Identification of CFDD (Common Regulatory Factor for DNA Replication and DREF Genes) and Role of Its Binding Site in Regulation of the Proliferating Cell Nuclear Antigen Gene Promoter*

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The Drosophila proliferating cell nuclear antigen (PCNA) gene promoter contains at least three transcriptional regulatory elements, the URE (upstream regulatory element), DRE (DNA replication-related element), and E2F recognition sites. In nuclear extracts of Drosophila Kc cells, we detected a novel protein factor(s), CFDD (common regulatory factor for DNA replication and DREF genes) that appeared to recognize two unique nucleotide sequences (5'-CGATA and 5'-CAATCA) and bind to three sites in the PCNA gene promoter. These sites were located at positions –84 to –77 (site 1), –100 to –93 (site 2) and –134 to –127 (site 3) with respect to the transcription initiation sites. Sites 2 and 3 overlapped with DRE and URE, respectively, and the 5'-CGATA matched with the reported recognition sequence of BEAF-32 (boundary element-associated factor of 32 kDa). Detailed analyses of CFDD recognition sequences and experiments with specific antibodies to DRE (DRE-binding factor) and BEAF-32 suggest that CFDD is different from DREF, UREF (URE-binding factor) and BEAF-32. A UV cross-linking experiment revealed that polypeptides of ~76 kDa in the nuclear extract interact directly with the CFDD site 1 sequence. Transient expression assays of chloramphenicol acetyltransferase (CAT) in Kc cells transfected with PCNA promoter-CAT fusion genes carrying mutations in CFDD site 1 and examination of lacZ expression from PCNA promoter-lacZ fusion genes carrying mutations in site 1, introduced into flies by germ line transformation, revealed that CFDD site 1 plays an important role for the promoter activity both in cultured cells and in living flies. In addition to the PCNA gene, multiple CFDD sites were found in promoters of the DNA polymerase α and DREF genes, and CFDD binding to the DREF promoter was confirmed. Therefore, CFDD may play important roles in regulation of Drosophila DNA replication-related genes.

The proliferating cell nuclear antigen (PCNA), 1 an accessory protein of DNA polymerase δ, is required for replication of simian virus 40 (1) as well as cellular DNA (2, 3). It has been proposed to function as a sliding clamp at DNA replication forks (4) and is also involved in DNA repair (5, 6) and cell cycle regulation (7–9). The amino acid sequence of the PCNA protein has been highly conserved and demonstrates essential similarities among a wide range of species from yeast to man (4, 10).

In previous studies of the Drosophila genes for PCNA and DNA polymerase α, we found a common 8-base pair palindromic sequence, named DRE (DNA replication-related element) (11), which appeared to be an important regulatory element not only for these two DNA replication-related genes but also for various other cell cycle (12)- and cell proliferation-related genes (13, 14). We also identified a specific DRE-binding factor (DREF) consisting of an 80-kDa polypeptide homodimer (11) and cloned its cDNA (15). Characterization of DRE in vivo has revealed that it is essential for the function of the PCNA gene promoter both in embryos and in larvae (16).

We have also identified two E2F recognition sites in the region downstream of the PCNA gene DRE (17). cDNAs have been cloned for Drosophila E2F and DP (18–20), these two proteins interacting with each other to fulfill sequence-specific DNA binding and optimal transactivation (19). Multiple E2F recognition sequences have been also identified in the promoters of the Drosophila DNA polymerase α 180-kDa subunit (18) and the 73-kDa subunit (21), and transcription of DNA polymerase α and PCNA genes is completely lost in E2F mutant embryos after division cycle 16 (22). Analyses with transgenic flies demonstrated that two E2F sites are essential for PCNA gene promoter activity throughout development (17). However, E2F sites alone proved to be insufficient for PCNA gene promoter activity during embryonic and larval stages, since deletion of the upstream region containing the DRE sequence completely abolished the promoter activity during these stages (17).

Another important regulatory element for the PCNA gene promoter is URE (upstream regulatory element) located in the region from nucleotide positions –168 to –119 (16). The URE, in addition to the E2F sites and DRE, is essential for activation of the PCNA gene promoter in larvae, and a protein factor, UREF (URE-binding factor) has been found, which specifically binds to its sequence (16). Thus, URE, DRE, and E2F sites likely cooperate to optimize activity of the PCNA gene promoter during development. However, the earlier studies could not exclude the possibility that there is another important regulato; URE, upstream regulatory element; UREF, URE-binding factor; CFDD, common regulatory factor for DNA replication and DREF genes; BEAF-32, boundary element-associated factor of 32 kDa; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; BTS, BEAF-32-binding site(s); GST, glutathione S-transferase; BrdUrd, 5'-bromo-2'–deoxyuridine; scs, special chromatin structure.

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1 The abbreviations used are: PCNA, proliferating cell nuclear antigen; DRE, DNA replication-related element; DREF, DRE-binding factor; URE, upstream regulatory element; UREF, URE-binding factor; CFDD, common regulatory factor for DNA replication and DREF genes; BEAF-32, boundary element-associated factor of 32 kDa; CAT, chloramphenicol acetyltransferase; PCR, polymerase chain reaction; BTS, BEAF-32-binding site(s); GST, glutathione S-transferase; BrdUrd, 5'-bromo-2'–deoxyuridine; scs, special chromatin structure.

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In the present study, we identified a novel factor that binds to the region between −87 and −62. This factor recognizes two unique nucleotide sequences, and although it binds to two additional sites overlapping with DRE and URE in the PCNA gene promoter, it appears to be different from DREF and UREF. We termed this factor CFDD (common regulatory factor between DREF and URE). Since it also binds to two E2F recognition sites in the adenovirus E2 gene (AdE2Fwt) and at least one of the CFDD sites plays an important role in PCNA gene promoter activity, we investigated the role of its binding site.

**EXPERIMENTAL PROCEDURES**

**Antibodies**—Monoclonal antibodies to DREF, Mab-1 and Mab-4, were raised as described previously (15). A polyclonal antibody that reacts with both BEAF-32A (23) and BEAF-32B (24) was purified from serum using E-Z-SEP (Pharmacia Biotech Inc.).

**Oligonucleotides**—The sequences of double-stranded oligonucleotides containing DRE (DRE-P), a 2-base-substituted derivative (DRE-m), or other derivatives in the PCNA promoter were as described earlier (25). The sequences of double-stranded oligonucleotides containing E2F recognition sites 1 and 2 in the PCNA promoter (E2F-P) and E2F recognition sites in the adenovirus E2 gene (AdE2Fwt) were also as reported previously (17).

The sequences of double-stranded oligonucleotides containing CFDD-binding sites or their derivatives in the PCNA promoter were defined as follows.

**FIG. 1.** Nucleotide sequences in and around CFDD recognition sites in the Drosophila PCNA gene and its related sequences in the DREF gene and the hsp70 gene. A, constructs of PCNA-lacZ (p5′-168DPCNAzW8HS and p5′-119DPCNAzW8HS) and PCNACAT (p5′-168DPCNACAT and p5′-119DPCNACAT) fusion genes are shown. The vertical lines with horizontal arrows indicate the transcription initiation site. The open and closed boxes indicate the 5′-untranslated and coding sequences of the PCNA gene, respectively. The dark stippled boxes indicate the DREF sequence. The dark hatched boxes indicate the URE sequence. The open and closed circles indicate E2F and CFDD recognition sites, respectively. The shaded and CFDD recognition sites, respectively. The black, open, and dark hatched boxes indicate E2F, and mutant PCNA genes are shown. Locations of each site relative to the transcription initiation site are indicated by numbers with vertical lines. Nucleotides with substitution for the wild-type sequence and those inserted into the wild-type sequence are shown by small letters. Nucleotide sequences of CFDD sites 1, 2, and 3 are indicated by boxed sequences. The 5′-CGATA sequences are marked by horizontal arrows, and the 5′-CAATCA sequence is marked by a dark box. B, nucleotide sequences in and around the CFDD site-related sequences of the DREF gene promoter and ses site region of the hsp70 gene are shown.

**FIG. 2.** Complex formation between the −87/−62 oligonucleotides and the Kc cell nuclear extract and competition by various oligonucleotides. Radiolabeled double-stranded −87/−62 oligonucleotides were incubated with Kc cell nuclear extract (4 μg of protein) in the presence or absence (0) of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane). −87/−62, oligonucleotides containing the CFDD site 1 from the PCNA gene promoter; URE, oligonucleotides containing the URE/CFDD site 3 from the PCNA gene promoter; E2F-P, oligonucleotides containing the E2F sites 1 and 2 from the PCNA gene promoter; DRE-P, oligonucleotides containing the DRE sequence/CFDD site 2 from the PCNA gene promoter; DRE-PM, DRE-P oligonucleotides having a mutation in the DRE sequence; AdE2Fwt, oligonucleotides containing two wild-type E2F sites from the adenovirus E2 gene promoter.
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For obtaining the fragment containing base substitutions at around position –72 of the PCNA gene promoter, the following primers were synthesized and used for the polymerase chain reaction (PCR).

-72BglII: AGGGGATATCCGTGCGAGATCTGTTGGCTTTTACACCTC
-80In2: GCTAGCAGATATCCGTGCGAGATCTGGCTTTTACACCTC

CAT-1: GCCCTGACAAATCTGGCAAGCTGAGC

For obtaining the fragment containing base substitutions at around position –72 of the PCNA gene promoter, the following primers were synthesized and used for PCR.

PI: GCTAGCAGATATCCGTGCGAGATCTGGCTTTTACACCTC
PIn20: GCTAGCAGATATCCGTGCGAGATCTGGCTTTTACACCTC

The sequences of double-stranded oligonucleotide containing BEAF-32-binding sites (BTS) in the sea' region of the Drosophila hsp70 gene were as described earlier (23).

The sequences of double-stranded oligonucleotide containing CFDD-binding sites or its base-substituted derivative in the DREF promoter were defined as follows.

+218/+253: TTGGGATGTTAATGCATAATCGCTCTTATGAGTTG

Plasmid Constructions—The plasmid p5'-16DPCNA CAT contains the PCNA gene fragment spanning from –168 to +23 placed upstream of the chloramphenicol acetyltransferase (CAT) gene in the plasmid pSKCAT (26). The plasmid p5'-119DPCNA CAT contains the PCNA gene fragment spanning from –119 to +23 (26).

The plasmids p5'-16DPCNA CAT and p5'-119DPCNA CAT were digested with EcoRV and BglII linkers (Toyobo) and insert were into the plasmids p5'-16DPCNA CAT and p5'-119DPCNA CAT, respectively. The plasmids p5'-16DPCNA CAT DNA was digested with BglII and then with mung bean nuclease. Plasmids p5'-16DPCNA CAT and p5'-16DPCNA CAT DNA, respectively obtained by these procedures, were digested with ClaI and SacI, and the isolated fragments were then inserted between

**Fig. 3. Effects of mutations in the DRE/CFDD site 2 on the complex formation with –87/–62 oligonucleotides or with DRE-P.** A, radiolabeled double-stranded –87/–62 oligonucleotides were incubated with Kc cell nuclear extract (4 μg of protein) in the presence or absence (0) of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane). B, radiolabeled double-stranded DRE-P oligonucleotides were incubated with Kc cell nuclear extract (0.8 μg of protein) in the presence or absence (0) of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane). –87/–62, oligonucleotides containing the CFDD site 2 from the PCNA gene promoter; DRE-P, oligonucleotides containing the DRE sequence/CFDD site 2 from the PCNA gene promoter.

The sequences of double-stranded oligonucleotides containing URE or their base-substituted derivatives in the PCNA promoter were defined as follows.

URE: tcgaccGTAAAGATGTAACATCAAAACCAGTGCAGGCA
ggCATTTTCTCACCTGTATGTTGGTCAACCGCTagtct
mut: tcgaccGTAAAGATGTAACATCAAAACCAGTgttac
ggCATTTTCTCACCTGTATGTTGGTCAACCGCTagtct
mutb: tcgaccGTAAAGATGTAACATCAAAACCAGTGGCAGAATACTCAAC
ggCATTTTCTCACCTGTATGTTGGTCAACCGCTagtct
mut2: tcgaccGTAAAGATGTAACATCAAAACCAGTGCAGGCA
ggCATTTTCTCACCTGTATGTTGGTCAACCGCTagtct
mutb2: tcgaccGTAAAGATGTAACATCAAAACCAGTGCAGGCA
ggCATTTTCTCACCTGTATGTTGGTCAACCGCTagtct

Pr-57: GGGATGTTAAGACC

For obtaining the fragment containing base insertions at either position –72 or position –80 of the PCNA gene promoter, the following

**Fig. 3. Effects of mutations in the DRE/CFDD site 2 on the complex formation with –87/–62 oligonucleotides or with DRE-P.** A, radiolabeled double-stranded –87/–62 oligonucleotides were incubated with Kc cell nuclear extract (4 μg of protein) in the presence or absence (0) of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane). B, radiolabeled double-stranded DRE-P oligonucleotides were incubated with Kc cell nuclear extract (0.8 μg of protein) in the presence or absence (0) of the indicated amounts of competitor oligonucleotides (indicated at the top of each lane). –87/–62, oligonucleotides containing the CFDD site 2 from the PCNA gene promoter; DRE-P, oligonucleotides containing the DRE sequence/CFDD site 2 from the PCNA gene promoter.
CAT, respectively.

A fragment from −102 to +24 having 4-base-substituted mutations was generated by the PCR method using p5′-168DPNCAT as a template with primers PI and CAT-1. The PCR product was digested with Clal and SacI, then replaced with the fragment between the Clal and SacI sites of p5′-168DPNCAT or p5′-119DmIDPNCAT to create the plasmids p5′-168mut1DPNCAT and p5′-119mut1DPNCAT, respectively. p5′-168mutIn2DPNCAT and p5′-119mutIn2DPNCAT were created in a similar way except that primers PIIn2 and CAT-T1 were used for the PCR. Similarly, p5′-168mut2DPNCAT and p5′-119mut2DPNCAT were constructed using primers −80in2 and CAT-1.

A fragment from −87 to +24 having an 8-base insertion at −72 was generated by the PCR method using p5′-168DPNCAT as a template with primers −72BglIII and CAT-1. The PCR product was digested with EcoRV and SacI, then replaced with the fragment between the EcoRV and SacI sites of p5′-119DmIDPNCAT to create the plasmid p5′-119mut8I−72DPNCAT.

The plasmid p5′-168DPNCATACZW88SHS contains the PCNA gene fragment spanning from −168 to +137 fused with the lacZ gene in a P-element vector (26). To create mutated derivatives in P-element vector backbones, fragments having various mutations in CFDD sites were isolated from CAT plasmids by digestion with SacI (−168) and SacI (+23) and inserted between the Xhol (−607) and SacI (+23) sites of p5′-607DPNCATACZW88SHS. The obtained plasmids were verified by nucleotide sequence analysis with synthetic primers.

pGST-DREF16−608 containing DREF cDNA fused with the glutathione S-transferase (GST) gene was constructed as described previously (15).

All plasmids were propagated in Escherichia coli XL-1 Blue and isolated by standard procedures (27). The isolated plasmids were further purified through two cycles of ethidium bromide/CsCl density gradient centrifugation.

**Band Mobility Shift Assay and Preparation of Nuclear Extracts—** Band mobility shift analysis was performed as described earlier (11) with minor modifications. 32P-Labeled probes (20,000 cpm, 500 ng) were incubated in 13 ml of reaction mixture containing 25 ml Hepes, pH 7.6, 150 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 0.5 μg of poly(dIdc), on ice for 5 min. When necessary, unlabeled DNA fragments were added as competitors at this step. Then, E. coli lysates containing GST-DREF fusion proteins or Kc cell nuclear extracts were added, and the reaction mixture was incubated for 15 min on ice. In experiments with antibodies, Kc cell nuclear extracts were preincubated with the antibody for 2 h on ice. DNA-protein complexes were electrophoretically resolved on 3% polyacrylamide gels in 100 mM Tris bated with the antibody for 2 h on ice. DNA-protein complexes were electrophoretically resolved on 3% polyacrylamide gels in 100 mM Tris borate, pH 8.3, 2 mM EDTA containing 2.5% (v/v) glycerol at 25 °C. The gels were dried and then autoradiographed.

**Expression of GST Fusion Proteins—** Expression of GST-DREF fusion proteins was carried out as described elsewhere (29) with minor modifications. GST-Labeled clones were isolated from phage plaques on a lawn of E. coli (strains DH5α, C8004, C36, and C8005) plated onto 2% agarose plates with 1.5 μg/ml each of pepstatin, leupeptin, and aprotinin. Lysates were cleared by centrifugation at 12,000 × g for 20 min at 4 °C and used for band mobility shift assays as described above.

**Determination of the CFDD Size by UV Cross-linking Analysis—** UV cross-linking analysis was carried out as described earlier (11) with modifications. Thirty ng of oligonucleotide UV-89 and 20 ng of oligonucleotide PR-57 were mixed in 41.5 μl of a solution containing 33 mM Tris acetate, pH 7.9, 10 mM magnesium acetate, 66 mM potassium acetate, and 0.5 mM dithiothreitol and incubated for 3 min at 95 °C, followed by 10 min at 25 °C. Then the solution was mixed with 8.5 μl of reaction mixture containing 118 μM each of dATP, dGTP, dCTP, and dTTP, 87/62 oligonucleotides (29) labeled 32P with 30 μg of DNA polymerase I large fragment (29). DNA was uniformly labeled with 32P and BrdUrd by incubation at 37 °C for 1 h and then chased for 15 min with 10 μM unlabeled dCTP. The 32P-labeled and BrdUrd-substituted probe (1.75 ng) was incubated with Kc cell nuclear extract (32 μg of protein) for 15 min on ice in 17 μl of the same buffer as that used in the band mobility shift analysis. Uncapped Eppendorf tubes containing the reaction mixtures were placed on an 8-cm distance from an inversely placed 254-nm ultraviolet transilluminator (model UVGL-58, UVP, Inc.) and irradiated on ice for 20 min. UV dose under these conditions was 4.19 kJ/m2. Solutions of CaCl2 and MgCl2 were added to final concentrations of 10 mM and 100 mM, respectively. Digestion by 2 units of DNase I and 9 units of exonuclease III was carried out at 30 °C for 30 min. The reaction was terminated by adding 20 μl of the loading buffer containing 100 mM Tris-HCl, pH 8.8, 4% SDS, 0.2% bromphenol blue, 20% (v/v) glycerol, and 0.2 mM dithiothreitol. The samples were heated and applied to 10% polyacrylamide gels containing 0.1% SDS. After electrophoresis, the gels were stained with Coomassie Brilliant Blue, photographed, dried, and autoradiographed.

The molecular weights of the protein bands were estimated by comparing their mobilities with those of marker proteins (Bio-Rad). The following molecular weights marker proteins were used: myosin (200,000), β-galactosidase (116,250), phosphorylase B (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,500), lysozyme (14,400).

**DNA Transfection into Cells, CAT Assays, and Luciferase Assays—** DNA transfection into cells was performed as described elsewhere (32). 32P-Labeled −87/62 oligonucleotides (lanes a–f) were incubated without (lanes a and f) or with an extract of E. coli producing GST-DREF (lanes b and g) or GST (lanes c and h, 0.1 μg; lanes d and i, 0.4 μg) or GST (lanes e and j, 0.4 μg). In some experiments (lanes b–e), 32P-labeled −87/62 oligonucleotides (lanes a–e) or GST-DREF oligonucleotides (lanes f–j) were incubated with Kc cell nuclear extract in the absence (lanes c and h) or presence of anti-DREF monoclonal antibody number 1 (Mab-1) (lanes b and g, 1 μl; lanes a and f, 2 μl of culture supernatant) or anti-DREF monoclonal antibody number 4 (Mab-4) (lanes d and i, 1 μl; lanes e and j, 2 μl of culture supernatant).

**Identification of CFDD and Role of Its Binding Site**
The luciferase assay was carried out by means of a PicaGene assay kit (Toyo Ink) as described previously (34). All assays were performed within the range of linear relation of the activity to incubation time and protein amount. CAT activity was normalized to the luciferase activity. The obtained values were essentially comparable with those normalized to protein amounts determined by Bio-Rad protein assay. Transfections were performed several times with at least two independent plasmid preparations.

**Establishment of Transgenic Flies**—Fly stocks were maintained at 25 °C on standard food. Canton S flies were used as the wild-type strain. P-element-mediated germ line transformation was carried out as described earlier (35), and G1 transformants were selected on the basis of white eye color rescue (36). Multiple independent lines were obtained for each of the various fusion genes. Established transgenic fly strains and their chromosomal linkages are listed in Table I.

**Analysis of Expression Patterns for PCNA-lacZ**—Quantitative measurement of β-galactosidase activity in extracts was carried out as described previously (37). Male transgenic flies were crossed with wild-type females, and groups of 50–100 individual dechorionated embryos, larvae, pupae, and adult flies were homogenized in 500 μl of ice-cold assay buffer (50 mm potassium phosphate, pH 7.5, 1 mm MgCl₂). Homogenates were centrifuged at 10,000 g for 5 min. For each assay, 50–200 μl of supernatant was added to 1 ml of assay buffer containing 1 mm chlorophenol red-β-D-galactopyranoside substrate (Boehringer Mannheim). Reaction incubations were at 37 °C in the dark. Substrate conversion was measured at 574 nm using a spectrophotometer 0.25, 0.5, 0.75, 1, and 1.5 h after addition of the extract, and the rate of color development was linear. The β-galactosidase activity was defined as absorbance units/h/mg of protein. To correct for endogenous β-galactosidase activity, extracts from the wild-type strain were included in each experiment, and this background reading was subtracted from readings obtained with each transformant line. Variation among independent strains was less than 30% (not shown). The protein concentrations of the extracts were determined by Bio-Rad protein assay.

**RESULTS**

**Detection of CFDD**—The Drosophila PCNA gene promoter is regulated by at least three transcriptional regulatory elements, URE (−149 to −118), DRE (−100 to −93), and E2F recognition sites (−56 to −36) (Fig. 1A). To analyze potential interactions of DREF and E2F, a fragment containing both DRE and E2F sites (−108 to −28) was used for band mobility shift analysis using Kc cell nuclear extracts. We thereby detected a novel protein factor that may have an affinity to the region between the DRE and E2F sites (data not shown).

To confirm this finding, an oligonucleotide containing the region from −87 to −62 (Fig. 1A) was chemically synthesized and used for the band mobility shift analysis. With this oligonucleotide, DNA-protein complexes were detected (Fig. 2), measured as described previously (33). The radioactivity of acetylated chloramphenicol on thin-layer plates was quantified with an imaging analyzer BAS2000 (Fuji Film).
which were diminished by adding an excess amount of unlabeled \(-87/–62\) oligonucleotide as a competitor but not by adding oligonucleotides containing E2F sites such as E2F-P or AdE2Fwt (Fig. 2, lanes a–e, j–m, and w–z). The oligonucleotides DRE-P and DRE-PM containing sequences related to the \(-87/–62\) oligonucleotide (Fig. 2A) competed for the binding (Fig. 2, lanes n–v). Unexpectedly, the URE oligonucleotide containing no related sequence to the \(-87/–62\) oligonucleotide also competed for complex formation (Fig. 2, lanes f–i). These results indicate that a common factor can bind to these DRE and URE oligonucleotides. We designated this factor CFDD, since it had an affinity to the DREF gene promoter in addition to the PCNA gene promoter as described below. The highest affinity of CFDD appears to be for the \(-87/–62\) oligonucleotide (Fig. 2).

**CFDD Is Different from DREF**—Oligonucleotides carrying various mutations in the DRE sequence (Fig. 1A) were added to the binding reaction in the band mobility shift analysis. When the \(^{32}\)P-labeled \(-87/–62\) oligonucleotide was used as a probe, oligonucleotides DRE-P, CIIA(-), and mut\(\Delta 1\)(–96) competed for binding as effectively as the wild-type \(-87/–62\) oligonucleotide (Fig. 3A, lanes a–e, j–r, and w–z). Both mut\(\Delta 3\) and mut\(\Delta 1\)(–98) competed less effectively (Fig. 3A, lanes f–i and s–v). In contrast, none of these mutant oligonucleotides competed with the complex formation between DNA and DREF when DRE-P was used as a probe in the band mobility shift analysis (Fig. 3B). The faint bands migrating more slowly than the DNA-DREF complex behaved similarly to those detected with the \(-87/–62\) oligonucleotide probe (Fig. 3B, complex), suggesting that they represent complexes between DRE-P and CFDD.

As shown in Fig. 4A, GST-DREF fusion proteins did not bind to the \(-87/–62\) oligonucleotide in the band mobility shift analysis under the examined conditions, although they had strong affinity for the DRE-P oligonucleotide. Furthermore, monoclonal antibodies to DREF exerted no effect on the complex formation between DNA and CFDD (Fig. 4B, lanes a–e), while they either inhibited (Fig. 4B, lanes f and g) or shifted (lanes i and j) the DRE-DREF complex. Taken together, these results indicate that CFDD is different from DREF, although CFDD has an affinity for the DREF sequence as well as the \(-87/–62\) oligonucleotide.

**Nucleotide Sequences Required for Binding to CFDD**—To determine the nucleotide sequence required for binding to CFDD, various mutations in the fragment between \(-87\) and \(-62\) were introduced (Fig. 1A) and used as competitors in the band mobility shift analysis. Internal deletion mutants mut\(\Delta 6\)(–77/–82) and mut\(\Delta 5\)(–81/–82) did not compete for the binding (Fig. 5, lanes j–n). Similarly, the 4-base substitution mutant mutI and its 2-base-insertional derivative mutI.In8(–81) did not compete at all (Fig. 5, lanes o–r and a’–c’). In contrast, the 6-base substitution mutants mutJ and mutK competed for the binding as effectively as the wild-type \(-87/–62\) oligonucleotide (Fig. 5, lanes g’–o’). The 8-base-insertional mutant mutIn8(–81) competed much less effectively for the complex formation (Fig. 5, lanes a–e). However, 2-base-insertional mutation at \(-81\) that recreated the sequence 5’-CGATA in both strands retained the competition ability (Fig. 5, lanes d’–f’). These results indicate that the sequence 5’-
CGATA plays an important role in the CFDD binding. In addition, one copy of the sequence 5’-CGATA appeared to be sufficient for the CFDD binding, since DRE-PM and mutA (98) competed for the binding (Figs. 2 and 3). We designated the CFDD recognition site in the region between -84 and -77 as CFDD site 1 and that overlapping with the DRE as CFDD site 2 (Fig. 1A).

**CFDD Is Different from BEAF-32**—A palindromic sequence 5’-CGATA-TATCG has been identified as a binding sequence for Drosophila BEAF-32 (23). This sequence contains one required for binding to CFDD, suggesting the possibility that CFDD is identical to BEAF-32. As shown in Fig. 6, lanes a and h–j, a BTS oligonucleotide containing the palindromic sequence 5’-CGATA-TATCG and one additional 5’-CGATA (23) effectively competed for the complex formation between CFDD and the -87/-62 oligonucleotide, indicating that CFDD has a strong affinity for the BEAF-32-binding sequence. The band that has less mobility than the DNA-CFDD complex was detected with the nuclear extract used in this experiment. Since this band was not competed out by any oligonucleotides, this very likely represents a complex between a probe and nonspecific DNA-binding proteins. When the BTS oligonucleotide was used as a probe, DNA-protein complexes were detected (Fig. 6, lane m). They were diminished by adding an excess amount of unlabeled BTS oligonucleotide and DRE-P as competitors (Fig. 6, lanes q–o). However, the -87/-62 oligonucleotide only marginally competed for the complex formation (Fig. 6, lanes m–p) and its mutant oligonucleotide mut1 did not compete at all (Fig. 6, lanes w and x). In addition, this complex migrated much faster than the complex between DNA and CFDD when analyzed in the same gels (data not shown). These results suggest that the protein factor detected with the BTS oligonucleotide probe is different from CFDD.

Addition of the anti-BEAF-32 antibody to the binding reaction with the BTS oligonucleotide probe inhibited the complex formation (Fig. 7, lanes d–f), indicating that the complex is formed between the BTS oligonucleotide and BEAF-32. However, when the -87/-62 oligonucleotide was used as a probe in the band mobility shift analysis, the anti-BEAF-32 antibody exerted no effect on the complex formation (Fig. 7, lanes p–r). These results also suggest that CFDD is different from BEAF-32.

**Identification of the CFDD Polypeptide by the UV Cross-linking Method**—Among the three CFDD-binding sites, CFDD site 1 appears to have the highest affinity for CFDD. A polypeptide(s) binding directly to the CFDD site 1 (Fig. 1) was identified by UV cross-linking experiments using UV-89 oligonucleotide as a probe. As shown in Fig. 8, a few polypeptides at around 76 kDa were specifically cross-linked with the radioactive probe. Lesser amounts of radiolabeled polypeptides were observed by adding increasing amounts of the -87/-62 oligonucleotide as a competitor (Fig. 8, lanes b–c), whereas the mut1 oligonucleotide carrying a 4-base substitution in the CFDD site 1 competed less effectively (Fig. 8, lanes f–h). Thus, the CFDD polypeptides are around 76 kDa in size, and therefore clearly different from BEAF-32, since it is composed of a single polypeptide of 32 kDa (23).

**Nucleotide Sequence Required for Binding to CFDD in the URE Region**—Although the URE sequence effectively competed for the complex formation between CFDD and the -87/-62 oligonucleotide (Fig. 2), no nucleotide sequence related to 5’-CGATA was found in the URE region. To determine the nucleotide sequence required for CFDD binding, a set of base
substitution mutations was introduced into the URE sequence (Fig. 1A), and mutated oligonucleotides were used as competitors in the band mobility shift analysis. As shown in Fig. 9, oligonucleotides muta, mutβ, and mute (lanes f–m and w–z) competed for the binding as effectively as the wild-type URE oligonucleotide. In contrast, mutg and mutd did not compete at all (Fig. 9, lanes n–v). Therefore, the nucleotide sequence from 2134 to 2127 appears to be essential for the CFDD binding. We designated this region CFDD site 3 (Fig. 1A).

**CFDD Binds to the DREF Gene Promoter**—We have cloned the DREF gene and mapped its promoter within the region between 121103 and 12253.2 In the nucleotide sequence analysis of the essential region for the DREF gene promoter activity, we found the nucleotide sequence 5′-CAATCA that matches to the CFDD site 3 of the PCNA gene promoter (Fig. 1). When the 1218/1262 oligonucleotide containing this sequence of the DREF gene promoter was used as a competitor in the band mobility shift analysis, strong inhibition of complex formation between CFDD and the 287/262 oligonucleotide of the PCNA gene was observed (Fig. 10, lanes e–g). However, the oligonucleotide mutB carrying base substitutions in the 5′-CAATCA does not compete as well as the 287/262 or 218/253 oligonucleotide, although some inhibition was observed (Fig. 10, lanes h–j). When the 218/253 oligonucleotide was used as a probe in the band mobility shift analysis, a shifted band was observed, which was diminished by adding the −87/−62 oligonucleotide (data not shown). In addition, anti-BEAF antibody exerted no effect on DNA-protein complex formation with the 218/253 oligonucleotide, as was the case with the −87/−62 oligonucleotide (Fig. 7, lanes m–x). These results indicate that CFDD has a strong affinity for the region spanning the 5′-CAATCA sequence of the DREF gene promoter.

**Effects of Mutations in the CFDD Site 1 on the PCNA Gene Promoter Activity in Kc Cells**—Since CFDD sites 2 and 3 overlap with DRE and URE, respectively (Fig. 1A), mutations in these sites would be expected to exert effects on binding of not only CFDD but also other factors such as DREF and UREF. Therefore, we focused our attention on the CFDD site 1 with the highest CFDD affinity. Various mutations were introduced in and around the site, and the mutated PCNA gene promoter was placed upstream of the CAT gene in a CAT vector. Plasmids carrying these constructs were then transfected into Kc cells, and CAT expression levels were determined. As shown in Fig. 11, various deletions, base substitutions, and an 8-base insertion in the CFDD site 1 all reduced the CAT expression. The extent of the reduction was slightly larger with constructs deleting the CFDD site 3 (Fig. 11B). However, the 2-base-insertional mutation at position −81 that recreated the 5′-CGATA sequence on both strands and had no effect on CFDD binding (Fig. 5) showed no reduction of CAT expression. These results indicate that CFDD site 1 plays an important role in the PCNA gene promoter activity in Kc cells.

**Role of the CFDD Site 1 in the Function of the PCNA Gene Promoter in Living Flies**—Although the results of CAT transient expression assay in Kc cells clearly demonstrated an

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*F. Hirose, N. Ohshima, Y. Takahashi, Y. Hayashi, M. Yamaguchi, and A. Matsukage, submitted for publication.*
Identification of CFDD and Role of Its Binding Site

In the work presented here, we identified a novel protein recognition sites (16, 17), to which the protein factors UREF, transcriptional regulatory elements, URE, DRE, and E2F recognize specifically and respectively bind. In the work presented here, we identified a novel protein factor(s), CFDD that binds to the region between −87 and −62 of the PCNA gene promoter. This site (CFDD site 1) is located between DRE and E2F sites. In addition, CFDD binds to two other sites (CFDD sites 2 and 3) overlapping with DRE and URE, respectively (Fig. 1A). Among these three sites, CFDD site 1 appears to have a highest affinity to CFDD.

While the nucleotide sequence of the CFDD site 2, in particular, perfectly matches the 8-base pair DRE sequence, we conclude that CFDD is different from DREF, for the following two reasons. First, DREF binds to the CFDD site 2/DRE but not to the CFDD site 1, and the nucleotide sequence required for the binding is clearly different between CFDD and DREF. Secondary, anti-DREF antibodies did not react with CFDD when they were added to the binding reaction for the band mobility shift analysis.

Although the UREF protein has not been fully characterized yet, its recognition sequence has been mapped to the region between −130 and −118 (data not shown) containing the reported binding consensus sequence for Drosophila snail gene product and its related proteins (5'-ANCACCTGTNNCA) (38, 39). Since this binding site does not exactly match to the CFDD site 3, CFDD is also very likely to be different from UREF.

Recognition of a single binding site by multiple transcription factors has been frequently observed for promoters of various genes. Although detailed mechanisms have yet to be determined, CFDD might regulate the PCNA gene promoter activity by competing against DREF for binding to the CFDD site 2/DRE and UREF for binding to the CFDD site 3/URE, respectively.

We conclude that CFDD is also different from BEAF-32, which was found to be able to bind to the CFDD site 2/DRE but not to the CFDD site 1. Furthermore, anti-BEAF-32 antibodies did not react with CFDD, and the molecular weight of BEAF-32 is much smaller than that of CFDD. Since BEAF-32 has a high affinity for CFDD and the PCNA gene promoter, it might play a role in regulation of the PCNA gene promoter activity.

BEAF-32 binds with high affinity to the scs' boundary element from the Drosophila 87A7 hsp70 locus, and therefore it has been suggested that this protein plays a critical role in establishing the chromosomal boundary (23). However, it is now known that scs' sequences, including the binding site of the BEAF-32 protein, are very likely to be within the promoter of the aura gene (40). Therefore, taken together with our results, BEAF-32 might have dual roles: one is establishment of the chromosomal boundary, the other is regulation of the promoter activity. From the same reasons, it can be suggested that CFDD plays a role in the establishment of chromosomal boundary and regulation of the aura gene promoter, since it has a high affinity for the BEAF-32-binding sites in the scs' boundary element.

In the present study of the analysis of CFDD site 1 functions, we observed several differences between results for cultured cells and transgenic flies. For instance, the effect of a 4-base substitution (mutI) at the CFDD site 1 on the promoter activity was far more prominent in the transgenic fly analysis than in cultured cells. In contrast, the P-element method provides only one copy of the transgene integrated into the chromosome. Therefore, the transgenic fly analysis is very likely to represent more faithfully the regulation in vivo.

In conclusion, we have identified three CFDD-binding sites within the PCNA gene promoter. We also found a CFDD site 3-like sequence in the DREF gene promoter. In addition to this sequence, there are two CFDD site 2/DRE sequences and one additional 5'-CGATA sequence within the DREF gene promoter. Therefore, in total 4 CFDD-binding sites exist in the DREF gene promoter. In addition to these two genes, there are three important roles for CFDD site 1 for the PCNA gene promoter activity, these observations have to be further confirmed in living flies. For this purpose, transgenic Drosophila provides an appropriate system to characterize transcriptional regulatory elements in vivo.

Previously, we established transgenic flies carrying PCNA (−168 to +137 or −119 to +137) and lacZ fusion genes (16). To examine its role in the PCNA gene promoter activity during Drosophila development, we generated PCNA-lacZ fusion genes carrying various mutations in the CFDD site 1. These fusion genes were then introduced into flies by germ line transformation. Established transgenic lines and their chromosomal linkages are listed in Table I. Activations of the modified promoters were then monitored by quantitative β-galactosidase assay at various developmental stages.

In flies carrying the PCNA gene promoter region up to the position −168, a 6-base deletion and a 4-base substitution in the CFDD site 1 reduced the lacZ expression in larvae, pupae, and adults, while no significant effect was observed in embryos (Fig. 12, left). In flies carrying the PCNA gene promoter region up to the position −119, various mutations in the CFDD site 1 all reduced the lacZ expression throughout development (Fig. 12, right). Thus, an important role of the CFDD site 1 for the PCNA gene promoter activity was confirmed in living flies.

DISCUSSION

The Drosophila PCNA gene promoter contains at least three transcriptional regulatory elements, URE, DRE, and E2F recognition sites (16, 17), to which the protein factors UREF, DREF, and E2F/Dp complex specifically and respectively bind. In the work presented here, we identified a novel protein factor(s), CFDD that binds to the region between −87 and −62 of the PCNA gene promoter. This site (CFDD site 1) is located between DRE and E2F sites. In addition, CFDD binds to two important roles for CFDD site 1 for the PCNA gene promoter activity, these observations have to be further confirmed in living flies. For this purpose, transgenic Drosophila provides an appropriate system to characterize transcriptional regulatory elements in vivo.

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**TABLE I**

| P-element plasmids | Strains | Chromosome linkage |
|--------------------|---------|-------------------|
| p5-168DPCNAlacZW8HS | 5A | II |
| ............................... | 21 | X |
| ............................... | 73 | I |
| ............................... | 89 | II |
| ............................... | 91 | III |
| ............................... | 91 | III |
| p5-168mutΔ6(−77–82)DPCNAlacZW8HS | 29 | II |
| ............................... | 55 | I |
| ............................... | 76 | II |
| ............................... | 28 | III |
| ............................... | 103 | II |
| ............................... | 128 | III |
| p5-168mutIDPCNAlacZW8HS | 29 | III |
| ............................... | 30 | III |
| ............................... | 40 | III |
| ............................... | 67 | II |
| p5-119DPCNAlacZW8HS | 20 | II |
| ............................... | 25 | II |
| ............................... | 52A | II |
| ............................... | 52B | II |
| ............................... | 121 | III |
| p5-119mutΔ2(−81–82)DPCNAlacZW8HS | 16 | X |
| ............................... | 74 | III |
| ............................... | 84 | II |
| ............................... | 87 | II |
| p5-119mutΔ6(−77–82)DPCNAlacZW8HS | 16 | X |
| ............................... | 37 | II |
| ............................... | 102 | II |
| ............................... | 56 | X |
| p5-119mutIn8(−81)DPCNAlacZW8HS | 33 | III |
| ............................... | 56 | X |

* A line whose lacZ expression pattern is different from those of other lines carrying the same fusion gene.
Identification of CFDD and Role of Its Binding Site

CFDD site 2/DRE sequences in the DNA polymerase α 180-kDa catalytic subunit gene promoter and one CFDD site 3 sequence, one CFDD site 2/DRE sequence, and two 5′-CGATA/DRE-related sequences in the DNA polymerase α 73-kDa subunit gene promoter. It is therefore likely that multiple CFDD-binding sites exist in promoters of various DNA replication-related genes in Drosophila.

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REFERENCES

1. Tsurimoto, T., Melendy, T., and Stillman, B. (1990) Nature 346, 534–539
2. Jaskulski, D., DeRiel, K., Mercer, W. E., Calabretta, B., and Baserga, R. (1988) Science 240, 1544–1546
3. Liu, Y. C., Marraccino, R. L., Keng, P. C., Bambara, R. A., Lord, E. M., Chou, W. G., and Zain, S. B. (1989) Biochemistry 28, 2967–2974
4. Kelman, Z. A. O., and O'Donnell, M. (1995) Nucleic Acids Res. 23, 3613–3620
5. Shivji, M. K. K., Kenny, M. K., and Wood, R. D. (1992) Cell 69, 367–374
6. Smith, M. L., Chen, I.-T., Zhan, Q., Bae, I., Chen, C.-Y., Gilmer, T. M., Kastan, M. B., O'Connor, P. M., and Forrester, A. J. (1994) Science 266, 1376–1380
7. Matsuoka, S., Yamaguchi, M., and Matsukage, A. (1994) J. Biol. Chem. 269, 11030–11036
8. Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994) Nature 369, 574–578
9. Flores-Rozas, H., Kelman, Z., Dean, F., Pan, Z.-Q., Harper, J. W., Elledge, S. J., O'Donnell, M., and Hurwitz, J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8655–8659
10. Moriuchi, T. (1990) Mol. Cell. Biol. 10, 911–915
11. Hirose, F., Yamaguchi, M., Handa, H., Inomata, Y., and Matsukage, A. (1993) J. Biol. Chem. 268, 2092–2099
12. Ohno, K., Hirose, F., Sakaguchi, K., Nishida, Y., and Matsukage, A. (1996) Nucleic Acids Res. 24, 3942–3946
13. Ryu, J.-R., Chui, T.-Y., Kwon, E.-J., Lee, W.-H., Nishida, Y., Hayashi, Y., Matsukage, A., Yamaguchi, M., and Yoo, M.-A. (1997) Nucleic Acids Res. 25, 794–799
14. Matsukage, A., Hirose, F., Hayashi, Y., Hamada, K., and Yamaguchi, M. (1995) Gene (Amst.) 166, 233–236
15. Hirose, F., Yamaguchi, M., Kuroda, K., Omori, A., Hachiya, T., Ikeda, M., Nishimoto, Y., and Matsukage, A. (1996) J. Biol. Chem. 271, 3930–3937
16. Yamaguchi, M., Hirose, F., and Matsukage, A. (1996) Genes Cells 1, 47–58
17. Yamaguchi, M., Hayashi, Y., and Matsukage, A. (1995) J. Biol. Chem. 270, 25159–25165
18. Ogihara, K., and Nevin, J. R. (1994) Mol. Cell. Biol. 14, 1603–1612
19. Dynlacht, B. D., Brook, A., Dembski, M., Yenush, L., and Dyson, N. (1994) Mol. Cell. Biol. 14, 3942–3946.

Fig. 12. Effects of mutations in CFDD site 1 on PCNA gene promoter activity in transgenic flies. Male transgenic flies (indicated in each panel) were crossed with female wild type flies, and extracts were prepared from Drosophila bodies at various stages of development. The β-galactosidase specific activities in the extracts are expressed as absorbance units per h per mg protein. Closed bars indicate the average values for independent transgenic strains carrying the indicated fusion gene. Numbers (n) of independent strains carrying the same fusion gene are given in each panel.
Identification of CFDD and Role of Its Binding Site

20. Hao, X. F., Alphey, L., Bandara, L. R., Lam, E. W.-F., Glover, D., and La Thangue, N. B. (1995) J. Cell Sci. 108, 2945–2954
21. Takahashi, Y., Yamaguchi, M., Hirose, F., Cotterill, S., Kobayashi, J., Miyajima, S., and Matsukage, A. (1996) J. Biol. Chem. 271, 14541–14547
22. Duronio, R. J., O’Farrell, P. H., Xie, J. E., Brook, A., and Dyson, N. (1995) Genes Dev. 9, 1445–1455
23. Zhao, K., Hart, C. M., and Laemmli, U. K. (1995) Cell 81, 879–889
24. Hart, C. M., Zhao, K., and Laemmli, U. K. (1997) Mol. Cell. Biol. 17, 999–1009
25. Yamaguchi, M., Hayashi, Y., Nishimoto, Y., Hirose, F., and Matsukage, A. (1995) J. Biol. Chem. 270, 15808–15814
26. Yamaguchi, M., Hirose, F., Nishida, Y., and Matsukage, A. (1991) Mol. Cell. Biol. 11, 4909–4917
27. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
28. Smith, D. B., and Johnson, K. S. (1988) Anal. Biochem. 67, 31–40
29. Wu, C., Wilson, S., Walker, B., Dawid, I., Paisley, T., Zimarino, V., and Ueda, H. (1987) Science 238, 1247–1253
30. Echalier, G., and Ohanessian, A. (1979) In Vitro 6, 162–172
31. Cross, D. P., and Sang, J. H. (1978) J. Embryol. Exp. Morphol. 45, 161–172
32. Di Nocera, P. P., and Dawid, I. B. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 7095–7098
33. Yamaguchi, M., Hayashi, Y., and Matsukage, A. (1988) Nucleic Acids Res. 16, 8773–8787
34. Yamaguchi, M., Hayashi, Y., Matsuoka, S., Tkaashi, T., and Matsukage, A. (1994) Eur. J. Biochem. 221, 227–237
35. Spradling, A. C. (1986) in Drosophila: A Practical Approach (Roberts, D. B., ed) pp. 175–197, IRL Press, Oxford
36. Robertson, H. M., Preston C. R., Phillips, R. W., Johnson-Schlitz, D. M., Benz, W. K., and Engels, W. R. (1988) Genetics 118, 461–470
37. Fridell, Y.-W. C., and Searles, L. L. (1992) Mol. Cell. Biol. 12, 4571–4577
38. Kasai, Y., Nambu, J. R., Lieberman, P. M., and Crews, S. T. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3414–3418
39. Fuse, N., Hirose, S., and Hayashi, S. (1994) Genes Dev. 8, 2270–2281
40. Glover, D. M., Leibowitz, M. H., McLean, D. A., and Parry, H. (1995) Cell 81, 95–105