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

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DREF, a transcriptional 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. Immunocytological 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 Mi1 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 DREF 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).

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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, DREF-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 DREF 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 DREF oligonucleotide-conjugated latex particles and affinity chromatography as described elsewhere (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% polyacrylamide gel, and electrophoretically blot-transferred to a sheet of Glassybond paper. Membrane-bound protein was subjected to in situ proteolytic cleavage using endoproteinase Lyso-C (Wako) in 0.1 mM 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; each of the 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 sequence analyzer.

Oligonucleotides—All oligonucleotides were chemically synthesized using an Applied Biosystems DNA synthesizer. Degenerate primers for reverse transcription PCR were synthesized depending on partial amino acid sequences of DREF. The synthesized oligonucleotide sequences were as follows: I, 5′-TT(C/T)GA(T/C)AA(T/C)GA(T/C)GICTAC(A/G)C(T)CC; II, 5′-TT(C/T)GA(T/C)CA(T/G)GA(T/C)GICTAC(A/G)C(T)CC; III, 5′-AA(A/G)GA(T/C)GICTAC(A/G)C(T)CC; IV, 5′-AA(A/G)GA(T/C)GICTAC(A/G)C(T)CC; V, 5′-ATCCATGA(T/C)GA(T/C)GCTGTTCC; five more oligonucleotides with complementary sequences to I–V were also synthesized (R, IIR, IIIR, IVR, and VR).

To amplify a sequence containing a complete open reading frame, a set of PCR primers with a BamHI site was synthesized. 5′-specific oligonucleotide, 5′-ACAGATGCAATGGAAGCAGGAAGGTTTACCA, and 3′-specific oligonucleotide, 5′-ATCCATGA(T/C)GA(T/C)GCTGTTCC, where sequences with underlining indicate the BamHI 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)$_{20}$ (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 DNA using Taq DNA polymerase (5 units) for 4 h at 72°C 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 each 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 Smal site of pbBlueScript II SK(−) (pCDREFl.8) and sequenced by the dyeodeoxy 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 pCDREFl.8 as a probe. The obtained cDNA clones (2.7 kb) had a single EcoRI site and, therefore, two EcoRI fragments of 1.0 and 1.7 kb were excised from λgt10 DNA. Each EcoRI fragment was subcloned into the EcoRI site of pbBlueScript II SK(−) (pCDREFl.0 and pCDREFl.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 clones, using full-length cDNA as a template and 5′- and 3′-specific oligonucleotides as primers. The resultant DNA fragment was digested with BamHI and subcloned into BamHI-Smal sites of pbBlueScript II SK(−) (pCDREFl.2).

Expression of GST–DREF Fusion Proteins in Escherichia coli.—To construct deletion mutant GST–DREF expressing plasmids, 27 mer oligonucleotides were synthesized. All primers for cloning and complementing strands were designed to contain BamHI and EcoRI 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 pCDREFl.2 with BamHI and EcoRI into BamHI and Smal sites of pGEX-2T. A construct containing amino acid residues 16–608 (pGST–DREF16–608) was obtained by excising a 1.8-kb DNA fragment from pCDREFl.18 with EcoRI and BamHI, filling the protruding-end with Klenow fragment and inserting it into the Smal 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 EcoRI and religating the plasmid DNA with T4 DNA ligase. CDNAS of the other GST–DREF mutants (see Fig. 2) were amplified by PCR with EcoRI-BamHI 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 μM KCl, 1 mM phenylmethylsulfonyl fluoride, and 1 μg/ml each of pepstatin, leupeptin, antipain, 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).**

**α-Phenanthroline–Copper Footprinting—The probe for α-phenanthroline–copper footprinting was prepared as described earlier (37). The DNA fragment excised from plasmid pH3 (−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 [γ-32P]ATP. The probe (1 ng, 5 × 10⁶ cpm) was incubated with an extract (1 μg of protein) of E. coli producing pGST–DREF16–608, 30 μg of calf thymus protein A (Sigma), 15 mM Heps (pH 7.6), 100 mM NaCl, 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 3 days. 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 mM NaCl, 20 mM EDTA, 1 mM of 1,10-phenanthroline 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 38–608 amino acids fused with GST. Monoclonal antibodies were screened by enzyme-linked immunoassay using the recombinant GST–DREF.
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 mg of p52 and 92168DPCNACAT as a reporter plasmid and 2 mg 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 21107DPOLACAT containing 21107 to 145 of the DNA polymerase alpha 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 mg of protein) was preincubated with 2 mg of control or anti-DREF IgG in 10 ml 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 ml of transcription mixture containing 50 mM Hepes (pH 7.6), 12% glycerol, 0.15 M KCl, 5 mM MgCl2, 4 mM each of ATP, GTP, CTP, and UTP, 25 units of ribonuclease inhibitor (Takara), and 0.5 mg of supercoiled 21107DPOLACAT. The reaction was allowed to proceed for 30 min at 30°C, after stopping by addition of 500 ml of a stop mixture consisting of 0.25 M NaCl, 1% SDS, 20 mM Tris-HCl, 5 mM EDTA, and 10 mg of glycogen, nucleic acids were extracted once with phenol/chloroform and precipitated with ethanol. The pellet was dissolved in 10 ml of H2O and mixed with 100 ml of DNase digestion mixture containing 20 mM Hepes (pH 7.6), 12% glycerol, 0.15 M KCl, 5 mM MgCl2, 4 mM each of ATP, GTP, CTP, and UTP, 25 units of ribonuclease inhibitor (Takara), and 0.5 mg of supercooled 21107DPOLACAT. The reaction was allowed to proceed for 30 min at 30°C after stopping by addition of 500 ml of a stop mixture consisting of 0.25 M NaCl, 1% SDS, 20 mM Tris-HCl, 5 mM EDTA, and 10 mg of glycogen, nucleic acids were extracted once with phenol/chloroform and precipitated with ethanol. The pellet was dissolved in 10 ml of H2O and mixed with 100 ml of DNase digestion mixture containing 25 mg of RNase-free DNase I, and the reaction was incubated at 37°C for 15 min. DNase digestion was completed by adding 100 ml of DNase stop buffer containing 20 mg of Tris-HCl (pH 8.0), 50 mM EDTA, 1% SDS, 0.3

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 acidic 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; RRRRTPPRK). The amino acids essential for DRE-binding (16–105 amino acids) are also indicated by a bracket.
Transcription Factor for DNA Replication Genes

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 μg from 1 g of Kc cells. Twenty μg 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 endoprotease 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 1R, respectively. The 1.8-kb cDNA fragment was used as a probe to screen cDNA libraries (λgt10) prepared with mRNAs extracted from 0–3-h or 3–6-h embryos. Eight clones were isolated from 4 × 10⁵ 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 (RRRTT) 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-
We used the 5′-end-labeled upper or lower strands of a 337-bp XhoI-BamHI 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.

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-BamHI 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.

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 32P-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-dhck primase antibody (6 μl); lanes 5 and 6, culture supernatant of mouse hybridoma cell line 16 (6 μl); lanes 7 and 8, culture supernatant of mouse hybridoma cell line 4 (6 μl); lanes 9 and 10, rabbit anti-DREF polyclonal antibody (50 μg/ml IgG, 6 μl). Nuclear extract from Kc cells (2 μg) was mixed with each antibody, incubated for 2 h on ice, added to mixtures containing 32P-labeled DRE-P oligonucleotides (106 cpm) and 1 μg of poly(dI-dC), incubated for 15 min on ice, and analyzed on a 4% polyacrylamide gel.

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.
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 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...
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-mediating 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 zerknullt 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 plasminos. 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 after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA demonstrated that nuclei after the nuclear division cycle 8. A pulse-labeling experiment of zygotic transcribed RNA...
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Drosophila DNA Replication-related Genes
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