

Isolation and Characterization of cDNA for DREF, a Promoter-activating Factor for *Drosophila* DNA Replication-related Genes*

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DREF, a transcription regulatory factor which specifically binds to the promoter-activating element DRE (DNA replication-related element) of DNA replication-related genes, was purified to homogeneity from nuclear extracts of *Drosophila* Kc cells. cDNA for DREF was isolated with the reverse-transcriptase polymerase chain reaction method using primers synthesized on the basis of partial amino acid sequences and following screening of cDNA libraries. Deduced from the nucleotide sequences of cDNA, DREF is a polypeptide of 701 amino acid residues with a molecular weight of 80,096, which contains three characteristic regions, rich in basic amino acids, proline, and acidic amino acids, respectively. Deletion analysis of bacterially expressed DREF fused with glutathione *S*-transferase (GST-DREF) indicated that a part of the N-terminal basic amino acid region (16–115 amino acids) is responsible for the specific binding to DRE. A polyclonal and four monoclonal antibodies were raised against the GST-DREF fusion protein. The antibodies inhibited specifically the transcription of DNA polymerase α promoter *in vitro*. Co-transfection experiments using Kc cells demonstrated that overproduction of DREF protein overcomes the repression of the proliferating cell nuclear antigen gene promoter by the *zerknüllt* gene product. These results confirmed that DREF is a trans-activating factor for DNA replication-related genes. Immunocytochemical analysis demonstrated the presence of DREF polypeptide in nuclei after the eighth nuclear division cycle, suggesting that nuclear accumulation of DREF is important for the coordinate zygotic expression of DNA replication-related genes carrying DRE sequences.

A number of enzymes involved in DNA replication have been suggested to form an enzyme complex for this purpose (1). Genes for these enzymes are expressed in proliferating cells and repressed in quiescent cells reaching confluency or in association with cellular differentiation (2). Therefore, it is of interest to clarify the genetic mechanisms governing the coordinate induction or repression of DNA replication-related genes in relation to growth or differentiation signals.

In budding yeast, the *MluI* cell cycle box and the specific binding factor, DSC-1, are responsible for cell cycle-dependent

transcription of a number of DNA replication-related genes (3–6).

The mRNAs for human DNA polymerase α , PCNA,¹ murine DNA polymerase α -primase complex, and thymidylate synthetase are present throughout the cell cycle and increase slightly prior to the S phase (7–10). The critical promoter regions of DNA polymerase α , dihydrofolate reductase (DHFR), and thymidine kinase genes contain binding sites for the E2F family (11–13). Mutagenesis of the promoter of the DHFR gene has provided strong evidence that the E2F element is responsible for the promoter activation in late G₁ (14). Furthermore, the E2F-binding site was shown to be involved in activation of the DHFR and thymidine kinase genes following serum stimulation (15, 16). The fact that the active form of E2F accumulates in late G₁ toward the S phase (17–19) provides further evidence that E2F family members are likely candidates for involvement in transcriptional regulation of specific G₁/S-phase-activated genes that are required for DNA replication. The E2F binding sites of the *Drosophila* genes for DNA polymerase α and PCNA are also important for their proliferation-related expression (20, 21). However, little is known about regulation mechanisms of DNA replication-related genes during the transition from G₀ into the cell cycle or the transition from proliferating to quiescent states.

We have analyzed upstream regulatory regions of *Drosophila* genes for the DNA polymerase α 180-kDa catalytic subunit (22) and the PCNA (23), and found a novel transcription regulatory sequence consisting of an 8-bp palindromic sequence (5'-TATCGATA), called DRE (DNA replication-related element) and a specific binding factor, DREF (DRE-binding factor) (24). Three DREs and one DRE are present in the DNA polymerase α and PCNA genes, respectively. Transient CAT expression and gel mobility shift assay using cultured Kc cells indicated that DRE stimulates the promoter activities and that DREF can bind specifically to DRE (24).

Another aspect is that promoters of *Drosophila* DNA replication-related genes are repressed by the product of the *zerknüllt* (*zen*) gene, a homeobox gene which regulates the differentiation of the optic lobe and the amnioserosa in the dorsal region of the *Drosophila* embryo (25–27). Repression of promoter activities by Zen protein has been observed not only in cultured Kc cells but also in transgenic flies carrying the PCNA gene promoter-directed *lacZ* gene (28, 29). Recently we obtained evidence indicating that overexpression of Zen protein results in reduction of DREF activities in the cell (29). There-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) D78373.

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¹ The abbreviations used are: PCNA, proliferating cell nuclear antigen; DRE, DNA replication-related element; DREF, DRE-binding factor; bp, base pair(s); kb, kilobase pair(s); CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; *zen*, *zerknüllt*; DAPI, 4,6-diamidino-2-phenylindole; HPLC, high performance liquid chromatography; GST, glutathione *S*-transferase.

fore, DREF may be one of the key transcription regulatory factors involved in proliferation- and differentiation-related control of DNA replication genes.

We earlier purified DREF as a homodimer of an approximately 86-kDa polypeptide to near homogeneity (24). In the present study, we isolated cDNA for DREF and demonstrated the involvement of DREF in the high level of expression of DNA replication-related genes with DRE sequences.

EXPERIMENTAL PROCEDURES

Cell Culture—Kc cells derived from *Drosophila* early embryos were grown in spinner culture at 25 °C using M3(BF) medium (30) supplemented with 2% fetal calf serum in the presence of 5% CO₂.

Purification of DREF—The DREF polypeptide was purified using DRE oligonucleotide-conjugated latex particles and gel filtration column chromatography as described earlier (24). It was then concentrated using a Centricon 30 (Amicon, Inc.) and precipitated by adding trichloroacetic acid to a final concentration of 10%.

Determination of the Partial Amino Acid Sequences—The trichloroacetic acid-precipitated DREF polypeptide (20 µg) was washed with ice-cold acetone, dissolved in sample buffer for SDS-polyacrylamide gel electrophoresis, boiled for 1 min, separated on an 8% SDS-polyacrylamide gel, and electrophoretically blotted onto a sheet of Glassybond paper. Membrane-bound protein was subjected to *in situ* proteolytic cleavage using endopeptidase Lyso-C (Wako) in 0.1 M NH₄HCO₃ at 37 °C for 24 h. The resulting oligopeptide mixture was separated with an HPLC apparatus equipped with a reverse-phase C4 column. Oligopeptide-containing fractions were collected manually into Eppendorf tubes. When necessary, each of the peak fractions was further purified with rechromatography on a reverse-phase C18 column. Samples were stored at -70 °C before analysis. Each sample was adsorbed to a disc of Glassybond, and the amino acid sequence was analyzed with an Applied Biosystems model 470A automated gas-phase protein sequencer.

Oligonucleotides—All oligonucleotides were chemically synthesized using an Applied Biosystems DNA synthesizer. Degenerate primers for reverse transcriptase-PCR were synthesized depending on the partial amino acid sequences of DREF. The synthesized oligonucleotides sequences were as follows: I, 5'-TT(C/T)GA(C/T)CIAA(T/C)GA(A/G)ATIC-CIAA(T/C)CC; II, 5'-TT(C/T)GA(C/T)AG(C/T)AA(T/C)GA(A/G)ATICCI-AA(T/C)CC; III, 5'-AA(A/G)TA(T/C)GA(A/G)GA(T/C)GTITCICA(A/G)-(T/C)T; IV, 5'-AA(A/G)TA(T/C)GA(A/G)GA(T/C)GTIAG(T/C)CA(A/G)(T/C)T; V, 5'-TA(T/C)TGG(C/A)GITA(T/C)TT(T/C)GGITT(T/C)CC; five more oligonucleotides with complementary sequences to I-V were also synthesized (IR, IIR, IIIR, IVR, and VR).

To amplify a sequence containing a complete open reading frame, a set of PCR primers with a *Bam*HI site was synthesized: 5'-specific oligonucleotide, 5'-ACAGGATCCAAGATGAGCGAAGGGGTACCA, and 3'-specific oligonucleotide, 5'-ATCCTAATTGTTGTATGATGCT, where sequences with underlining indicate the *Bam*HI site, translation initiation codon, and stop codon of the DREF cDNA, in that order.

Cloning of cDNA for DREF—Poly(A) tail-containing RNA was isolated using an mRNA separator kit (Clontech) from Kc cell total RNA. Poly(A)-RNA (1 µg) was reverse-transcribed for 1 h at 37 °C using SuperScript II reverse transcriptase (1 unit) (Life Technologies, Inc.) with oligo(dT)₁₂₋₁₈ (0.5 µg) as a primer in a solution (50 µl) containing 50 mM Tris-HCl (pH 8.3), 40 mM KCl, 8 mM MgCl₂, 1 mM dithiothreitol, 0.1 mg/ml bovine serum albumin, and 50 µM each of dATP, dCTP, dGTP, and dTTP. The products were ethanol-precipitated in the presence of 0.3 M ammonium acetate (pH 7.0), washed with 80% ethanol, and redissolved in 50 µl of Tris-EDTA. Aliquots (5 µl) were used to amplify DREF cDNA using *Taq* DNA polymerase (5 units) in a solution (50 µl) containing 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatin, 0.1% Triton X-100, 100 µM each of dATP, dCTP, dGTP, and dTTP, and 100 µM of the pair of PCR primers with the sequences detailed above. cDNA products were generated by 30 cycles of PCR (1 min at 94 °C, 30 s at 55 °C and 2 min at 72 °C), separated on a 1% agarose gel, and recovered from the gel by the DE-81 method (33). Obtained DNA fragments were cloned into the *Sma*I site of pBluescript II SK(-) (pDCDREF1.8) and sequenced by the dideoxy sequencing method (34).

Full-length cDNA clones for DREF were isolated by screening two kinds of λgt10 cDNA libraries constructed from mRNAs obtained from 0–3-h-old and 3–12-h-old *Drosophila* embryos under high stringency conditions using a ³²P-labeled 1.8-kb cDNA fragment excised from pDCDREF1.8 as a probe. The obtained cDNA clones (2.7 kb) had a single *Eco*RI site and, therefore, two *Eco*RI fragments of 1.0 and 1.7 kb

were excised from λgt10 DNA. Each *Eco*RI fragment was subcloned into the *Eco*RI site of pBluescript II SK(-) (pDCDREF1.0 and pDCDREF1.7) and sequenced.

A cDNA containing a complete open reading frame without 5'- and 3'-untranslated sequences was obtained with 30 cycles of PCR (1 min at 94 °C, 1 min at 55 °C, and 2 min at 72 °C) using 10 ng of DNA of λgt10 clone carrying full-length cDNA as a template and 5'- and 3'-specific oligonucleotides as primers. The resultant DNA fragment was digested with *Bam*HI and subcloned into *Bam*HI-*Sma*I sites of pBluescript II SK(-) (pDCDREF2.2).

Expression of GST-DREF Fusion Proteins in *Escherichia coli*—To construct deletion mutant GST-DREF expressing plasmids, 27 mer PCR primers were synthesized. All primers for coding and complementary strands were designed to contain *Bam*HI and *Eco*RI sites, respectively.

We produced recombinant DREF fused to GST. A construct containing the full-length DREF (amino acids 1–701; pGST-DREF1–701) was created by inserting a 2.2-kb cDNA fragment from pDCDREF2.2 with *Bam*HI and *Eco*RI into *Bam*HI and *Sma*I sites of pGEX-2T. A construct containing amino acid residues 16–608 (pGST-DREF16–608) was obtained by excising a 1.8-kb cDNA fragment from pDCDREF1.8 with *Eco*RI and *Bam*HI, filling the protruding-end with Klenow fragment and inserting it into the *Sma*I site of pGEX-3X. This expression plasmid was used for large scale preparation of the DREF polypeptide, which was then applied as an antigen to raise antibodies. Extraction and purification of recombinant protein were carried out as described previously (35).

Constructs containing the N-terminal basic region (amino acids 16–242; pGST-DREF16–242) and C-terminal acidic region (amino acid 240–608; pGST-DREF240–608) were prepared by partially digesting pGST-DREF16–608 with *Eco*RI and religating the plasmid DNA with T4 DNA ligase. cDNAs of the other GST-DREF mutants (see Fig. 2) were amplified by PCR with *Eco*RI-*Bam*HI sites and inserted into pGEX-2T. All constructs were sequenced. DREF-fusion proteins were produced in *E. coli* essentially as described earlier (36). Lysates of cells were prepared by sonication in buffer D containing 0.6 M KCl, 1 mM phenylmethylsulfonyl fluoride, and 1 µg/ml each of pepstatin, leupeptin, and aprotinin. Lysates were clarified by centrifugation at 12,000 × *g* for 20 min at 4 °C, and used for analysis by SDS-polyacrylamide gel electrophoresis, gel mobility shift assay, or footprinting.

Gel Mobility Shift Assay—Preparation of nuclear extracts from Kc cells and conditions of gel mobility shift assay were as described elsewhere (24).

***o*-Phenanthroline-Copper Footprinting**—The probe for *o*-phenanthroline-copper footprinting was prepared as described earlier (37). The DNA fragment excised from plasmid pHP3 (–292 to +45 region of the DNA polymerase α gene) (24) was labeled at the 5'-end of either the upper or the lower strand using T4 polynucleotide kinase and [³²P]ATP. The probe (1 ng, 5 × 10⁴ cpm) was incubated with an extract (1 µg of protein) of *E. coli* producing GST-DREF16–242 in 30 µl of solution containing 15 mM Hepes (pH 7.6), 100 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, 1 µg of poly(dI-dC), and 1 µg of sonicated calf thymus DNA on ice for 20 min. The resulting complexes were resolved on a 3% polyacrylamide gel. After electrophoresis, one of the glass plates was removed. The gel attached to the other glass plate was transferred to a glass dish containing 200 ml of 10 mM Tris-HCl (pH 8.0), and equilibrated for 1 h at room temperature. Then, 20 ml of 0.9 mM CuSO₄, 4 mM 1,10-phenanthroline solution were added to the equilibrated gel. The cleavage reaction was initiated by adding 20 ml of 0.5% 3-mercaptopropionic acid, allowed to proceed for 1 min at room temperature, and stopped by adding 10 ml of 1.2% 2,9-dimethyl-1,10-phenanthroline. The gel was rinsed twice with distilled water and exposed to x-ray film for 4 h at 4 °C. Portions of gel containing DNA-protein complexes or free DNA probe were excised and incubated in 500 µl of DNA elution buffer containing 1% SDS, 0.2 M NaCl, 20 mM EDTA, and 1 mg/ml tRNA overnight at 37 °C to elute the DNA. The elution buffer was removed from the gel fragment and extracted once with phenol/chloroform, and the DNA was ethanol-precipitated. The DNA was then dissolved in formamide-loading dye solution and resolved on a 6% denaturing polyacrylamide gel in parallel with products of Maxam-Gilbert sequencing reactions (38) using the same DNA used as a probe for sequencing controls. After electrophoresis, gels were dried and autoradiographed.

Preparation of Antibodies against DREF—Rabbit antiserum and mouse monoclonal antibodies against DREF polypeptide were raised against the bacterially produced recombinant DREF consisting of 16–608 amino acids fused with GST. Monoclonal antibodies were screened by enzyme-linked immunoassay using the recombinant GST-DREF

A

280 ACAGTCAGACGTAATTGAAATAAAAAGCGGTGACTCTCATATTTAGCAGTCTGTAATAAAT
 340 AATCTCAATAAATGAGAGTGCAATTTGAAGCATTTAGCAAGCTTTTATTTGCTTATTCGAT
 400 TGACGGCGGTGCGCGTGGCAACTCTGAAGTCTACTCTGATACAGATAAAAAATTTGAC
 460 TATTGAACCGTCTATCTATAATCGAGAAATTTGCGCGTAACTATCGCATTTGGGCACAGCA
 520 ATCGTTGTGCAATCTAATCAAAAACAAAGAGATGCCCAATCCCCACAGAGCAAG
 580 ATGAGCGAAGGGGTACCAAGCTGCGCCGTGGCATTTGGCGAATCAAGTACGAGGATGTG
 1 M S E G V P A S P V A I G E L K Y E D V
 640 TCGCAGCTAAATTTTCCAACTGTACTGCCCAAGATGAAAGCGTATACTGGCGCTAC
 21 S Q L N F S K L Y S P K M K S V Y W R Y
 700 TTTGGATTCCTCGAAGCAATAATGAGGTGATTACCAACAGAATGTGGTCTGGATT
 41 F G F P S N D N N E V I T K Q N V V W I
 760 AAGTGTCAAGGTGCTGACCAACATGGCAACACCAATTTGCGTGGCGATGCTCAG
 61 K C H K V L T N L N T N L R A H V Q
 820 CACCGACACAAGGATCTGTTCAAGAGGCTGTCCAGGAGGACGACATCATGTGCCCGG
 81 H R H K D L F K E L C Q E H D I H V P P
 880 CGCAAGCGCGCTAATGTATCCATCCACCGCTGTCCAAGCGGAATTTCTCTCGCG
 101 R K T P R N V S H P P L S K R N V S S R
 940 CGGTCAAACTGGAGTTCATTAATAACCGGAAGTGGAGCGAGCGGTGGCACTCGCG
 121 R V K L E F I N N R N W T K Q P W Q L R
 1000 CCATCGAGCGGAGGAGGACGCTCTCGCAACCATGCTGTACGAGGCGATGGTCCCT
 141 P C R R R R T P P R K P C C T R P W C P
 1060 TCACCTACGAGGCGCAAACTGGGAGAGGAGGAGGAGCGCTAGTTATGGAACAAA
 161 S P T T R P K T G R G G G A P S Y G T K
 1120 GTACGGACGCAACGCAAGGTGGCACTCATCGAGTGCCTGATGACGGCGGTGTAT
 181 V R T Q T Q S G H S I E C P D A R A C H
 1180 CAAGCACGAGGAGCGGCTATGCCCGCGTGGCAATCGCAATCTGCCCGGAAGCCCTT
 201 Q A R G S G Y A A V A N I A N L A E A L
 1240 ACGGACATTTGTGATTAAGGATTGGCAATGTGATTCTCTGTACGATGCGCGTTTGGT
 221 T D I V I K D L R N V D S L Y D A G F G
 1300 GAATTCCTGCGTCAAGTCTAGGCAATTCGGCAGCATGCCGAGCACAAGATCGAC
 241 E F L R Q V L G N S A A M P E P H K I D
 1360 TCTTTGATCAATGAGTGCAGCGCTCAAGTTTGGAGATTGGCGAAATCACCGCGCAT
 261 S L I N E M H A S K F L E I G E I T R D
 1420 TTCACCTCCGAGAAGCCCTCTCTCGCGCTTTCAGATGGGTGAATGTTGAACAGCGG
 281 F T S E K P S L A F Q M W V N E V Q R
 1480 CGCTTCCTAGCATCTTCATCACTTTCTGGACGAGGAGCGCACTAGTTCCGCGTATG
 301 R F L S I F H S F L D E E T H S F V R G M
 1540 CTGTATGCCACGTCGAGTCAACAGCTACATCTCTTTGACGATCTGCTGACGCACTT
 321 L Y A T V E Y N D Y I V F D D L L T D F
 1600 TACTTGGCAATTCACACTGGCCATCAAACTACGACGAGGAGAGGACCTTTTGAC
 341 Y L A N C T L A I I N Y D E E E D L L H
 1660 ACCTATCTGCGAGAGAAAAATATACCATTTCTGCTGTCTGCTTTGGTAATTGACAAA
 361 T Y L R E K N I P I S L C Y V S V I D K
 1720 TGTTCGGTGTGTGTTTGAGATCGAAGAGGTGGCCACTTTATTTGGAGCAGGTGAAGGAC
 381 C L R R V F E I E E V A T L L E Q V K D
 1780 CTAATGACGCTATTCAACGGAGATTGCCCTCAAGGTCTCCGAGGTGCCCATGCCACG
 421 Y N E H F P W S T V R N I E V L C R V P
 1840 TACAACGAGCACTTCCCTGGAGCACTGTACGAAACATTGAAGTCTTTTGGCAGTTCCA
 441 Y L G P R T W I T W L Y Q P R R C T E A
 1900 TATCTTGGTCCGAGGACATGGATCACCTGGTTATATCAGCAGACGGTGTACGAGGGCC
 461 L S A L V I A L H T L R G E D I P L C S
 1960 CTGAGTGTCTGGTGTCTGCTTTGCACACGCTGCGTGGCAGGACATTCCCTTGTGTAGC
 481 M L S P I T S K I L I K K L G I A E Q D
 2020 ATGCTTTCGCAATCACTTCAAGATTCTTATCAAGAGTTGGCGATCGCCGAGGAGGAC
 501 D P L M M N L K R T I S S V L Q A H V I
 2080 GATCCCTAATGATGAATTTGAAGCGGACATTTCAAGTCTCCAGGCGCACGTTATA
 521 S N D N L T A A A L L D P R F H R L T T
 2140 TCCAATGACAATCTGACCGTGTCTGATTGTTGGACCCAGCTTCCACCGCTGACACC
 541 I D N L E R T V R M L T H K Y N I N F G
 2200 ATTGACAACCTAGAGCGAAGCTTGTATGTCGACCCATAAGTATAACATTAACTTTGGT
 561 G V G G G E S N E V A A T S S V V A I K
 2260 GGAGTGGGCGGAGGGGAATCAACGAAGTGGCAGCTACCTCCAGCGTGGTGGCCATTAG
 581 S E P R V V D G S A P K K L G L K L L F
 2320 TCGAAGCGAGATACCAATCTCCGAGCGAGATGGGACGACGCGTGGAGTCCGAT
 601 D S N E I P N P P K R D A D S T V E S D
 2440 CTTAAGCGATATCGCAACGAGGTGGTCTGTCAGTGGATGAGTCCGCCATCGAGTGGTGG
 621 L K R Y R N E V V V Q L D E S P I E W W
 2500 CTCAGATGGGACACATTTATGGAACGCTGCGGATTTGGCAGCGCTGACCAAGTGTG
 641 L K M G H I Y G T L R D L A S L Y H S V
 2560 CCCGGCTGGTGAAGTCTCAAGAGGCGCTGAGAGACCAATATACGACTTCAAC
 661 P G V V T L S F K K A L R D Q I Y D F N
 2620 AAGGATTCATGCTCAGCGTAGTCAATCGACGCACTCTCTTTTGCATCATCAAC
 681 K R F M L T G S H I D A I L F L H H N
 2680 AATTAGTCTGACTGGCGACTACTGTTCTTATATGCAAACTTTTATTACTTTACGT
 701 N *
 2740 ACTGTGTATGAAATGACTACGAGGCGAGGCAATAGTAATACGATTGGCAGTCACTTT
 2800 TAACCAAAAGCAGCGCTTGGGAGTACAGGAATCTTAAGTATCGACGATTAAATGTT
 2860 AAGGGAACATTTTGACATCATATTTCAATCAACAGATAATCTAAGAAATTTGTTTA
 2920 ATAAATCCATAACGAAAAAAGAAAAA

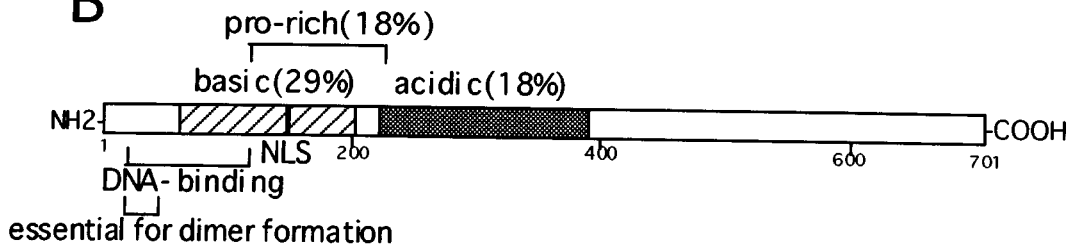
B

FIG. 1. **cDNA and schematic structure of DREF.** A, nucleotide sequence and the deduced amino acid sequence for DREF cDNA. The open reading frame with initiation and stop codons, at nucleotide numbers 571 and 2683, respectively, encodes a protein of 701 amino acid residues (shown in single letter code below the nucleotide sequence). Peptide sequences determined by microsequencing after lysyl-endopeptidase digestion of DREF are underlined. A putative polyadenylation signal is shown by **bold letters**. B, schematic structure of DREF. The region rich in basic amino acids (61–203 amino acids) and the region rich in acidic amino acids (218–390 amino acids) are shown by the hatched and shaded boxes, respectively. The proline-rich region (99–217 amino acids) is also indicated. NLS represents a putative nuclear localizing sequence (143–151 amino acids; RRRRTTPPRK). The amino acids essential for DRE-binding (16–105 amino acids) are also indicated by a bracket.

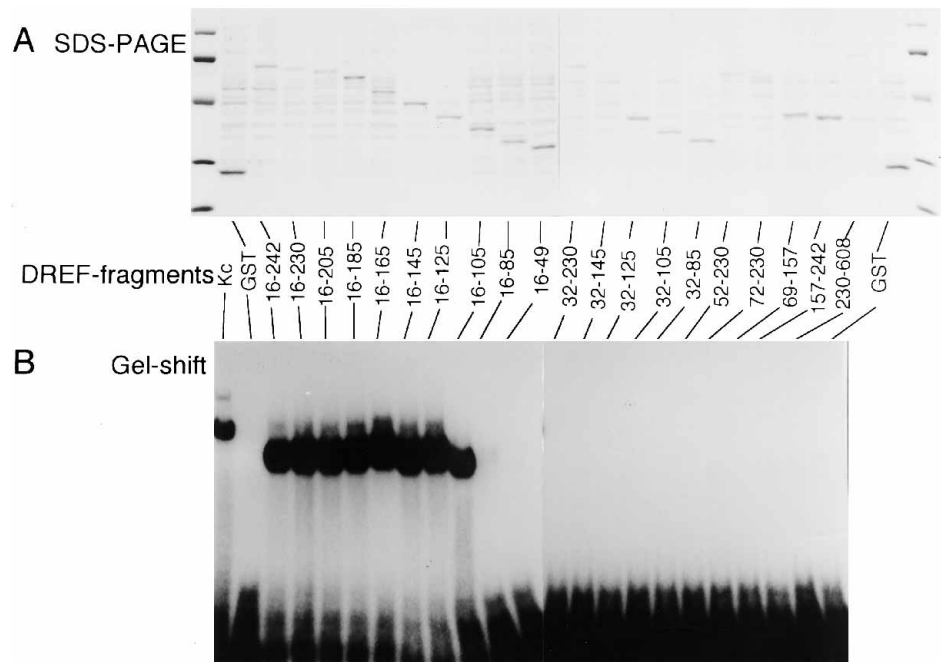
polypeptide. If necessary, each of monoclonal antibodies was purified from culture medium of hybridomas using E-Z-SEP (Pharmacia Biotech Inc.). Polyclonal antibody specific for DREF polypeptide was purified from antiserum using a GST-DREF-conjugated Sepharose column after passing through a GST-conjugated-Sepharose column. The obtained IgG was precipitated at 50% ammonium sulfate saturation, dissolved in phosphate-buffered saline, and dialyzed against phosphate-buffered saline.

Expression Plasmid, DNA Transfection, and CAT Assay—The expression plasmid pUAS-DREF1-701 contained the full-length DREF coding sequence placed under the promoter consisting of five Gal-4 binding sites and the hsp70 TATA box (pUAST vector). The plasmid pAct-Gal-4 plasmid expressed full-length yeast Gal-4 protein under control of *Drosophila* actin 5C promoter (pAct-GEM3 vector). DNA transfection was carried out by the calcium phosphate coprecipitation technique described earlier (23). Two μ g of p5'–168DPCNACAT as a reporter plasmid and 2 μ g of pAct-Gal-4 were cotransfected with indicated amounts of pUAS-DREF1-701 and pAct5C-*zen* as expression plasmids. Total amount of effector plasmid was kept constant by addition of the expression vectors pAcGEM3 and pUAST. At 48 h after transfection, cell extracts were prepared and CAT activity was measured as described previously (23). The CAT activity was quantified with an imaging analyzer BAS2000 (Fuji film). CAT activity was normalized

to the protein amount.

In Vitro Transcription Reaction—The template used in *in vitro* transcription assay was the plasmid –1107DPOLACAT containing –1107 to +45 of the DNA polymerase α gene in the plasmid pOLCAT as described earlier (24). *In vitro* transcription was performed with Kc cell nuclear extracts prepared as detailed previously (24). Kc nuclear extract (40 μ g of protein) was preincubated with 2 μ g of control or anti-DREF IgG in 10 μ l of solution containing 50 mM Hepes (pH 7.6), 12% glycerol, 0.15 M KCl, 1 mM EDTA, 1 mM dithiothreitol for 30 min on ice, and then the mixture was added to 40 μ l of transcription mixture containing 50 mM Hepes (pH 7.6), 12% glycerol, 0.15 M KCl, 5 mM $MgCl_2$, 4 mM each of ATP, GTP, CTP, and UTP, 25 units of ribonuclease inhibitor (Takara), and 0.5 μ g of supercoiled –1107DPOLACAT. The reaction was allowed to proceed for 30 min at 30 °C, and after stopping by addition of 500 μ l of a stop mixture consisting of 0.25 M NaCl, 1% SDS, 20 mM Tris-HCl, 5 mM EDTA, and 10 μ g glycogen, nucleic acids were extracted once with phenol/chloroform and precipitated with ethanol. The pellet was dissolved in 10 μ l of H_2O and mixed with 100 μ l of DNase digestion mixture containing 20 mM Hepes (pH 7.6), 50 mM NaCl, 10 mM dithiothreitol, 5 mM $CaCl_2$, 5 mM $MgCl_2$, and 5 μ g of RNase-free DNase I, and the reaction was incubated at 37 °C for 15 min. DNase digestion was completed by adding 100 μ l of DNase stop buffer containing 20 mM Tris-HCl (pH 8.0), 50 mM EDTA, 1% SDS, 0.3

FIG. 2. DRE binding of deletion mutants of GST-DREF. A, expression of deletion mutants of the GST-DREF fusion protein. *E. coli* carrying plasmids expressing deleted DREF fused to GST were inoculated in 2 ml of LB medium containing 50 μ g/ml ampicillin, and synthesis of GST-DREF was induced at 37 °C for 1 h by adding 1 mM β -D-isopropyl-thiogalactopyranoside. *E. coli* were pelleted by centrifugation from 100 μ l of culture medium, resuspended in SDS sample buffer, boiled for 3 min and subjected to electrophoresis on an 8% SDS-polyacrylamide gel. B, DRE binding activities of deletion mutants of GST-DREF fusion protein. *E. coli* lysates expressing GST-DREF were prepared as described under "Experimental Procedures." A gel mobility shift assay with 32 P-labeled DRE-P oligonucleotide (DRE-containing sequence from the PCNA gene) (24) was performed using Kc cell nuclear extract (Kc), lysates of *E. coli* containing GST-DREF fusion proteins and *E. coli* lysate containing GST (GST).



M NaCl. After phenol/chloroform extraction, RNA was precipitated with ethanol. Primer extension analysis was carried out essentially as described earlier (39) using an oligonucleotide (28 mer) synthesized based on the nucleotide sequence of the CAT gene as a primer, 5'-GTGG-TATATCCAGTGATTTTTTCTCCA. Quantification was achieved with an image analyzer BAS2000 (Fuji film).

Northern Analysis—Total RNA was extracted from Kc cells by the acid guanidinium thiocyanate-phenol-chloroform method (31). Twenty micrograms of total RNA were separated on a 1% agarose gel containing formaldehyde and blotted onto a sheet of GeneScreen Plus membrane (DuPont). Probes were radiolabeled using the random primer method (32). Hybridization and washing conditions were the same as described elsewhere (22). Blots were exposed to Kodak X-Omat XAR films and also to a Fuji-imaging plate, and relative radioactivity was quantified using a Fuji BAS 2000 imaging analyzer.

Immunocytochemical Staining of Embryos—Canton S embryos (0–3 h old) were collected, dechorionated, fixed, and devitellinized as described earlier (40). Embryos were blocked with Tris-buffered saline containing 20% normal donkey serum, and 0.15% Triton X-100, and then incubated in Tris-buffered saline with either 5 μ g/ml of rabbit anti-DREF IgG (used at a dilution 1:100) or hybridoma culture supernatant. Secondary antibodies were used at a dilution of 1:500 and color development was performed as for the Western blotting. Samples were examined under a microscope (Olympus BX-50) and photographed using TMAX 100 films (Eastman Kodak Co.).

RESULTS

Cloning of cDNA for DREF—DREF was affinity-purified from the nuclear extract of Kc cells using DRE-conjugated latex particles and chromatography on a gel filtration column as described earlier (24). The purity of the DREF polypeptide, which has an apparent molecular mass of 86 kDa, was greater than 90%. This polypeptide specifically bound to the DREs from DNA polymerase α and PCNA genes as described previously (24). Yield of the DREF polypeptide was about 1 μ g from 1 g of Kc cells. Twenty μ g of purified DREF were subjected to SDS-polyacrylamide gel electrophoresis, and the 86-kDa polypeptide was isolated from the gel. Automated Edman degradation revealed that the N-terminal amino acid was blocked. Thus, we determined amino acid sequences of oligopeptides obtained by digesting the DREF polypeptide with endopeptidase Lyso-C. Oligopeptides were separated by reverse-phase HPLC and subjected to microsequence analysis. Three oligopeptide sequences were obtained as indicated with *underlining* in Fig. 1.

Reverse-transcriptase-PCR with all possible combinations of degenerated primers deduced from amino acid sequences was performed to isolate cDNA fragments. PCR products of 1.7 and 1.8 kb were obtained with primer III in combination with primers VR and IIR, respectively. The 1.8-kb cDNA fragment was used as a probe to screen cDNA libraries (λ gt10) prepared with mRNAs extracted from 0–3-h or 3–6-h embryos. Eight clones were isolated from 4×10^5 phages.

Southern blot analysis of *Drosophila* genomic DNA using the 1.8-kb cDNA fragment as a probe indicated that the DREF gene is present as a single copy (data not shown).

Structure of DREF—The nucleotide sequence containing a complete DREF-coding region which was determined from three cDNA clones is shown in Fig. 1. This 2.7-kb cDNA fragment appears to be nearly full-length since Northern analysis demonstrated a single mRNA species of approximately 2.8 kb (see Fig. 6), and nucleotide sequence analysis revealed that 5'-ends of eight cDNA clones independently isolated were mapped within 200 bp. In a primer extension experiment, the major transcription start site was mapped to 570 bp upstream from the ATG translation initiation codon (data not shown).

The open reading frame encodes a polypeptide of 701 amino acid residues with a predicted molecular weight of 80,096 and pI of 8.5. A data base search using the FASTA program did not cover any significant homology with reported conserved motifs.

DREF contains three characteristic regions, 1) rich in basic amino acids (28.7% between amino acid residues 61 and 203), 2) rich in proline (17.9% between amino acid residues 99 and 217), and 3) rich in acidic amino acids (18.0% between amino acid residues 218 and 390), as shown in Fig. 1B. DREF also contains a putative consensus sequence for the nuclear localization signal (RRRRTPPRK) at 143–151 residues. However, typical structures reported to be responsible for DNA binding, transactivation, and dimerization were not found.

DRE Binding Potential of DREF Produced in *E. coli*—To ascertain the DRE binding activity, we overexpressed DREF protein in the form of a fusion protein with GST in *E. coli*. We tested DNA binding using *E. coli* extracts carrying expression plasmids GST-DREF16–242 and GST-DREF230–608 by a gel mobility shift assay in which the DRE-P oligonucleotide was used as a probe. DRE binding activity was found to be associ-

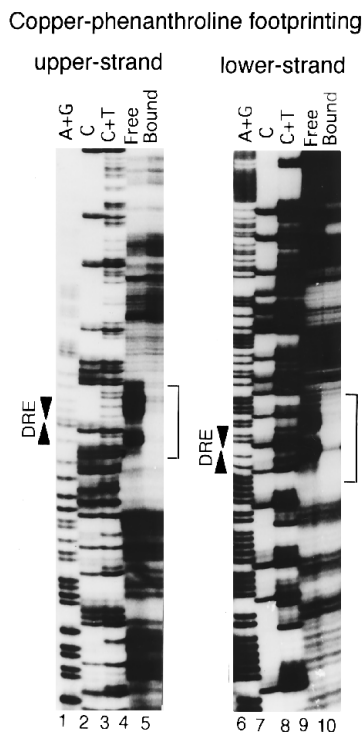


FIG. 3. **Copper-phenanthroline footprinting of GST-DREF.** A gel mobility shift assay was performed on the 5'-end-labeled upper or lower strands of a 337-bp *XhoI*-*Bam*HI fragment containing the promoter region of the *Drosophila* DNA polymerase α gene (positions -292 to +45) with GST-DREF16-242 fusion protein. After DNA cleavage with copper and *o*-phenanthroline in the gel, DNA was eluted from the portions of the gel containing DNA-protein complexes (*Bound*) and the free probe (*Free*), and subjected to electrophoresis on a sequencing gel. The regions protected from cleavage are indicated by brackets. The position of DRE are indicated by triangles. Maxam-Gilbert A + G, C, C + T sequencing reactions were performed in the adjacent lanes.

ated with the fragment of 16-242 amino acid residues but not with that of 230-608 amino acid residues (see Fig. 2B). Prior to fine mapping the DNA binding domain, the binding specificity of the recombinant DREF polypeptide to the DRE sequence was examined by the *o*-phenanthroline-copper footprinting method (Fig. 2). We used the 5'-upstream region of the *Drosophila* DNA polymerase α gene containing three DRE sequences (DRE- α I, α II, and α III) as a probe (24) and the *E. coli* extract containing GST-DREF16-242. The footprinting patterns containing the DRE-sequence at the centers were indistinguishable from those obtained using Kc cell nuclear extract (24) and purified DREF (data not shown).

Determination of the DNA Binding Region of DREF—Plasmids expressing a set of deletion mutants of GST-DREF were constructed using PCR to examine the specificity of DRE binding activity. Analysis of extracts of *E. coli* carrying expression plasmids by SDS-polyacrylamide gel electrophoresis showed that similar amounts of GST-DREF polypeptides were produced (Fig. 2A).

The DRE binding activities of these mutant polypeptides were examined by gel mobility shift assay using DRE-P as a probe (Fig. 2B). Truncated polypeptides encoding from the 16th amino acid residue up to the C termini at 242, 230, 205, 185, 165, 145, and 125 amino acid residues exhibited DNA binding activities as effective as GST-DREF16-608. C-terminal deletion up to amino acid residue 105 resulted in a reduction of DNA binding activity to approximately half that of the 16-125 construct, and deletion up to amino acid residue 85 resulted in only weak activity, less than 1% that of the 16-125 form. Further C-terminal deletion up to residue 49 caused complete

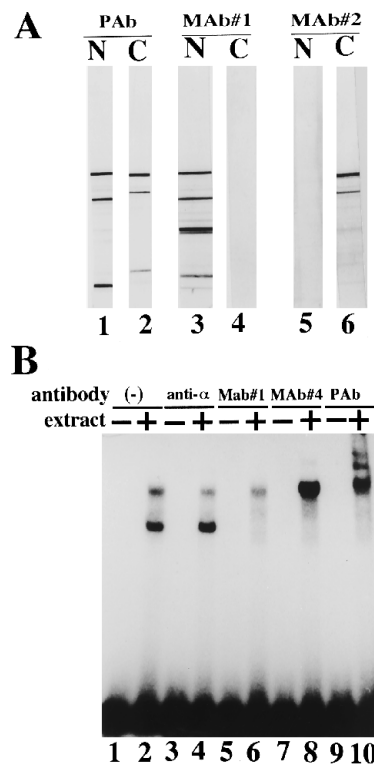


FIG. 4. **Characterization of antibodies against DREF.** A, immuno-Western blotting analysis. Crude extracts from *E. coli* with plasmids expressing GST-DREF16-242 (N) (lanes 1, 3, and 5) and GST-DREF240-608 (C) (lanes 2, 4, and 6) were fractionated by SDS-polyacrylamide gel electrophoresis, transferred onto a poly(vinylidene fluoride) membrane sheet, and immunostained with anti-DREF antiserum (lanes 1 and 2), culture supernatants of hybridoma lines 1 (lanes 3 and 4) and 2 (lanes 5 and 6) as primary antibodies and alkaline phosphatase-conjugated second antibodies. B, effects of antibodies on DRE-DREF complex formation. A gel mobility shift assay was performed using 32 P-labeled DRE-P oligonucleotide as the probe incubated without (lanes 1, 3, 5, 7, and 9) or with (lanes 2, 4, 6, 8, and 10) Kc cell nuclear extract in the absence (lanes 1 and 2) or presence (lanes 3, 4, 5, 6, 7, 8, 9, and 10) of various antibodies. Used antibodies were: lanes 3 and 4, culture supernatant of a mouse hybridoma cell line (4-2D) which produces anti-chick primase antibody (6 μ l); lanes 5 and 6, culture supernatant of mouse hybridoma cell line 1 (6 μ l); lanes 7 and 8, culture supernatant of mouse hybridoma cell line 4 (6 μ l); lanes 9 and 10, rabbit anti-DREF polyclonal antibody (50 μ g/ml IgG, 6 μ l). Nuclear extract from Kc cells (2 μ g) was mixed with each antibody, incubated for 2 h on ice, added to mixtures containing 32 P-labeled DRE-P oligonucleotides (10^4 cpm) and 1 μ g of poly(dI-dC), incubated for 15 min on ice, and analyzed on a 4% polyacrylamide gel.

loss of DNA binding activity. These results indicate that the region essential for strong DRE binding activity is located between amino acid residues 16 and 115, although amino acid residues 16-85 are sufficient for weak DRE binding.

Deletion of 32 amino acid residues from the N terminus resulted in an almost complete ablation of DNA binding activity, although we detected very weak signals of DRE-protein complexes with N-terminal deletion mutants, 32-230, 32-145, 32-125, and 32-105 after long-term exposure of gels to the imaging plate. Interestingly, their mobilities in the gel were clearly faster than those of DREF mutants with similar sizes carrying the 16-32 region. One possible explanation is that amino acid residues 16-32 are required for dimer formation and also for the strong DRE binding activity.

Characterization of Antibodies against DREF—We generated a rabbit antiserum and four mouse hybridoma lines producing antibodies against DREF polypeptide using GST-DREF16-608 fusion protein as an antigen. The specificities of these antibodies were confirmed by immuno-Western blotting

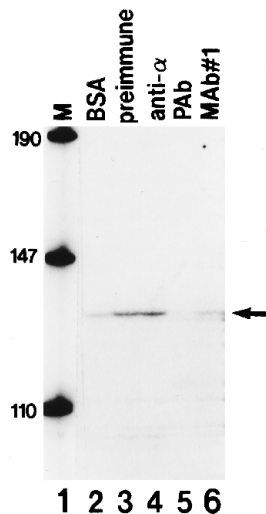


FIG. 5. Requirement of DREF for *in vitro* transcription. Kc cell nuclear extract (40 μ g of protein) was mixed with 2 μ g of BSA (lane 2), preimmune rabbit IgG (lane 3), anti-chick DNA polymerase α monoclonal IgG, anti-DREF polyclonal IgG (lane 4), or anti-DREF monoclonal 1 IgG (lane 5) and incubated for 30 min on ice, and then supplemented with a mixture for *in vitro* transcription using supercoiled plasmid containing DNA polymerase α promoter as a template. Transcripts were detected by primer extension. Arrow indicates the position of precisely transcribed RNA. DNA size markers (M) are shown in lane 1.

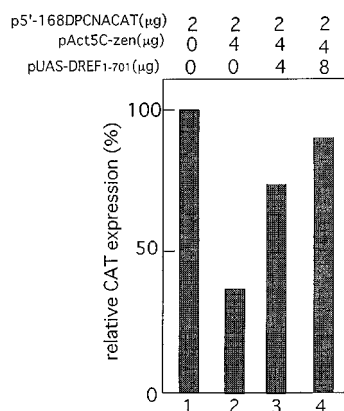


FIG. 6. Effect of DREF overproduction on repression by *zen*. Two- μ g aliquots of p5'-168DPCNACAT plasmid were cotransfected with the indicated amounts of pAct5C-*zen* and pUAS-DREF1-701 plasmids into Kc cells. After 48 h, cell extracts were prepared to determine the CAT expression. CAT activity was normalized to the protein amount. Quantification was with an imaging analyzer BAS 2000. Averaged values obtained from four independent transfections are given as CAT activity relative to that of transfection without effector plasmids.

analysis and gel mobility shift assay. An example of the results of the former is shown in Fig. 4A. *E. coli* extracts expressing GST-DREF16-242 (N) and GST-DREF240-608 (C) were electrophoretically separated and blotted, followed by analysis of their immunoreactivities. The antiserum reacted with both N and C polypeptides, whereas culture supernatants with monoclonal antibodies 1 and 2 reacted only with N and C, respectively. Immunoblotting analysis using culture supernatants of the other hybridoma lines revealed that both 3 and 4 monoclonal antibodies specifically recognize GST-DREF240-608 (data not shown).

Effects of antibodies on DRE-DREF complex formation were examined by gel mobility shift assay, in which the Kc cell nuclear extract was incubated with each antibody prior to adding the probe (Fig. 4B). Monoclonal antibody 1 inhibited complex formation. Western blotting analysis with a set of

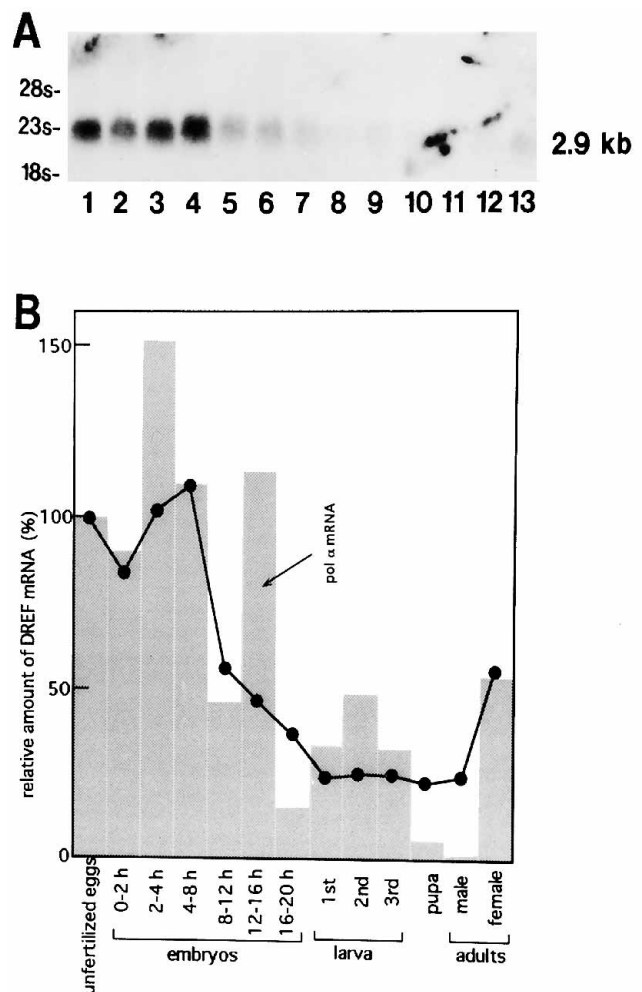


FIG. 7. Changes in DREF mRNA during *Drosophila* development. Twenty μ g of RNAs prepared from *Drosophila* bodies at various developmental stages were fractionated on a 1% agarose gel containing formaldehyde, transferred to a sheet of GeneScreen Plus filter, and subjected to hybridization. A, RNA blot hybridized with the 32 P-labeled 1.8-kb cDNA fragment derived from pDCDREF1.8. Used RNAs were derived from: lane 1, unfertilized eggs; lane 2, 0-2-h embryos; lane 3, 2-4-h embryos; lane 4, 4-8-h embryos; lane 5, 8-12-h embryos; lane 6, 12-16-h embryos; lane 7, 1st instar larva; lane 8, 2nd instar larva; lane 9, 3rd instar larva; lane 10, pupa; lane 11, adult male flies; lane 12, adult female flies. B, amounts of DREF mRNA as determined using an imaging analyzer (closed circles). The amount of DNA polymerase α mRNA is also shown by the shaded bars, determined by rehybridization of the filter with a 1.5-kb *Pst*I fragment of DNA polymerase α cDNA.

truncated GST-DREF proteins indicated that the epitope for monoclonal antibody 1 is located within the DNA binding region between amino acid residues 72 and 125 (data not shown), confirming that this region plays a role in DNA binding. Addition of monoclonal antibody 4 as well as the polyclonal antiserum resulted in supershifts of the DRE-DREF complexes. Monoclonal antibodies 2 and 3 had no effect on DRE-DREF complex formation (data not shown). The results confirmed that the cDNA isolated in this study encodes the DREF polypeptide.

Trans-activating Property of the DREF Protein—To confirm the trans-activation property of DREF, cotransfection experiments were carried out using the DREF expression plasmid as an effector and CAT plasmids directed by various promoters with or without DRE elements as reporters. In such experiments, overproduction of DREF neither enhanced nor reduced CAT expression (data not shown). We supposed that the amount of DREF might be almost saturated in Kc cells so that

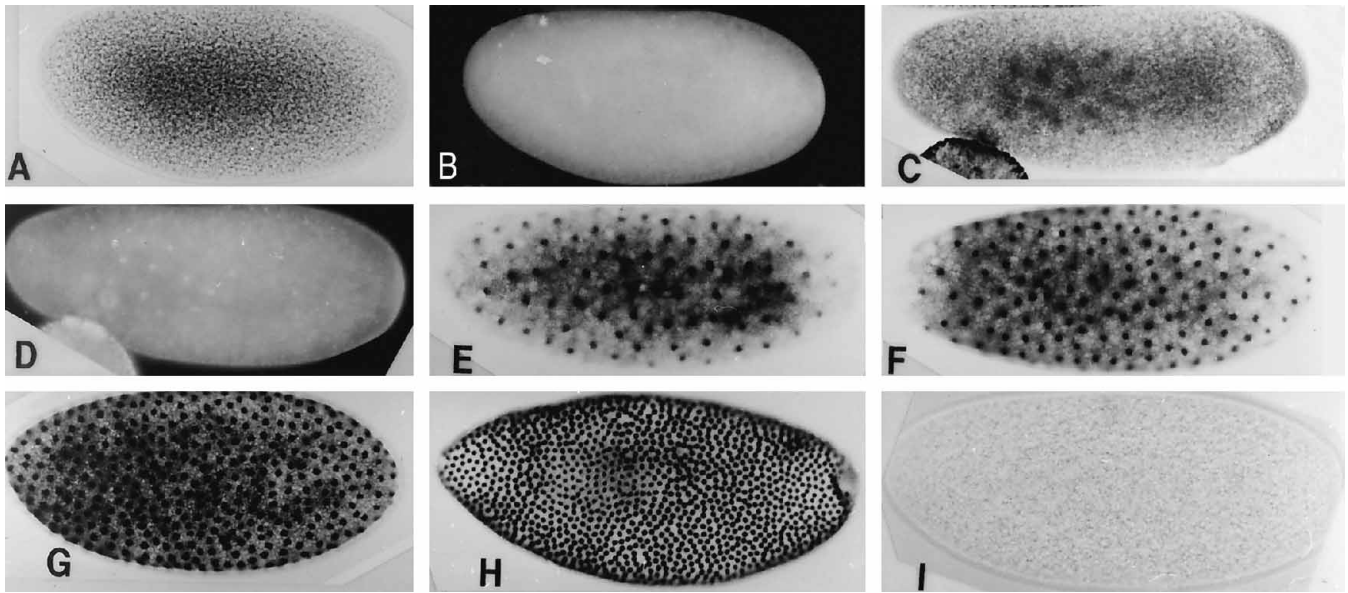


FIG. 8. **Immunocytochemical localization of DREF in *Drosophila* early embryos.** Polyclonal antibody against DREF and alkaline phosphatase-conjugated goat anti-rabbit IgG were used as the primary and secondary antibodies, respectively. Stages in nuclear division cycles were determined by counting the numbers of DAPI-stained nuclei. A, unfertilized egg; B, DAPI-staining of the unfertilized egg shown in A; C, cycle 5; D, DAPI-staining of the embryo shown in C; E, cycle 8; F, cycle 9; G, cycle 10; H, cycle 11; I, embryo stained without the primary antibody. Views at an internal focal plane (A–D) or at a surface focal plane (E–I) are shown. In all cases, the anterior ends of the embryos are on the left. Magnification $\times 284$.

the effect of additional DREF expression is minimal. Therefore, we employed an *in vitro* transcription assay using the DNA polymerase α gene promoter as a template with the Kc cell nuclear extract to investigate the DREF requirement. Nuclear extracts were preincubated with control or anti-DREF antibodies and added to the reaction mixture for *in vitro* transcription, and transcripts were analyzed by the primer extension method (Fig. 5). The sizes of the products revealed that transcription faithfully started at the cap site on the DNA polymerase α gene as determined previously (22). Preincubation with the preimmune rabbit IgG or the control mouse IgG (lanes 3 and 4) slightly stimulated transcription activity, while treatment of the nuclear extract with the anti-DREF polyclonal antibody (lane 5) or monoclonal antibody 1 (lane 6) resulted in almost complete and 60% loss of transcription activity, respectively. The results clearly confirm the contribution of DREF polypeptide to transcription on DNA polymerase α promoter.

DREF Overproduction Overcomes the DRE/DREF-mediated Repression by zen—Previously we suggested that the Zen protein represses PCNA and DNA polymerase α gene promoters by reducing DREF in the transfected cells (29). We investigated whether overproduction of DREF protein suppresses the repression of the promoter by Zen protein. As expected, the DREF expression plasmid overcame the repression of PCNA promoter by Zen protein in a dose-dependent manner (Fig. 6). However, the CAT activity never increased beyond that without *zen* and DREF expression plasmids, supporting our idea that the DREF amount in Kc cells is almost saturated, as mentioned above.

Changes in the Amount of DREF mRNA during Development—We measured the level of DREF mRNA with Northern hybridization analysis using RNA extracted from *Drosophila* bodies at various developmental stages. Using DREF cDNA as a probe, a single transcript of 2.9 kb was detected. A cDNA for the DNA polymerase α was also used as a reference probe, since this gene (and also the PCNA gene) is expressed in correlation with cell proliferation during development and is suggested to be under the control of the DRE/DREF system (22, 23). As shown in Fig. 7, DREF mRNA was detected at the highest level

in 4–8 h embryos, and at a relatively high level in unfertilized eggs, 2–4 h embryos and adult female flies. mRNA at a low level was detected in larvae, pupae, and adult male flies. Fluctuations of DREF mRNA were roughly similar to those of DNA polymerase α mRNA and PCNA mRNA through most developmental stages. However, discrepancies appeared in 12–16 h embryos and second larvae and adult male flies.

Immunocytochemical Localization of the DREF Polypeptide in Early Embryos—Expression of DREF was determined by immunocytochemical study in embryos during the first 13 nuclear division cycles (Fig. 8). Embryos were also stained with 4,6-diamidino-2-phenylidole (DAPI) to judge their nuclear division cycles. No significant staining was observed in any stage when normal rabbit IgG was used as a primary antibody (Fig. 8I, and data not shown).

The ooplasm of unfertilized eggs was weakly stained with the antibody (Fig. 8A), confirming maternal storage of DREF. Up to nuclear division cycle 7, the nuclei and the surrounding cytoplasmic region were stained weakly, but still more strongly than other regions of the syncytial cytoplasm (Fig. 8C). The signals differed from those observed with anti-PCNA antibody, which was uniformly strong in nuclei at these cycles (39). After cycle 8, strong and uniform nuclear staining was observed (Fig. 8E). By cycle 11, staining in the cortical cytoplasm underneath surface nuclei had mostly faded, suggesting that most maternally stored DREF had been translocated into nuclei from the cytoplasm by this point.

DISCUSSION

In our previous studies, promoters of DNA replication-related genes such as those encoding PCNA and DNA polymerase α 180-kDa catalytic subunit were found to be positively regulated by DRE and a specific binding factor, DREF (24, 41). We have searched for the TATCGATA sequence in a *Drosophila* data DNA base and found 60 genes carrying this sequence within 600-bp upstream regions from the transcription initiation sites. Interestingly, more than 30 of these genes are related to cell proliferation, suggesting that DRE is a common regulatory element responsible for the coordinated expression

of many proliferation-related genes (42). Furthermore, overexpression of the *zerknüllt* gene product results in repression of promoters of both PCNA and DNA polymerase α genes by impeding the DREF activity in cells (29). These findings suggest that DNA replication related genes are both positively and negatively regulated via DRE and DREF. The present isolation of cDNA for DREF, and preparation of specific antibodies should provide important clues toward further understanding how DREF functions in this regulation.

The DREF polypeptide contains three characteristic domains, respectively rich in basic amino acids, proline, and acidic amino acids. Although no significant similarity with any other proteins in data bases was found, these characteristic regions may be required for the function of DREF as a transcription regulatory factor. The DNA binding domain was mapped between 16–105 amino acid residues in the basic amino acid-rich region of 90 amino acid residues, but no characteristic feature similar to those previously reported could be identified. Structural analysis with nuclear magnetic resonance imaging is now under way using the DNA binding domain of the recombinant DREF polypeptide.

Transactivation of DRE-containing promoters by DREF overproduction was not observed in the co-transfection experiment with DREF expression and CAT plasmids. We suppose that DREF might be almost saturated in Kc cells. However, anti-DREF antibodies inhibited *in vitro* transcription activity of Kc cell nuclear extract, indicating that DREF is required for the high level of transcription from the DNA polymerase α gene promoter. In addition, over-expression of DREF protein in Kc cells overcame repression of the PCNA gene promoter by Zen protein. We have obtained similar results with the DNA polymerase α gene promoter (data not shown). The evidence indicates that DREF is one of the positive factors required for DNA replication-related genes and that it might be an important transcription regulatory factor involved in proliferation- and differentiation-related control. We have isolated the gene for DREF and analysis of its promoter region is in progress. We preliminary obtained results suggesting that *zen* protein represses the DREF promoter.

In the present study, the fluctuation of DREF mRNA content during development was similar to those of DNA polymerase α and PCNA, providing further evidence that DREF is an important transcription regulatory factor for DNA replication-related genes. The DREF polypeptide is distinctly localized in nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic-transcribed RNA demonstrated that nuclei become competent for transcriptional activation at cycle 10 while synthesis of rRNA, tRNAs, 5sRNA, snRNA, and poly(A) RNA is first detectable during cycles 11 or 12 (43). Transcripts for *Krüppel*, *hunchback*, and *hairy* genes, members of the gap gene and pair-rule gene families, are first detected at cycle 11 and their products begin to be expressed at cycles 13–14, (44–46). The histone genes become transcriptionally competent during cycle 10 (43). Northern hybridization analysis has revealed that the amounts of mRNAs for both PCNA and DNA polymerase α start to increase significantly in embryos by about 2 h after fertilization (cycles 12–15) (22). Therefore, the appearance of DREF in nuclei at and after cycle 8 might contribute to such zygotic transcription of DNA replication-related genes.

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