Transcriptional regulation of the Drosophila moira and osa genes by the DREF pathway

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ABSTRACT

The DNA replication-related element binding factor (DREF) plays an important role in regulation of cell proliferation in Drosophila, binding to DRE and activating transcription of genes carrying this element in their promoter regions. Overexpression of DREF in eye imaginal discs induces a rough eye phenotype in adults, which can be suppressed by half dose reduction of the osa or moira (mor) genes encoding subunits of the BRM complex. This ATP-dependent chromatin remodeling complex is known to control gene expression and the cell cycle. In the 5’ flanking regions of the osa and mor genes, DRE and DRE-like sequences exist which contribute to their promoter activities. Expression levels and promoter activities of osa and mor are decreased in DREF knockdown cells and our results in vitro and in cultured cells indicate that transcription of osa and mor is regulated by the DRE/DREF regulatory pathway. In addition, mRNA levels of other BRM complex subunits and a target gene, string/cdc25, were found to be decreased by knockdown of DREF. These results indicate that DREF is involved in regulation of the BRM complex and thereby the cell cycle.

INTRODUCTION

Promoter regions of DNA replication-related genes contain a common regulatory sequence, 5’-TATCGATA, named the DNA replication-related element (DRE) (1). A specific DRE-binding factor (DREF) has been identified (2) and numerous studies have revealed that the DREF/DRE system is required for the expression of many genes involved in cell proliferation and the cell cycle (3). Computational analysis of Drosophila gene promoters revealed that DRE is one of the most prevalent motifs in core promoters (4). In fact immunostaining of polytene chromosomes with anti-DREF antibody revealed that DREF binds to hundreds of interband regions on the polytene chromosomes (5). In addition SAGE analyses revealed that DRE exists in upstream regions of many genes expressed in proliferating cells in eye imaginal discs (6). RNAi-mediated knockdown of DREF in growing tissues also provided direct evidence that it is necessary for cell cycle and cell growth control (7,8).

It is reported that DREF is a component of the TATA box-binding protein-related factor 2 (TRF2) complex (9), which interacts with basal transcription machinery (10). This complex is directed to promoters of the PCNA and DNA polymerase ε 180 kDa genes and upregulates their expression (9). DNA-binding activity of DREF is inhibited by dMi-2, which is the ATPase of an ATP-dependent chromatin remodeling complex (5). In addition, a homeodomain protein Dll interacts with the DNA-binding domain of DREF and prevents its DRE-binding activity (11).

Overexpression of the DREF in eye imaginal discs induces ectopic S phase, apoptosis and inhibited photoreceptor cell differentiation, resulting in a rough eye phenotype in adults. Enhancement of this phenotype was observed in crosses of DREF-overexpressing flies with Dll mutants. This observation combined with molecular and biochemical analyses suggested that Dll is a negative regulator of DREF (11,12).

By genetic screening of mutations that modify the rough eye phenotype, we searched for other genes that genetically interact with DREF. For example, the rough eye phenotype was suppressed by half dose reduction of the trithorax group genes brahma (brm), osa and moira (mor) (12) known to maintain expression patterns of homeotic genes (13). The three genes encode subunits of the BRM complex, a SWI/SNF class ATP-dependent chromatin remodeling complex in Drosophila, altering nucleosome structures to activate or repress gene transcription (14,15). BRM complexes can be subclassified into BAP and PBAP types depending on their signature subunits (16). OSA is specific to BAP, and Polybromo and BAP170 are specific to PBAP complex. BRM, MOR and five other proteins (Snrl, BAP111, BAP60, BAP55 and Actin) are shared with both complexes (16). Recent studies have revealed

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functional differences between BAP and PBAP in cell cycle regulation (17), only BAP promoting G2/M progression. BRM complexes also genetically interact with cell cycle regulators, E2F and cyclin E (18,19), suggesting a role in negatively controlling G1/S progression.

To clarify the relationship between DREF and BRM complexes, we searched for DRE or DRE-like sequences in 5′ flanking regions of brm, osa and mor, and found examples in the osa and mor genes. Binding of DREF to DRE in both genes could be demonstrated in vitro and in vivo and luciferase transient expression assays confirmed that DRE and DREF are important in their promoter activities. Furthermore, mRNA levels of osa and mor were reduced in DREF knockdown cells. These results indicate that transcription of osa and mor is regulated by the DRE/DREF regulatory pathway. In addition, mRNA levels of other BRM complex subunits and of its target gene, DREF regulatory pathway. In addition, mRNA levels of other BRM complex subunits and of its target gene, string/cdc25, were decreased by knockdown of DREF. These results taken together revealed that DREF is involved in regulation of BRM complexes and consequently the cell cycle.

MATERIALS AND METHODS

Fly strains

Fly strains were maintained at 25°C on standard food. Canton S flies were used as the wild-type strain. The UAS-
other BRM complex subunits and of its target gene, DREF regulatory pathway. In addition, mRNA levels of
GAL4 on the X chromosome (20,21). osa2
the transgenic fly line (line number 16) carrying GMR-
Fly strains

Fly strains were maintained at 25°C on standard food. Canton S flies were used as the wild-type strain. The UAS-dDREF transgenic fly line was described earlier (12) as was the transgenic fly line (number 16) carrying GMR-GAL4 on the X chromosome (20,21). osa2/TM6B, P[PZ]/osa00090/TM3 and mor1/TM6B were obtained from the Drosophila Genetic Resource Center.

Scanning electron microscopy

Adult flies were anesthetized, mounted on stages and observed under a VE-7800 (Keyence Inc., Osaka, Japan) scanning electron microscope in the high vacuum mode.

Oligonucleotides

For RT-PCR these primer pairs were chemically synthesized.

osac-F, 5′-CCCTGTCCCTGTTCTCTC
osac-R, 5′-GATGGAACACCGGTAAACCC
mor-F, 5′-GCACAAGGACGATGAAAGAG
mor-R, 5′-CGCTGATGATGATGTGGAAC
Rp49-F, 5′-GCTTTCTGGTTTCCGGAACGGTCT
Rp49-R, 5′-GACCTCAAAGCTCGCGCACGTT
ACCAGAGAC
DREF-F, 5′-GCCAATCTCGTACAAACAGCATCGATT
DREF-R, 5′-TTCCACTTCGAGAAGGCCCT
β-tubulin-F, 5′-AGTTCCAGCGATCATCG
β-tubulin-R, 5′-CGCAAAACATTGATCGAG
brm-F, 5′-TGTCGACCACTACCCGTTAT
brm-R, 5′-GTCATGCTTATCGAGTCTCG
snr1-F, 5′-ACAAGAAGTTACCCAGGAAAT
snr1-R, 5′-GACATACTCCTGCTCGT
BAP55-F, 5′-GGACATCTATTCTCCGAGT
BAP55-R, 5′-GGAACGCTGACGTTTGGGT
BAP60-F, 5′-GTAGCAAAAGATGTCGCAACG
BAP60-R, 5′-CCCGGAACAGTTGAGTATG
polymbromo-F, 5′-CCTGAAACCGGTATTTACGC
polymbromo-R, 5′-ATGTTCGGAGATTTTCTGATG
BAP170-F, 5′-GCATCATCCATCCCATCATCC
BAP170-R, 5′-GACCTGTGTCACCAACAGC
string-F, 5′-GTGGAGGAAACAACACTCG
string-R, 5′-TCCATGCTCATCAGTTCCAG
The following oligonucleotides were used for electrophoretic mobility shift assays as probes and competitors.
osadRE1,
5′-GCCAATCTCGTACATCGATACATCG
TGCGTCTACCCGC
osadRE1 comp,
5′-GCCGGTACGACGATAGATCGATACG
GTACGAGTATGCGGC
osaDRE2,
5′-CCCCAGAGTGCGGAAATTCGATATCGG
CAACTCATTTCCTGTC
osaDRE2 comp,
5′-GACGCGAAAATGATGTTGCTCA
morDRE1,
5′-CGATAACGACTGTCACAACACATCGATT
AAATACCATCTC
morDRE2,
5′-CAAGTGAGGTATTAAAATCGATGGTTGTA
CAGTCGTTATCG
morDRE3,
5′-GCTCTCAAACTGTACAATCGATGCTGTT
ACCTAGCCCTAG
morDRE3 comp,
5′-CGATGTGTTGTGACGCTTTATCGAT
TTCAGTCTTCCACT
morDRE3, 5′-GCTCTCAAAACGTGAAAATCCGATGCTGTT
ACTAGCCTAG
morDRE3 comp, 5′-CGTACGCGCTATAGTAACTCAGCATCGG
TACAGTTGGAGCG
To carry out chromatin immuno precipitation assays, the following oligonucleotides were used. The reverse primer to amplify morDRE1, 2 was mor3HindIII as described below.
osadRE1-F, 5′-CGAAACCGGACGACCGATAATG
osadRE1-R, 5′-ACGAGAAGCGAGCCATCGTAC
osadRE2-F, 5′-GACCAGCGTATAATTTTGA
osadRE2-R, 5′-CTGGCGAATTAGCGATATTT
morDRE1,2-F, 5′-GCTTCAACCAACAAATAGGT
morDRE1,2-R, 5′-CTACGTGCTTCGATTTTC
morDRE3, 5′-CGAAGAAAGCTGACCATCACAG
morDRE3, 5′-ACATGTCACCACTAACCCACAAC
brmDRE1-R, 5′-CTAGAGCTACTGTTTGTCAG
brmDRE2-F, 5′-CGAAACGCTGTTGAAATCTCG
brmDRE2-R, 5′-CCCAGGATCACAGTTTACG
brmDRE3-F, 5′-GGATTGTTAATCTCACAAC
brmDRE3-R, 5′-GGAAGGTTACCCAAATTGAC
BAP55DRE-F, 5′-TaAGGCCTAGAAGCGTAC
BAP55DRE-R, 5′-CCGCAAAATCTCCTGCGT
BAP60DRE-F, 5′-TGATACACAAATTCTTCCAG
BAP60DRE-R, 5′-CAAGACGCGTACGTTGAGC
BAP170DRE-F, 5′-CCAAATCTAGATGGGATCG
BAP170DRE-R, 5′-CCACGCGCCTGACGTTGAGC

To carry out chromatin immunopreparation assays, the following oligonucleotides were used. The reverse primer to amplify morDRE1, 2 was mor3HindIII as described below.

osadRE1-F, 5′-CGAAACCGGACGACCGATAATG
osadRE1-R, 5′-ACGAGAAGCGAGCCATCGTAC
osadRE2-F, 5′-GACCAGCGTATAATTTTGA
osadRE2-R, 5′-CTGGCGAATTAGCGATATTT
morDRE1,2-F, 5′-GCTTCAACCAACAAATAGGT
morDRE1,2-R, 5′-CTACGTGCTTCGATTTTC
morDRE3, 5′-CGAAGAAAGCTGACCATCACAG
morDRE3, 5′-ACATGTCACCACTAACCCACAAC
brmDRE1-R, 5′-CTAGAGCTACTGTTTGTCAG
brmDRE2-F, 5′-CGAAACGCTGTTGAAATCTCG
brmDRE2-R, 5′-CCCAGGATCACAGTTTACG
brmDRE3-F, 5′-GGATTGTTAATCTCACAAC
brmDRE3-R, 5′-GGAAGGTTACCCAAATTGAC
BAP55DRE-F, 5′-TaAGGCCTAGAAGCGTAC
BAP55DRE-R, 5′-CCGCAAAATCTCCTGCGT
BAP60DRE-F, 5′-TGATACACAAATTCTTCCAG
BAP60DRE-R, 5′-CAAGACGCGTACGTTGAGC
BAP170DRE-F, 5′-CCAAATCTAGATGGGATCG
BAP170DRE-R, 5′-CCACGCGCCTGACGTTGAGC
**Plasmid construction**

To construct the plasmids p5'-415osawt-luc and p5'-894morwt-luc, PCR was performed using *Drosophila* genomic DNA as a template and the following primers in combination:

- osa5'KpnI, 5'-GGTACCCACGAGAT AAGGCTTCTAG and osa3'HindIII, 5'-AAGCTTCTGACGCTCATACT; mor5'KpnI, 5'-GGTACCTCGCTCTTCTTCTTC and mor3'HindIII, 5'-AAGCTTACGCTCATACTACT.
- morDRE1mut, 5'-AAGCTTACGCTCATACTACTACT; morDRE3mut, 5'-AAGCTTACGCTCATACTACTACT.
- osaDRE2mut comp, 5'-AAGCTTACGCTCATACTACTACT; morDRE2mut, 5'-AAGCTTACGCTCATACTACTACT.
- osaDRE1mut, 5'-AAGCTTACGCTCATACTACTACT; morDRE1mut comp, 5'-AAGCTTACGCTCATACTACTACT.
- osaDRE2, 5'-AAGCTTACGCTCATACTACTACT; morDRE2mut, 5'-AAGCTTACGCTCATACTACTACT.
- osaDRE2mut, 5'-AAGCTTACGCTCATACTACTACT; morDRE2mut comp, 5'-AAGCTTACGCTCATACTACTACT.
- osaDRE1, 5'-AAGCTTACGCTCATACTACTACT; morDRE1mut, 5'-AAGCTTACGCTCATACTACTACT.
- CAGCTATCTAGTAACTCAGGcTCGcTGTACAGTTTGAAGGC.

**Cell culture**

Schneider (S2) cells were cultured in M3(BF) medium (22), supplemented with 10% fetal bovine serum (FBS) at 25°C in 5% CO2.

**DNA transfection into cells and luciferase assays**

Approximately 1 x 10⁵ S2 cells were plated 24 h before transfection, then 500 ng of reporter plasmid and 1 ng of pAct5C-seapansy as an internal control were cotransfected into the cells using CellFectin reagent (Invitrogen). At 48 h after transfection, cells were harvested and luciferase activities were measured using the Dual-Luciferase Reporter assay system (Promega, Madison, WI, USA). All assays were performed within the range of linear relation of activity to incubation time and values were normalized to *Renilla* luciferase activity. Transfections were performed several times with at least two independent plasmid preparations.

**Western immunoblot analysis**

S2 cells (1 x 10⁶) were collected and lysed as described earlier (23). They were subjected to 10% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA, USA) in transfer buffer (50 mM borate–NaOH, pH 9.0, 20% methanol). The blotted membranes were incubated with anti-DREF monoclonal antibodies (2) at 1:10 000 dilution or antiTubulin monoclonal antibodies (2) at 1:5000 dilution or monoclonal antibodies (2) at 1:10 000 dilution or antiTubulin IgG (Sigma, St Louis, MO, USA) at 1:5000 for 16 h at 4°C. The bound antibodies were detected with peroxidase-conjugated goat antimouse IgG and the ECL system (GE Healthcare, Little Chalfont, UK) according to the manufacturer’s recommendations, and images were analyzed with a Lumivision Pro HSII image analyzer (Aisin Seiki, Aichi, Japan).

**Electrophoretic mobility shift assays**

Electrophoretic mobility shift assays were performed as reported previously (1), with minor modifications. Preparation of Kc cell nuclear extracts was described elsewhere (1). They were mixed with double-stranded ³²P-labeled synthetic oligonucleotides (100 000 cpm) in a reaction buffer [15 mM HEPES, pH 7.6, 60 mM KCl, 0.1 mM EDTA, 1 mM DTT, 12% glycerol and 0.1 mg/ml BAP170DRE-R, 5'-ACACGAGGTTGACATCCACG actin5C-F, 5'-CTCCATCATGAAGTGTTA GTG actin5C-R, 5'-CTGACTCTGCTTGGGACGTC. For double-stranded RNA (dsRNA) interference experiments, 30 μg of DREF dsRNA or LacZ dsRNA in FBS-free M3(BF) medium were added to 1 x 10⁶ S2 cells for 1 h. dsRNA-free incubation was conducted as a control for 1 h in FBS-free M3(BF). After the incubation, 4 v of M3(BF) medium containing 10% FBS were added. After 72 h of RNAi treatment, 1 x 10⁵ cells were cotransfected with reporter genes, 2 μg of reporter plasmid and 1 ng of pAct5C-seapansy plasmids, with the aid of Cell-Fectin reagents (Invitrogen, Carlsbad, CA, USA). At 48 h after transfection, luciferase activity was measured as described above.
poly(dI-dC), and incubated for 15 min at 0°C. When necessary, unlabeled oligonucleotides were added as competitors at this step. DNA–protein complexes were electrophoretically resolved on 4% polyacrylamide gels in 50 mM Tris-borate, pH 8.3, 1 mM EDTA and 2.5% glycerol. The gels were dried and autoradiographed.

Electrophoretic mobility shift assays were also performed in the presence of antiDREF monoclonal antibody 1, antiDREF monoclonal antibody 4 (2) or normal mouse IgG as a control. Kc cell nuclear extracts were mixed with each antibody, incubated for 2 h at 0°C and added to mixtures containing 32P-labeled synthetic oligonucleotides (100 000 cpm). After 15 min incubation at 0°C, electrophoresis was carried out as described above.

Chromatin immunoprecipitation assays

We performed chromatin immunoprecipitation using a Chip Assay kits as recommended by the manufacturer (Upstate, Lake Placid, NY, USA) (24). Approximately 1 x 107 S2 cells were fixed in 1% formaldehyde at 37°C for 10 min and then quenched in 125 mM glycine for 5 min at 25°C. The cells were then washed in PBS containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatin A) and lysed in SDS lysis buffer. Lysates were sonicated to break DNA and the sonicated cell supernatants were diluted 10-fold in chip dilution buffer. Supernatant was pre-cleared with Protein A Agarose/Salmon Sperm DNA (50% slurry) for 30 min at 4°C. After brief centrifugation, each supernatant was incubated with 1 μg of the rabbit IgG or antiDREF polyclonal antibody for 16 h at 4°C. Protein A Agarose/Salmon Sperm DNA (50% slurry) was added, with incubation for 1 h at 4°C. After washing, immunoprecipitated DNA was eluted with elution buffer (1% SDS, 0.1 M NaHCO3). Then the protein–DNA crosslinks were reversed by heating at 65°C for 4 h. After deproteinization with proteinase K, DNA was recovered by phenol–chloroform extraction and ethanol precipitation.

The immunoprecipitated DNA fragments were detected by quantitative real-time PCR using SYBR Premix Ex Taq (Takara, Kyoto, Japan) and the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems, Tokyo, Japan). Primer pairs for PCR were described above.

Quantitative RT-PCR

S2 cells were exposed to 30 μg of dsRNA and at 5 days after dsRNA treatment total RNA was isolated using Trizol® Reagent (Invitrogen). The cDNA was prepared with a Takara high fidelity RNA PCR kit using the oligo dT primer (Takara) for PCR with SYBR Premix Ex Taq (Takara) and the ABI PRISM® 7000 Sequence Detection System (Applied Biosystems). Ct values were normalized to that of the Rp49 gene.

RESULTS

Half reduction of the osa or mor gene dose suppresses the DREF-induced rough eye phenotype

Overexpression of DREF-induced ectopic DNA synthesis and apoptosis, and inhibited photoreceptor cell

Figure 1. osa and mor genes genetically interact with DREF. Scanning electron micrographs of adult eyes. DREF overexpressing flies (GMR-GAL4/GMR-GAL4; UAS-DREF/UAS-DREF) were crossed with osa or mor mutant flies and developed at 28°C. (A, B) GMR-GAL4/+; UAS-DREF/+; +/+; (C, D) GMR-GAL4/+; UAS-DREF/+; osa2+/+. (E, F) GMR-GAL4/+; UAS-DREF/+; P[PB]osa00090+/+. (G, H) GMR-GAL4/+; UAS-DREF/+; mor1/+. (A, C, E, G) Scale bars are for 50 μm. (B, D, F, H) Scale bars are for 12.5 μm.
Differentiation in eye imaginal discs so that adult flies exhibited a severe rough eye phenotype (12) (Figure 1A and B), but their viability and fertility were normal. Therefore, we utilized these flies as a genetic tool for screening of mutations that can modify the rough eye phenotype (11.25–27).

Previously we reported that half dose reduction of the *trithorax* group genes, *osa* and *mor* suppressed the DREF-induced rough eye phenotype (12). Here, we further confirmed that the F1 progeny of crosses between the DREF target genes, *osa* and *mor* exhibited a severe rough eye phenotype (12) (Figure 1A and B). Although their eyes were modestly rough, fused ommatidia were not apparent, suggesting that *osa* and *mor* are DREF target genes or positive regulators of the DRE/DREF regulatory pathway.

The 5' flanking regions of the *osa* and *mor* genes contain DRE and DRE-like sequences

On the basis of our evidence that *osa* and *mor* might be DREF target genes, we searched for DRE or DRE-like sequences within 1.4 kb genomic regions from the transcription initiation sites of each of these genes. It has been described that DRE within such regions can upregulate transcription, a central 6 bp of DRE being sufficient for DREF to bind and activate the promoter (1,28).

In the 5' flanking region of the *osa* gene, one DRE and two DRE-like sequences were identified, and named DRE1 (−14 to −21), DRE2α (−243 to −250) and DRE2β (−249 to −256) (Figure 2A). DRE1 was found to perfectly match the 8 bp DRE sequence, 5'-TATCGATA, while DRE2α and DRE2β each, matched 7 out of 8 bp.

In the promoter region of the *mor* gene, one DRE and two DRE-like sequences were found. They were named DRE1 (−2 to −9), DRE2 (−26 to −33) and DRE3 (−718 to −725) (Figure 2B). DRE2 perfectly matching the consensus DRE sequence and DRE1 and DRE3 showing identity in 6 out of 8 bp. Since all of these sites were detected in located at positions within 1.4 kb of the transcription initiation site, they are good candidate transcriptional regulatory sites for the *osa* and *mor* genes.

Roles of DRE sites in *osa* and *mor* gene promoter activities

To examine the roles of the DREs in *osa* promoter activity, we carried out luciferase transient expression assays in cultured *Drosophila* S2 cells. We constructed a *osa* promoter–luciferase fusion plasmid and derivatives carrying mutations in one or more of DRE1, DRE2α and DRE2β. These plasmids were transfected into S2 cells and luciferase activities were measured. Mutation in DRE1 (osa-DRE1mut) reduced the promoter activity by 49% relative to that of the wild-type promoter. Mutations in DRE2α (osa-DRE2αmut) and DRE2β (osa-DRE2βmut) reduced the promoter activity by 21 and 19%, respectively. Mutations in both DRE2α and DRE2β (osa-DRE2αβmut) reduced it by 20%. Mutations in all DREs (osa-DRE1 and 2αβmut) reduced the promoter activity by 76% (Figure 3A). These results indicate that DRE1 mainly acts for activation of the *osa* promoter while DRE2 might have a supporting role.

The roles of DREs in *mor* promoter activity were examined in a similar way. Mutations in any one of three DREs alone exerted no effects on *mor* promoter activity and only slight reduction was noted with mutations in any two of the three. However, when all three DREs were mutated simultaneously, promoter activity was decreased by 73% (Figure 3B). These results revealed that all DREs can contribute to *mor* promoter activation and any one appears to be sufficient for activity.

Effects of knockdown of the DREF gene on *osa* and *mor* gene promoter activity

Endogenous *osa* and *mor* gene expression in RNAi-mediated DREF knockdown cells was examined to demonstrate that *osa* and *mor* are DREF target genes. Total RNAs from dsRNA-treated S2 cells were isolated and quantitative RT-PCR was carried out to measure the level of mRNAs. The DREF mRNA level was reduced by 95% in DREFdsRNA-treated cells, but was not changed in LacZdsRNA-treated cells (Figure 4A). Under these conditions, the level of *osa* mRNA was decreased to 32% and that of *mor* mRNA to 14% relative to those with no RNAi treatment (Figure 4A). The β-tubulin gene was used as a negative control and its expression was not affected by DREFdsRNA treatment (Figure 4A).

To further investigate the requirement of DREF for *osa* and *mor* promoter activity, we performed luciferase transient expression in DREF knockdown cells. DREFdsRNA or LacZdsRNA were added to S2 cells, and reduction of DREF protein was confirmed by western blotting. DREF protein was not detectable at 2, 4 and 6 days after DREFdsRNA treatment, while no change was evident in mock and LacZdsRNA-treated cells (Figure 4B). The luciferase reporter plasmids were transfected into S2 cells at 3 days after RNAi treatment, and luciferase activities were determined. In DREFdsRNA-treated cells, the wild-type *osa* promoter activity was decreased to 61% and *mor* promoter activity to 51% of those in non-dsRNA treatment cells. The promoter activities of both genes were not changed in LacZdsRNA-treated cells (Figure 4C).

To further confirm dependence of promoter activation by DREF on DREs, we measured promoter activities of all DRE-mutant promoters of both *osa* and *mor* genes. Although we expected that the promoter activities of DRE mutants would not change in DREF knockdown cells, *osa*
and mor promoter activities were slightly but significantly decreased in DREFdsRNA-treated cells (Figure 4C). These results suggest that DREF not only directly but also indirectly regulates the osa and mor gene transcription. It may be possible that some DREF target gene products activate the osa and mor gene transcription.

**DREF binds to the DRE sequence in vitro**

Binding of DREF to DRE is essential for transcriptional activation. We therefore investigated whether DREF has binding ability to the DRE sequences in the osa and mor promoter regions by electrophoretic mobility shift assays. 

32P labeled DRE-containing oligonucleotides were mixed with Drosophila Kc cell nuclear extracts and DNA–protein complexes were detected with oligonucleotides containing osaDRE1 (Figure 5A) or osaDRE2 (Figure 5B). On addition of non-labeled DRE oligonucleotides as competitors, shifted bands were diminished (Figure 5A and B). Addition of a competitor carrying mutations in each DRE did not affect the complex formation with osaDRE1 and osaDRE2 (Figure 5A and B). The shifted band with osaDRE1 was decreased by the addition of a 100-fold excess of DRE1mut competitor, while those with osaDRE2 were not, suggesting the DRE1mut competitor oligonucleotide to possess an additional DREF-binding site. To confirm that the DRE–protein complex contains DREF, we added antiDREF monoclonal antibodies to the binding reaction. The addition of antiDREF 1, which binds to the DNA-binding domain of DREF (2), inhibited the complex formation with osaDRE1 and osaDRE2 (Figure 5A and B). Furthermore, the band with osaDRE1 or osaDRE2 was

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**Figure 3.** Effects of mutations in DRE sites in the 5' flanking region of the osa and mor genes on their promoter activities. osa or mor promoter–luciferase fusion plasmids were transfected into S2 cells. Luciferase activities are expressed relative to wild-type osa (A) and mor (B) promoters. Mean values with standard deviations from three independent transfections are shown. The white box, wild-type DRE; the black box, mutant DRE.
supershifted by addition of antiDREF 4 (2) (Figure 5A and B). These results indicate that DREF indeed form complexes with osaDRE1 and osaDRE2.

We also examined whether DREF can bind to DREs of the mor gene in the similar way. DNA–protein complexes were detected with the oligonucleotides morDRE1, morDRE2 and morDRE3 as probes (Figure 6A–C). Since more nuclear extract was required to detect the DNA–protein complex with morDRE1, DREF appears to have lower affinity for morDRE1 than for the others. Each of the shifted bands disappeared on adding non-labeled DRE oligonucleotides as competitors, while competitor oligonucleotides carrying mutations in the DRE sequences exerted no effect (Figure 6A–C). To further verify the existence of DREF in the complex with morDRE oligonucleotides, antiDREF monoclonal antibodies were added to the binding reaction. The addition of the antiDREF 1 inhibited the complex formation with morDRE2 and morDRE3 (Figure 6B and C). The shifted bands with morDRE2 and morDRE3 were supershifted by adding antiDREF 4 (Figure 6B and C). These results indicate that DREF form complexes with the oligonucleotides morDRE2 and morDRE3.

Although addition of antiDREF 1 to the binding reaction only marginally affected the complex formation with morDRE1, the shifted band was at least partially supershifted by adding antiDREF 4 (Figure 6A). These results suggest that not only DREF but also other protein(s) can form complexes with morDRE1.

DREF binds to the genomic regions containing DRE and DRE-like sequences of the osa and mor in vivo

Based on the observation that DREF has ability to bind to osa and mor DRE sequences in vitro, we further
examined DREF binding under cellular conditions by chromatin immunoprecipitation assays using affinity purified antiDREF polyclonal antibodies. osaDRE1 and osaDRE2 could be considerably amplified from the immunoprecipitates with antiDREF IgG. Amplification of osaDRE1 was 21-fold and that of osaDRE2 was 14-fold compared with amplification from the immunoprecipitates with control IgG, while amplification of Act5C genomic region was only 1.5-fold (Figure 7). Thus, we concluded that DREF binds to the osaDRE1-and osaDRE2-containing genomic region in cultured cells.

Amplification of the region containing morDRE1 and 2 from the immunoprecipitates with antiDREF IgG was 3.3-fold in comparison with amplification of Act5C gene locus, but morDRE3 was amplified only 0.9-fold (Figure 7). We therefore conclude that DREF does not bind to the genomic region containing morDRE3 in vivo. It should be noted that morDRE3 matches only six out of the eight bases canonical DRE and appeared to have only a weak affinity for DREF in the electrophoretic mobility shift assay in vitro (Figure 6C).

Knockdown of DREF in S2 cells reduces expression of several genes encoding components of BRM complexes

In the 5' flanking regions of the genes coding for BAP55, BAP60 and BAP170, DRE or DRE-like sequences were found. Although no DRE was found in the 5' flanking region of brm, its second intron contains three DRE-like sequences, suggesting that these sequences might act as enhancer elements. The snr1, BAP111 and polybromo genes carry no DRE or DRE-like sequences in their 5' flanking regions or introns (Table 1). These findings suggest that DREF plays a role in activating the transcription of several genes encoding subunits of both BAP and PBAP complexes.

To examine whether DREF affects expression of these genes, mRNA levels of brm, BAP55, BAP60, snr1, polybromo and BAP170 in DREF knockdown cells were measured by quantitative RT-PCR. The brm expression level in DREFdsRNA-treated cells was reduced to 25%
relative to non-RNAi-treated cells. The mRNA levels for **BAP55** and **BAP60** were decreased to 24 and 29%, respectively (Figure 8A). **PBAP**-specific **polybromo** and **BAP170** were also decreased to 12 and 20%, respectively (Figure 8A). DREF is assumed to control **PBAP** expression as well as **BAP**. Expression of the **snr1** gene was not affected by knockdown of DREF (Figure 8A).

To investigate whether DREF directly regulates **brm**, **BAP55**, **BAP60** and **BAP170**, we carried out chromatin immunoprecipitation assays. Genomic regions containing **brmDRE1** (+881 to +874), **brmDRE2** (+2208 to +2201) and **brmDRE3** (+7120 to +7113) were not amplified from immunoprecipitates with antiDREF IgG (Figure 8B). It is therefore not likely that these DREs in the second intron of the **brm** gene act as enhancer elements. DRE-containing regions of **BAP55**, **BAP60** and **BAP170** were amplified by 9-, 12- and 18-fold, respectively (Figure 8B). Therefore, DREF appears to bind to DREs in these genes and directly activates **BAP55**, **BAP60** and **BAP170** transcription.

It is reported that the OSA-containing BAP complex is necessary for G2/M progression through **string** (**stg**)/**cdc25** promoter activation (17). We therefore examined mRNA levels of **stg** in DREF knockdown cells. Quantitative RT-PCR revealed 80% decrease (Figure 8C).

**DISCUSSION**

In this study, we demonstrated that both **osa** and **mor** are DREF target genes. Thus **osa** and **mor** promoters exhibited decreased activities when carrying mutations in their DREs and after knockdown of DREF in cultured cells. In addition, levels of **osa** and **mor** mRNAs were reduced in DREF knockdown cells. Third, DREF can bind to DREs of **osa** and **mor** in vitro, and binding of DREF to the genomic regions containing DREs of both genes was observed in cultured cells. These results showed that DRE and DREF are important for **osa** and **mor** promoter activation. Promoters having mutations in all DREs of both **osa** and **mor** genes, however, still retained some activity. It is therefore possible that another element(s) and/or unknown factor(s) regulated by DREF are involved in **osa** and **mor** transcriptional activation. The observed rescue of the DREF-induced rough eye phenotype by a reduction in the **osa** and **moira** gene dosage is consistent with the idea that the **osa** and **moira** gene transcription is activated by DREF. However, we cannot exclude the possibility that the rescue could also be affected by a mechanism involving protein–protein interactions.}

Table 1. DRE or DRE-like sequences in and around the genes coding for subunits of BRM complexes

| Gene  | DRE or DRE-like | Position |
|-------|----------------|----------|
| **BAP**<br> brm | 5’-cATCGATA | +881 to +874 |
| **PBAP**<br> **mor**<br> BRM55 | 5’-cATCGATG | +2208 to +2201 |
| **mor**<br> BRM55 | 5’-cATCGATA | +7120 to +7113 |
| **BAP55**<br> BRM55 | 5’-aATCGATA | -9 to -2 |
| **BAP60**<br> BRM55 | 5’-ATATCGATA | -33 to -26 |
| **BAP170**<br> BRM55 | 5’-gATCGATA | -277 to -270 |
| **snr1**<br> BRM55 | None | - |
| **BAP170**<br> BRM55 | None | - |
| **OSA**<br> BRM55 | 5’-TATCGATA | -21 to -14 |
| **OSA**<br> BRM55 | 5’-TATCGATG | -250 to -243 |
| **OSA**<br> BRM55 | 5’-aATCGATA | -256 to -249 |
| **PBAP**<br> **BAP170**<br> BRM55 | 5’-ATATCGATA | -16 to -9 |
| **PBAP**<br> **BAP170**<br> BRM55 | 5’-aATCGATA | -29 to -22 |
| **PBAP**<br> **BAP170**<br> BRM55 | 5’-aATCGATA | -442 to -435 |

Figure 8. Effects of DREF knockdown on mRNA levels of other BRM complex components. (A) Total RNA was isolated from S2 cells treated with dsRNA and cDNA was prepared. mRNA levels were determined by quantitative RT-PCR. Fold differences versus no dsRNA treatment are shown as mean values with standard deviations from three independent dsRNA transfections. (B) DRE-containing regions were amplified from immunoprecipitates with antiDREF IgG. (C) mRNA level of **stg** in DREFdsRNA-treated cells.
interactions between DREF and BAP/PBAP at the promoters of cell cycle-regulated genes. Further analyses are necessary to address this point.

Both oma and mor encode components of the BRM complex (16,29–31), which is a SWI/SNF type ATP-dependent chromatin remodeling complex conserved from yeast to human (14), with two forms, BAP and PBAP. OSA is a signature subunit of BAP, while PBAP contains Polybromo and BAP170 in its place (16). Localization patterns of OSA and Polybromo on polytene chromosomes differ, though several sites overlap (16). Whole-genome expression analysis also demonstrated that BAP and PBAP differentially regulate gene expression (17). For example, OSA negatively regulates expression of the Wingless-target genes and the achaete/scuté gene (32,33). OSA, Polybromo and BAP170 are all required for function of BRM complex (17). It is thought that OSA functions in recruitment of BAP to its target genes. MOR, a subunit common to both BAP and PBAP, is presumed to be essential for complex integrity, since its absence results in degradation of both forms (17). SRG3, which is a homolog of MOR in mammals, also acts for complex stabilization by protecting against proteosomal degradation (34). Therefore, OSA and MOR are essential subunits for function and stabilization of BRM complexes and DREF may control integrity of the BRM complex through activating oma and mor gene expression.

BAP and PBAP share seven subunits, BRM, MOR, Snr1, BAP111, BAP60, BAP55 and Actin (16). BRM is a catalytic subunit harboring the ATPase domain (35) and it was previously reported that reduction of the brm gene dose suppressed the DREF-induced rough eye phenotype (12). We also found the mRNA level of brm to be decreased in DREF knockdown cells. However, DRE-like sequences in the second intron, do not appear to function as regulatory elements, since DREF does not bind to the genomic region containing these sites in vivo. DREF may therefore indirectly control brm gene expression.

The genes coding for BAP55 and BAP60, common subunits for BAP and PBAP, also contain DRE or DRE-like sequences in their 5’ flanking regions and are affected by DREF knockdown. DREF binds to the genomic regions containing their DREs in vivo and it is, therefore, possible that BAP55 and BAP60 are directly regulated by DREF. Furthermore, the PBAP-specific subunit BAP170 carries a DRE in its 5’ flanking region. Reduction of mRNA levels of oma, polybromo and BAP170 in DREF knockdown cells also is evidence that DREF contributes to the transcriptional regulation of both BAP and PBAP complexes. Therefore, DREF may regulate expression of genes coding for most subunits for both BAP and PBAP complexes and influence expression of many genes through chromatin remodeling.

It is reported that OSA-containing BAP complexes are necessary for G2/M progression through stg promoter activation while PBAP complexes are not (17). stg encodes a CDC25 phosphatase, which is required for G2/M progression (36). It is well known that DREF predominantly regulates the transcription of DNA replication-related genes (1,25,37,38). Reduced stg mRNA has been reported in DREF-eliminated cells (8) and we also observed reduction of stg mRNA levels in DREF knockdown cells, as with brm, oma and mor. In addition to regulation of S phase entry, DREF thus appears to play an important role in G2/M transition by activating the BAP complex to promote cell cycling. We found two DRE-like sequences in the stg gene upstream region, −219 to −212 (5’aATCGAtg) and −591 to −584 (5’-TATCGAti). Therefore, DREF could regulate stg gene expression directly via binding to DRE-like and/or indirectly via activation of genes coding for BAP complexes. Further analysis is necessary to distinguish these possibilities.

BRM complexes are thought to inhibit S phase entry and mutations of brm, oma and mor suppress the rough eye phenotype induced by E2F/DP/p35 overexpression (18). The rough eye phenotype of a cyclin E hypomorphic mutant was also suppressed by BRM complex mutation through increase in the S phase (19). Therefore, BRM complexes appear to negatively regulate S phase entry, while DREF activates E2F gene transcription and promotes G1/S progression (39). Although oma is ubiquitously expressed in eye imaginal discs, it is most intensely expressed anterior to the morphogenetic furrow where cells enter the G1 phase (40). Similarly, DREF is strongly expressed in this region (12). It is conceivable that DREF simultaneously activates both positive and negative regulators of G1/S progression. This kind of regulation may be necessary for fine tuning of cell cycle progression to inhibit excess S phase induction.

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