The DNA replication-related element (DRE)–DRE-binding factor (DREF) system may be involved in the expression of the *Drosophila melanogaster TBP* gene

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Abstract The TATA box binding protein (TBP) is a general transcription factor required for initiation by all three eukaryotic RNA polymerases. Previously, we found that the promoter region of the *Drosophila melanogaster TBP* gene contains three sequences similar to the DNA replication-related element (DRE) (5′-TATCGATA). In the present study, we found that the DRE-like sequences are also present in the promoter of the *Drosophila virilis TBP* gene, suggesting a role for these sequences in TBP expression. Band mobility shift assays revealed that oligonucleotides containing sequences similar to the DRE of *D. melanogaster TBP* gene promoter form specific complexes with a factor in a Kc cell nuclear extract and with recombinant DRE-binding factor (DREF). Furthermore, these complexes were either supershifted or diminished by monoclonal antibodies to DREF. Transient luciferase assays demonstrated that induction of mutations in two DRE-related sequences at positions −223 and −63 resulted in an extensive reduction of promoter activity. Thus, the DRE–DREF system appears to be involved in the expression of the *D. melanogaster TBP* gene.

Key words: TATA box-binding protein; DNA replication-related element; Gene expression; Promoter activity; *Drosophila melanogaster*

1. Introduction

In eukaryotes, there are three nuclear RNA polymerases, RNA polymerase I, II, and III, each of which transcribes different classes of genes. The genes in each class contain their unique promoters, which require a distinct set of transcription factors to initiate their transcription. The transcription factor, TATA box binding protein (TBP), was originally found to be involved in TATA-box dependent transcription by RNA polymerase II. However, it has since been shown to be necessary for the transcription initiation of all three classes of RNA polymerases, even though it behaves in functionally distinct ways [1,2]. The association of TBP with polymerase-specific transcription factors gives the specificity of RNA polymerases for each class of promoters. TBP is involved as a subunit of SL1, TFIID, and TFIIIB complexes in the transcription initiations of RNA polymerase I, II, and RNA III, respectively.

Although the functional roles of TBP have been extensively studied, the regulation of *TBP* gene expression is not well understood. Since TBP is required for most gene expression, one could expect that TBP is constitutively expressed in the cell and does not need specific regulation of its gene expression. However, there are several reports which suggest that *TBP* gene expression is regulated. For example, the levels of TBP mRNA are very different in different tissues [3,4], and higher levels of TBP mRNA were observed in several carcinomas [5]. It was also reported that the level of human TBP mRNA increases rapidly after the addition of serum to starved cells [5]. The analysis of human TBP promoter revealed that it is regulated by the Ets family proteins [6], which have been implicated in the developmental process and cellular proliferation [7]. These results raise the possibility that *TBP* gene expression is regulated in a tissue- and/or developmental-specific manner.

Previously, we isolated and characterized the *TBP* gene of *Drosophila melanogaster* [8]. The promoter of the *D. melanogaster TBP* gene does not have the TATA box consensus sequence, but does have AT-rich sequences [8]. The 401 bp long region (−261 to +138 with respect to the transcription initiation site) is sufficient for efficient expression [9]. A serial deletion analysis of the region between −261 and −207 suggested that at least two *cis*-regulatory elements in this region are involved in the expression of the *D. melanogaster* TBP gene [10]. One of the elements is a 11 bp palindromic sequence, CTTTTGAAAAG, centered at −243, and the other possible element is located in the region between −225 and −214. By site-directed mutagenesis and electrophoretic mobility shift assay, we have previously shown that the 11 bp palindromic sequence is involved in the stimulation of the *D. melanogaster* TBP expression and is recognized by one or more proteins in nuclear extract [10].

The region between −225 and −214 contains the sequence TATCGATT, which is similar to the DNA replication-related element (DRE) sequence, TATCGATA [11]. Two additional DRE-related sequences are also found downstream of this

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Abbreviations: DRE, DNA replication-related element; DREF, DRE-binding factor; TBP, TATA box binding protein; GST, glutathione *S*-transferase

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region. DRE is known to be important for DNA replication-related genes and various cell cycle- and cell proliferation-related genes in *D. melanogaster* and *Bombyx mori* [11–15]. The transcription factor, DRE-binding factor (DREF), which specifically binds to the DRE sequence, has been isolated as a homodimer of a 80 kDa polypeptide [11], and its cDNA has been cloned [16]. The importance of the role of the DRE–DREF regulatory system was demonstrated by the finding that DRE–DREF is a target of differentiation signals, such as Zen, a homeodomain-containing protein [17,18]. Zen is known to be involved in the differentiation of the amnioserosa and the optic lobe [19–21]. Zen expression in cultured cells results in the repression of DNA replication-related genes by reducing DREF activity [17,18]. Furthermore, it has been shown that the DRE–DREF system activates transcription of the *D. melanogaster E2F* gene, an important transcription regulator of cell cycle-related genes [22]. The DRE–DREF system also appears to function as a common regulatory system for transcription of DNA replication-related genes. Therefore, it was of interest to determine whether the DRE–DREF system plays a role in the expression of the *TBP* gene. In this study, we provide results which indicate that the DRE–DREF system may be involved in the expression of the *D. melanogaster TBP* gene.

2. Materials and methods

2.1. Oligonucleotides

The sequences of double-stranded oligonucleotides containing potential DRE sites or their base-substituted derivatives present in the promoter region of *D. melanogaster TBP* gene are defined as follows: TBP-DRE1-wt: 5′-GCCATTTTAGTTATCGATTGGCAACGACG-T-3′, 3′-CGGTAAAATCAATAGCTAACCGTTGCTGCA-5′; TBP-DRE1-mut: 5′-GCCATTTTAGTTtagGATTGGCAACGACGT-3′, 3′-CGGTAAAATCAAatcCTAACCGTTGCTGCA-5′; TBP-DRE2-wt: 5′-TGCTAAGAACGTAACGATAAACGTAAGACT-3′, 3′-ACGATTCTTGCATTGCTATTTGCATTCTGA-5′; TBP-DRE2-mut: 5′-TGCTAAGAACGTtAgGATAAACGTAAGACT-3′, 3′-ACGATTCTTGCAaTcCTATTTGCATTCTGA-5′; TBP-DRE3-wt: 5′-ACTCAGCCAGCTATCGATAGTTGCGGCAAA-3′, 3′-TGAGTCGGTCGATAGCTATCAACGCCGTTT-5′; TBP-DRE3-mut: 5′-ACTCAGCCAGCTtagGATAGTTGCGGCAAA-3′, 3′-TGAGTCGGTCGAatcCTATCAACGCCGTTT-5′, where potential DRE sites are underlined and mutated bases are designated as lowercase letters. Three DRE sites are located at −223 (TBP-DRE1), −63 (TBP-DRE2), and −36 (TBP-DRE3), upstream from the transcription initiation site [8]. *Draf*-DRE-wt and *Draf*-DRE-mut1 oligonucleotides [14] were also used as controls.

2.2. Isolation of Drosophila virilis TBP gene

A *D. virilis* genomic library obtained from Dr. J. Tamkun (University of California, Santa Cruz, CA, USA) was screened under conditions of reduced stringency using *D. melanogaster* TBP cDNA [23] as a probe. Hybridization at reduced stringency was performed by first pre-hybridizing overnight at 37°C in 40% formamide, 5×SSC (1×SSC: 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0), 5×Denhardt's (1×Denhardt's: 0.2 mg/ml each of Ficoll, bovine serum albumin, and polyvinylpyrrolidone), 0.1% sodium dodecyl sulfate (SDS), 1 mM EDTA, and 50 μg/ml denatured herring sperm DNA. Hybridization was performed overnight using the pre-hybridizing conditions, but with the radiolabeled ^{32}P probes added to the pre-hybridization buffer. After hybridization, the filters were washed three times for 20 min in 2×SSC, 0.1% SDS at 50°C.

2.3. Band mobility shift assay

Preparation of nuclear extracts from *D. melanogaster* Kc cells was performed as described elsewhere [11]. Kc cell nuclear extracts or glutathione *S*-transferase (GST)–DREF fusion proteins containing the DNA-binding domain of DREF (DREF1–125) [22], and unlabeled competitor oligonucleotides, were incubated in 20 μl of reaction mixture containing 10 mM HEPES (pH 7.6), 50 mM KCl, 1 mM EDTA, 5% glycerol, 0.5 μg of poly(dI-dC) and 1 μg of sonicated herring sperm DNA for 10 min at room temperature (RT). The ^{32}P-end-labeled TBP-DRE oligonucleotides (2×10^4 cpm) were then added and the mixture further incubated for 20 min at RT. In the case of the gel shift assay performed with anti-DREF monoclonal antibody No. 1 (mAb 1) and No. 4 (mAb 4) [16], Kc cell nuclear extract was incubated in the reaction mixture described above with or without the mAb 1 or mAb 4 for 20 min before the addition of the radiolabeled oligonucleotides. The retarded bands caused by DNA–protein binding were electrophoretically resolved on a 4% non-denaturing Tris–borate–EDTA polyacrylamide gel. The gels were then dried and autoradiographed.

2.4. Construction of luciferase expression plasmids

The plasmid pT-luc was used as a parental plasmid for the construction of the promoter–luciferase fusion plasmids [24]. A DNA fragment of 549 bp, spanning −415 to +134 with respect to the transcription start site of the *D. melanogaster TBP* gene, was amplified by PCR and cloned into the *Bam*HI site of pT-luc. The resulting recombinant plasmid was designated pDRE-wt. The site-directed mutagenesis of the DRE-like sequences was performed using the procedure described by Kunkel [25]. The M13mp19 DNA containing the fragment of 549 bp (−415 to +134) and the mutagenic oligonucleotides, ACGTCGTTGCCAATCCTAAACTAAAATGGCGCA (mut1), AGTCTTACGTTTATCCTAACGTTCTTAGCATTT (mut2), and TTTTGCCGCAACTATCCTAAGCTGGCTGAGTCTT (mut3), were used for mutant construction. Mutants were identified by subjecting several randomly selected phage clones to nucleotide sequence analysis. The mutagenized M13 DNA fragments of 549 bp (−415 to +134) were then subcloned into the *Bam*HI site of pT-luc.

2.5. DNA transfection and luciferase assay

Dimethyldioctadecyl-ammonium bromide-mediated transfection of *D. melanogaster* Schneider Line 2 was performed using 24-well tissue culture plates as described previously [26]. Each transfection mixture contained 5 ng of the appropriate reporter plasmid and 100 ng of pcopia LTR-lacZ internal control plasmid. Three days after transfection, cell extracts were prepared by adding 20 μl of Cell Lysis reagent (Promega) to each well after removing the media by aspiration. The 24-well plate was shaken gently on a rotary shaker for 5 min, and cell extracts were transferred to a microcentrifuge tube. After centrifugation for 5 min, 2 and 1 μl of the supernatant were subjected to β-galactosidase and luciferase assays, respectively. The β-galactosidase assay was performed using a colorimetric method as described previously [27]. Luciferase assay was performed with a luminometer (TD-20/20, Turner Designs). Normalized luciferase activities were calculated by determining the luciferase/β-galactosidase activity ratios and by averaging the values from duplicate experiments. Each transfection was repeated 3–8 times and the average values and standard deviations were calculated.

3. Results

3.1. The palindromic sequence and the DRE-related sequences are conserved in the promoter regions of the D. melanogaster and D. virilis TBP genes

Previously, we showed that the two kinds of elements, the 11 bp long palindromic sequence, CTTTTGAAAAG, centered at −243, and the DRE-related sequence between −225 and −214, might be involved in the expression of the *D. melanogaster TBP* gene [10]. One of the ways of verifying the importance of the *cis*-regulatory elements in the expression of the *TBP* gene involved determining whether these elements are conserved in related species such as *D. virilis*. The *D. melanogaster* TBP cDNA was used to screen a *D. virilis* genomic library containing approximately three genomic equivalents under reduced stringency hybridization conditions (see Section 2), and this yielded two positive clones. The isolated clones were digested with various restriction enzymes and hybridized to the *D. melanogaster* TBP cDNA. 1.8-kb

A

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-521                      A AGCTTATGTA TGTAAAAGGG CCAAAGAATA AAATCATTTA
                           HindIII
-480  AACACATATT TTAAGCATAC TAAATAAACA AAAAACAAGA AAAAACAATG TTAACTGAAA
-420  CTTTTGTGAA ACCACACACT CTTTTTCTGG TATTATTGCA TCAATATATA TATTCTACAT
-360  GTGTCTTACA TTACTATTTT TTTATATATG CTGCATATGA TAACTTAAAT GTGTATGTGT
-300  ATCCTAACTG TTAATTATTT ATTAAGTCAG AAACACTTAA ATAAAGCCGT TAGAAGCATT
-240  TCATTCTTTG TACATATCTA AATCAACTGT GTTTATTGCC TTACTTTTCA AAGGTGTTGA
-180  CCGTTATGCG TCGAAGTTTT TTCGATTTCT TGATACATTT TGAAAATAGA ACTATCGAAA
-120  CTAATGATAT TTGTGTATAT CGATAAAAAT ACCTAAACGA ACTAATAATC AAATGAACGA
 -60  ACATATGGGA ACAACGGAAC TAACTCAGTG TATTTGGACT ACACACACAC ATGAAATTCA
       tsp
   1  TTTAGTGTTT AGTGTGAATG CAACCCTGAG GAGCGACGCG GCTTTCCATT TACGCTCACA
  61  TATTTTTGTT GAATTTTCAC CCAGCTTTGC ATTAATACGA ATTTGCGGCT CCAAGAATTA
 121  AATAAAACAA CGTCACAATG GATGACATTA TGAGCCCGAA CTTTTCCATA CCCAGCATTG
                                  Primer 1
 181  GGCATGCTCC AACGCCGATA CACCACAGCC TCGACGCCGA TCAACAAATT GAAGCATTAC
 241  CACATCAGGT CTATCACCCA GCCGCACTGG ATCCAACGCC AACAGCTACC ACA
                                  Primer 2
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B

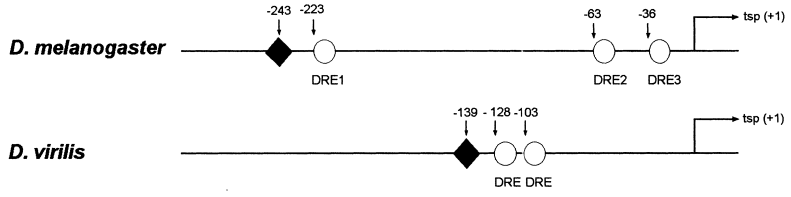

Fig. 1. The nucleotide sequence of the 5′-flanking region of the *D. virilis TBP* gene and the relative position of the conserved palindromic sequences and the DRE-related sequences in the 5′-flanking region of the *D. melanogaster* and the *D. virilis TBP* gene. A: The transcription start point (tsp) is indicated by an arrow, the translation initiation codon is shaded and the primers (primer 1 and primer 2), used for 5′-RACE, are underlined. DRE-related sequences are boxed and the conserved palindromic sequence adjacent to DRE is underlined. The EMBL/GenBank data library accession number of this sequence is AF22062. B: The conserved palindromic sequences and the DRE-related sequences of the *D. melanogaster* and *D. virilis TBP* genes are shown by the filled boxes and the open circles, respectively. The tsp is indicated by an arrow. The numbers above the boxes and circles indicate distances in nucleotides from the tsp.

*Hin*dIII fragments commonly present in both isolated genomic clones were detected. This fragment was partially sequenced and found to contain about 500 bp of promoter region DNA of the *D. virilis TBP* gene, which was enough to make a comparison with that of the *D. melanogaster TBP* gene.

Fig. 1A shows the sequence of the promoter region of the *D. virilis TBP* gene. The transcription start point was determined by a 5′RACE analysis using poly(A)$^+$ RNA extracted from *D. virilis* whole adult bodies. From the alignment of the nucleotide sequences in the 5′-flanking regions of the TBP genes, we found a highly conserved 19 bp long sequence, which includes the palindromic sequence, CTTTTGAAAAG in *D. melanogaster* and ATTTTGAAAAT in *D. virilis* (Fig. 1A,B). This palindromic sequence was previously found to be important for the expression of the *D. melanogaster TBP* gene [10]. We also found two DRE-related sequences at −103 (TATCGATA) and −128 (TATCGAAA) in the 5′-flanking region of the *D. virilis TBP* gene (Fig. 1A,B). The conservation of the palindromic and DRE-related sequences in the two *Drosophila* species implies their importance in the expression of the *TBP* genes, and supports our previous results obtained by promoter analysis of the *D. melanogaster TBP* gene [10].

3.2. DREF binds to the three DRE-related sequences in the promoter region of the D. melanogaster TBP gene

The promoter region of the *D. melanogaster TBP* gene contains one sequence identical to DRE (TATCGATA) at position −36 and two sequences similar to DRE at positions −223 (TATCGATT) and −63 (TAACGATA), with respect to the transcription initiation site (Fig. 1B). We named these DRE-related sequences DRE1, DRE2, and DRE3, respectively (Fig. 1B).

We then examined whether these three DRE-related sequences are recognized by DREF, by performing band mobility shift assays using Kc cell nuclear extracts and radiolabeled probes (Fig. 2). The oligonucleotide *Draf*-DRE-wt, containing two overlapping DRE sequences of the *D. melanogaster raf* gene, was used as a positive control [28,29]. *Draf*-DRE-wt was previously confirmed to be bound by DREF. The oligonucleotide, *Draf*-DRE-mut1, containing two 3 bp substitutions in *Draf*-DRE-wt, was used as a negative control [14]. Shifted bands were observed when the assays were per-

formed with the radiolabeled TBP-DRE1-wt, TBP-DRE2-wt, and TBP-DRE3-wt oligonucleotides as probes (Fig. 2A–C, lanes 2 and 7, respectively). These bands were diminished by adding excess amounts of unlabeled TBP-DRE1-wt, TBP-DRE2-wt, TBP-DRE3-wt, and *Draf*-DRE-wt (Fig. 2A–C, lanes 3 and 4 and lanes 8 and 9). However, the mutated oligonucleotides (TBP-DRE1-mut, TBP-DRE2-mut, TBP-DRE3-mut, and *Draf*-DRE-mut1) failed to compete for binding, even though these competitors were used at up to twofold more than the wild type competitors (Fig. 2A–C, lanes 5 and 6 and lanes 10 and 11). A comparison of the binding affinity of DREF to the three DRE-related sequences, by measuring the radioactivities of the shifted band under the same conditions, showed that the order of binding affinities was DRE1 > DRE3 > DRE2.

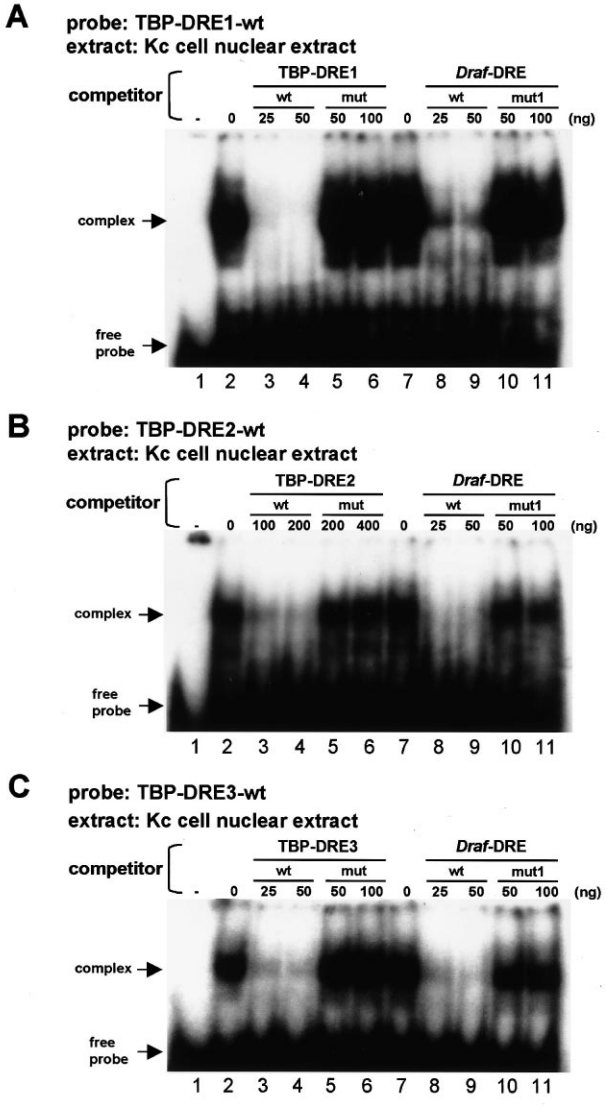

Fig. 2. Complex formation between the three potential DRE sites and Kc cell nuclear extracts. Radiolabeled TBP-DRE1-wt (A), TBP-DRE2-wt (B) or TBP-DRE3-wt (C) oligonucleotides were incubated with Kc cell nuclear extracts in the presence of increasing amounts of competitor oligonucleotides. The following competitors were used: TBP-DRE1-wt, TBP-DRE2-wt and TBP-DRE3-wt (A–C, lanes 3 and 4), oligonucleotides containing the putative DRE sites 1, 2 and 3 of the *Drosophila* TBP gene promoter; TBP-DRE1-mut, TBP-DRE2-mut and TBP-DRE3-mut (A–C, lanes 5 and 6), oligonucleotides containing 2 bp substitutions in the putative DRE sites 1, 2 and 3 of the *Drosophila* TBP gene promoter; *Draf*-DRE-wt (A–C, lanes 8 and 9), oligonucleotides containing the DRE sequence from the *Drosophila raf* promoter; *Draf*-DRE-mut1 (A–C, lanes 10 and 11), oligonucleotides containing two 3 bp substitutions in the DRE from the *Drosophila raf* promoter; lane 1 of A–C, no extract added; lanes 2 and 7 of A–C, no competitors added.

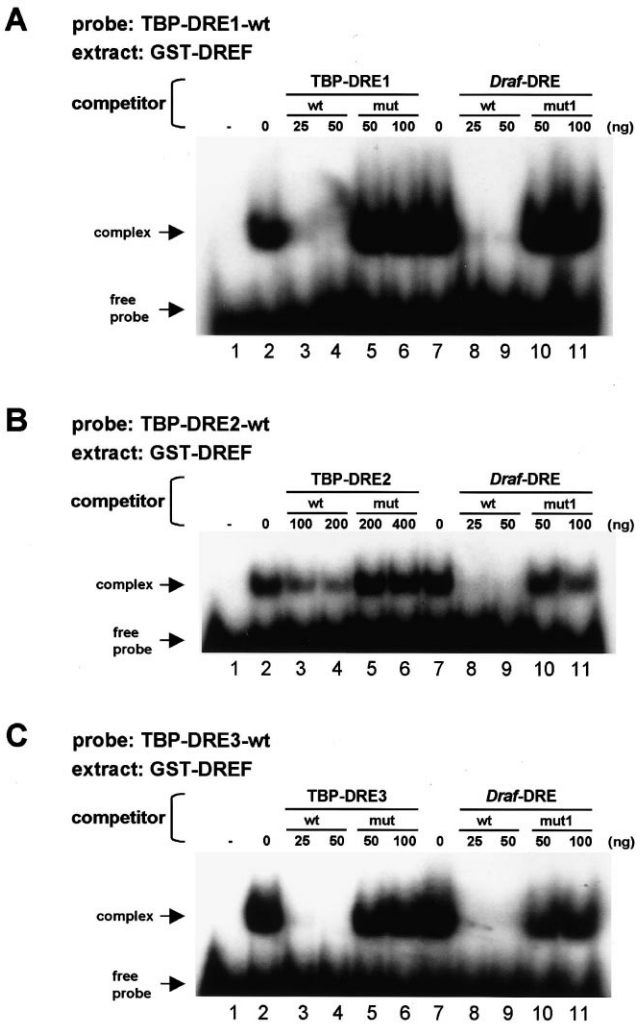

Fig. 3. Complex formation between three potential DRE sites and GST-DREF1–125. Radiolabeled TBP-DRE1-wt (A), TBP-DRE2-wt (B) or TBP-DRE3-wt (C) oligonucleotides were incubated with GST-DREF (1–125) fusion protein in the presence of increasing amounts of competitor oligonucleotides. The following competitors were used: TBP-DRE1-wt, TBP-DRE2-wt and TBP-DRE3-wt (A–C, lanes 3 and 4), oligonucleotides containing the putative DRE sites 1, 2 and 3 of the *Drosophila* TBP gene promoter; TBP-DRE1-mut, TBP-DRE2-mut and TBP-DRE3-mut (A–C, lanes 5 and 6), oligonucleotides containing 2 bp substitutions in the putative DRE sites 1, 2 and 3 of the *Drosophila* TBP gene promoter; *Draf*-DRE-wt (A–C, lanes 8 and 9), oligonucleotides containing intact DRE from the *Drosophila raf* promoter; *Draf*-DRE-mut1 (A–C, lanes 10 and 11), oligonucleotides containing two 3 bp substitutions in the DRE from the *Drosophila raf* promoter; lane 1 of A–C, no extracts added; lanes 2 and 7 of A–C, no competitors added.

Band mobility shift assays were also performed using a recombinant fusion protein of GST, and the N-terminal DRE-binding domain (1–125 amino acid residues) of DREF (GST-DREF1–125) [22]. As expected, the recombinant pro-

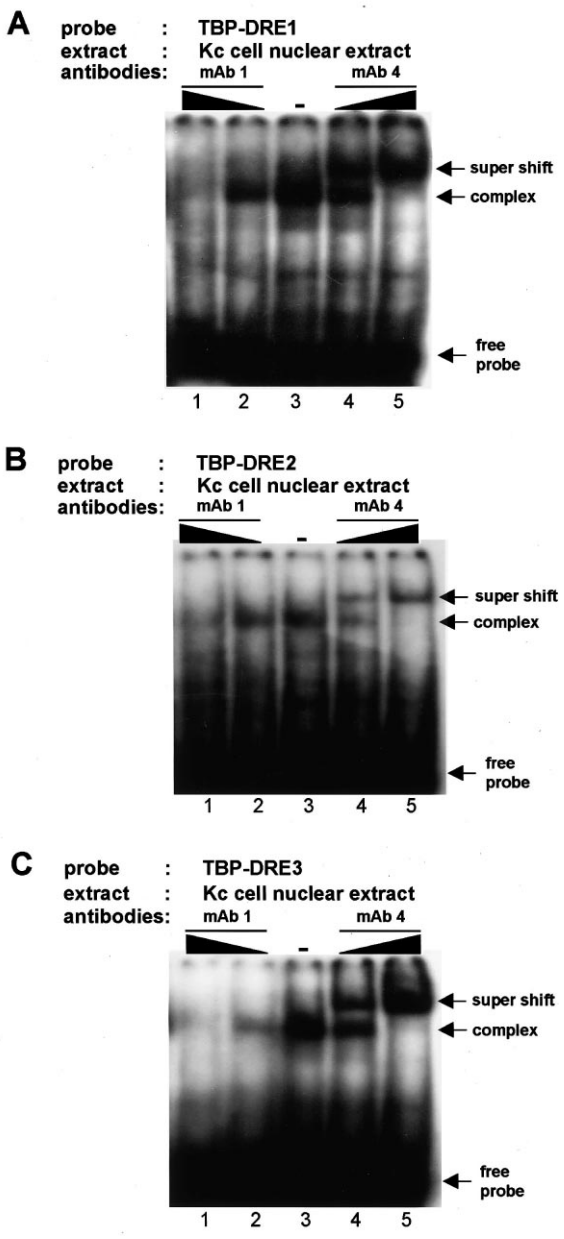

Fig. 4. Effects of monoclonal antibodies on the binding between three potential DRE sites and Kc cell nuclear extracts. Radiolabeled TBP-DRE1-wt (A), TBP-DRE2-wt (B) or TBP-DRE3-wt (C) oligonucleotides were incubated with Kc cell nuclear extracts in the absence (lane 3 of A–C) or presence (lanes 1,2 and 4,5 of A–C) of anti-DREF monoclonal antibody No. 1 (3 and 1.5 μl of culture supernatant) or anti-DREF monoclonal antibody No. 4 (0.4 and 0.8 μl of culture supernatant); mAb 1, anti-DREF monoclonal antibody No. 1; mAb 4, anti-DREF monoclonal antibody No. 4.

teins formed a complex with the TBP-DRE1-wt, TBP-DRE2-wt, and TBP-DRE3-wt oligonucleotides (Fig. 3A–C, lanes 2 and 7), and the shifted bands were diminished by adding wild type competitors (Fig. 3A–C, lanes 3 and 4 and lanes 8 and 9). In addition, as was found in the case of Kc cell nuclear extracts, the complexes between GST-DREF and TBP-DRE-wt were not diminished by the addition of mutated oligonucleotide competitors (Fig. 3A–C, lanes 5 and 6 and lanes 10 and 11).

To examine whether the shifted bands with Kc cell nuclear extracts, described above, actually contain DREF, anti-DREF monoclonal antibodies (mAb 1 and mAb 4) were added in the binding reaction. It was previously reported that mAb 1 inhibits the binding of DREF to DRE sites, while mAb 4 supershifts the complex [14,16]. As shown in Fig. 4, all of the three TBP-DRE-wts showed the same effect of anti-DREF antibodies, i.e. diminished by mAb 1 (Fig. 4A–C, lanes 1 and 2) and supershifted by mAb 4 (Fig. 4A–C, lanes 4 and 5). These results indicate that DREF recognized all of the three DRE sites in the promoter region of the *D. melanogaster TBP* gene.

3.3. *Effects of mutations in the DRE-related sequences on the promoter activity of the TBP gene*

To examine the role of DRE-related sequences on promoter activity, the activity of wild type TBP promoter was compared with that of the TBP promoter containing the same mutations in the DRE-related sequences as those used for the band mobility shift assay. For this purpose, we constructed luciferase expression plasmids containing the promoter region from −415 to +134, and mutagenized the three DRE-related se-

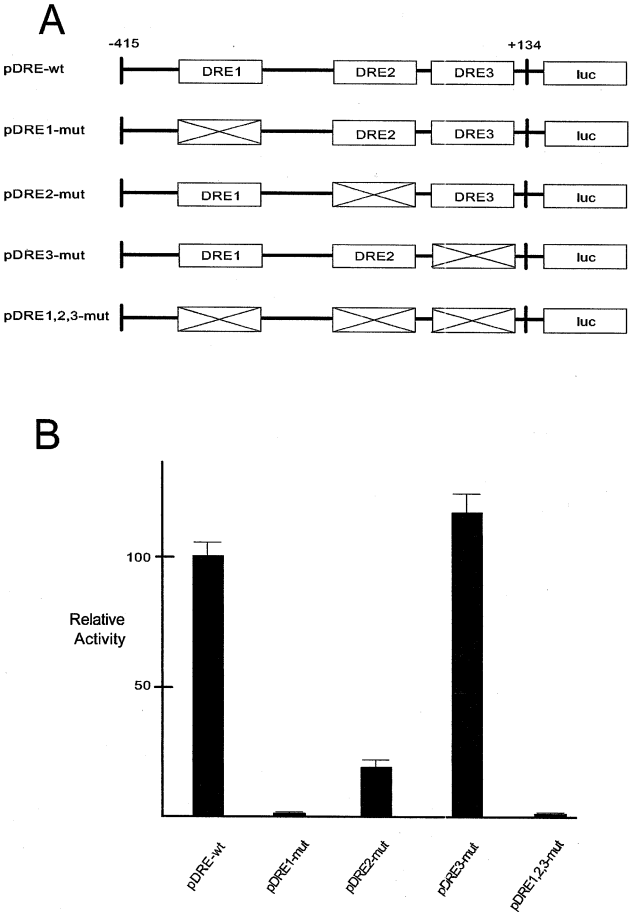

Fig. 5. Roles of DRE-related sequences upon the promoter activity of the *D. melanogaster TBP* gene. A: Schematic features of the promoter–luciferase fusion plasmids are illustrated. DRE-related sequences are indicated by open boxes and mutated DREs are marked by crossed boxes. B: Relative luciferase activities of the cells transfected with the promoter–luciferase fusion plasmid. Luciferase activities of the cells transfected with the plasmids are compared to that of the transfected cells by pDRE-wt. The transfection efficiency was normalized by cotransfection with the pcopia-lacZ plasmid, a β-galactosidase expression vector. Standard deviations of the means are indicated by error bars.

quences as shown in Fig. 5A. Each expression plasmid was transfected into *D. melanogaster* Schneider Line 2 cells, and the transient expression of luciferase was measured. As shown in Fig. 5B, the mutation in DRE1 almost completely abolished the expression of luciferase, and the mutations in DRE2 reduced this expression to 30%, but mutations in DRE3 did not affect the expression. Actually it increases the expression by approximately 20%. These results suggest that DRE1 and DRE2 are important for the promoter activity of the *D. melanogaster TBP* gene.

4. Discussion

In the present study, we have shown that the DRE–DREF system may be involved in the expression of the *D. melanogaster TBP* gene. Band mobility shift assays using either Kc cell nuclear extract and the anti-DREF monoclonal antibodies, or recombinant DREF, showed that DREF binds to DRE-related sequences in the promoter of the *D. melanogaster TBP* gene (Figs. 2–4). The binding affinities of DREF to the DREs do not seem to be directly related to the promoter activity, since the transient transfection assay using mutations in the DREs did not show any relevant changes in promoter activities which depended on the affinities of DREF to the DREs (Fig. 5). Even though DRE3 has a higher affinity to DREF than DRE2, DRE3 makes a lower contribution to promoter activity than DRE2. DRE1 seems to have a critical function in the expression of the *D. melanogaster* TBP gene, because the mutation in DRE1 abolished promoter activity (Fig. 5). This result is consistent with our previous study, which showed that the deletion of the promoter region between −261 and −207, containing DRE1, dramatically decreases the promoter activity [9,10]. DRE2 also contributes considerably to the promoter activity of the *TBP* gene, since its mutations decreased the promoter activity to about 30%. However, the mutations in DRE3 had no deleterious effect on promoter activity (Fig. 5). Therefore, we conclude that DRE1 and DRE2 but not DRE3 are necessary for the efficient expression of TBP, even though all three DREs can be recognized by DREF. The *D. virilis TBP* gene also contains the two DREs in its 5′-flanking region, which suggests that DREs are important in the expression of the *TBP* gene, and the two DREs may be necessary and sufficient for such expression.

It may be possible that the palindromic sequence interacts with the DRE–DREF system. DRE1 lies 15 bp away from the 11 bp long palindromic sequence in *D. melanogaster*, but in *D. virilis* it is 4 bp away. The difference, therefore, between the two species in terms of the distance of the DRE sequence from the palindromic sequence is 11 bp, about one helix turn. Further analysis should be undertaken to investigate the possible role of the palindromic sequence in the expression of the *TBP* gene.

In *D. melanogaster*, the DRE–DREF system is known to be involved in the expression of proliferating cell nuclear antigen genes, the 180 kDa and 73 kDa subunits of DNA polymerase α, cyclin A, *D-raf*, and E2F [11–14,22]. The presence of the DRE sequences in these proliferation-related genes suggests that the DRE–DREF system is a common regulatory element for the genes involved in cell division and/or cell proliferation. The involvement of the DRE–DREF system in *TBP* gene expression suggests that the expression of TBP is also related to cell division and cell proliferation. In addition, TBP is known to be a major target for the regulation of the expression of genes related to cell proliferation, because of its interaction with transcription factors such as retinoblastoma (RB) protein [30], p53 [31,32], E1A [33], c-Myc [34], and v-Myb [35]. For these reasons we believe that the regulation of *TBP* gene expression is likely to be important for cell division and/or proliferation. The regulation of human *TBP* gene expression by Ets proteins also supports this idea [6]. A study focused upon TBP expression related to cell proliferation is now in progress.

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