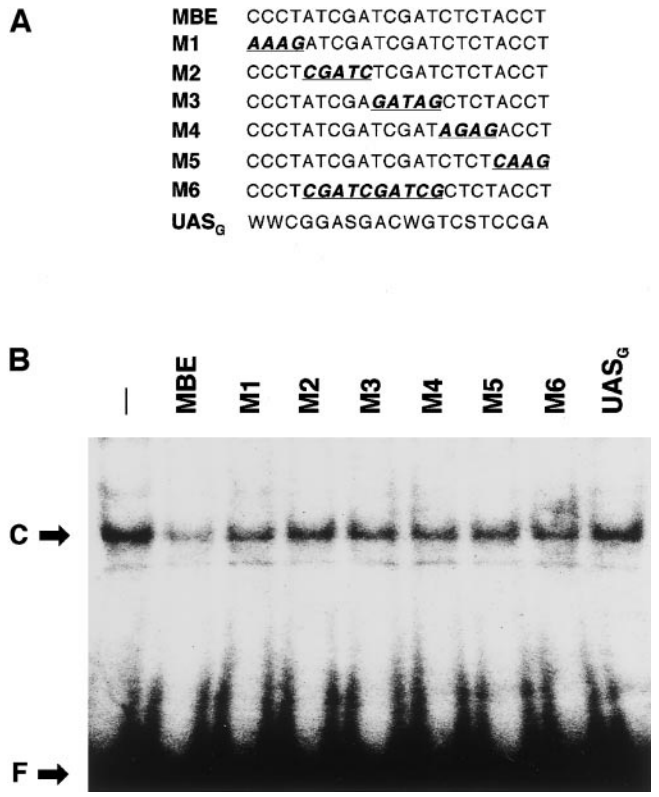

FIG. 3. Competition experiments using mutant MBE nucleotides. (A) Mutant MBE oligonucleotides. The bold and italic letters indicate the mutated sequences in each mutant oligonucleotide. (B) Competition experiments. EMSA was performed with ^{32}P-labeled MBE probe, recombinant GST-full length Mblk-1, and with MBE, USA$_G$ or one of six unlabeled mutant oligonucleotides (M1-M6) at a 40-fold molar excess over the probes. (—) indicates experiment without competitor. C and F indicate positions of protein/DNA complex and free probe, respectively.

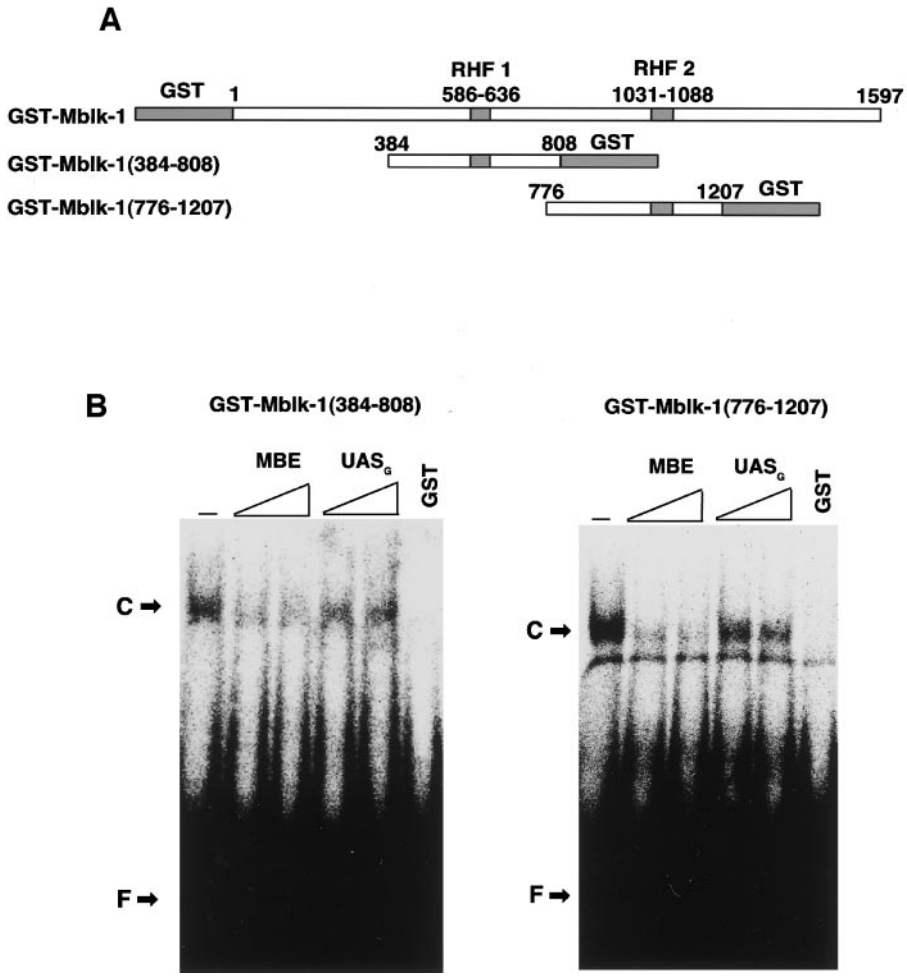

FIG. 4. MBE-binding abilities of truncated Mblk-1 mutants. Truncated GST-Mblk-1 mutant proteins were expressed in *E. coli* and tested for DNA-binding activity by EMSA. (A) Schematic representation of GST-Mblk-1 fusion proteins. GST-Mblk-1 comprises the entire Mblk-1 protein, while GST-Mblk-1(384-808) and GST-Mblk-1 (776-1207) contain the RHF 1 and RHF 2 domains, respectively. (B) Each 0.3 μg of purified recombinant GST-Mblk-1(384-808) or GST-Mblk-1(776-1207) or 1.2 μg of GST alone was incubated with ^{32}P-labeled MBE and subjected to EMSA. The triangles indicate that 10- and 20-fold excess of MBE or UAS$_G$ was added as a competitor to each samples, from left to right. C and F indicate positions of protein/DNA complex and free probe, respectively.

3A). These findings indicated that M1–M6 failed to compete with the MBE-Mblk-1 binding efficiently, indicating that both the central palindromic motif and the flanking sequences of MBE are important for recognition by Mblk-1.

Truncated Mblk-1 Proteins Containing the RHF 1 or RHF 2 Also Bind to MBE

Mblk-1 contains two putative DNA binding motifs, termed RHF 1 and 2, that share 40% sequence identity (18/45 amino acid residues). Therefore, we examined whether both of these motifs bind to MBE. Truncated Mblk-1 containing either RHF 1 or RHF 2 were expressed separately as GST-fusion proteins [GST-Mblk-1(384-808) and GST-Mblk-1(776-1207), respectively; Fig. 4A] and tested using EMSA. Both truncated proteins (each 300 ng) bound to MBE, and the addition of increasing amounts of unlabeled MBE, but not UAS$_G$, resulted in a diminution of the complex formation, indicating that both GST-Mblk-1(384-808) and GST-Mblk-1(776-1207) bound MBE specifically (Fig. 4B). In contrast, GST alone (1.2 μg) did not bind significantly.

A large amount of GST-Mblk-1(384-808) or GST-Mblk-1(776-1207) was needed for efficient complex formation (300 ng) compared with GST-full-length (12 ng). Accordingly, the apparent dissociation constants of GST-full-length Mblk-1 for MBE (K_{eq} = 18 nM) are 426- and 716-fold lower than those for the GST-Mblk-1(384-808) (K_{eq} = 7.5 μM) or GST-Mblk-1 (776-1207) (K_{eq} = 13 μM). These results indicated that RHF 1 and 2 are DNA-binding motifs of Mblk-1 and that the ternary structure of Mblk-1, such as cooperative interaction between RHF 1 and 2, is important for full binding.

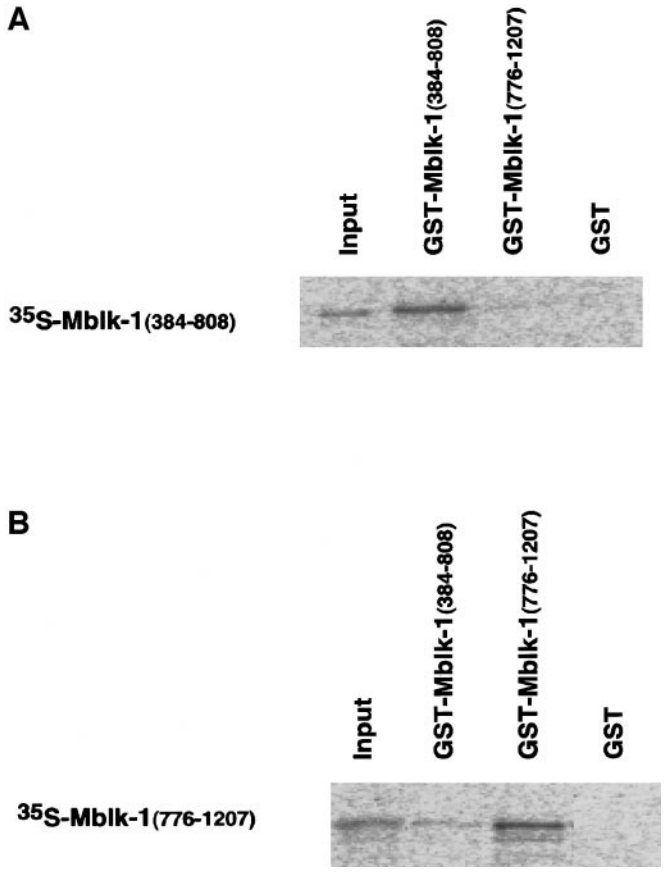

FIG. 5. Homodimeric binding of Mblk-1(384-808) and Mblk-1(776-1207) *in vitro*. *In vitro* translated and ^{35}S-Met-labeled Mblk-1(384-808) (A) or Mblk-1(776-1207) (B) was incubated with glutathione-Sepharose bound with either GST-Mblk-1(384-808), GST-Mblk-1(776-1207) or GST and bound proteins were subjected to SDS–polyacrylamide gel electrophoresis followed by autoradiography. Input indicates 1/10 volume of ^{35}S-Met labeled proteins.

Both DNA-Binding Motifs of Mblk-1 Afford Homodimeric Binding

To determine the possible cooperative interaction of RHF 1 and 2, we examined whether Mblk-1(384-808) or Mblk-1(776-1207) formed homo- or hetero-dimers using an *in vitro* pull-down assay. When ^{35}S-labeled Mblk-1(384-808) was incubated with glutathione–Sepharose beads bound with GST-Mblk-1(384-808), but not with GST-Mblk-1(776-1207) or GST alone, a significant amount of the input protein bound to the beads (Fig. 5A). In contrast, when ^{35}S-labeled Mblk-1(776-1207) was incubated with glutathione–Sepharose beads bound with the GST-Mblk-1(776-1207), a significant amount of input material bound to the beads (Fig. 5B). Although, ^{35}S-labeled Mblk-1(776-1207) also bound weakly with GST-Mblk-1(384-808) weakly, no significant binding was observed with GST alone. These results indicate that both RHF 1 and RHF 2 afford homophilic interactions rather than mutual interaction, suggesting that Mblk-1 functions as a dimer.

DISCUSSION

In the present study, we identified a binding sequence for Mblk-1 (MBE) using the BSS method. MBE contained a palindromic motif and CT-rich flanking regions. The competition experiments using mutant oligonucleotides indicated that both the palindromic motif and CT-rich flanking regions were important for recognition by Mblk-1. These findings are consistent with the facts that (i) the sequences selected by BSS are frequently longer than the 20-bp randomized sequence and (ii) the 5′ and 3′ ends of the sequences frequently contain CT-rich sequences, indicating that Mblk-1 recognizes the flanking nucleotides as well as the central palindromic motif.

MBE was recognized not only by the full-length Mblk-1 but also by truncated Mblk-1 mutants containing either RHF 1 [Mblk-1(38-808)] or RHF 2 [Mblk-1(776-1207)] (Fig. 4), indicating that both RHF 1 and RHF 2 function as DNA-binding domains. The affinity of these interactions, however, was remarkably lower than that of full-length Mblk-1. This is consistent with the fact that we failed to detect any amplified products using the BSS method with Mblk-1(776-1207), even after six rounds of selection (data not shown). An *in vitro* pull-down assay demonstrated that both RHF 1 and RHF 2 afford homodimeric binding. It is possible that the ternary structure of Mblk-1 containing both of these domains is required for full DNA-binding and the dimerization of Mblk-1 might lead to a more stabilized interaction with the target DNA.

The palindromic motif of MBE, 5′-ATCGATCGAT-3′, is similar to the binding sequence for the human Cut-like protein, 5′-ATCGATCG-3′ (15). The human Cut-like protein contains the homeodomain and three other DNA-binding regions called Cut repeats. Both the Cut repeat and homeodomain are required for high-affinity binding to the target sequence. Moreover, this protein binds to DNA as homo-dimers. Thus, Mblk-1 might have a similar mode of action with Cut-like protein, although they have no significant sequence similarity.

Although Mblk-1 has the highest sequence similarity with a *Drosophila* nuclear factor CG18389/*E93* protein (9), its biochemical characteristics have not been examined. This is the first report of the biochemical analysis of Mblk-1, including its homologues, in various animals. The identified binding sequence for Mblk-1 should facilitate analysis of the biologic functions of Mblk-1 and its homologues, including the target genes that they regulate.

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