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Genetic screening for modifiers of the DREF pathway in *Drosophila melanogaster*: identification and characterization of HP6 as a novel target of DREF

Hiroyuki Ida[1,2], Noriyuki Suzusho[1,2], Osamu Suyari[1,2], Hideki Yoshida[1,3], Katsuhito Ohno[1], Fumiko Hirose[4], Masanobu Itoh[1,2] and Masamitsu Yamaguchi[1,2,*]

[1]Department of Applied Biology, [2]Insect Biomedical Research Center, [3]Venture Laboratory, Kyoto Institute of Technology, Matsugasaki, Sakyo-ku, Kyoto 606-8585 and [4]Department of Life Science, Graduate School of Life Science, University of Hyogo, Kamigori, Hyogo 678-1297, Japan

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ABSTRACT

The DNA replication-related element-binding factor (DREF) regulates cell proliferation-related gene expression in *Drosophila*. By genetic screening, taking advantage of the rough eye phenotype of transgenic flies that express DREF in the eye discs, we identified 24 genes that suppressed and 12 genes that enhanced the rough eye phenotype when heterozygous for mutations. Five genes, *HP6*, *pigeon*, *lace*, *X box binding protein 1* and *guftagu* were found to carry replication-related element (DRE) sequences in their 5′-flanking regions. Of these, the *HP6* gene carries two sequences that match seven out of eight nucleotides of DRE and two additional sequences that match six out of eight nucleotides of DRE in the 5′-flanking region. Band mobility shift assays using *Drosophila* Kc cell nuclear extracts demonstrated DREF binding to two of these sites and chromatin immunoprecipitation using anti-DREF antibodies confirmed that this occurs *in vivo*. Knockdown of DREF in *Drosophila* S2 cells decreased the HP6 mRNA level. The results, taken together, indicate that DREF directly regulates expression of the *HP6* gene. HP6 mRNA was detected throughout development by RT-PCR with highest levels in adult males. In addition, immunostaining analyses revealed colocalization of HP6 and DREF in nuclei at the apical tips in the testes.

INTRODUCTION

Promoters of many DNA replication- and proliferation-related genes in *Drosophila* contain a common 8 bp palindromic sequence, 5′-TATCGATA, named the DNA replication-related element (DRE) (1–10). The requirement of DRE for promoter activity has been confirmed in both cultured cell and transgenic fly systems (1,11,12) and a specific DNA replication-related element-binding factor (DREF) has been identified. Molecular cloning of its cDNA has led to confirmation that DREF is a transcriptional activator of DRE-containing genes (1). It is also reported that DREF is a component of a transcription initiation complex containing TRF2 (13). In addition, the chromatin remodelling factor dMi-2 and a homeodomain protein Distal-less can bind to the DNA-binding domain of DREF to inhibit its DNA-binding activity (14,15).

Searches of the *Drosophila* genome database have revealed the presence of 277 genes containing DRE-like sequences within their promoter regions (16,17) and immunostaining of polytene chromosomes of salivary glands with anti-DREF monoclonal antibodies demonstrated binding of DREF to a hundred discrete interband

*To whom correspondence should be addressed. Tel: +81 75 724 7781; Fax: +81 75 724 7760; Email: myamaguc@kit.ac.jp
Present addresses:
Hiroyuki Ida, Environmental Research Laboratory of Public Health, Kankyo Eisei Yakuhin Co. Ltd., 3-6-2 Hikaridai, Seika-cho, Soraku-gun, Kyoto 619-0237, Japan
Hideki Yoshida, Terrence Donnelly Centre for Cellular and Biomolecular Research, University of Toronto, 160 College St., Toronto, Ontario, M5S 3E1, Canada
Katsuhito Ohno, Department of Molecular Biology and Biochemistry, Nelson Biology Laboratory, Rutgers University, 604 Allison Road, Piscataway, NJ 08854, USA

regions of polytene chromosomes (14). In addition, serial analysis of gene expression (SAGE) showed that many genes selectively expressed in dividing cells located anterior to the morphogenetic furrow of the eye imaginal disc carry DRE in their 5′-flanking regions (18). DREF may therefore regulate the expression of many genes and play multiple roles *in vivo*.

Ectopic expression of the dominant-negative form of DREF using the GAL4-UAS targeted expression system causes inhibition of both endo-replication in larval salivary gland cells and mitotic DNA replication in eye imaginal disc cells (19). Ectopic expression of full length DREF in eye imaginal discs causes ectopic DNA synthesis and apoptosis in otherwise post-mitotic cells, and inhibits photoreceptor cell differentiation that results in a severe rough eye phenotype (20). RNAi mediated knockdown of DREF in growing tissues has also provided direct evidence that it is necessary for cell cycle and cell growth control (21,22).

In order to identify novel targets of DREF, we have carried out a screening, taking advantage of the rough eye phenotype of the transgenic flies that express full length DREF in the eye imaginal discs. Our previous screen identified the *dE2F*, *brahma*, *moira* and *osa* gene as suppressors and the *Distal-less* gene as an enhancer of the DREF-induced rough eye phenotype (20). E2F is a transcription factor regulating the genes involved in cell cycle, while Brahma, Moira and Osa are components of the chromatin-remodelling Brahma (BRM) complex (23). Suppression of the DREF-induced rough eye phenotype by reduction of dosage of the *brahma*, *moira*, or *osa* suggests that the genes coding for the BRM complex are targets of DREF (20). These observations combined with molecular and biochemical analyses indicate that DREF is involved in transcriptional regulation of the genes coding for the BRM complex (24). In this study, we further identified 24 suppressors and 12 enhancers of the DREF-induced rough eye phenotype. One of the strongest suppressors was a mutant for the *HP6* (*CG15636*) gene, which carries multiple DRE-like sequences in its 5′-flanking region. The present results indicate that the *HP6* gene is one of the targets of the DRE/DREF regulatory system with major physiological significance.

MATERIALS AND METHODS

Fly stocks

Fly stocks were maintained at 25°C on standard food. The Canton S fly was used as a wild type strain. $dp^{ovlR}/SM5$ and $dp^{D}/SM1$ were obtained from the Kyoto Institute of Technology, *Drosophila* Genetic Resource Center (Japan). The UAS-DREF transgenic fly line was described earlier (19) as was the transgenic fly line (line number 16) carrying pGMR-GAL4 on the X chromosome (25). All other stocks used in this study were obtained from the Bloomington, Indiana, stock centre.

Establishment of transgenic flies

P-element-mediated germ line transformation was carried out as described earlier (26). F1 transformants were selected on the basis of white-eye colour rescue (27). Two independent lines were established for the pUAS-*HP6*. We used line 2 carrying UAS-*HP6* on the third chromosome in the present study.

Oligonucleotides

To obtain a cDNA for the *HP6* (*CG15636*) gene, the following polymerase chain reaction (PCR) primers were chemically synthesized:

5′Bgl2P,
5′-CGATATCTAAAAGATCTCGGAAGATGCC
3′Kpn1P,
5′-CGGTGCGGTACCGTTTTATGGACTAGG
5′BamH1P,
5′-TCTGGATCCATGCCCAGCTC
3′Xho1P,
5′-GTTTCTCGAGCTAGGCATTTCG

The sequences of double-stranded oligonucleotides containing DRE (DRE-P) in the PCNA gene were as described earlier (11). The DRE-PM oligonucleotide is a two-base substitution derivative of DRE-P (11). For band mobility shift assays, the following oligonucleotides were synthesized. The DRE and DRE-like sequences are shown in bold letters and the substituted bases in the *HP6* gene promoter are shown in small letters.

DRE2,
5′-CTTACACAAA**AATCGATT**AAATTGAAGAAC
3′-GAATGTGTTT**TTAGCTAA**TTTAACTTCTTG
DRE2Mut,
5′-CTTACACAAA**AcgCGAgT**AAATTGAAGAAC
3′-GAATGTGTTT**TgcGCTcA**TTTAACTTCTTG
DRE1,
5′-TGCCA**CATCGAAA**GGGTTGCCAAAGCA**TGT**
CGATACCTACAGT**TATCGAAA**CTGA
3′-ACGG**TGTAGCTTT**CCCAACGGTTTCGT**ACAG**
CTATGGATGTCA**ATAGCTTT**GACT
DRE1Mut,
5′-TGCCA**CcgCGAAc**GGGTTGCCAAAGCA**TGg**
CGAgcCCTACAGT**TcgCGAAc**CTGA
3′-ACGGT**GgcGCTTg**CCCAACGGTTTCGT**ACc**
GCTcgGGATGTCA**AgcGCTTg**GACT
DRE1αMutβγ,
5′-TGCCA**CcgCGAAc**GGGTTGCCAAAGCA**TGTC**
GATACCTACAGT**TATCGAAA**CTGA
3′-ACGGT**gTAGCTtT**CCCAACGGTTTCGT**ACAG**
CTATGGATGTCA**ATAGCTTT**GACT
DRE 1βMutαγ,
5′-TGCCA**CATCGAAA**GGGTTGCCAAAGCA**TGg**
CGAgcCCTACAGT**TATCGAAA**CTGA
3′-ACGGT**GTAGCTTT**CCCAACGGTTTCGT**ACc**
GCTcgGGATGTCA**ATAGCTTT**GACT
DRE 1γMutαβ,
5′-TGCCA**CATCGAAA**GGGTTGCCAAAGCA**TGT**
CGATACCTACAGT**TcgCGAAc**CTGA
3′-ACGGT**GTAGCTTT**CCCAACGGTTTCGT**ACAG**
CTATGGATGTCA**AgcGCTTg**GACT
DRE1γ,
5′-ACAGT**TATCGAAA**CTGAAAAATAAT
3′-TGTCA**ATAGCTTT**GACTTTTTATTA

DRE1γMut,
5′-ACAGT**TcgCGAAc**CTGAAAAATAAT
3′-TGTCA**AgcGCTTg**GACTTTTTATTA

To carry out chromatin immunoprecipitation, the following PCR primers were chemically synthesized:

PCNAP,
5′-GATGAATGATTAACGTGGGCTG
PCNAantiP,
5′-GAAATAAATATACTCTGTAAAAAGTGT GAAC
CG15636DRE1P,
5′-ATCGAAAGGGTTGCCAAAGC
CG15636antiDRE1P,
5′-GCGTAGCCAATTGTCACGTT
CG15636DRE2P,
5′-CTGGAATACATACACACCGAG
CG15636antiDRE2P,
5′-TGGGCGCACAATTTAAAGCAG
RP49P,
5′-AGCGCACCAAGCACTTCATC
RP49antiP,
5′-CGTTCTCTTGAGAACGCAGG

To carry out RT-PCR, the following PCR primers were chemically synthesized:

CG15636P,
5′-ATGCCCAGCTCCACTTTGAC
CG15636antiP,
5′-CTAGGCATTTCGTGATCGTTTCTTC

RP49 primers used for RT-PCR were the same as used for chromatin immunoprecipitation.

For quantitative real time PCR, the following oligonucleotides were synthesized:

DREF-F, 5′-GGCAATCTCCGTTGAATGACG
DREF-R, 5′-TTCACCTCCGAGAAGCCCTT
β-tubulin-F, 5′-AGTTCACCGCTATGTTCA
β-tubulin-R, 5′-CGCAAAACATTGATCGAG
RP49-F, 5′-GCTTCTGGTTTCCGGCAAGCTTCAAG
RP49-R, 5′-GACCTCCAGCTCGCGCACGTTGTGCA CCAGGAAC

CG15636 primers used for quantitative real time PCR were the same as used for RT-PCR.

Plasmid construction

To construct the pUAS-HP6 plasmid, PCR was performed using *Drosophila* genomic DNA as a template and primers 5′Bgl2P and 3′Kpn1P in combination. PCR products were digested with *Bgl*II and *Kpn*I and inserted between the *Bgl*II and *Kpn*I sites ofthe pUAST plasmid (28).

To construct the pGST-HP6 plasmid for expression of GST-HP6 fusion protein in *Escherichia coli*, PCR was performed using pUAS-HP6 as a template and primers 5′BamH1P and 3′Xho1P in combination. PCR products were digested with *Bam*HI and *Xho*I and inserted between the *Bam*HI and *Xho*I sites of pGex6p-1 (GE healthcare).

Expression of GST fusion proteins and purification of HP6 protein

Expression of GST-HP6 fusion proteins in *E. coli* BL21 was carried out as described elsewhere (29). Lysates of cells were prepared by sonication in PBS containing 1 mM PMSF, and 1 μM each of pepstatin and leupeptin. Lysates were cleared by centrifugation at 12 000*g* for 20 min at 4°C and applied to glutathione-Sepharose (GE healthcare). The columns were washed with PBS containing 0.5 M NaCl and 0.1% Triton X-100, then with a buffer containing 150 mM NaCl, 50 mM Tris–HCl pH 7.2, 1 mM EDTA and 1 mM dithiothreitol (DTT). The included GST-HP6 fusion proteins were treated with Precision protease (GE healthcare) for 16 h at 4°C (30) and then eluted with PBS.

Antibodies

The purified HP6 protein were used to elicit polyclonal antibody production in rabbit. Polyclonal antibodies reacting with HP6 were affinity-purified from anti-serum using the N-hydroxysuccinimide (NHS)-activated Sepharose HP (GE healthcare) coupled with GST-HP6 fusion protein after passage through GST-conjugated Sepharose HP. Preparation of anti-DREF monoclonal antibodies was as described previously (1,31).

Western immunoblot analysis

Adult males of *Canton S*, a line carrying the *Act5C*-GAL4 transgene and a line carrying both *UAS-Flag-HP6* and *Act5C*-GAL4 transgenes were frozen in liquid nitrogen and homogenized in a solution containing 50 mM Tris–borate (pH 6.8), 2% SDS, 6% β-ME, 10% glycerol and 0.1% bromophenol blue. Homogenates were centrifuged at 17 800*g* at 4°C for 5 min, and extracts (100 μg of protein) were electrophoretically separated on SDS-15% polyacrylamide gels and transferred to polyvinylidene difluoride (PVDF) membranes (Bio-Rad) in a solution containing 25 mM Tris, 190 mM glycine and 20% methanol for 1 h at 25°C. Blotted membranes were blocked with Tris–buffered saline (TBS) solution (20 mM Tris–HCl, pH 7.4 and 150 mM NaCl) containing 0.05% Tween 20 and 5% skim milk for 1 h at 25°C and then incubated with an anti-HP6 polyclonal antibody at a 1 : 500 dilution, or the anti-FLAGM5 antibody (Sigma) at a 1 : 2000 dilution at 4°C for 16 h. After washing with TBS containing 0.05% Tween 20, the blots were incubated with horseradish peroxidase-labelled anti-mouse IgG and anti-rabbit IgG (GE healthcare) at a 1 : 20 000 dilution for 1 h at 25°C. Detection was performed with ECL Western blotting detection reagents (GE healthcare).

Scanning electron microscopy

Adult flies were anesthetized, mounted on stages and observed under a Hitachi S-3000 scanning electron microscope in the low vacuum mode.

Band mobility shift assays

Band mobility shift analysis was performed as reported previously (4), with minor modifications. Kc cell nuclear

extracts were prepared as described elsewhere (4) and incubated in a reaction mixture containing 15 mM Hepes, pH 7.6, 60 mM KCl, 0.1 mM EDTA, 1 mM DTT, 12% glycerol, 0.05 mg/ml poly(dI-dC), 0.05 mg/ml Salmon sperm DNA (average size 0.2 kb) and double-stranded ^{32}P-labelled synthetic oligonucleotides (10 000 cpm) for 15 min at 0°C. When necessary, unlabelled 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 at 25°C. Gels were dried and autoradiographed.

Band mobility shift assays were also performed in the presence of anti-DREF monoclonal antibody 1, anti-DREF monoclonal antibody 4 (1) or anti-GST monoclonal antibody 1 as a control. Kc cell nuclear extracts were mixed with each antibody, incubated for 2 h on ice, added to mixtures containing ^{32}P-labelled synthetic oligonucleotides (10 000 cpm) and 0.05 mg/ml poly(dI-dC), 0.05 mg/ml Salmon sperm DNA (average size 0.2 kb) and then incubated for 15 min at 0°C as described above.

Immunostaining of polytene chromosomes

Polytene chromosome spreads were prepared according to the protocol of Zink *et al.* from Canton S wild-type wandering third instar larvae (32) and stored in PBS-0.05% Tween 20-1% bovine serum albumin (BSA) at 4°C for 16 h before incubation with anti-DREF monoclonal antibody at a 1 : 1000 dilution at 4°C for 16 h. After extensive washing with PBS-0.05% Tween 20-1% BSA, samples were incubated at 25°C for 1 h with anti-mouse IgG conjugated with Alexa 594 (Invitrogen) at a 1 : 400 dilution. The chromosomes were then washed with PBS-0.05% Tween 20-1% BSA and mounted in Fluoroguard Antifade Reagent (Bio-Rad) for microscopic observation.

Immunostaining of testes

Preparation of testes from 1-day-old adult males for immunostaining was as described (33). After blocking with PBS containing 10% normal goat serum, the preparations were incubated with anti-DREF monoclonal antibody at a 1 : 1000 dilution or with an anti-HP6 polyclonal antibody at a 1 : 500 dilution at 4°C for 16 h. After extensive washing with PBS, samples were incubated at 25°C for 2 h with anti-rabbit IgG conjugated with Alexa 594 (Invitrogen) or anti-mouse IgG conjugated with Alexa 488 (Invitrogen) at a 1 : 400 dilution. The samples were mounted in Fluoroguard Antifade Reagent (Bio-Rad) for microscopic observation.

Chromatin immunoprecipitation

We performed chromatin immunoprecipitation using a Chip Assay kit as recommended by the manufacturer (Upstate). Approximately 1×10^7 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. Cells were washed twice in PBS containing protease inhibitors (1 mM PMSF, 1 μg/ml aprotinin and 1 μg/ml pepstatin A) and lysed in 2 ml of SDS lysis buffer. Lysates were sonicated to break DNA into fragments of less than 1 kb and centrifuged at 15 300*g* for 10 min at 4°C. The sonicated cell supernatants were diluted 10-fold in Chip Dilution Buffer and precleared with 80 μl Salmon Sperm DNA/Protein A agarose-50% slurry for 30 min at 4°C. After brief centrifugation, each supernatant was incubated with 1 μg of the rabbit IgG or anti-DREF polyclonal antibody for 16 h at 4°C. Salmon Sperm DNA/Protein A agarose-50% slurry was added, followed by incubation for 1 h at 4°C. After washing, immunoprecipitated DNA was eluted with elution buffer containing 1% SDS and 0.1 M $NaHCO_3$. 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.

Immunoprecipitated DNA fragments were detected by quantitative real time PCR using SYBR Green I (Takara) and the Applied Biosystems 7500 Real Time PCR system (34). The ΔΔCT value for each sample was calculated by subtracting the CT value for the input sample from the CT value obtained for the immunoprecipitated samples. Fold differences relative to the controls using non-immune IgG were then calculated by raising 2 to the ΔΔCT power. The ΔΔCT was calculated by subtracting the ΔCT value for that for the sample immunoprecipitated with control IgG.

Quantitative RT-PCR

1×10^6 S2 cells were plated in 6-well dishes in 2 ml M3 medium containing 30 μg/well of DREF double stranded RNAs (DREFdsRNA) or LacZdsRNA for 1 h. After the incubation, 3 ml of 10% FBS-M3 medium was added to each well. At 5 days after the dsRNA treatment, total RNA was isolated from cells using TrizolReagent (Invitrogen) and 1 μg aliquots were reverse transcribed with oligo dT primer using a Takara high fidelity RNA PCR kit (Takara). Then, real time PCR was performed with a SYBR Green I kit (Takara) and the Applied Biosystems 7500 Real Time PCR system using one μl of reverse transcribed sample per reaction. Levels of mRNAs in the DREFdsRNA or LacZdsRNA treated cells and in no dsRNA treated cells were investigated by the C_T comparative method (35). The *β-tubulin* gene was chosen as a negative control. Rp49 was used as an endogenous reference gene. Experiments were performed in triplicate for each of three RNA batches isolated separately.

Developmental RT-PCR

Total RNAs from *Drosophila* bodies at various developmental stages were purified with TRIZOL (Invitrogen). For RT-PCR, mRNAs were purified using an Oligotex-dT30 <Super> mRNA Purification kit (Takara Bio) and then were used for cDNA synthesis using an oligo d(T) primer and *Bca* PLUS RTase (Takara Bio) according to the manufacturer's instructions. *HP6* and *RP49* DNA were amplified by PCR using *Pyrobest*™ DNA Polymerase (Takara Bio) with primer oligonucleotides CG15636P and CG15636antiP for *HP6* and RP49P and RP49antiP for *RP49*. The PCR conditions included one cycle of 2 min at 94°C followed by 25 cycles of 94°C for 30 s, 52°C or 55°C for 30 s and 72°C for 1 min. All the

PCR reactions were performed within the range of linear amplification and PCR products were separated on 2% agarose gels.

RESULTS

Genetic screening of modifiers of the DREF-induced rough eye phenotype and identification of an *HP6* mutation as a dominant suppressor

As reported previously, we have established transgenic fly lines bearing *GMR-GAL4* and DREF cDNAs under the control of a GAL4-binding sequence ($UAS\text{-}DREF_{1\text{-}709}$) (19,28). Over-expression of DREF induced ectopic DNA synthesis and apoptosis, and inhibited the photoreceptor cell differentiation in eye imaginal discs and adult flies exhibited a severe rough eye phenotype (36). Since the eye phenotype does not impair viability or fertility (20), these flies serve as a genetic tool to screen for modifying mutations. Previous studies identified 5 and 17 deletion regions that modify the DREF-induced rough eyes phenotype in the X and the second chromosome, respectively (20). In order to identify genes in these genomic regions that are responsible for modification of the DREF-induced rough eye phenotype, various mutants mapped in and around the 22 genomic regions (5D1-2; 5E, 7D1; 7D5-6, 9B1-2; 10A1-2, 11A2; 11B9, 19A5; 19D3, 21A1; 21B7-8, 21B8-C1; 21C8-D1, 21D2-3; 21F2-22A1, 25D2-4; 26B2-5, 32F1-3; 33F1-2, 35D1; 35D4, 35D2; 35F1-2, 35D2-4; 35E2-6, 36A8-9; 36E1-2, 36E4-36F1; 38A6-7, 37B2-12; 38D2-5, 37C2-5; 38B2-C1, 37D1-2; 38C1-2, 41A, 48A-B, 55A-55F, 57B4; 58B) were collected and used to cross with transgenic flies expressing DREF (Table 1).

Out of 238 independent mutant lines examined, 27 lines suppressed, while 19 lines enhanced the rough eye phenotype when they were heterozygous for the mutations (Figure 1D to F, Table 1). Under the scanning electron microscope, eyes of these heterozygous mutant flies appeared normal (data not shown). The other mutant lines apparently exerted no detectable effects on the DREF-induced rough eye phenotype. Cytological locations of these negative lines are listed in Supplementary Table 1. Data base search revealed that 24 genes are responsible for the suppression and 12 genes for the enhancement. One of the strongest levels of suppression of the rough eye phenotype was observed with the P-element insertion line *P{w + mGT = GT1}CG15636* (Figure 1B). The suppression could be reverted under dysgenic conditions (Figure 1C), suggesting the mutation induced by the P-element insertion to be truly responsible for the suppression. The Berkeley *Drosophila* genome project database (http://www.fruitfly.org/blast) revealed that the P-element is inserted 43 bp upstream of the termination codon of the *HP6* (*CG15636*) gene (Figure 2) and Greil *et al*. (37) reported that the mutant is semi-lethal. In contrast coexpression of HP6 further enhanced the DREF-induced rough eye phenotype in compared with the control flies coexpressing LacZ (Figure 1G and H), despite that overexpression of HP6 alone in the eye imaginal disc exerted only a marginal effect on the adult eye morphology (Figure 1I).

We searched for DRE like sequences in the 5′-flanking region of the *HP6* gene from the NCBI database, and found two sequences that match seven out of the eight nucleotides of DRE and two additional sequences that match six out of the eight nucleotides within the 1.4 kb upstream region (Figure 2). We named these sites as DRE1α (–161 to –154), DRE1β (–139 to –132), DRE1γ (–123 to –116) and DRE2 (–1013 to –1006) with respect to the translation initiation codon (Figure 2). It is reported that stimulatory effects of DRE can be observed at positions within at least 2.5 kb from the transcription initiation site (4) and sequences matching six out of eight nucleotides of DRE have promoter activity (11,38). Therefore, all of these DRE-like sequences of the *HP6* gene likely play roles in regulation of the *HP6* gene promoter activity.

DREF binds to the chromosomal region containing the *HP6* gene

To examine whether DREF locates to the chromosomal region containing the *HP6* gene, we carried out immunostaining of salivary gland polytene chromosomes in third instar larvae with anti-DREF monoclonal antibodies. DREF signals are detected in a number of discrete regions throughout the polytene chromosomes (14). Careful inspection allowed the mapping of signals for DREF at the *HP6* gene locus, 25A1, on the 2L chromosome (Supplementary Figure 1). The Berkeley *Drosophila* genome project database revealed that only two genes (*HP6* and *dumpy*) are located in this 25A1 locus. Since *HP6* is located in the intron of *dumpy* (*dp*), P-element insertion in line *P{w + mGT = GT1}CG15636* may affect not only *HP6* but also *dp* gene expression. We therefore crossed DREF-overexpressing flies with two independent X ray-induced homozygous lethal *dp* mutant strains, $dp^{\circ vlR}/SM5$ and $dp^{D}/SM1$. However no effect on the DREF-induced rough eye phenotype by *dp* mutation was observed (Supplementary Figure 2). Furthermore there is no DRE like sequence within the 2 kb 5′-flanking region of the *dp* gene. It is therefore very likely that DREF binds to DRE-like sequences in the 5′-flanking region of the *HP6* gene in the salivary glands.

DREF binding activity *in vitro*

To examine this directly, oligonucleotide DRE1 containing the region from DRE1α (–161 to –154) to DRE1γ (−123 to −116), oligonucleotide DRE1γ containing the DRE1γ (−123 to −116) region and oligonucleotide DRE2 containing the DRE2 (−1013 to −1006) region (Figure 2) were chemically synthesized and used for band mobility shift assays. As previously noted (4), specific DNA protein complexes could be detected with Kc cell nuclear extracts and the oligonucleotide DRE-P carrying the DRE sequence in the PCNA gene (Figure 3A). The shifted bands were effectively diminished by adding unlabelled oligonucleotides DRE1 and DRE2. Although DRE1 carrying mutations in either DRE1α or DRE1β also effectively competed against DRE-P, oligonucleotide

Table 1. Summary of genes that genetically interact with the *DREF* gene

Cytological location	gene	CG number	Allele(s) tested	Type of allele	Known function	Effect on rough eye phenotype
5D1-5D2; 5E			***Df(1)sqh***	**Deficiency**		**Suppression**
5E3-5E4	*Lag1*	CG3576	$Lag1^{G0365}$	P-element insertion	Unknown	Suppression
5E4	*Ubi-p5E*	CG32744	$l(1)G0287^{G0287}$	P-element insertion	Ubiquitin-dependent protein catabolic process	Suppression
7D1; 7D5-7D6			***Df(1)C128***	**Deficiency**		**Enhancement**
7B7	*Tom40*	CG12157	$Tom40^{G0216}$	P-element insertion	Transmembrane transporter activity	Enhancement
7C3	*l(1)G0155*	CG1515	$l(1)G0155^{G0155}$	P-element insertion	Unknown	Enhancement
7D3-7D5	*fs(1)h*	CG2252	$fs(1)h^{G0093}$	P-element insertion	Regulation of transcription	Enhancement
7D5	*mys*	CG1560	$mys^{KG02930}$	P-element insertion	Calcium-dependent cell-cell adhesion	Enhancement
7D5	*mys*	CG1560	mys^{G0281}	P-element insertion	Calcium-dependent cell-cell adhesion	Enhancement
7E6-7E7, 7E7-7E9	*CG32711, Trf2*	CG32711, CG18009	$l(1)G0219^{G0219}$	P-element insertion	Unknown, RNA polymerase II transcription factor activity	Enhancement
7E6-7E7, 7E7-7E9	*CG32711, Trf2*	CG32711, CG18009	$l(1)G0228^{G0228}$	P-element insertion	Unknown, RNA polymerase II transcription factor activity	Enhancement
7E6-7E7, 7E7-7E9	*CG32711, Trf2*	CG32711, CG18009	$l(1)G0295^{G0295}$	P-element insertion	Unknown, RNA polymerase II transcription factor activity	Enhancement
7E6-7E7, 7E7-7E9	*CG32711, Trf2*	CG32711, CG18009	$l(1)G0332^{G0332}$	P-element insertion	Unknown, RNA polymerase II transcription factor activity	Enhancement
7E6-7E7, 7E7-7E9	*CG32711, Trf2*	CG32711, CG18009	$l(1)G0372^{G0372}$	P-element insertion	Unknown, RNA polymerase II transcription factor activity	Enhancement
7E6-7E7, 7E7-7E9	*CG32711, Trf2*	CG32711, CG18009	$l(1)G0425^{G0425}$	P-element insertion	Unknown, RNA polymerase II transcription factor activity	Enhancement
19A5; 19D3			***Df(1)16-2-19***	**Deficiency**		**Suppression**
18D13-18E1	*dome*	CG14226	$dome^{G0199b}$	Loss of function	JAK/STAT signaling pathway	Enhancement
19C1	*CG9577*	CG9577	$CG9577K^{G09994}$	P-element insertion	Unknown	Enhancement
19C1	*sw*	CG18000	*P{SUPor-P}KG05547*	P-element insertion	Microtubule motor activity	Suppression
19C5-19C6	*l(1)G0004*	CG11738	$l(1)G0004^{G0004}$	P-element insertion	Unknown	Suppression
20B3	*l(1)G0196*	CG14616	$l(1)G0196^{G0196}$	P-element insertion	Unknown	Enhancement
21B8-C1; 21C8-21D1			***Df(2L)al***	**Deficiency**		**Suppression**
21C4-21C5	*ex*	CG4114	$l(2)k06506^{k06506}$	P-element insertion	Hippo signaling pathway	Suppression
21C4-21C5	*ex*	CG4114	$l(2)k07308^{k07308}$	P-element insertion	Hippo signaling pathway	Suppression
21D1	*cbt*	CG4427	$l(2)k08915^{k08915}$	P-element insertion	JNK signaling pathway	Enhancement
21D2-21D3; 21F2-22A1			***Df(2L)S3***	**Deficiency**		**Suppression**
21E2	*ds*	CG17941	$l(2)01855^{01855}$	P-element insertion	Calcium-dependent cell-cell adhesion	Enhancement
21E4	*S*	CG4385	S^{k09530}	P-element insertion	Effector of Egfr signalling	Enhancement

(continued)

Table 1. Continued

Cytological location	gene	CG number	Allele(s) tested	Type of allele	Known function	Effect on rough eye phenotype
21F1-2			*l(2)10685*^{k05810}	P-element insertion		Suppression
25D2-25D4; 26B2-26B5			***Df(2L)cl-h3***	**Deficiency**		**Suppression**
26B2	*lid*	CG9088	*lid*^{k06801}	P-element insertion	Trithorax grop protein trimethyl H3K4 demethylase	Mild suppression
26B2	*eIF-4a*	CG9075	*eIF-4a*^{k01501}	P-element insertion	Translation initiation factor activity	Suppression
26B2	*eIF-4a*	CG9075	*eIF-4a*^{02439}	P-element insertion	Translation initiation factor activity	Strong suppression
26D1-26D2			*l(2)k06107*^{k06107}	P-element insertion		Enhancement
32F1-32F3; 33F1-33F2			***Df(2L)Prl***	**Deficiency**		**Enhancement**
33A1-33A2	*crol*	CG14938	*crol*^{k05205}	P-element insertion	Transcription of a number of ecdysone-induced genes	Mild suppression
33C4	*Rab6*	CG6601	*Rab6*^{k13606}	P-element insertion	GTPase activity	Suppression
33F3	*CG5776, Å@spict*	CG5776, CG12292	*l(2)k05448*^{k05448}	P-element insertion	Unknown, negative regulation of BMP signaling pathway	Suppression
35D1; 35D4			***Df(2L)TW116(R)GW2***	**Deficiency**		**Suppression**
35C5-35D1	*gft*	CG11861	*gft*^{06430}	Loss of function	Ubiquitin-protein ligase activity	Mild suppression
35D2; 35F1-35F2			***Df(2L)TW116(R)GW13***	**Deficiency**		**Suppression**
35D2-35D4; 35E2-35E6			***Df(2L)b83d29a***	**Deficiency**		**Enhancement**
35D2	*lace*	CG4162	*lace*^{k05305}	P-element insertion	Serine C-palmitoyltransferase activity	Mild suppression
35E1-35E2			*P{lacW}J29*	P-element insertion		Suppression
36A8-36A9; 36E1-36E2			***Df(2L)H20***	**Deficiency**		**Enhancement**
36A11	*Cyt-c-d*	CG13263	*Cyt-c-d*^{bln1}	Loss of function	Cytochrome C proteins	Strong suppression
36E4-36F1; 38A6-38A7			***Df(2L)TW50***	**Deficiency**		**Enhancement**
36F4	*RpS26*	CG10305	*RpS26*^{04553}	P-element insertion	Structural constituent of ribosome	Suppression
37C7	*pigeon*	CG10739	*pigeonP1*	hypomorph	unknown	Enhancement
37B2-37B12; 38D2-38D5			***Df(2L)pr-A16***	**Deficiency**		**Enhancement**
38C5	*CG16798*	CG16798	l(2)k07219^{k07219}	P-element insertion	Unknown	Suppression
55A-55F			***Df(2R)PC4***	**Deficiency**		**Suppression**
55B5-55B7	*stau*	CG5753	*stau*^{ry9}	Loss of function	RNA binding	Mild suppression
55B7-55B8	*Hsf*	CG5748	*Hsf*^{03091}	Loss of function	RNA polymerase II transcription factor activity	Suppression
55F3-55F4	*l(2)08717*	CG15095	*l(2)08717*^{08717}	P-element insertion	Plasma membrane protein	Suppression
57B4; 58B			***Df(2R)Pu-D17***	**Deficiency**		**Enhancement**
57B12	*CG9350*	CG9350	*l(2)03050*^{03050}	P-element insertion	Unknown	Suppression
57C3-57C4	*Xbp1*	G9415	*Xbp1*^{k13803}	Loss of function	Regulation of transcription	Suppression
57E6-57E8	*CG10496*	CG10496	*CG10496*^{07128a}	P-element insertion	Unknown	Suppression
57E8-57E9	*MESK2*	CG15669	*MESK2*^{k00119}	P-element insertion	Unknown	Suppression

Bold characters indicate deficiency lines used in the previous study (20)

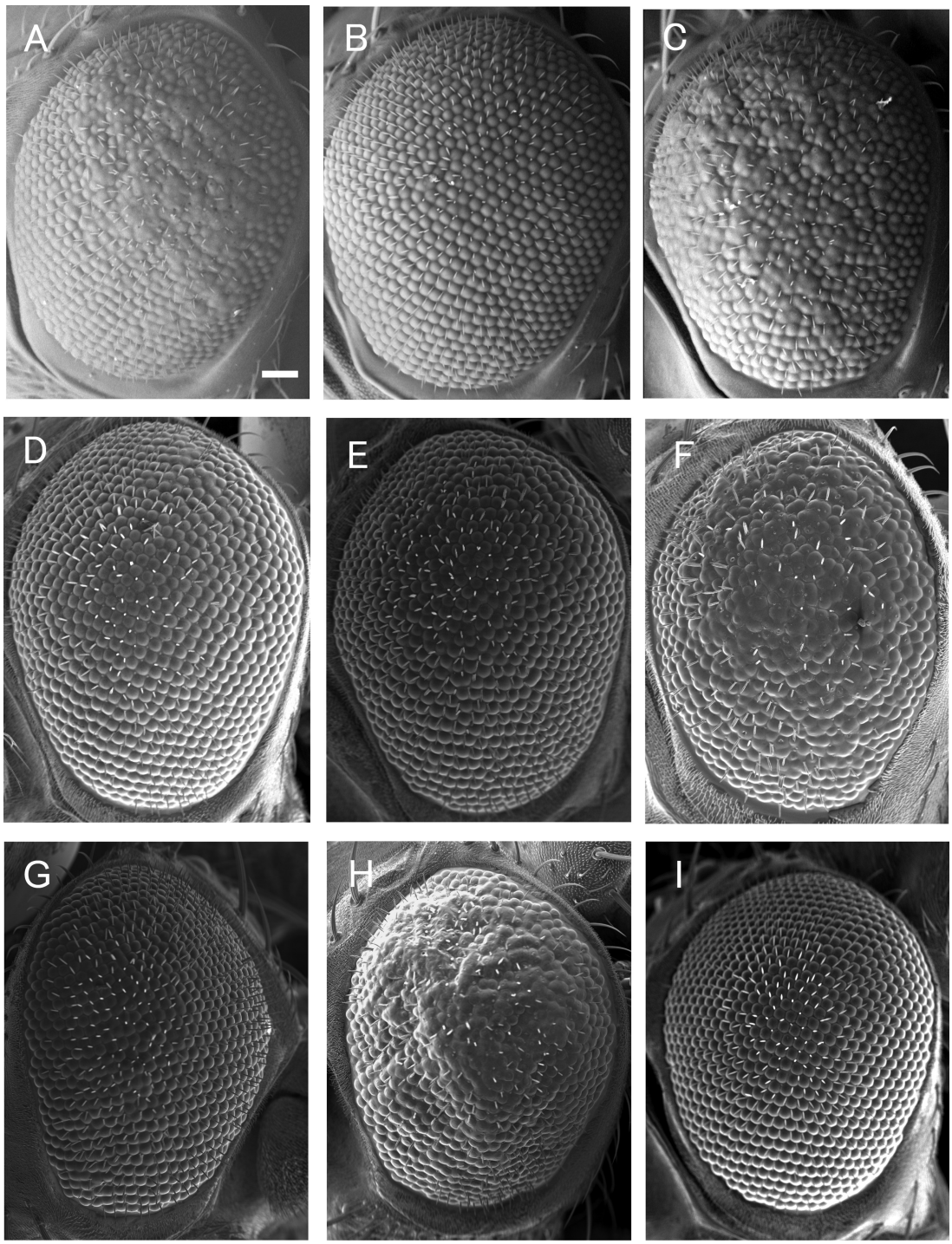

Figure 1. Scanning electron micrographs of adult eyes. (**A**) *GMR-GAL4/+; UAS-DREF/+; +/+*. (**B**) *GMR-GAL4/+; UAS-DREF/P{w+mGT=GT1}CG15636; +/+*. (**C**) *GMR-GAL4/+; UAS-DREF/P{w+mGT=GT1}CG15636rev; +/+*. (**D**) *GMR-GAL4/+; UAS-DREF/rps26; +/+*. (**E**) *GMR-GAL4/+; UAS-DREF/pepck; +/+*. (**F**) *GMR-GAL4/+; UAS-DREF/star; +/+*. (**G**) *GMR-GAL4/+; UAS-DREF/+; UAS-nlslacZ/+*. (**H**) *GMR-GAL4/UAS-HP6; UAS-DREF/+; +/+*. (**I**) *GMR-GAL4/UAS-HP6; +/+; +/+*. Bar indicates 50 μm.

DRE1 carrying mutations in DRE1γ less effectively competed. These results suggest that DREF has affinity for the region containing DRE1γ and DRE2.

When the oligonucleotides DRE2 or DRE1γ were mixed with Kc cell nuclear extracts, specific DNA-protein complexes were detected [Figures 3B (lane 1) and 3C (lane 1)], which were diminished by addition of an excess amount of unlabelled DRE2 and DRE1γ oligonucleotides as competitors [Figures 3B (lanes 2 and 3) and 3C (lanes 2 and 3)] but not of oligonucleotides carrying mutations in the DRE-like sequences [Figures 3B (lanes 4 and 5) and 3C (lanes 4 and 5)]. Furthermore, the specific

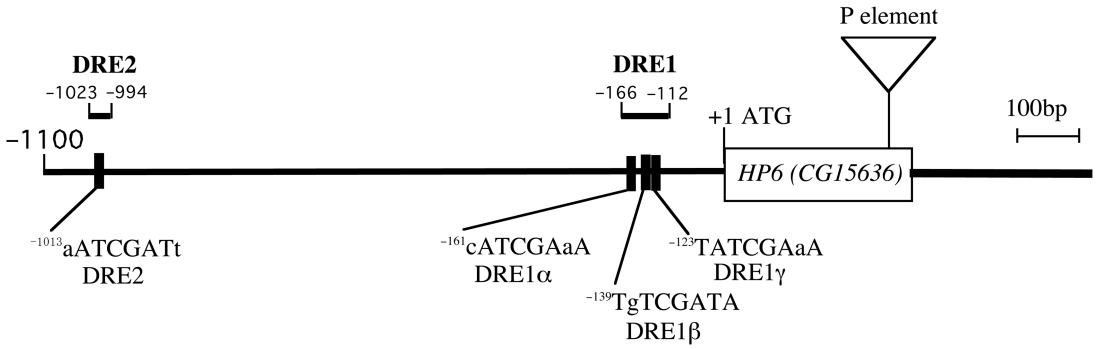

Figure 2. DRE and DRE-like sequences in the 5′-flanking regions of the *HP6* gene. The translation initiation site is numbered as +1. DRE and DRE-like sequences are located at positions –1013 to –1006 (DRE 2), –161 to –154 (DRE 1α), –139 to –132 (DRE 1β) and –123 to –116 (DRE1γ). DRE1 comprises DRE 1α, DRE 1β and DRE 1γ. Nucleotides that do not match to DRE consensus sequences are shown in small letters. A P-element is inserted 43 bp upstream of the termination codon of the *HP6* gene. The regions (DRE1 and DRE2) used as probes for band mobility shift assays are indicated.

DNA-protein complexes were either diminished or super shifted by adding anti-DREF monoclonal antibodies, but not by adding the control anti-GST monoclonal antibody [Figures 3B (lanes 7 and 8) and 3C (lanes 7 and 8)]. These results indicate that DREF can bind to DRE1γ and DRE2 sequences in the *HP6* gene promoter *in vitro*.

DREF binds to the DRE2- and DRE1-containing genomic region *in vivo*

To further examine DREF-binding to the DRE1- and DRE2-containing region of the *HP6* gene, primers to amplify the region from –66 to −167 and –856 to −1060 (Figure 2) were chemically synthesized and used for chromatin immunoprecipitation assays with anti-DREF polyclonal antibodies. It is well established that the *Drosophila PCNA* gene is regulated by the DREF pathway (1,11,12). Amplification of the *PCNA* gene promoter region containing the DRE in immunoprecipitates with the anti-DREF polyclonal antibody was 27-fold higher than with control rabbit IgG (Figure 4). In contrast, no amplification of the *Actin 5C* gene region was observed (Figure 4). Amplification of the *HP6* gene promoter region containing the DRE1 in the immunoprecipitates with anti-DREF polyclonal antibody was 28-fold and that containing DRE2 was 13-fold (Figure 4). These results indicate that DREF binds to the genomic region containing DRE1 and DRE2 of the *HP6* gene in S2 cells.

Effects of knockdown of the *DREF* gene on *HP6* gene expression in cultured cells

Endogenous *HP6* gene expression in RNAi-mediated DREF knockdown cells was examined to further demonstrate that *HP6* is a DREF target gene. Total RNAs from double-stranded RNA (dsRNA)-treated S2 cells were isolated and quantitative RT-PCR was carried out (Figure 5). The DREF mRNA level was reduced by 82% in DREFdsRNA-treated cells, but not changed with LacZdsRNA-treatment. Under these conditions, the level of endogenous *HP6* mRNA was decreased to 39%, while LacZdsRNA treatment exerted no effect (Figure 5). Expression of the *β-tubulin* gene employed as a negative control was not affected by DREFdsRNA treatment. These results indicate that DREF is required for *HP6* gene expression.

Levels of *HP6* mRNA are highest in *Drosophila* adult testes

We carried out RT-PCR to determine the *HP6* expression pattern during *Drosophila* development (Figure 6). *HP6* mRNA could be detected throughout all developmental stages but with the highest expression in adult males. Furthermore, the HP6 mRNA was expressed at least 6.3-fold higher in testes than in other parts of the body (Figure 6B). The observed HP6 expression pattern is consistent with the results reported by Greil *et al.* (37) and FlyAtlas (http://flyatlas.org/atlas.cgi?name=FBgn0031613). Relatively high expression of both HP6 and DREF proteins in nuclei at the apical tips of testes was observed with immunostaining using anti-HP6 and anti-DREF antibodies (Figure 7B) and the specificity of anti-HP6 antibody binding was confirmed by western blot analysis with extracts from adult male flies expressing Flag-HP6 fusion protein (Figure 7A). The results suggest some specific role of HP6 during spermatogenesis.

DISCUSSION

The present genetic screening of modifiers of the DREF-induced rough eye phenotype and identified 24 suppressors and 12 enhancers (Table 1 and Figure 1). Although these modifier genes are not necessarily transcriptional targets of DREF as reported previously (14), they could be critical genes in positive or negative regulation of the DREF pathway. By data base search, five genes, *HP6*, *pigeon*, *lace*, *X box binding protein 1* (*Xbp-1*) and *guftagu* were found to carry DRE sequences in their 5′-flanking regions. These genes are therefore candidate DREF target genes. Nucleotide positions of DRE and DRE like sequences in the 5′-flanking regions of these genes are listed in the Supplementary Table 2.

The *fat* gene, one of the suppressors of the rough eye, encodes nonclassical cadherin (39,40) and genetically

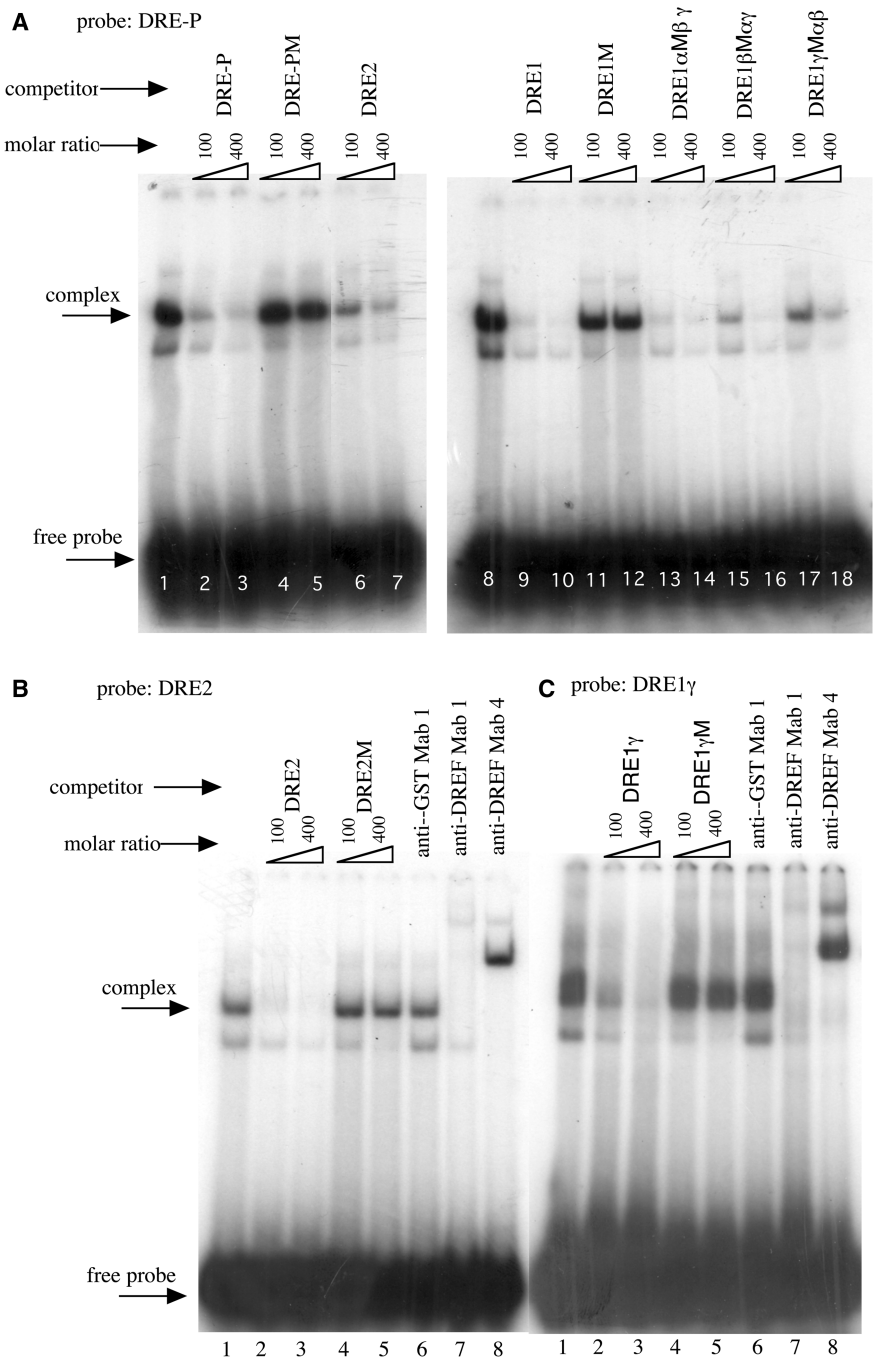

Figure 3. Complex formation between DRE in the *PCNA* gene promoter and Kc cell nuclear extracts. ^{32}P-labelled double stranded oligonucleotides DRE-P (**A**), DRE2 (**B**) and DRE1γ (**C**) were incubated with Kc cell nuclear extracts in the presence of the indicated competitor oligonucleotides or anti-DREF monoclonal antibodies. The amounts of competitors were 100- or 400-fold molar ratios. Anti-GST Mab1, anti-GST monoclonal antibody 1; anti-DREFMab1, anti-DREF monoclonal antibody 1; anti-DREFMab4, anti-DREF monoclonal antibody 4; DRE-P, oligonucleotide containing the DRE sequence of the *Drosophila PCNA* gene; DRE-PM, DRE-P having a mutation in the DRE sequence; DRE2, oligonucleotide containing the DRE2 sequence of the *HP6* gene; DRE2M, DRE2 having mutations in the DRE-like sequence; DRE1, oligonucleotide containing the DRE1 sequence of the *HP6* gene; DRE1M, DRE1 having mutations in the DRE1αβγ sequences; DRE1αM, DRE1 having mutations in the DRE1α sequence; DRE1βM, DRE1 having mutations in DRE1βsequence; DRE1γM, DRE1 having mutations in the DRE1γ sequence.

interacts with *armadillo* (41), a *Drosophila* homologue of mammalian β-catenin and downstream effecter of the Wnt signal transduction pathway (42). Interaction with *fat* in the eye confirms the ability of this gene to modify cytoplasmic Armadillo level (41). When sufficient Armadillo protein accumulates in the cell, it forms a complex with Pangolin, a *Drosophila* homologue of mammalian T-cell factor (43). Previously we demonstrated that the Armadillo/Pangolin complex activates transcription of the *DREF* gene (44). We therefore suggest that

suppression of the DREF-induced rough eye phenotype is caused by decrease of the Armadillo protein accumulation by half reduction of the *fat* gene dosage. The present screen also identified the *lace* gene as another suppressor. The *lace* gene encodes a membrane protein similar to the yeast protein LCB2, a subunit of serine palmitoyltransferase (SPT), which catalyses the first step of sphingolipid biosynthesis (45). It is now well known that sphingolipids trigger elevated levels of apoptosis via the modulation of known signaling pathways (46). Previously we reported that DREF is involved in regulation of vein formation through the activation of *raf*, downstream of Egfr signaling in the *Drosophila* wing imaginal discs (21). In accordance with this, the present genetic screen identified the *star* gene as one enhancer of the DREF-induced rough eye (Table 1). It encodes an integral membrane protein that is expressed in cells secreting Spitz and is localized in the early endoplasmic reticulum and nuclear envelope (47). Star interacts directly with Spitz, an activating ligand for Egfr (48), and regulates its protein expression (49).

The *Xbp-1* gene is also a suppressor of the DREF-induced rough eye phenotype. The *Xbp-1* gene encodes a 'bZIP'-containing transcription factor and plays a key role in the unfolded protein response, an evolutionarily conserved signalling pathway activated by an overload

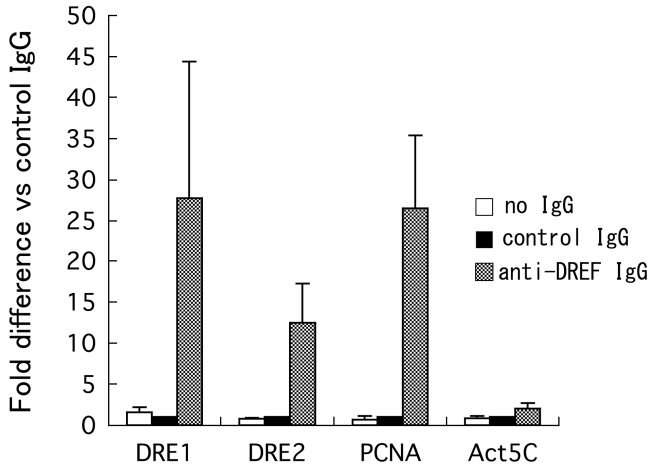

Figure 4. Binding of DREF to DRE-containing genomic regions of the *HP6* gene. Cross-linked chromatin of S2 cells was immunoprecipitated with anti-DREF IgG, control rabbit IgG or no IgG. The genomic regions containing DRE1 of the *HP6* gene, DRE2 of the *HP6* gene, DRE of the *PCNA* gene and Act5C gene were amplified by real time PCR and compared with the amplification products from the immunoprecipitates with the control IgG.

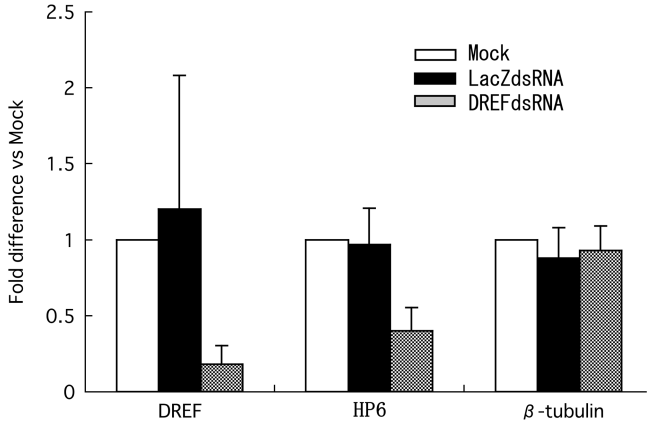

Figure 5. Effects of dsRNA treatment on mRNA levels of HP6 in S2 cells. cDNAs were prepared from total RNA isolated from dsRNA treated S2 cells and levels of *DREF*, *HP6* and *β-tubulin* mRNAs were measured by quantitative RT-PCR. Fold differences against the amplification with no treatment (Mock) are shown with standard deviations from three independent dsRNA treatments.

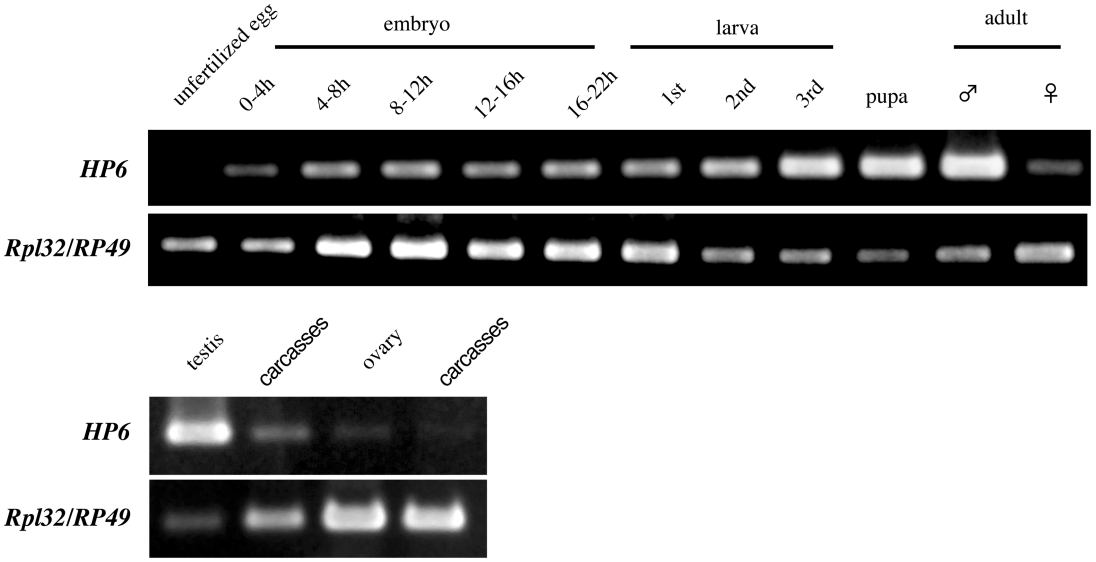

Figure 6. Developmental RT-PCR. Total RNA was extracted from *Drosophila* bodies or the indicated tissues at various developmental stages and RT-PCR was carried out. The upper panels represent the *HP6* mRNA levels and lower panels the *Rpl32*/*RP49* mRNA levels as a control.

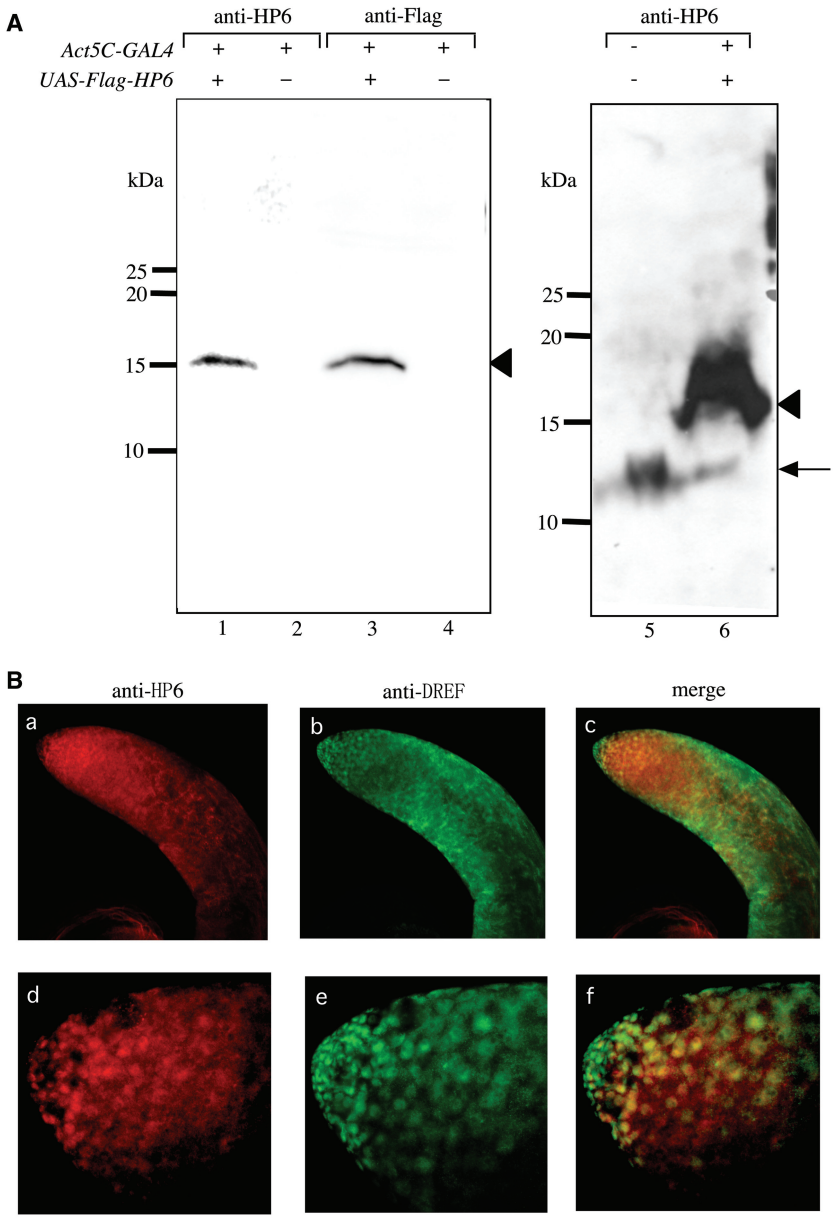

Figure 7. Specificity of anti-HP6 rabbit polyclonal antibody examined by western blot analysis and immunostaining of testes. (**A**) Extracts were: from *w*; +; *Act5C-GAL4*/+ adult male flies for immunoblotting with anti-HP6 antibody (lane 2) or anti-FlagM5 antibody (lane 4); from *w*; +; *Act5C-GAL4*/*UAS-HP6* adult male flies for immunoblotting with anti-HP6 antibody (lanes 1 and 6), or anti-FlagM5 antibody (lane 3); from wild type adult male flies for immunoblotting with anti-HP6 antibody (lane 5). The arrowheads correspond to the Flag-HP6 protein and the arrow corresponds to endogenous HP6 protein. The 100 µg aliquots of protein were used for lanes 1–4, 500 µg for lane 5 and 300 µg for lane 6. (**B**) Immunostaining of testis with anti-HP6 antibody (a and d) or anti-DREF antibody (b and e). Merged images of HP6 and DREF signals (c and f). (d to f) Higher magnification images of a to c.

of misfolded proteins in the endoplasmic reticulum ER (50). The *guftagu* gene is an other suppressor that encodes the *Drosophila* Cullin-3 homologue (d-Cul3) (51) whose function impinges on the activity of many different signalling pathways and developmental events via targeted destruction or modification of specific proteins (51). Recently, we have reported that the *Drosophila skpA* gene is a target of DREF (52). The *skpA* gene encodes a component of the SCF complex that functions in combination with the ubiquitin conjugating enzyme UbcD1 and is involved in cell cycle regulation. Moreover regulation of the gene encoding the proteasome regulator REGγ by the DRE/DREF system has also been reported by others (53). The ubiquitin-proteasome pathway plays key roles in many basic cellular processes, including immune responses, development and programmed cell death (45,46). In addition to degradation of defective or misfolded proteins, a critical regulatory role has been defined in studies of the cell cycle (54–56). Some major signal transduction pathways that are of great importance during development are known to be controlled in a coordinated way,

in which the DRE/DREF pathway may be intimately involved (57).

The *eukaryotic initiation factor 3p40* (*eIF3p40*), the *dribble* and the *ribosomal protein S26* genes were included in the other suppressors identified in the screening and they are all associated with protein synthesis (Table 1). The dribble protein encodes a novel KRR1p-like KH domain protein (58) and *krr1* mutations affect biogenesis of 18S rRNA and its precursors and 40S ribosomal subunits(59). The eIF3p40 protein encodes the p40 subunit of the eIF3 complex which facilitates charging of the 40S ribosomal subunit with the ternary complex (eIF2, Met-tRNAMet, GTP) and bridging with the eIF4G subunit of the cap-binding complex, eIF4F and inhibiting the association of 40S and 60S ribosomal subunits (60,61). The *ribosomal protein S26* gene encodes a *Drosophila* ribosomal protein (RP) with homology to rat RP S26 (62). A slow growth rate and an altered adult size are thought to be the result of a reduced capacity for protein synthesis and this phenotype has been demonstrated to disrupt genes that encode RPs (63). Recently we identified the *eIF4A* gene, encoding a member of the DEAD box family of ATP-dependent RNA helicases (64), as another target of DREF. eIF4A is proposed to function in cap(m^7GpppN)-dependent initiation of protein synthesis by unwinding the secondary structure of 5′-untranslated regions of mRNA (65,66). Since genes responsible for degradation of defective or misfolded proteins are targets of DREF as described above, DREF apparently promotes both protein synthesis and degradation by directly or indirectly activating genes involved in these processes. This is presumably associated with the active protein metabolism typical of proliferating cells.

A number of other genes are of obvious interest given their physiological significance. Among the strongest suppressors of the DREF-induced rough eye phenotype was the mutated *HP6* gene. The present studies clearly demonstrate that *HP6* gene is one of the targets of DREF. Although HP6 is not a modifier of position effect variegation as are several of other *Drosophila* HPs, it carries chromo shadow domain (37). It has been shown that chromo shadow domain in HP1 is highly conserved across species and crucial for interaction with many proteins such as the SUV39H1 (67), SP100 (68,69), TIF1-β(KAP-1) (70), Ku70 (71), lamin B receptor, HP1 itself (72), Ki-67 (73) and HP1/origin recognition complex-associated protein (HOAP) (74). It has further been reported that HP6 directly interacts with the Caravaggio protein in a two-hybrid assay (75). The *caravaggio* gene is otherwise known as *Drosophila* HOAP. We here found the expression level of *HP6* mRNA to the highest in adult males and it much higher in testes than other sites. DREF is also expressed in the testis (1). The present study revealed that both proteins at least partially co-localize in nuclei at the apical tips of testes where cell proliferation actively occurs, suggesting some roles of HP6 in regulation of cell proliferation or transcription of the meiosis-related genes in testis.

We have searched for DRE sequences in the 5′-flanking regions of other five HP family genes in *Drosophila* on the genome database and found that examples in promoters in all cases (Table 2). In this context it should be noted that DREF is also involved in transcriptional regulation of genes coding for the chromatin remodeling BRM complex (24). Moreover, the present genetic screen identified the *little imaginal discs* (*lid*) gene as a suppressor of the DREF-induced rough eye phenotype. The *lid* encodes a histone H3 trimethyl-Lys4 demethylase, a regulator of the chromatin structure (76–78). Therefore DREF may influence expression of many genes through regulation of genes involved in alteration of chromatin structures.

Table 2. DRE or DRE-like sequences in 5′-flanking region of the *Drosophila HP*family genes

Gene	DRE or DRE-like	Position
HP1	5′-cATCGATt	−462 to −469
	5′-aATCGATt	−470 to −477
	5′-taTCGATA	−503 to −510
	5′-TcTCGATc	−979 to −986
HP2	5′-aATCGATt	−489 to −495
HP3	5′-TATCGATt	−134 to −141
	5′-TATCGATt	−186 to −194
	5′-gATCGAgA	−475 to −482
	5′-TATCGAcA	−920 to −927
HP4	5′-TATCGATA	−366 to −373
	5′-atTCGATA	−536 to −543
HP5	5′-TATCGATt	−670 to −677
HP6	5′-TATCGAaA	−116 to −123
	5′-TgTCGATA	−132 to −139
	5′-cATCGAaA	−154 to −161
	5′-aATCGATt	−1006 to −1013

Five suppressor genes for the DREF-induced rough eye phenotype; *HP6*, *pigeon*, *lace*, *Xbp-1* and *guftagu* are candidate DREF target genes, since they carry DRE sequences in their 5′-flanking regions. These five genes have distinct functions as described above. Overexpression of DREF in eye imaginal discs induced multiple effects such as induction of DNA synthesis and apoptosis, inhibition of photoreceptor cell differentiation and loss of pigment cells (20). Although suppression of the rough eye phenotype by mutation of each suppressor genes appeared to be strong by examination with a scanning electron microscopy, inspection of horizontal sections of adult fly eyes showed that the suppression is still partial in most cases (20). Therefore suppression of the DREF-induced rough eye phenotype could be resulted from disturbance of multiple pathways in which many suppressor genes might be involved.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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