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Binding of *Drosophila* maternal Mamo protein to chromatin and specific DNA sequences

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ABSTRACT

Alterations in chromatin structure dynamically occur during germline development in *Drosophila* and are essential for the production of functional gametes. We had previously reported that the maternal factor Mamo, which contains both a BTB/POZ domain and C_2H_2 zinc-finger domains and is enriched in primordial germ cells (PGCs), is required for the regulation of meiotic chromatin structure and the production of functional gametes. However, the molecular mechanisms by which Mamo regulates germline development remained unclear. To evaluate the molecular function of Mamo protein, we have investigated the binding of Mamo to chromatin and DNA sequences. Our data show that Mamo binds to chromatin and specific DNA sequences, particularly the polytene chromosomes of salivary gland cells. Overexpression of Mamo affected the organization of polytene chromosomes. Reduction in maternal Mamo levels impaired the formation of germline-specific chromatin structures in PGCs. Furthermore, we found that the zinc-finger domains of Mamo directly bind to specific DNA sequences. Our results suggest that Mamo plays a role in regulating chromatin structure in PGCs.

1. Introduction

In many animals, mature gametes transmit genetic information to the next generation. Alterations in chromatin structure occur dynamically during germline development and are essential for the production of functional gametes. However, the developmental programs that regulate germline-specific chromatin structures remain unclear.

In *Drosophila*, the germ plasm localized in the posterior pole region of the early embryo is inherited in PGCs. The PGCs migrate within the embryo to reach the gonads, where they later differentiate into functional gametes. The germ plasm contains maternal factors sufficient for germline development. Several maternal factors have been identified thus far, and their functions are required for cellular events in germline development. For example, mitochondrial large ribosomal RNA and Germ cell-less protein are both required for PGC formation. The maternal Nanos protein, a CCHC zinc-finger protein that is a component of a translational repressor complex, functions in the arrest of cell division, repression of apoptosis, transcriptional quiescence in PGCs, and repression of somatic differentiation of PGCs. The Polar granule component protein, which is a transcriptional repressor, is required for the maintenance of PGCs [1].

We previously found that the maternal factor Mamo, which contains both a BTB/POZ domain and C_2H_2 zinc-finger domains and is enriched in PGCs, is necessary for the execution of meiosis and production of functional gametes [2]. Because many BTB/POZ-zinc finger proteins bind to DNA and alter the chromatin structure to exert their effects, Mamo may promote germline development through the regulation of chromatin structure. However, the molecular mechanisms by which Mamo regulates germline development remained unclear.

To determine whether Mamo could regulate chromatin structure, we have investigated the binding of Mamo protein to chromatin and DNA sequences. Using expression analysis, we found that Mamo preferentially accumulates in the nuclei of salivary gland cells and binds to polytene chromosomes. Random oligonucleotide selection and electrophoretic mobility shift assay (EMSA) showed that the zinc-finger domains of Mamo directly bind to specific DNA sequences. Furthermore, inhibition of the function of maternal Mamo impaired the formation of germline-specific chromatin structures in PGCs. Our results suggest mechanisms by which

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Mamo participates in the regulation of the chromatin structure in PGCs.

2. Material and methods

2.1. Fly stocks

The wild-type strain used was Oregon-R (OR). *mamo*^{SVA53} has been described previously [2]. *nanos-Gal4* was a gift from Dr. R. Lehmann. *P{hs-Gal4(II)}* (stock number 2077), *P{hs-Gal4(III)}* (stock number 1799), and *w ovo*^{D1} *v*^{24} *P{FRT}101; P{hsFLP}38* were obtained from the Bloomington Stock Center. All stocks were maintained in a standard *Drosophila* medium.

2.2. Transgenic flies

Different FLAG-tagged Mamo fragments were amplified from the pBluescript-*mamo*-FLAG plasmid [2] by PCR. The MZD-FLAG fragment was amplified using the MZD-FLAG-F (5′-CGCGGATCCATGGATGCCATGCCCGTGATT-3′) and T3 (5′-AATTAACCCTCACTAAAGGG-3′) primers; this step was followed by digestion with *Bam*HI and *Xba*I and subcloning into the *Bam*HI/*Xba*I sites of the pUASp vector. The L-POZ-FLAG fragment was amplified using the POZ-FLAG-F (5′-CGGGGTACCATGGAACAAAAACTCATCTCAGAAGAGGATCTGATGGGCAGTGAGCACTAC-3′) and L-POZ-FLAG-R (5′-CTAGTCTAGATTATTTATCATCATCATCTTTATAATCCTCGTCATCCGAGTGCG-3′) primers. The S-POZ-FLAG fragment was amplified using the POZ-FLAG-F and S-POZ-FLAG-R (5′-GCTCTAGATATTTATCATCATCATCTTTATAATCGTTCGTCATCTCGGCCAG-3′) primers. The underlined text indicates the recognition sites of restriction enzymes. These fragments were digested with *Xba*I and *Kpn*I and then subcloned into the *Xba*I and *Kpn*I sites of the pUASp vector, respectively. The constructs were injected into *y w* flies by using a standard procedure [3].

2.3. Immunostaining

Salivary glands were dissected from third instar larvae expressing FLAG-tagged Mamo fragments under the control of the *hs-Gal4* driver and stained using standard techniques. Embryos expressing Mamo-FLAG under the control of the *nanos-Gal4* driver were fixed and dissected using tungsten needles to enhance antibody penetration into the embryonic gonads. Then, the embryos were stained as described [4]. The following primary antibodies were used: mouse anti-FLAG M2 (1:200, Sigma), mouse anti-histone H3 CMA301 (1:10, H. Kimura), and rabbit anti-Vasa (1:500, S. Kobayashi). Alexa Fluor 488- and 568-conjugated second antibodies were used at a 1:1000 concentration. Hoechst or propidium iodide (PI) was used to visualize DNA. Stained salivary glands and embryos were observed under confocal microscopy (TCS NT, Leica Microsystems; or FV2000, Olympus). Polytene squashes and staining were performed using a standard protocol [5]. Rabbit anti-FLAG antibody (Sigma) preabsorbed with fixed embryos was used at 1:200 for polytene immunostaining.

2.4. Expression and purification of GST-MZD protein

FLAG-tagged Mamo fragment (AA 798–1089) was amplified from pBluescript-*mamo*-FLAG by PCR with the primers GST-MZD-F (5′-CGGGATCCCCCCACTGCACATGTTTCCGT-3′) and T3 (5′-AATTAACCCTCACTAAAGGG-3′). This fragment was digested with *Xba*I and blunted. The blunt-ended fragment was digested with *Bam*HI and then subcloned into the *Bam*HI/*Sma*I sites of pGEX-5X-1. *Escherichia coli* BL21 was used as the host strain for transformation. GST-MZD expression was induced by adding IPTG (0.5 mM final concentration) and incubating at 37 °C for 2 h. The bacterially expressed GST-MZD fusion protein was purified using glutathione-Sepharose 4B beads. The purified protein was analyzed by western blotting using a mouse anti-FLAG antibody (1:1000).

2.5. Random oligonucleotide selection

Random oligonucleotides (5′-CGCTCGAGGGATCCGAATTCN$_{32}$-TCTAGAAAGCTTGTCGACGC-3′, 0.27 μg) were mixed with the *Sal*I-*Xba*I primer (5′-GCGTCGACAAGCTTTCTAGA-3′, 0.5 μg), annealed, and converted into double-stranded DNA by using EX Taq polymerase (Takara). The double-stranded random oligonucleotides were mixed with purified GST-MZD fusion protein (1 μg) and incubated in binding buffer (20 mM HEPES, 50 mM KCl, 2 mM $MgCl_2$, 10% glycerol, 1 mM DTT, 1 mM PMSF, 0.1 mM $ZnSO_4$, 1 μg Poly[-dI-dC]) for 30 min at 25 °C. Then, glutathione-Sepharose 4B beads were added, and the mixture was further incubated for 30 min at 25 °C. The beads were washed 4 times with the binding buffer. Bound oligonucleotides were eluted and PCR-amplified using the *Sal*I-*Xba*I primer and *Xho*I-*Eco*RI primer (5′-CGCTCGAGGGATCCGAATTC-3′). Five rounds of binding and amplification were performed. Products from the final amplification were gel purified, subcloned, and sequenced.

2.6. EMSA

The no. 77 sense probe (5′-GTCCATGCTTGGGTTGTGTGCGTTTGGTTG-3′) and no. 77 antisense probe (5′-CAACCAAACGCACACAACCCAAGCATGGAC-3′) were annealed. Then, the double-stranded DNA was end-labeled by digoxigenin-11-ddUTP using the DIG gel shift kit, 2nd generation (Roche). The DIG-labeled probes (30 fmol) were incubated with 60 ng GST or GST-MZD protein in a reaction buffer (20 mM HEPES, pH7.6, 1 mM EDTA, 10 mM $[NH_4]_2SO_4$, 1 mM DTT, 0.2% Tween 20, 30 mM KCl, 1 μg poly[d(I-C)], 0.1 μg poly L-lysine) at 25 °C for 15 min. After incubation, the protein DNA complexes were separated by 7% nondenaturing polyacrylamide gel electrophoresis. The protein DNA complexes were transferred to positively charged nylon membranes (Roche) by electroblotting and detected using the DIG gel shift kit. For binding competition assays or super shift assays, 25 pmol of unlabeled DNA or 1 μg of mouse monoclonal anti-FLAG antibodies was added to the reaction mixture.

3. Results

3.1. Nuclear localization of the Mamo protein in salivary gland cells

To evaluate the cellular and molecular function of Mamo protein, we performed expression and functional analyses of several FLAG-tagged Mamo fragments (Fig. 1). Because the small size of embryonic cells makes observing Mamo fragment localization difficult, we used larval salivary gland cells, which are larger than most other fly cells. We examined the intracellular localization of the different fragments of Mamo in the salivary gland cells by using the *upstream activation sequence* (*UAS*)-*Gal4* system [6]. When *UAS-Mamo-FLAG* was expressed under the control of a *heat shock-Gal4* driver, *hs-Gal4 (III)*, Mamo-FLAG was detected in salivary gland cells without heat shock treatment because of leaky expression. We found that Mamo-FLAG preferentially accumulated in the nuclei of salivary gland cells (Fig. 1B). The MZD-FLAG fragment, which contains the C_2H_2 zinc-finger domains but lacks the BTB/POZ domain, was also localized to the nuclei of salivary gland cells (Fig. 1B). In contrast, the L-POZ-FLAG and S-POZ-FLAG fragments did not localize to the nuclei. These results suggest that both the full-length Mamo protein and MZD fragment predominantly

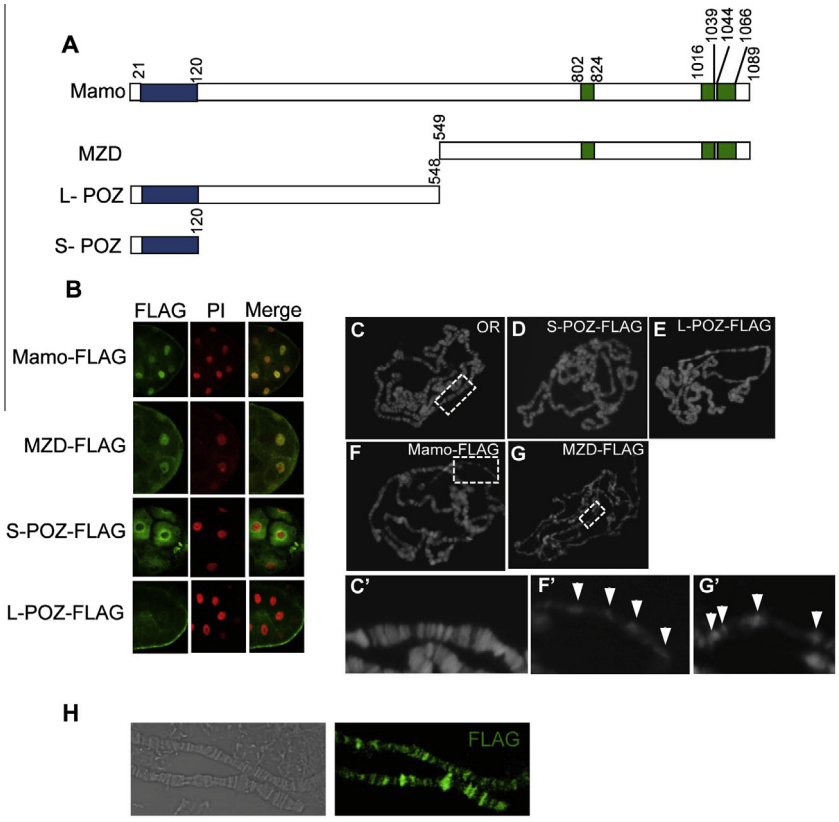

Fig. 1. Mamo localizes to the nuclei of salivary gland cells and binds to polytene chromosomes. (A) Schematic representation of FLAG-tagged Mamo constructs. Mamo contains the BTB/POZ domain (blue) and C_2H_2 zinc-finger domains (green). Numbers indicate amino acid residues. In all the constructs, the FLAG-tag was added at the C-terminus. (B) Salivary glands cells expressing Mamo-FLAG, MZD-FLAG, S-POZ-FLAG, and L-POZ-FLAG were stained with an anti-FLAG antibody (green) and PI (red). (C–G) Polytene chromosomes from wild type (C), salivary glands expressing S-POZ-FLAG (D), L-POZ-FLAG (E), Mamo-FLAG (F) and MZD-FLAG (G), were stained with Hoechst. (C′, F′ and G′) Enlargements of outlined region in (C), (F) and (G). Arrows indicate bands. Overexpression of Mamo-FLAG or MZD-FLAG causes polytene chromosomes to lose their banding patterns. (H) Polytene chromosomes from salivary glands cells expressing Mamo-FLAG were stained with an anti-FLAG antibody (green).

localize to the nuclei of salivary gland cells, which implies that Mamo possesses a nuclear function.

3.2. Mamo binds to polytene chromosomes and affects the organization of polytene chromosomes

Unexpectedly, we found that expression of full-length Mamo or MZD resulted in defects in the organization of polytene chromosomes in salivary glands (Fig. 1F and G). When L-POZ-FLAG or S-POZ-FLAG fragments were expressed, the size of the salivary glands was comparable to that of the wild type. The polytene chromosomes from salivary gland cells expressing L-POZ-FLAG or S-POZ-FLAG were indistinguishable from those of the wild type (Fig. 1C–E). However, salivary glands expressing Mamo-FLAG or MZD-FLAG were smaller than wild-type salivary glands. Moreover, the expression of Mamo-FLAG or MZD-FLAG affected the structure of polytene chromosomes; the fixed polytene chromosome squashes from the salivary glands expressing Mamo-FLAG or MZD-FLAG were thinner than those of the wild type, and their banding pattern was disrupted (Fig. 1F′ and G′). These data imply that Mamo may affect the organization of polytene chromosomes through direct binding to polytene chromosomes. To determine whether Mamo could bind to chromosomes, we examined the distribution of Mamo-FLAG in polytene chromosomes from salivary glands expressing Mamo-FLAG. Because Mamo overexpression severely impaired the organization of polytene chromosomes, we could not examine the distribution of Mamo in polytene chromosomes under the condition described above. Therefore, we used a weaker Gal4 driver, *hs-Gal4 (II)*, to express *UAS-Mamo-FLAG* in salivary gland cells and examined Mamo-FLAG localization on polytene chromosomes. Immunostaining showed that Mamo binds to polytene chromosomes, as numerous Mamo signals were observed along the chromosome arms (Fig. 1H). Mamo bound to discrete regions of polytene chromosomes. These data suggest that Mamo binds to chromatin and that it plays a role in the regulation of chromatin structure.

3.3. Mamo is required for a germline-specific chromatin structure in PGCs

Maternal Mamo is enriched in PGCs and exhibits a punctate staining pattern in the nuclei [2]. To confirm this distribution, we expressed *UAS-Mamo-FLAG* in PGCs under the control of the *nanos-Gal4* driver. Immunostaining of *nanos-Gal4 > UAS-Mamo-FLAG* embryos showed punctate nuclear staining in the PGCs (Fig. 2A). This expression pattern, in conjunction with the phenotypes in Mamo-overexpressing salivary gland cells, indicates that Mamo may be involved in the regulation of chromatin structure in PGCs. We stained wild-type embryos by using an anti-histone H3 monoclonal antibody [7] to examine chromatin structure in PGCs. Immunostaining showed that germline-specific chromatin condensation occurs in PGCs at embryonic stage 15 (Fig. 2B). Condensed chromatin was observed in PGCs but not in the surrounding somatic cells. Next, we investigated the chromatin structure in *mamo*-PGCs, which were formed in embryos derived from germline clones homozygous for the $mamo^{SVA53}$ mutation

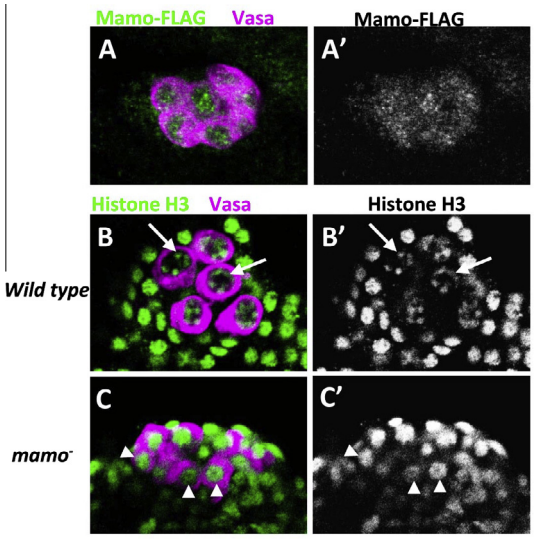

Fig. 2. Maternal *mamo* mutation affects germ cell-specific chromatin condensation in PGCs. (A) PGCs in *nanos-Gal4>UAS-Mamo-FLAG* embryos were stained with anti-FLAG (green) and anti-Vasa (magenta) antibodies. (A′) FLAG channel is shown separately. (B and C) PGCs in wild-type (B) and *mamo*$^{-}$ embryos (C) at stage 15 were stained with anti-histone H3 (green) and anti-Vasa (magenta) antibodies. (B′ and C′) Histone H3 channel is shown alone. Germline-specific chromatin condensation occurs in wild-type PGCs (arrows). Less-condensed chromatin was observed in *mamo*-PGCs (arrowheads).

[2]. The germline-specific chromatin structure was affected in *mamo*-PGCs. Chromatin condensation was observed in most nuclei of wild-type PGCs at embryonic stages 15–16 (99.3%, $n = 299$). In contrast, less-condensed chromatin, which is similar to the chromatin in somatic nuclei, was observed in 45.5% of nuclei of *mamo*-PGCs ($n = 200$, $P < 0.001$; Fig. 2C). These results show that Mamo plays a role in regulating the germ cell-specific chromatin structure.

3.4. MZD directly binds to specific DNA sequences

The C_2H_2 zinc-finger domain is shared by DNA-binding proteins and serves as an interface for DNA recognition [8]. To determine whether the C_2H_2 zinc-finger domains of the Mamo protein directly bind to DNA, we generated and characterized a recombinant fusion protein containing the zinc-finger domains. We chose a Mamo fragment (AA 798–1089) that contains 3 zinc-finger domains and has a predicted molecular weight of 29 kDa. This fragment was appended to the C-terminus of the GST protein, which is predicted to contribute 55 kDa to an in-frame fusion protein (GST-MZD). We introduced the FLAG-tag at the C-terminus of GST-MZD. The bacterially expressed protein was affinity-purified and analyzed by SDS–PAGE and by western blotting using an anti-FLAG antibody (Fig. 3A, B). Purified GST-MZD protein was used for the following assays. To address the DNA-binding activity of MZD and to identify its binding sequences, we performed random oligonucleotide selection using the GST-MZD protein [9,10]. The 49 oligonucleotides obtained by random oligonucleotide selection were subcloned and sequenced. Our visual inspection showed that the sequences had a strong preference for guanine (Fig. 3C). To identify a possible consensus sequence for binding of MZD, the sequences of the 49 oligonucleotides were aligned using Multiple Em for Motif Elicitation (MEME). Most of the sequences were found to contain at least 1 TGCGT (Fig. 3C, D). Next, we chose DNA sequence no. 77, which contains 1 possible consensus sequence, and examined the binding of MZD to the DNA sequence by EMSA. EMSA analyses showed that GST-MZD specifically binds to the sequence. In contrast, GST alone showed no DNA-binding activity, and GST-MZD was bound to the DNA sequence (Fig. 3E). To determine whether the binding of GST-MZD was sequence-specific, we performed competition experiments. Excess unlabeled probe abolished the mobility shift. In addition, to determine whether the mobility shift was specific for GST-MZD, anti-FLAG antibody was included in the EMSA reaction. The FLAG antibody supershifted the high-molecular-weight DNA–protein complexes. To determine whether the possible consensus sequence (TGCGT) is necessary for GST-MZD binding, we deleted the sequence from the probe (no. 77ΔTGCGT probe) and performed EMSA. When probe no. 77ΔTGCGT was used as a competitor, the mobility shift was not abolished (data not shown). In addition, when we performed EMSA using the no. 77 ΔTGCGT probe and GST-MZD, the shifted DNA–protein complex band was hardly detectable (Fig. 3F). These data demonstrate that MZD can directly bind to specific DNA sequences.

4. Discussion

In this study, we found that Mamo protein is predominantly localized to the nuclei of salivary gland cells and binds to polytene chromosomes. Overexpression of Mamo in salivary gland cells affects the organization of the polytene chromosome. Reduction in maternal Mamo function impairs the formation of germline-specific chromatin structure in PGCs. Furthermore, we found that MZD directly binds to specific DNA sequences. These findings support the roles of maternal Mamo protein in the regulation of chromatin structure in PGCs. Thus, Mamo constitutes a maternal protein that is enriched in PGCs and binds to chromatin and specific DNA sequences.

We found that Mamo protein accumulates in the nuclei of salivary gland cells. Proteins localized to the nucleus usually contain classical nuclear localization signals (cNLS), i.e., monopartite or bipartite cNLSs, which are recognized by the importin α-mediated nuclear transport system [11]. To determine whether Mamo contains cNLSs, we analyzed the Mamo primary structure by using the search algorithm from PSORT II [12] and found that Mamo does not contain cNLSs. Thus, the nuclear import of Mamo may be mediated by other systems. Signal transducer and activator of transcription (STAT) 1 does not contain cNLSs but is recognized and imported to the nucleus via an importin-α mediated-system [13]. It is therefore plausible that nuclear localization of Mamo may be mediated by importin α. In contrast to the results for salivary gland cells, a considerable amount of Mamo was also present in the cytoplasm of PGCs [2] (Fig. 2). Thus, the nuclear localization of Mamo may be regulated by a germline-specific mechanism [1].

Here, we have shown that Mamo is required for the formation of germline-specific chromatin structures in PGCs. A similar germline-specific chromosome condensation in PGCs has been reported in *Caenorhabditis elegans*, which indicates that the chromatin architecture in PGCs is conserved and may be essential for germline development [14,15]. The oocytes derived from the PGCs with reduced maternal Mamo activity fail to form meiosis-specific chromosomal configurations [2]. Thus, Mamo may have several functions in the regulation of chromatin structure during germline development. Because BTB/POZ-zinc finger proteins are known to form complexes with chromatin regulators to exert their function [16,17], Mamo may recruit chromatin factors to condense chromatin in PGCs. It is also possible that Mamo is involved in the regulation of gene expression of chromatin factors in PGCs.

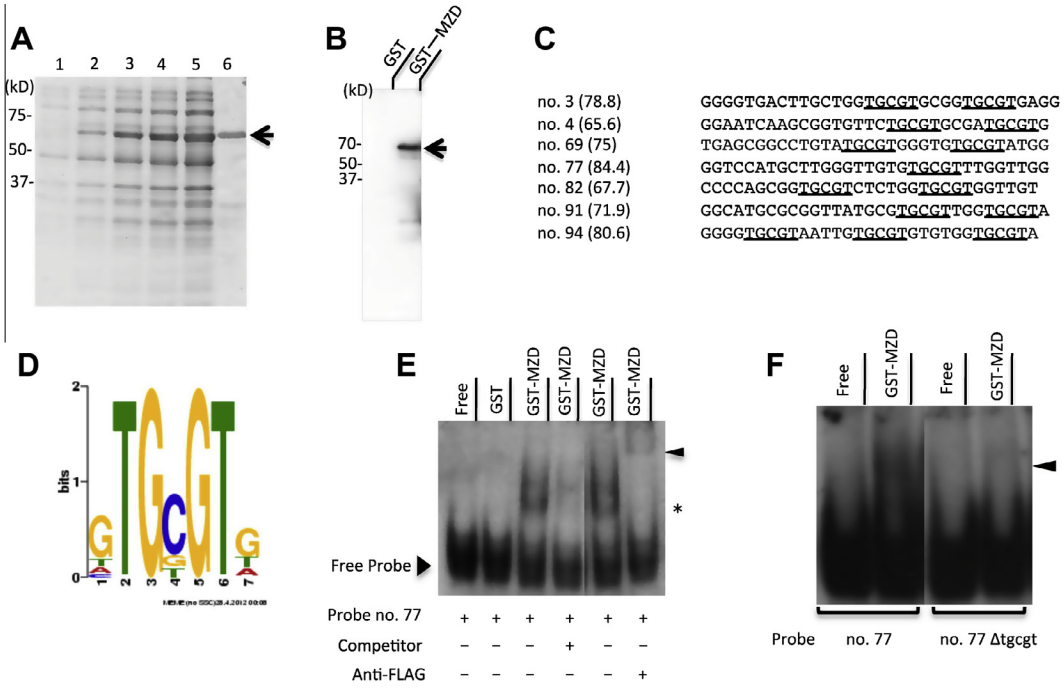

Fig. 3. MZD directly binds to specific DNA sequences. (A) Bacterially expressed and purified GST-MZD was analyzed by SDS–PAGE. lane 1, uninduced control; lane 2, 0.5 h induced; lane 3, 1 h induced; lane 4, 1.5 h induced; lane 5, 2 h induced; and lane 6, purified protein. (B) Purified GST-MZD protein was detected by western blotting using an anti-FLAG antibody. The arrows in A and B indicate GST-MZD. (C) Sequences of oligonucleotides selected by GST-MZD binding have a high GC content (65.6–84.4%). Possible consensus sequences (TGCGT) are underlined. (D) A possible consensus sequence for MZD binding. The sequences selected by random oligonucleotide selection were aligned with MEME. (E) Electrophoretic mobility shift assay (EMSA) analysis shows that GST-MZD specifically binds to the selected oligonucleotide (no. 77). The labeled probe without protein (Free) or with GST served as a negative control. The bound (asterisk) and supershifted (arrowhead) probes are indicated. (F) EMSA assay showing that the consensus sequence (TGCGT) is necessary for GST-MZD binding.

We found that the GST-MZD fusion protein binds specific DNA sequences that are rich for guanine. These data are consistent with previous reports showing that the consensus binding sites for C_2H_2 zinc-finger proteins contain guanine-rich sequence [18,19]. Mamo may regulate chromatin structure in PGCs through direct binding to specific DNA sequences and/or target genes.

Acknowledgments

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