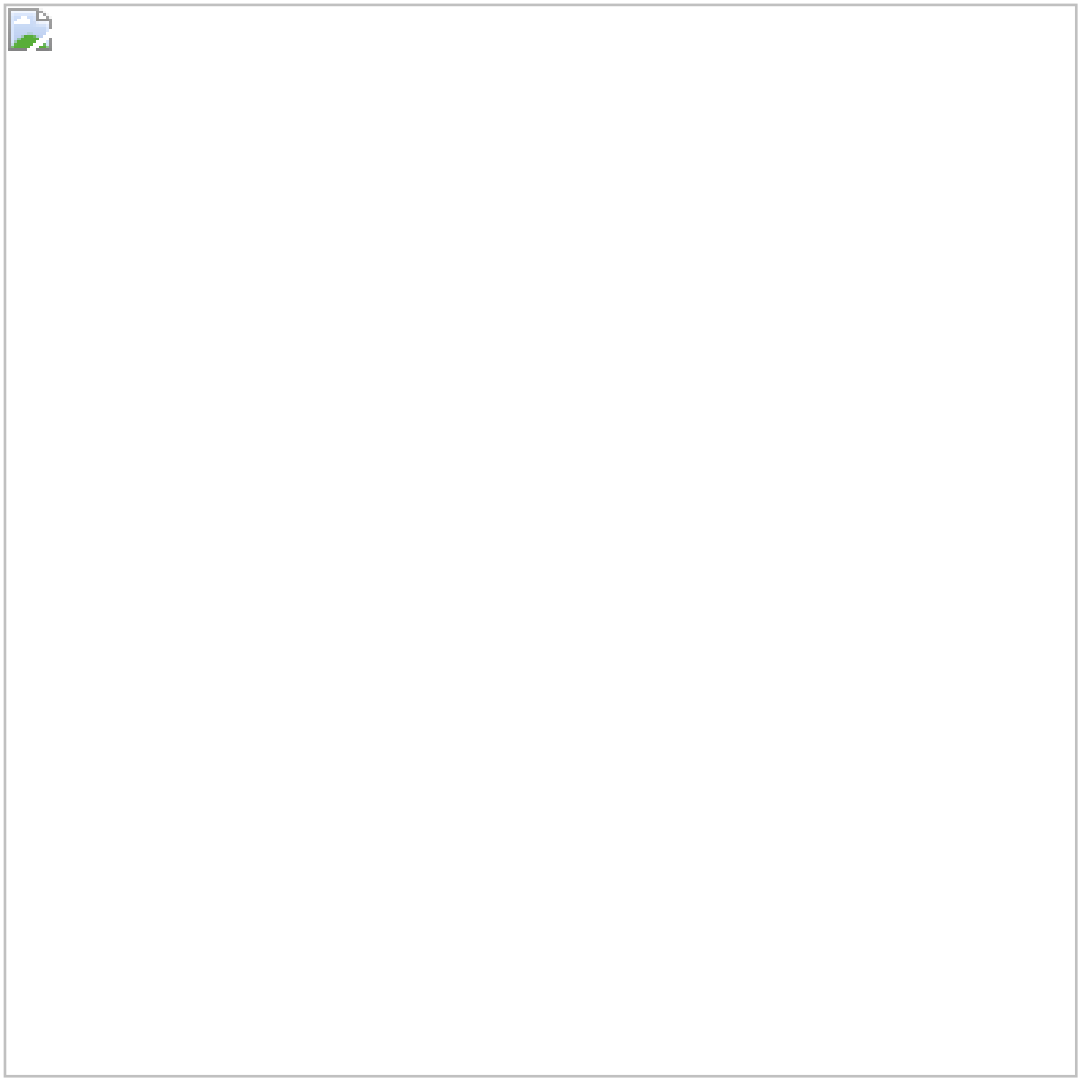


# Expression, purification and characterization of methyl DNA binding protein from Bombyx mori

Authors: Uno, Tomohide, Nomura, Yuka, Nakamura, Masahiko, Nakao, Atsushi, Tajima, Shoji, et al.

Source: Journal of Insect Science, 5(8) : 1-8

Published By: Entomological Society of America

URL: https://doi.org/10.1673/031.005.0801

The BioOne Digital Library (https://bioone.org/) provides worldwide distribution for more than 580 journals and eBooks from BioOne's community of over 150 nonprofit societies, research institutions, and university presses in the biological, ecological, and environmental sciences. The BioOne Digital Library encompasses

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

Uno T, Nomura Y, Nakamura M, Nakao A, Tajima S, Kanamaru K, Yamagata H, Iwanaga Y. 2005. Expression, purification and characterization of methyl DNA binding protein from *Bombyx mori*. 8pp. *Journal of Insect Science*, 5:8, Available online: insectscience.org/5.8

Journal of Insect Science

insectscience.org

# Expression, purification and characterization of methyl DNA binding protein from *Bombyx mori*

**Tomohide Uno[1], Yuka Nomura[1], Masahiko Nakamura[2], Atsushi Nakao[1], Shoji Tajima[2], Kengo Kanamaru[1], Hiroshi Yamagata[1], Yousuke Iwanaga[1]**

*[1]Laboratory of Biological Chemistry, Department of Biofunctional Chemistry, Faculty of Agriculture, Kobe University, Nada-ku, Kobe, Hyogo, 657–8501, Japan*

*[2]Institute for Protein Research, Osaka University, Suita, Osaka, 565-0871, Japan*

uno_tom@yahoo.co.jp

Received 17 May 2004, Accepted 9 October 2004, Published 28 March 2005

## Abstract

A cDNA clone encoding methyl DNA binding domain-containing protein (bMBD2/3) was obtained by homology searches using a *Bombyx mori* fat body cDNA library. The cDNA encoded a polypeptide with 249 amino acids sharing 54% similarity with the methyl DNA binding protein from *Drosophila melanogaster*. To characterize the biochemical properties of bMBD2/3, the clone was expressed in *Escherichia coli* as His-tagged protein. The recombinant protein was purified to homogeneity using Ni-NTA superflow resin and heparin agarose. The protein showed specific methyl DNA binding activity and was phosphorylated by protein kinase *in vitro*. Immunoblotting using the purified antibody indicated that bMBD2/3 was expressed in almost all tissues. Using west-western blotting analysis, some proteins that interact with bMBD2/3 were identified in the brain. This is the first report that insect MBD is phosphorylated and is present in adult tissues. These results suggest that bMBD2/3 plays important roles in the DNA methylation-specific transcription of *Bombyx mori*.

*Keywords:* DNA methylation, phosphorylation

*Abbreviation:*
EMSA Electro Phoretic Mobility Shift Assay

## Introduction

In mammals, cytosine methylation at CpG dinucleotides represents the most common form of DNA modification (Rein *et al*. 1998). Approximately 2–10% of all cytosine residues in genomic DNA are methylated. This methylation has been implicated in the regulation of a number of genetic activities during mammalian cell differentiation and embryonic development (Li *et al*. 1992; Okano *et al*. 1999). These activities include tissue-specific gene transcription, cellular defense against viral agents, tumorigenensis, genomic imprinting and X-chromosome inactivation (Bartolemes *et al*. 1997; Constancia *et al*. 1998; Heard *et al*. 1997).

DNA methylation in several invertebrate species including insects have been reported (Field *et al*. 2000; Patel *et al*. 1987). Recently DNA methylation was also detected in *Drosophila melanogaster* by highly sensitive chemical means (Lyko *et al*. 2000; Gowher *et al*. 2000). Cytosine methylation in *D. melanogaster* is reported to be considerably more abundant during very early development. Further, the enzyme responsible for this methylation, DNA cytosine methyl transferase, was identified from *D. melanogaster* using database searches (Hung *et al*. 1999). Overexpression of this clone resulted in genomic DNA methylation of *D. melanogaster* S2 cells (Reddy *et al*. 2003). This result suggests that DNA methylation in insects is important during embryonic development.

In vertebrates the regulatory information encoded by DNA methylation is interpreted by a family of proteins with a conserved DNA binding surface termed a methyl-CpG binding domain, or MBD (Bird *et al*. 1999; Siegfried *et al*. 1997; Hendrich *et al*. 1998). MeCP2, MBD1, MBD2, MBD3, and MBD4 constitute a family of vertebrate proteins that have a methyl binding domain. These MBD proteins could preferentially bind to methyl CpG-containing DNA, recruit histone deacethylase-containing complexes and thus cause chromatic condensation. This is very likely one major scheme involved in the functioning of vertebrate DNA methylation.

Several recent reports have also noted the identification of insect MBD proteins in *D. melanogaster* (Ballestar *et al*. 2001; Roder *et al*. 2000). Only one type of MBD protein was found, compared

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

to the five types of vertebrate MBD proteins described above. The MBD protein of *Drosophila* was expressed during early development as two alternative spliced types, and repressed transcription in both mammalian and insect cells. Further MBD2/3 from *Acheta domesticus* (aMBD2/3) was isolated by degenerate RT-PCR (Tweedie *et al*. 1999). The expressed protein in *E. coli* showed methyl DNA binding, but its function and distribution in tissues is unknown.

*Bombyx mori* is a major experimental insect for genetic and biochemical analysis. Because a *B. mori* data base (silk base) has been established, transgenic insects can be produced and various genetic mutants have been isolated. Cytosine residues in genomic DNA of the silk gland of *B. mori* were reported to be methylated at a level of 0.15–0.2%. However, little detectable genome 5-methyl cytosine has been found in adult *D. melanogaster* (Patel *et al*. 1987). *B. mori* is, therefore, useful to clarify the molecular mechanism of insect DNA methylation.

In this report, we identified a clone as MBD protein from *B. mori.* This clone was expressed as an His-tagged thioredxin fusion protein in *Escherichia coli* and purified using affinity chromatography. The recombinant protein specifically bound methylated DNA, interacted with proteins in the brain and was phosphorylated by protein kinase. Immunoblotting indicates that this protein was expressed in almost all tissues, which is different from what is seen in *D. melanogaster*.

## Materials and Methods

### *Materials*

Thrombin, ECLplus Western blotting detection reagent and [γ-$^{32}$P] ATP (>1,000 Ci/mmol) were from Amersham Pharmacia Biotech (www.apbiotech.com). Heparin agarose was from Sigma (www.sigmaaldrich.com). Calmodulin-dependent protein kinaseII and PKA were from New England BioLab, www.neb.com. Goat peroxidase-conjugated anti mouse IgG was from Cell Signaling Technology, Inc. (www.cellsignal.com) PKC was from Upstate Biotechnology (www.upstate.com). Ni-NTA superflow resin was from Qiagen (www.qiagen.com). The other chemicals were of the purest grade commercially available.

### *Construction of expression plasmid and expression in E. coli*

The cDNA fragments containing the entire coding sequence of bMBD 2/3 were kindly provided by Dr. Mita of the National Institute of Entomological and Sericultural Science. Sequence analysis was done using an ABI sequencer 377 (www.appliedbiosystems.co.jp). Sequence homologies were done by BLAST search (Altschul *et al*. 1990). The cDNA fragments containing the entire coding sequence of bMBD2/3 were amplified by PCR with primers containing *Bam*HI or *Eco*RI. The amplified fragments were digested with *Bam*HI and *Eco*RI. The digested fragment was inserted into the *Bam*HI and *Eco*RI sites of an expression vector, pET32a. This cDNA was transformed into *E. coli* strain BL21. Transformed *E. coli* cells (BL21) were incubated overnight in LB medium. The medium was diluted to 1:100 and incubated for 3 h. Expression of thioredoxine-fusion protein was then induced by adding 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), followed by additional incubation for 24 h at 16 °C. The cells were collected by centrifugation at 5,000 *g* for 5 min, and then stored at –80 °C.

### *Purification of bMBD2/3 protein*

All procedures were carried out at 4 °C. The frozen cells (26.5 g) were suspended in phosphate-buffered saline (PBS) [140 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, and 1.8 mM $KH_2PO_4$ (pH 7.3)], disrupted by sonication and then cleared by centrifugation at 12,000 *g* for 30 min. The fusion protein was bound to a Ni-NTA superflow resin equilibrated with buffer A (50 mM $NaH_2PO_4$, 300 mM NaCl, and 10 mM imidazole; pH 8.0). After washing, proteins were eluted with a step-wise increase in imidazole. The 0.15 M imidazole eluate was dialyzed against PBS. To remove His-tagged thioredxin, the protein was incubated with thrombin at 22 °C for 16 hr. After centrifugation the supernatant was applied to a Ni-NTA column equilibrated with buffer A. The passed-through fraction was applied to a heparin agarose equilibrated with 25 mM Hepes-NaOH (pH 7.6). After washing, proteins were eluted with a step-wise increase in NaCl. The 0.3 M NaCl eluate was recovered. The sample was stored at –80 °C. Proteins were determined using BSA (Fraction V, Sigma) as the standard by the method of Lowry *et al*. (1951).

### *SDS–PAGE*

SDS–polyacrylamide gel analysis was performed according to the method of Laemmli (1970), using a 4.5% stacking gel and a 15% separating gel, at a constant current of 16 mA. The proteins in a gel were visualized with Coomassie Blue stain.

### *Electrophoretic mobility shift assay (EMSA)*

The sequence of oligonucleotides used in EMSA is 5'-cc$^{m}$ggcc$^{m}$ggcc$^{m}$ggcc$^{m}$gg-3' as the methylated DNA probe and 5'-ccggccggccggccgg-3' as the unmethylated DNA probe. The oligonucleotides were labeled with $^{32}$P at their 5' ends and gel purified prior to EMSA. The purified protein was incubated in 10 mM Tris HCl (pH 8.0) containing 3 mM $MgCl_2$, 50 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.1% NP-40, 5% glycerol, 0.4 mg/ml BSA and one pico mole of radiolabeled probe. Samples were incubated for 30 min at 25 °C. Thirty picomoles of competitor DNA were used per binding reaction. To separate the protein-DNA complexes, the reaction mixtures were loaded onto a running nondenaturating 6% polyacrylamide gel. Electrophoresis was carried out. Gels were dried and band patterns were analyzed using a bioimaging analyzer (BAS 1000; FUJIX).

### *Preparation of brain crude homogenate from Bombyx mori*

Crude protein kinase fraction was obtained from the brain of *Bombyx mori*. First, the brain was rapidly dissected out of 3-day-old larvae of 5th instar in Ringer's solution. The brain was homogenized in the homogenization buffer (25 mM Tris-HCl [pH 7.5], 50 mM mercaptoethanol, 2 mM EGTA, 1mM PMSF, and 0.25 M sucrose). The homogenate was used as the brain extract.

### *Protein kinase assay*

The typical reaction mixture with a total volume of 20 *u*l contained 50 mM Tris-HCl (pH 7.5), 6.25 mM $MgCl_2$, 0.125 mM $CaCl_2$, 2 mM dithiothreitol, 0.1 mM EDTA, 0.1 *u*g bMBD2/3 protein, 50 units protein kinase and 50 μM [γ-$^{32}$P] ATP. After incubation at

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

25 °C for 25 min, the samples were treated with SDS–sample buffer. After electrophoresis, the gel was stained with Coomasie Brilliant Blue and dried. The band patterns were analyzed using a bioimaging analyzer. The position of bMBD2/3 protein on a gel was identified by Coomasie Brilliant Blue staining. Protein kinase C was assayed by addition of phosphatidyl serine, and dioleine. Ca/CaM dependent protein kinase II was assayed by addition of 1.2 µM calmodulin.

*Western blotting*

The recombinant protein was dialyzed against distilled water and freeze-dried. The protein was mixed with complete adjuvant and immunized into the rabbit. After boost injection, the serum was isolated. The antiserum was subjected to immunoaffinity chromatography with bMBD2/3 immobilized on the affinity column (2-fluoro 1-methyl pyridinium toluene-4-sulfonate activated cellulofine; Seikagaku [www.seikagaku.com]). The tissue was homogenized in the homgenization buffer [10 mM Hepes (pH 7.6), 10% glycerol, 0.1 M KCl, 3 mM, $MgCl_2$, 0.1 mM EDTA, 1 mM $Na_3VO_4$, and 1 mM DTT]. After SDS–PAGE, the proteins were transferred to a nitrocellulose membrane (Advantec, www.advantecmfs.com). After blocking, the primary antibody was incubated (1:5000). After washing, goat peroxidase-conjugated anti rabbit IgG (H+L) was incubated as secondary antibody (1:5000). After washing, protein bands were visualized using ECLplus Western blotting detection reagent and analyzed by Typhoon 9400 (Amersham Pharmacia Biotech).

*West-western blotting*

The brain was homogenized as described above. After SDS–PAGE, the protein was transferred to a nitrocellulose membrane. After blocking, the purified bMBD2/3 protein (10 *u*g/ml) was incubated in 10 mM sodium phosphate buffer (pH 7.4) containing 0.1M NaCl, 0.2mM, $NaN_3$, and 0.2 mM EGTA. After washing, the primary antibody was incubated (1:5000). After washing, goat peroxidase-conjugated anti rabbit IgG (H+L) was incubated as secondary antibody (1:5000). After washing, protein bands were visualized using ECLplus Western blotting detection reagent and analyzed by Typhoon 9400 (Amersham Pharmacia Biotech).

## Results and Discussion

*Molecular cloning of bMBD2/3*

Multiple EST clones were identified by Blast search using the MBD2b sequence. One of the clones, bMBD2/3, originating from a Baculovirus-infected *Bombyx mori* fat body cDNA library was identified and sequenced (Figure 1). Its insert is 2.4 kbp long, including the poly A tail. The carboxyl terminal non-coding region was 1.5 kbp long. bMBD2/3 bears an open reading frame corresponding to aa 249. In insects, two MBDs (from *A. domesticus* [aMBD 2/3] and *D. melanogaster* [dMBD2/3]) were previously isolated and cloned. The expressed recombinant proteins were characterized (Ballestar *et al*. 2001; Roder *et al*. 2000; Tweedie *et al*. 1999). bMBD2/3 showed 67.6 and 53.7% similarity to aMBD2/3 and dMBD2/3, respectively. The methyl -CpG- binding domain of bMBD2/3 closely resembles that of aMBD 2/3 but differed from that of dMBD2/3 (Figure 2). However, the carboxyl-terminal domain was almost conserved among them. The carboxyl-domain is a transcriptional repression domain, which changes the local chromatin structure, mainly through recruitment of histone deacetylase. bMBD was suggested to bind DNA and regulate the transcription of *B. mori*.

*Expression and purification of the recombinant protein*

To characterize bMBD2/3, cDNA for bMBD2/3 was expressed in *E.coli* and purified using affinity chromatography. The cDNA for bMBD2/3 was inserted into an expression vector pET32a and expressed in *E. coli* as a His–tagged thioredoxin fusion protein. The fusion protein recovered in the soluble fraction was bound by a Ni-superflow column. The fusion protein was eluted after washing. After dialysis, the fusion protein was cut to remove thioredoxin and the soluble fraction was recovered. This fraction was then applied to a heparin agarose. SDS–PAGE analysis showed that the purified protein was homogeneous (Figure 3). The evaluated molecular mass on SDS–PAGE was 26 kDa. About 0.1 mg protein was recovered from 10 liter culture of *E. coli*.

*EMSA of bMBD2/3*

Whether the purified bMBD2/3 specifically binds methyl DNA or not was examined. As shown in Figure 4, lane 5, and lane 6, bMBD2/3 did not bind non-methyl-DNA, but did bind to methyl-DNA (Figure 4, lane 1 and lane 2). The DNA binding was inhibited by methyl DNA, but not inhibited by non-methyl DNA (Figure 4, lane 3, and lane 4). This result indicates that bMBD2/3 specifically bound methyl DNA.

*Western blotting of bMBD2/3*

The availability of an antibody against dMBD2/3, which shows weak methyl DNA binding, was shown, but the identification of aMBD2/3 using immuno blotting, which shows a strong binding, was not done. To ascertain the distribution of bMBD2/3 in the tissues of *B. mori,* immunoblotting of several tissues was done using the purified antibody as the primary antibody (Figure 5). One band around 27 kDa was detected in almost all tissues examined including the brain and silk gland (lanes 4 and 8).

Immunodetection of dMBD2/3 in *D. melanogaster* revealed two signals, one at Mr 30,000 and one at Mr 25,000, which represent the long and the short isoforms of the protein, respectively (Marhold *et al*. 2002). Early embryonic stages contained only the long isoform while late embryonic stages contained only the short isoform. Both proteins were present during embryogenesis. As described above, *D. melanogaster* genomic methylation is restricted to the early stage. It is apparent that the methyl DNA-related protein is expressed during embryogenesis in *D. melanogaster*.

In *B. mori*, methyl DNA was contained in DNA of larval silk glands (Patel *et al*. 1987). The DNA CpG methylation site of *B. mori* was recently identified using PCR (Tatematsu *et al*. 2003). We will try to clarify the relationships between bMBD2/3 and methylated CpG islands in the *B. mori* genome. Immunoblotting demonstrated that bMBD2/3 was expressed in the brain (Figure 5, lane 8). Methyl DNA binding protein plays important roles in brain development (Armstrong 1997). Further Blast search indicated that partial sequences for DNA methyl transferase were isolated from the brain cDNA library of *B. mori* (unpublished observations). bMBD2/3 and DNA methyl transferase may regulate DNA

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

**Figure 1.** Nucleotide and deduced amino acids sequences of bMBD2/3. The nucleotide and predicted amino acid sequences of the cDNA clone encoding the protein, bMBD2/3 are shown.

```
GGCGTGGGCTCACCACAAACCACAAACATACCTGTTTCAGTGTTTACATAAAATGTGTAGTAGGTACATACTCAAAACATTCTCTAAATT    90
GAAATTGGAATATTATTGTGGTTTGATTTATTAAGAAGAAAGAAATAAACCGTAAATATGAACATATCTATCGAAAGAAAACGATCCGAT   180
                                                          M  N  I  S  I  E  R  K  R  S  D
TGTTCAGCGTTACCCAAAGGCTGGCAAAGAGAGGAAGTAGTTAGAAAAACAGGTCTGTCGGCAGGGAAGGTTGATGTTTATTATTACAGT   270
 C  S  A  L  P  K  G  W  Q  R  E  E  V  V  R  K  T  G  L  S  A  G  K  V  D  V  Y  Y  Y  S
CCAACAGGTAAAAAGTTTCGCAGTAAGCCAGAGCTTGTTCGTTATCTCGGTGATAGTGTGGACCTGTCGTGTTTCGACTTTCAGCAAGGT   360
 P  T  G  K  K  F  R  S  K  P  E  L  V  R  Y  L  G  D  S  V  D  L  S  C  F  D  F  Q  Q  G
CAGATAAACACAATGTTGCTGTGCAAGGCCAAGAAAGCACGAGCGCAGTTTGACTACAGGGGTGTACGTAATGATGCCTCATTAGTGCCA   450
 Q  I  N  T  M  L  L  C  K  A  K  K  A  R  A  Q  F  D  Y  R  G  V  R  N  D  A  S  L  V  P
CCAATTCGTCAAACAGCATCAATATTCAAACAGCCTGTGACGGTCTACAAAACTCAAGACAGTAAAGTTAAAACAGATCTCAAACATGGG   540
 P  I  R  Q  T  A  S  I  F  K  Q  P  V  T  V  Y  K  T  Q  D  S  K  V  K  T  D  L  K  H  G
ACACAGGAAAAGCCTAAACAATTATTTTGGGAAAAAAGACTAGAGGGGTTGACAGCTTGTGATGCAAATGGTGTAATTGGAACTACGAGT   630
 T  Q  E  K  P  K  Q  L  F  W  E  K  R  L  E  G  L  T  A  C  D  A  N  G  V  I  G  T  T  S
CTACCAAAATACATCAAATCCTTGGGACCATACACTTCTGATGCCACCACTATCCAGTCATTGGCTACAGCACTTCATGTATCTTCACAA   720
 L  P  K  Y  I  K  S  L  G  P  Y  T  S  D  A  T  T  I  Q  S  L  A  T  A  L  H  V  S  S  Q
CCTATAACTGGACAAATAGGATCGAAACAAGCCATAAAAGACAATCCAGGTGTTTTTTTAAACCCAGAACAACCCCTGATTGCTGCAGTG   810
 P  I  T  G  Q  I  G  S  K  Q  A  I  K  D  N  P  G  V  F  L  N  P  E  Q  P  L  I  A  A  V
ACAATCACTAAGGAAGATGTGCGACGCCAAGAGGAGCGCGTGAAGCGAGCTCGCCAGCGATTGCGACAAGCTCTATCGGTGGCCTAGGGC   900
 T  I  T  K  E  D  V  R  R  Q  E  E  R  V  K  R  A  R  Q  R  L  R  Q  A  L  S  V  A  *
TGCGACGGCGGCGGTGAGCCTAGTGTGGAAACCGCTGCCGACCTTTCTATGTCCGCTATGCTTCCCGACTCTACACAGGACTCCCCCAAG   990
TCCACAGAGTCTAATAGAAATGACTAAAATGGTTTTTACTTTTTGTAAAACCATATTGCAGGATATCGAATTCCACTAAATAAGATGTAA  1080
TTCCCGATTGAATTAATTGAACTCAATAGTAATGTAACAAGACACAATTTGATGTTGTCTGAACAGATGATAACAAAAACTTCTTGTTAG  1170
TCCTCAATAAGTAACGGGAAGTAGTAATTATTGCCTCGTTATTTGGTTAGATACGCTGGATACTGCACCAGGTCCCGATTTGACTCACAA  1260
CTGGAGTCTGTAATCTCTCTAGATATGGTATTTGGTACCTAAGAGTTCTTGTTTTTTCTGGTATGTGTATAGAACCCTTGGGGCCTCAAT  1350
GCGAGAAAATAGTGATTTAGGTCCACTGGGAATTTCCACATTTCCTTATTTAGCTATAGTCATGTCATTTTAATCATTTTTAAAATGCCA  1440
ACATTGGTCTCATAACAGAATTGCTATGACATAATATGAAAACAGTTTTATATCATAATTATCTTACTATCCTATTTAATAAGTTTAAGA  1530
TTAGATATACAAATCTACTTATCTTTAGACATGGCCTAATTAATTTAAAGAAAATCAATCAGTCTAGCGTCGCATGGTCACATCAACAGC  1620
AACCACAGTGACAGGCTATTGAGTAAAAGTCGTACACAGTTTTTACACATTAATTTATTAAAAGGCAATTGTTGCAATTATTGTGTATTC  1710
TATCACTATCTGTAATTGCAAGACAGTTCACAACCATAAATTTTTAAAACAGCTTTTAGGTAAAACTTCTAAATTGCATGAAAATAAGCA  1800
TAGTCGTATATGCAAAGTGTACAGAATTATACGCGGGGGTGTAAAGATACCGATCCCGCAACGGAAACGGAAATGAACTTGAAAACATTG  1890
ATTTTTTTAAAAAAGATTTGTATTACGATGTTTACATTTTGTGAATAACCCCTGATTGCATATTCCGCGTGTTTGCCGATTATAATTGTG  1980
TTTATTGACAAAAAATATGGCACAAATTGGGAAAATTCTAATGTTTTTCTTACTTGTAGGCTGCAGAAAATAAAGTAATATAATTATTAT  2070
CTAATTTAAAAGATTTTCGGGATCGGTTCCAGTACACCCTTTATAGGTGAGATATAAGGCGTAATTGAAATTCATTAAAGGTAGATTTAA  2160
ATTTGTACAAAACCTAGTGATTGCAATATTTAAAATGGACGGCGCGAAATGACGGACAAGCAGAATGTCTAAGATATTAAACTTTAGTAG  2250
TAAATATAGTATTTAAGTTTTGATAACTATATATTTAGTTTTTTATGGTGTTAATGTCTTATTTCTATCAATAAAAATAGTGAAAATAAA  2340
TTAAAAAAAAAAAAA
```

**Figure 2.** Comparison of the deduced amino acid sequence of *Bombyx mori* bMBD2/3 with those of *Drosophila melanogaster* (dMBD2/3) and *Acheta domesticus* (aMBD2/3). Identical amino acids are indicated by asterisks. The methyl-CpG binding domain is highlighted in yellow.

```
bMBD2/3    1:--MNIS--IERKRSDCSALPKGWQREEVVRKTG-----------LS--A------GKVDVYYYSPTGKKFRSKPELVRYLGDSVDLSCF
aMBD2/3    1:--M--S--VEKKKFECSALPKGWQREEVIRKSG-----------IS--A------GKVD-YYYSPNGKKFRSKPQLARYLGDALDLATF
dMBD2/3    1:MQMNPSVTIERKRVDCSVLPKGWQRDEV-RKSGSSANNNASSNNNSSATASSNNNNNKVDVFYY--------S-----R----------
                  *  *   * *   ** ******* ** ** *            *  *       ***  **        *     *

bMBD2/3   67:DFQQGQINTMLLCKAKKAR-AQFDY-RGVRNDASLVPPIRQTASIFKQPVTV---YKTQ-DSKVKTDLKHGTQEKPKQLFWEKRLEGLTA
aMBD2/3   64:DFRSGKINSLLLRKNKKQRGTQFDYSRGVRNDASLVPPIRQTASIFKQPVTV---YKTQ-EGKVKADFKHGAQEKPKQLFWEKRLEGLRA
dMBD2/3   66:---------AL------R---TD----V----SLVPPIRQTASIFKQPVTVIRNHK-QDPAKAKNEPKHGTREKPKQLFWEKRLERLRA
                       *      *    *    *    ******************    * *   * *   ***  ************* * *

bMBD2/3  151:C-DANGVIGTTSL-----PKYIKSL---GP----YTSDATIQSLATALH-----VSSQPITGQIGSKQA--IKDNPGVFLNPEQPL-IA-
aMBD2/3  150:C-DLDGFEFDAML----EP---RALRAVGPHVGEET---LLQSVATALH-----VSTQPVTGQTGSRSA-LEK-NPGVFLNPDQPLVQAV
dMBD2/3  128:CHD-SG----EELDDISLPKTI---RTVGPNVNEQT---VLQSVATALHMLNAGV-----HGQ-SSTKADLTK-NAMAFMNPEQPL--MH
             * *  *      *     *         **     *     ** *****     *      **  *  *   * *   * ** ***

bMBD2/3  219:AVTITKEDVRR-QEERVKRARQRLRQ-AL--SVA
aMBD2/3  222:AV--ADEDIKR-QEERVATARKKL-QDALKRFVA
dMBD2/3  198:AV-IISEDDIRKQEDRVGVARRKL-QDALK---T
             **    **  * ** **  **  * * **
```

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

**Figure 3.** SDS–PAGE analysis of bMBD2/3. Lane 1, molecular weight markers; lane 2, purified bMBD2/3 (1*u*g).

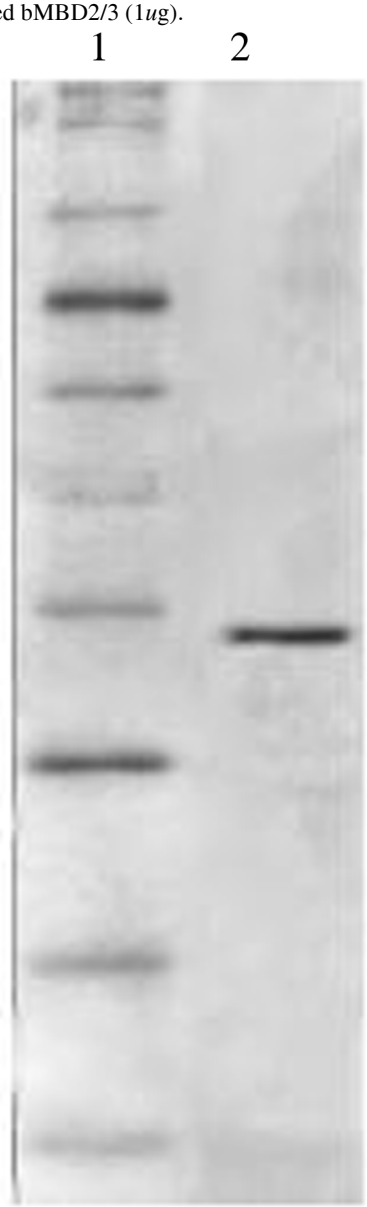
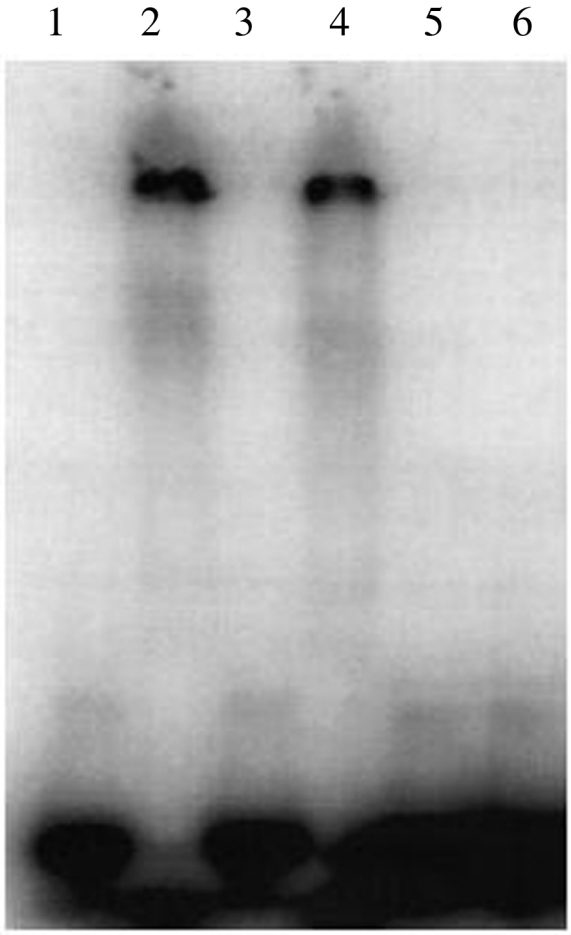



**Figure 4.** EMSA of the purified bMBD2/3. bMBD2/3 was examined for the ability to bind to methylated or unmethylated DNA. Binding reactions were performed as described in Materials and Methods. Lanes 1–4 of each gel contain radiolabelled, methylated DNA as a probe and lanes 5–6 contain radiolabelled unmethylated DNA. For each gel, lanes 1 and 5 contain only the probe without any added protein. Lanes 2 and 6 contain 50 *n*g of protein. Binding was competed with methylated DNA (lane 3) or unmethylated DNA (lane 4) as competitors.




methylation and the transcription of neuropeptides in the brain of *B. mori* because mammalian methyl DNA binding protein regulates the transcription of brain-derived neurotrophic factor (BDNF) (Chen *et al*. 2003).

*West-western blotting analysis using bMBD2/3*

In mammals, some functional proteins that interact with methyl DNA binding protein have been identified using a two hybrid system (Kokura *et al*. 2001; Screaton *et al*. 2003; Lembo *et al*. 2003). But there are no reports of such proteins in insects. We tried a west-western blotting analysis to determine if interacting proteins for bMBD2/3 exist in *B. mori*. In the mammalian brain, methyl DNA binding proteins play important roles in gene expression (Chen *et al*. 2003). So brain extracts were used as proteins to interact with bMBD2/3. As shown in Figure 6, 10 protein bands interacting with bMBD2/3 were detected. Their molecular weights were 25, 29.5, 30, 31, 33, 41, 46, 55, and 65, respectively. One protein band was detected around 27 kDa. This protein was suggested to be bMBD2/3 because western blotting showed the band at about 27 kDa (Figure 6, lane 2). These results suggest that proteins that interact with bMBD2/3 exist in the insect brain.

*Phosphorylation of bMBD2/3 in vitro*

DNA binding proteins such as nuclear receptors, are well known to be phosphorylated by protein kinase such as PKC, cdk/cyclin, MAPK and PKA (Rochette-Egly 2003). Phosphorylation changes DNA binding, ligand binding, recruitment of chromatin remodellers and modifiers to decompact repressive chromatin. Finally

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

**Figure 5.** Immuno blotting analysis of bMBD2/3 expression in the tissues of *Bombyx mori*. Lane 1, control (silk gland; Non-immune serum was used as first antiserum), lane 2, control (antibody adding antigen was used as primary antibody), lane 3, control (rabbit IgG was used as the primary antibody), lane 4, silk gland; lane 5, epidermis; lane 6, fat body; lane 7, gut; lane 8, brain.

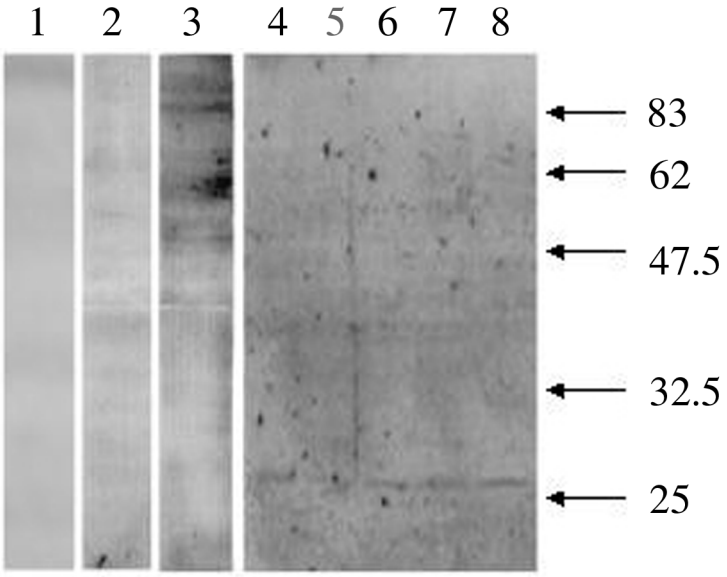



**Figure 7.** Phosphorylation of bMBD2/3 *in vitro*. The reaction mixture containing bMBD2/3 proteins and protein kinases [lane 1, Protein kinase A; lane 2, protein kinase C; lane 3, Ca/CaM dependent protein kinaseII; lane 4, brain extract; lane 5 brain extract (-bMBD2/3)] were incubated with [$\gamma$-$^{32}$P]ATP. The samples were treated with SDS–sample buffer. After electrophoresis, the gel was analyzed using a bioimaging analyzer (upper lanes). The bands stained with Coomassie Brilliant Blue after electrophoresis (lower lanes).

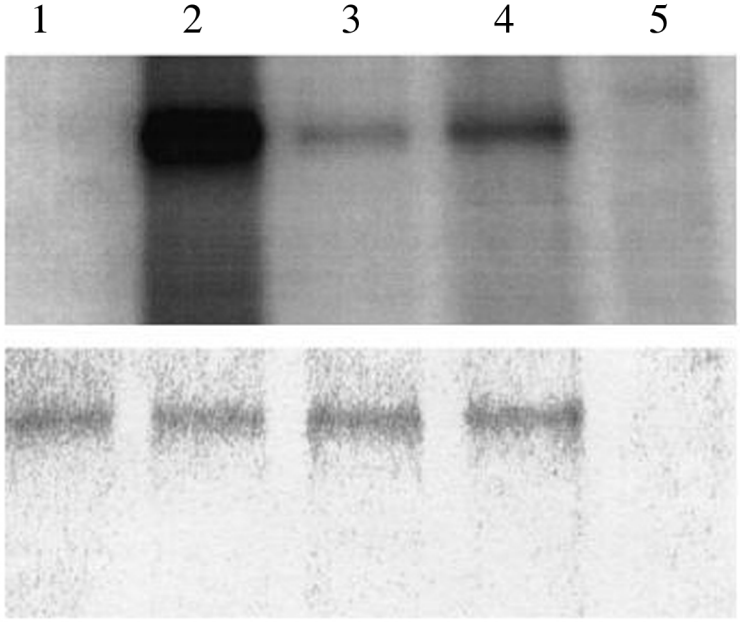
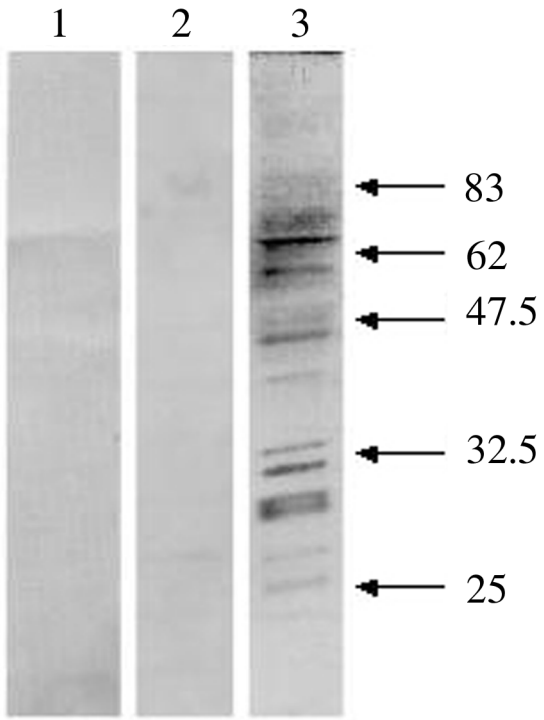



**Figure 6.** West-western blotting analysis. Protein from brain extract was transferred to a nitrocellulose membrane after electrophoresis. The membrane was incubated with bMBD2/3 or GST. After washing, the membrane was incubated with the purified antibody against bMBD2/3. Lane 1, GST protein was added as the protein interacting the proteins transferred on the membrane. Lane 2, No protein to interact was added. Lane 3, bMBD2/3 protein was used as interacting protein.




phosphorylations can regulate transcription positively or negatively. Recently, methyl DNA binding protein was reported to be phosphorylated by protein kinase (Sakai *et al*. 2002). MeCP2, which is abundantly expressed in the central nervous system, was phosphorylated by calcium-dependent protein kinase (Chen *et al*. 2003). We examined the phosphorylation of bMBD2/3 by protein kinase and brain extract *in vitro*. The brain extract from the *B. mori* exhibited protein kinase activity of bMBD2/3 (Figure 7, lane 5). Using mammalian protein kinase, PKC and CaM kinase phosphorylated bMBD2/3 (Figure 7; lanes 3 and 4).

What is the significance of the phosphorylation of bMBD2/3? Phosphorylation may change the affinity for the methylated DNA and interactions with proteins. Phosphorylation of the DNA binding region may produce the release of bMBD2/3 from methylated-DNA and activate transcription. Actually, mammalian MeCP2 was phosphorylated in the brain and released from the promoter of BDNF. The determination of phosphorylated amino acid residues of bMBD2/3 must be determined. Further, *in vivo* phosphorylation of bMBD2/3 must be defined using radiolabeled insect brain.

MBD protein, except MBD4, interact to the various transcriptional regulatory protein such as histone deacetylase, ATPase, and DNA methyl transferase (Tatematsu *et al*. 2000; Brehm *et al*. 2000). Activated PKC was known to enter the nucleus from the cytosol and function. Phosphorylation of bMBD2/3 in the nucleus may change the interaction of these proteins. Regulatory proteins

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

such as ATPase and deacetylase have been shown to be present in insects by enzymatic assays (Tweedie *et al*. 1999). As described above, bMBD2/3 was distributed in almost all tissues of adult *B. mori* (Figure 5) and some proteins that interact with bMBD2/3 in the insect tissues were detected using west-western blotting analysis (Figure 6). So it would be possible to identify and purify the regulatory proteins that interact with bMBD2/3 directly, using bMBD-bound affinity chromatography. Such information may clarify the relationship between the phosphorylated bMBD2/3 and the regulatory proteins.

Methylation of DNA has been reported in various insects. Insect MBD protein was reported to be only one type, although vertebrates have five types. The mammalian proteins each have specific characters. Mutations in MeCP2 are the cause of Rett syndrome, an X-linked progressive neurological disorder, and MeCP2 functions as a selective regulator of neuronal gene expression (Armstrong 1997; Chen *et al*. 2001). MBD3 plays an important role in embryo development and MBD2 influences some forms of behavior (Jung *et al*. 2003; Hendrich 2001). MBD4 regulates the removal of thymine or uracil from a mismatched CpG site (Hendrich *et al*. 1999). Recently, proteins that specifically interact with MBD were identified using a two hybrid system (Kokura *et al*. 2001; Screaton *et al*. 2003; Lembo *et al*. 2003). These interacting proteins probably determine the physiological specificity of MBD proteins as described above. Identification of interacting proteins with bMBD2/3 will help to clarify the physiological role of bMBD2/3 in the insect cell and discover new functions specific to insect MBD.

## Acknowledgments

We thank Dr. Mita of National Institute of Entomological and Sericultural Science for providing cDNA clones of bMBD2/3.

## References

Altschul L, Stephen F, Warren G, Webb M, Eugene WM, David JL. 1990. Basic local alignment search tool. *Journal of Molecular Biology* 215: 403–410.

Armstrong DD. 1997. Review of Rett syndrome. *Journal of Neuropathological Experimental Neurology* 56: 843–849.

Ballestar E, Pile LA, Wassarman DA, Wolffe AP, Wade PA. 2001. A *Drosophila* MBD family member is a transcriptional corepressor associated with specific genes. *European Journal of Biochemistry* 268: 5397–5406.

Bartoleme MS, Tilghman SM. 1997. Genomic imprinting in mammals. *Annual Reviews of Genetics* 31: 493–525.

Bird AP, Wolffe AP. 1999. Methylation–induced repression belts, braces and chromatin *Cell* 99: 451–454.

Brehm A, Langst G, Kehle J, Clapier CR, Imhof A, Eberharter A, Muller J, Becker PB. 2000. dMi-2 and ISWI chromatin remodeling factors have distinct nucleosome binding and mobilization properties. *EMBO Journal* 19: 4332–4341.

Chen RZ, Akbarian S, Tudor M, Jaenisch R. 2001. Deficiency of methyl-CpG binding protein-2 in CNS neurons results in a Rett-like phenotype in mice. *Nature Genetics* 27: 327–331.

Chen WG, Chang Q, Lin Y, Meissner A, West AE, Griffith EC, Jaenisch R, Greenberg ME. 2003. Derepression of BDNF transcription involves calcium-dependent phosphorylation of MeCP2. *Science* 302: 885–889.

Constancia M, Pickard B, Kersey G, Reik W. 1998. Imprinting mechanism. *Genome Research* 8: 881–900.

Field LM. 2000. Methylation and expression of amplified esterase genes in the aphid *Myzus persicae* (Sulzer). *Biochemical Journal* 349: 863–868.

Gowher H, Leismann O, Jeltsch A. 2000. DNA of *Drosophila melanogaster* contains 5-methyl cytosine. *EMBO Journal*. 19: 6918–6923.

Heard E, Clerc P, Avner P. 1997. X-chromosome inactivation in mammals. *Annual Review of Genetics*. 31: 571–610.

Hendrich B, Bird A. 1998. Identification and characterization of a family of mammalian methyl-CpG binding proteins. *Molecular and Cellular Biology*. 18: 6538–6547.

Hendrich B, Harderland U, Ng HH, Jiricny J, Bird A. 1999. The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. *Nature* 401: 301–304.

Hendrich B, Guy J, Ramsahoye B, Wilson VA, Bird A. 2001. Closely related proteins MBD2 and MBD3 play distinctive but interacting roles in mouse development. *Genes and Development* 15: 710–723.

Hung MS, Karthikeyan N, Huang B, Koo HC, Kiger J, Shen CKJ. 1999. *Drosophila* proteins related to vertebrate DNA (5-cytosine) methyltransferase. *Proceedings of National Academic Sciences USA*. 96: 11940–11945.

Jung BP, Zhang G, Nitsch R, Trogadis J, Nag S, Eubanks JH. 2003. Differential expression of methyl CpG binding domain containing factor MBD3 in the developing and adult rat brain. *Journal of Neurobiology*. 55: 220–232.

Kokura K, Kaul SC, Wadhwa R, Nomura T, Khan MM, Shinagawa T, Yasukawa T, Colmenares C, Ishii S. 2001. The ski protein family is required for MeCP2-mediated transcriptional repression. *Journal of Biological Chemistry* 276: 34115–34121.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 227: 680–685.

Lembo F, Pero R, Angrisano T, Vitiello C, Iuliano R, Bruni CB, Chiariotti L. 2003. MBDin, a novel MBD2-interacting protein, relieves MBD2 repression potential and reactivates transcription from methylated promoters. *Molecular and Cellular Biology* 23: 1656–1665.

Li E, Bestor T, Jaenisch R. 1992. Targeted mutation of the DNA methyltransferase gene results in embryonic lethality. *Cell* 69: 915–926.

Lowry OH, Rosenbrough NJ, Farr AL, Randall RJ. 1951. Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* 193: 265–275.

Lyko F, Ramsahoye BH, Jaenisch R. 2000. DNA methylation in *Drosophila melanogaster*. *Nature* 408: 538–540.

Marhold J, Zbylut M, Lankenau DH, Li M, Gerllich D, Ballestar E, Mechler BM, Lyko F. 2002. Stage-specific chromosomal association of *Drosophila* dMBD2/3 during genome activation. *Chromosoma* 111: 13–21.

Okano M, Bell DW, Haber DA, Li E. 1999. DNA methyltransferase Dnmt3a and Dnmt3b are essential for *de novo* methylation

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use

and mammalian development. *Cell* 99: 247–257.

Patel CV, Gopinathan KP, 1987. Determination of trace amounts of 5-methyl cytosine in DNA by reverse-phase high-performance liquid chromatography. *Analytical Biochemistry* 164: 164–169.

Reddy MN, Tang LY, Lee TL, Shen C-KJ, 2003 A candidate gene for *Drosophila* genome methylation. *Oncogene* 22: 6301–6303.

Rein T, DePamphilis ML, Zorbas H. 1998 Identifying 5-methylcytosine and related modification in DNA genomes. *Nucleic Acids Research* 26: 2255–2264.

Rochette-Egly C. 2003. Nuclear receptors: Integration of multiple signalling pathways through phosphorylation. *Cellular Signalling*15: 355–366.

Roder K, Hung MS, Lee TL, Lin TY, Xiao H, Isobe K, Juang JL, Shen CKJ. 2000. Transcriptional repression by *Drosophila* methyl-CpG binding proteins. *Molecular and Cellular Biology* 20: 7401–7409.

Sakai H, Urano T, Ookata K, Kim MH, Hirai Y, Saito M, Nojima Y, Ishikawa F. 2002. MBD3 and HDAC1, two components of the NuRD complex, are localized at Aurora-A positive centrosomes in M phase. *Journal of Biological Chemistry* 277: 48714–48723.

Screaton RA, Kiessling S, Sansom OJ, Millar CB, Maddison K, Bird A, Clarke AR, Frisch SM. 2003. Fas-associated death domain protein interacts with methyl-CpG binding domain protein 4: A potential link between genome surveillance and apoptosis. *Proceedings of National Academic Sciences USA* 100: 5211–5216.

Siegfried Z, Cedar H.1997 DNA methylation: A molecular lock. *Current Biology* 7: 305–307.

Tatematsu K, Yamazaki T, Ishikawa F. 2000. MBD2-MBD3 complex binds to hemi-methylated DNA and forms a complex containing DNMT1 at the replication foci in late S phase. *Genes to Cells* 5: 677–688.

Tatematsu K, Hirokawa M, Kosegawa E. 2003. A novel method, PCR-mediated amplification of methylated sites, detects methylated CpG islands in the genome of *Bombyx mori*. *Journal of Insect Biotechnology and Sericology* 72: 191–195.

Tweedie S, Ng HH, Barlow AL, Turner BM, Hendrich B, Bird A. 1999. Vestiges of a DNA methylation system in *Drosophila melanogaster*? *Nature Genetics* 23: 389–390.

Downloaded From: https://staging.bioone.org/journals/Journal-of-Insect-Science on 23 Mar 2025
Terms of Use: https://staging.bioone.org/terms-of-use