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Involvement of the DNA Replication-Related Element (DRE) and DRE-Binding Factor (DREF) in Transcriptional Regulation of the *Bombyx mori* PCNA Gene[1]

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The promoter region of the *Bombyx mori* gene encoding the proliferating cell nuclear antigen (PCNA), and its activating factor(s) were analyzed to ascertain similarities with *Drosophila* regulatory elements. Full promoter activity was established to reside within the region from −466 to +347 base pairs with respect to the transcription initiation site. Within this region, we found a sequence similar to the DNA replication-related element (DRE), 5′-TATCGATA, which is a promoter-activating sequence common to promoters of the *Drosophila* genes for DNA replication-related factors, including PCNA. A mutation in the DRE-like sequence of the *B. mori* PCNA gene promoter caused reduction of the promoter activity and also binding to the recombinant *Drosophila* DRE-binding factor (DREF). Furthermore, a factor(s) binding to the DRE sequence was detected in extracts of *B. mori* BmN4 cells. Monoclonal antibodies against *Drosophila* DREF inhibited the binding activity of the factor, as shown by gel mobility shift assays, and allowed specific detection of a 100 kDa protein on immuno Western blot analysis. These results suggest that the *B. mori* DREF homolog binds to DRE to regulate transcription of the PCNA gene.

Key words: *Bombyx*, DNA replication, PCNA, promoter region, transcriptional regulation.

Proliferating cell nuclear antigen (PCNA) is an auxiliary protein for DNA polymerase δ (*1, 2*), and is one of the essential factors for leading strand replication of simian virus 40 DNA (*3-5*). It is also essential for cellular DNA synthesis and cell cycle progression (*6, 7*). PCNA can bind to various cell cycle regulators such as cyclins and p21/WAF1/CIP1 (*8*), an inhibitor of the cyclin-dependent protein kinases (*9, 10*). Recent studies have shown that PCNA is involved in excision repair (*11*) and also mismatch repair (*12*). The level of mRNA for PCNA, like those for other replication-related factors such as DNA polymerase α and DNA topoisomerase I, increases prior to DNA synthesis (*13, 14*), and therefore, transcription of these genes is likely to be regulated through a common mechanism.

In budding yeast, the promoter regions of many DNA replication-related genes contain a common nucleotide sequence (5′-ACGCGT) named MCB (*15*) (*Mlu*I cell cycle box), and the specific transcription factor, MBF (MCB-binding factor), is required for their transcription at the G1-S boundary (*16, 17*). In mammalian cells, the transcription factor, E2F, binds to the E2F-binding site (5′-TTTCGCGC), and positively regulates the transcription of genes whose products are required for cell proliferation (*18, 19*), such as DNA polymerase α, dihydrofolate reductase (DHFR), thymidine kinase, c-Myc, c-Myb, cdc2, PCNA, cyclin D, and cyclin E (*20-23*).

We have analyzed transcriptional regulation of *Drosophila* genes for the DNA polymerase α 180 kDa subunit and 73 kDa subunit, PCNA, cyclin A, and D-raf (*24-27*). The promoter regions of these genes contain a common 8 base pair (bp) palindromic sequence (5′-TATCGATA), named the DNA replication-related element (DRE). The requirement of DREs for promoter activity has been confirmed in both cultured cells (*24-27*) and transgenic flies (*28*). We have also found a specific DRE-binding factor (DREF) consisting of an 80 kDa polypeptide homodimer (*24*), and isolated its cDNA (*29*). Since this DRE/DREF transcriptional regulatory system plays a key role in regulation of the proliferation-related genes in *Drosophila*, it is of interest to determine whether this system functions in other organisms. As a first step to generalize our findings in *Drosophila*, we have initiated a search for the DRE/DREF system in *Bombyx mori*.

In the present study, we found that the promoter region of the *B. mori* PCNA gene contains one DRE and one E2F-binding site, and that a specific binding factor for the DRE is detectable in cell extracts. A mutation in the DRE sequence resulted in a reduction of the promoter activity.

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Abbreviations: bp, base pair(s); GST, glutathione *S*-transferase; PCNA, proliferating cell nuclear antigen; PCR, polymerase chain reaction; DRE, DNA replication-related element; DREF, DRE-binding factor.

The obtained results thus suggest that the DRE/DREF transcriptional regulation system also functions in *B. mori*.

MATERIALS AND METHODS

Cell Culture—BmN4 cells derived from *B. mori* were grown at 27°C in TC-100 medium supplemented with 10% fetal calf serum (*30*).

Oligonucleotides—To obtain a fragment containing the promoter of the PCNA gene, the following polymerase chain reaction (PCR) primers were chemically synthesized: BmPP-1,5′-CCCTCTAGATGTATCACTTCAATAATTTT (containing an *Xba*I site) (see Fig. 4); and BmPP-2,5′-CCCTCTAGAGTTTATCTGAAAGTTGATGT (containing an *Xba*I site) (see Fig. 4). Two oligonucleotides were used as primers for base-substitution mutation at the E2F site by means of PCR-aided mutagenesis: BmE2FmutC, 5′-GAAGAATTGGCTCGACACTTGTCCATTTG; and BmE2FmutN, 5′-AATGGACAAGTCTCGAGCCAATTCTTCCCG.

To determine the transcription initiation site, the following oligonucleotide was used in primer extension experiments (see Figs. 1 and 4): P.E. primer, 5′-ATAATTTCGCTAACGAGGCAGCGCGACCCCTAACTT.

The sequences of double-stranded 30 bp oligonucleotides containing the DRE sequence or its base-substituted derivatives in the PCNA gene promoter were defined as follows:

BmDRE-P, 5′-
GATCCATGCCAGTATCGATTGTCTTTATA-3′
3′-GGTACGGTCATAGCTAACAGAAATATCTAG-5′
BmDRE-PM, 5′-
GATCCATGCCAGTAg CGAg g GTCTTTATA-3′
3′-GGTACGGTCATc GCTc c CAGAAATATCTAG-5′
DcDRE-P, 5′-
GATCCCTGCCTGCTATCGATAGATTCAGGA-3′
3′-GGACGGACGATAGCTATCTAAGTCCTCTAG-3′
DcDRE-PM, 5′-
GATCCCTGCCTGCTt a CGATAGATTCAGGA-3′
3′-GGACGGACGAa t GCTATCTAAGTCCTCTAG-3′

Nucleotides different from in the wild type sequence are shown by lowercase letters, and Bm and Dc indicate *B. mori* and *Drosophila meranogaster*, respectively. The DRE sequence and its derivatives are underlined.

Isolation of B. mori cDNA and the Gene for PCNA—The λgt11 cDNA library, constructed from mRNA of 4-day-old *B. mori* embryos, was a gift from Dr. Kokubo (*31*). cDNA clones for PCNA were isolated by screening the library with a *Drosophila* PCNA cDNA (pDcPCNA01) as a probe (*32*) under high-stringency hybridization conditions (10% dextran sulfate, 1% SDS, and 1 M NaCl at 65°C). The genomic library, which was constructed by *Sau*3AI partial digestion of *B. mori* DNA and ligation into the *Bam*HI site of λEMBL3, was provided by Dr. Adachi (*33*). Genomic clones were isolated by screening the library with the *B. mori* PCNA cDNA probe under high-stringency hibridization conditions.

Southern and Northern Blot Analysis—DNA was extracted from silkmoth larvae as described previously (*34*), digested with restriction enzymes, and then separated by agarose gel electrophoresis and blotted onto a GeneScreen Plus membrane (New England Nuclear) using a VacuGene blotting apparatus (LKB). The membrane was sequentially hybridized with the ^{32}P-labeled probe which contained the PCNA cDNA of *Drosophila* or *B. mori* under high-stringency hybridization conditions.

Total cellular RNA was isolated from BmN4 cells by the acid-guanidinium thiocyanate-phenol-chloroform extraction method (*35*), and then blotted onto a GeneScreen Plus membrane (New England Nuclear). The membrane was then hybridized with a ^{32}P-labeled *B. mori* PCNA cDNA probe. The hybridization and washing conditions were as described elsewhere (*36*).

Plasmid Construction—All nucleotide positions of the PCNA gene referred to below are expressed with respect to the transcription initiation site, which was determined in the present study. To construct a plasmid containing PCNA cDNA, λcBmPCNA1A was digested with *Eco*RI, and then the cDNA fragment was inserted into the *Eco*RI site of pBluescript II to create plasmids pBmPCNA1Aup and pBmPCNA1Adown.

To construct a plasmid containing the PCNA gene, λgBmPCNA was digested with *Sal*I and *Eco*RI, and then the obtained fragment containing the gene sequence was inserted between the *Sal*I and *Eco*RI sites of pBluescript II to create plasmid pBmgPCNA.

To isolate the promoter region of the PCNA gene, PCR was performed using plasmid pBmgPCNA as a template, and the combination of primers T-7 and BmPP-2. The PCR product was digested with *Xba*I and *Hin*dIII, and then inserted between the *Xba*I and *Hin*dIII sites of plasmid pBluescript II. This plasmid was digested with *Kpn*I and *Sac*I, and then the obtained insert was placed between the *Kpn*I and *Sac*I sites of plasmid pGV-B to create plasmid pBmPLuc, which contains the gene region from −466 to +347 and firefly luciferase cDNA.

To construct plasmid pBmPLucCla(−), containing a mutation in the DRE sequence, pBmPLuc was digested at the center of the DRE sequence with *Cla*I and then blunt-ended using T4 DNA polymerase, followed by self-ligation with T4 DNA ligase. With this treatment, two base pairs, GC, were inserted at the center of the DRE sequence. To construct plasmid pBmPLucE2F(−), containing a mutation in the E2F site, a first PCR was performed using pBmgPCNA as a template with the following combinations of primers: T-7 and BmE2FmutC, and BmPP-2 and BmE2FmutN. The resultant products were named E2Fmutup and E2Fmutdown, respectively. A second PCR was performed using E2Fmutup and E2Fmutdown as templates with primers T-7 and BmPP-2. Under these conditions, only the DNA fragment produced on hybridization of two templates was amplified, and the hybridized region contained the mutated E2F site. The product was digested with *Bam*HI and *Hin*dIII, and then used to replace the region carrying the wild type sequence between the *Bam*HI and *Hin*dIII sites of pBmPLuc to create plasmid pBmPLucE2F(−).

DNA Sequencing—In order to determine the nucleotide sequences of the cDNA and genomic clones, DNA fragments were cloned into pBluescript II. A series of unidirectional deletion derivatives was constructed using a Erase-a-Base system (Promega). The nucleotide sequence of each clone was determined by the dideoxy-sequencing method using a Sequenase kit (United States Biochemical) with the T-3 or T-7 primer. When necessary, chemically synthe-

sized oligonucleotides with sequences derived from the gene or cDNA were used as sequencing primers.

Primer Extension—Total cellular RNA was isolated from BmN4 cells by the acid-guanidinium thiocyanate-phenol-chloroform extraction method (*35*). A 34 mer primer (P.E. primer) complementary to the downstream 5′ end of cDNA (Fig. 1) was chemically synthesized. Primer extension analysis was performed as described (*25*).

Gel Mobility Shift Analysis—The expression plasmid for the GST-DREF fusion protein was constructed and expressed in *Escherichia coli* as described previously (*29*). The *E. coli* cells were collected and suspended in a solution comprising 25 mM Hepes (pH 7.9), 1 mM EDTA, 0.02% 2-mercaptoethanol, 10% glycerol, 0.1% Tween 80, and 0.2 M KCl, sonicated, and then centrifuged. The supernatant was collected and used for the gel mobility shift analysis. Whole cell extracts of BmN4 cells were prepared as described (*37*), with minor modifications. A ^{32}P end-labeled oligonucleotide containing the DRE sequence was used as a probe. Gel mobility shift analysis was performed as described (*24*), with minor modifications. The ^{32}P-labeled probe (10,000 cpm) was added to 14 μl of a reaction mixture comprising 15 mM Hepes (pH 7.6), 60 mM KCl, 0.1 mM EDTA, 1 mM dithiothreitol, 12% glycerol, and 0.5 μg of poly(dI-dC) on ice for 5 min. When necessary, unlabeled oligonucleotides were added as competitors at this step. Then, an extract of *E. coli* or BmN4 cells was introduced and the mixture was incubated for 45 min on ice. DNA-protein complexes were electrophoretically resolved on 3% polyacrylamide gels in 50 mM Tris/borate (pH 8.3) containing 1 mM EDTA and 2.5% glycerol at 25°C. The gels were dried and autoradiographed.

DNA Transfection and Luciferase Assay—BmN4 cells (2×10^6) were grown in a 25-cm^2 flask for 24 h and then transfected with 10 μg of reporter plasmid DNA by lipofection as described previously (*38*). Cells were harvested 48 h after DNA transfection and extracts were prepared for determination of luciferase activity (*39*). The assay was carried out with a PicaGene assay kit (Toyo) as described previously (*40*). All assays were performed within the linear activity range with regard to incubation time and protein amount. Luciferase activitiy was normalized as to the contained protein.

Immuno Western Blot Analysis—A monoclonal antibody against *Drosophila* DREF was produced as described previously (*29*), and purified from the culture medium of hybridoma cells using E-Z-SEP (Pharmacia). GST-DREF fusion protein was expressed in *E. coli* as described above. GST-DREF or GST was allowed to bind to glutathione-Sepharose beads for 1 h at room temperature, which were then washed. The monoclonal antibody was incubated with glutathione-Sepharose, GST-DREF bound glutathione-Sepharose, or GST bound glutathione-Sepharose for 1 h at room temperature. The supernatants were then used as the sources of primary antibodies for immuno Western blot analysis. Whole cell extracts of *Drosophila* Kc cells or *B. mori* BmN4 cells were subjected to electrophoresis (50 μg of cellular protein per lane) on 8% polyacrylamide gels containing 0.1% SDS, and then transferred to PVDF membranes (Bio-Rad). The membranes were blocked in TBST (150 mM NaCl, 10 mM Tris-HCl, pH 8.0, and 0.05% Tween 20) containing 10% skim milk for 1 h, and then incubated with a 1:2,000 diluted monoclonal antibody in TBS containing 20% FCS for 1 h, both at room temperature. After washing with TBST and exposure to alkaline phosphatase-conjugated rabbit anti-mouse IgG, bands were visualized with an NTB (nitro blue tetrazolium)/BCIP (5-bromo-4-chloro-3-indolyl phosphate) color development system (Promega), according to the manufacturer's in-

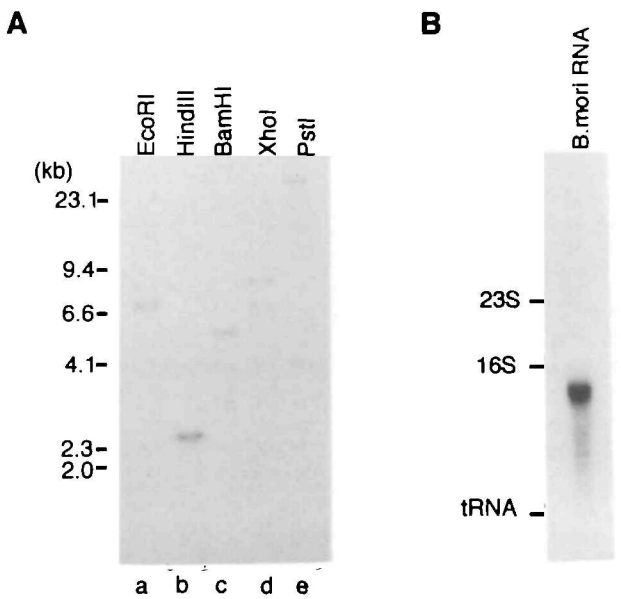

Fig. 2. **Southern and Northern blot analyses.** (A) Southern blot analysis of DNA from *B. mori*. Genomic DNA from *B. mori* larvae (lanes a to e) was digested with *Eco*RI, *Hind*III, *Bam*HI, *Xho*I, or *Pst*I. The 0.3 kb fragment was used as a probe (see Fig. 1). (B) Northern blot analysis of RNA from *B. mori* BmN4 cells. The 0.3 kb fragment was used as a probe.

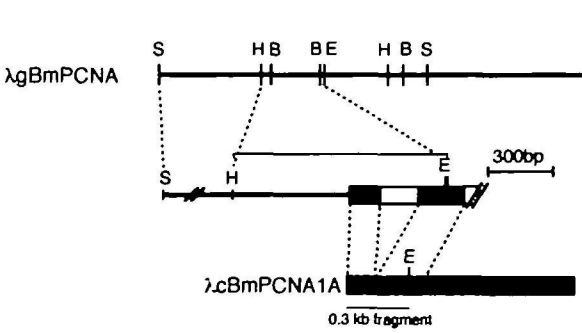

Fig. 1. **Structures of the gene and cDNA for *B. mori* PCNA.** A restriction map of the genomic clone, λgBmPCNA, is presented at the top. The structure of the 5′ region of the PCNA gene is shown in the middle. The nucleotide sequence indicated by the bracket was determined and is shown in Fig. 4. The structure of the full length cDNA clone λcBmPCNA1A is shown at the bottom. The 0.3 kb fragment used as a probe for Southern and Northern analyses and screening for genomic clones is indicated. The coding regions and introns are indicated by solid and open boxes, respectively. The 5′ and 3′ untranslated regions are indicated by shaded boxes. Restriction sites: B, *Bgl*II; E, *Eco*RI; H, *Hind*III; S, *Sal*I.

structions. The molecular masses of the protein bands were estimated by comparing their mobilities with those of prestained marker proteins (Bio-Rad) which migrated differently from their true molecular masses. The apparent molecular masses of the prestained marker proteins were as follows; β-galactosidase (112 kDa), BSA (84 kDa), ovalbumin (53 kDa), and carbonic anhydrase (36 kDa).

RESULTS

Cloning of cDNA and the Gene for B. mori PCNA—An 0.7 kb DNA fragment isolated from the insert of plasmid pDcPCNA01, containing cDNA for *Drosophila* PCNA, was used as a probe to screen the *B. mori* cDNA library. One positive clone, designated as λcBmPCNA1A, contained a 1.1 kb insert, and another, designated as λcBmPCNA9A, contained an 0.75 kb insert. The 0.3 kb fragment containing the 5′-untranslated region and a part of the translated region of the *B. mori* PCNA gene (Fig. 1) was used as a probe to screen the *B. mori* genomic library. A genomic clone, λgBmPCNA containing an insert of about 16 kb, was obtained. The restriction map and relationships of these clones are shown in Fig. 1. The 0.3 kb fragment was used as a probe for Southern blot hybridization analysis of *B. mori* genomic DNA. A single band was detected on the blot of DNA digested with *Eco*RI, *Bam*HI, *Hind*III, or *Pst*I (Fig. 2A, lanes a, b, c, and e). When DNA was digested with *Xho*I, double bands were detected (Fig. 2A, lane d). Bands of similar sizes were also observed when *Drosophila* PCNA cDNA was used as a probe (data not shown). The results

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                                                                    CGA
   ACGTCGAACGGTTACGAACCTACTCCTTTGTCATTTATTTTACACAGTTGTTTTAATTTGTTTGTAAGTTA
   GGGGTCGCTGCCTGTTAGCGTTATTATTAATTTTCAACAAAATTATTGAAGTGATACATTAAAATATAAAC

   1 ATG TTT GAG GCA CGT TTA CTC CGA AGC TCT ATC TTA AAG AAG GTC TTG GAG GCT
   1 Met Phe Glu Ala Arg Leu Leu Arg Ser Ser Ile Leu Lys Lys Val Leu Glu Ala

  55 ATT AAA GAT CTG CTG ACA CAG GCC ACT TTC GAT TGC GAT GAC AAT GGA ATT CAG
  19 Ile Lys Asp Leu Leu Thr Gln Ala Thr Phe Asp Cys Asp Asp Asn Gly Ile Gln

 109 TTA CAG GCT ATG GAC AAC TCT CAT GTG TCT CTC GTG TCA CTC ACT CTC CGA GCT
  37 Leu Gln Ala Met Asp Asn Ser His Val Ser Leu Val Ser Leu Thr Leu Arg Ala

 163 GAT GGC TTT GAC AAG TAC CGC TGC GAT AGG AAC ATC TCA ATG GGC ATG AAT CTA
  55 Asp Gly Phe Asp Lys Tyr Arg Cys Asp Arg Asn Ile Ser Met Gly Met Asn Leu

 217 GGC AGC ATG TCA AAG ATT CTC ATT TTT GCT GGA GAT AAG GAT ACA GCC ACA ATA
  73 Gly Ser Met Ser Lys Ile Leu Ile Phe Ala Gly Asp Lys Asp Thr Ala Thr Ile

 270 AAA GCA CAG GAT AAT GCT GAC AAT GTC ACA TTT GTT TTT GAG AGC CCA AAT CAA
  91 Lys Ala Gln Asp Asn Ala Asp Asn Val Thr Phe Val Phe Glu Ser Pro Asn Gln

 325 GAG AAA GTC TCT GAT TAC GAG ATG AAG CTT ATG AAT TTG GAT CTT GAA CAT TTA
 109 Glu Lys Val Ser Asp Tyr Glu Met Lys Leu Met Asn Leu Asp Leu Glu His Leu

 379 GGT ATT CCA GAG ACT GAA TAC AGC TGC ACT ATC CGC ATG CCA AGT TCT GAA TTT
 127 Gly Ile Pro Glu Thr Glu Tyr Ser Cys Thr Ile Arg Met Pro Ser Ser Glu Phe

 433 GCT AGA ATC TGC CGG GAT CTC TCA CAG TTT GGA GAA TCA ATG GTG ATT TCA TGC
 145 Ala Arg Ile Cys Arg Asp Leu Ser Gln Phe Gly Glu Ser Met Val Ile Ser Cys

 487 ACA AAA GAA GGA GTA AAG TTC TCG GCA ACA GGC GAC ATC GGC TCA GCG AAC GTC
 163 Thr Lys Glu Gly Val Lys Phe Ser Ala Thr Gly Asp Ile Gly Ser Ala Asn Val

 541 AAG CTG GCC CAG ACC GCT TCT ATT GAC AAA GAG GAA GAG GCA GTC GTC ATT GAA
 181 Lys Leu Ala Gln Thr Ala Ser Ile Asp Lys Glu Glu Glu Ala Val Val Ile Glu

 595 ATG GAA GAG CCC GTC ACT CTG ACG TTT GCC TGC CAG TAC CTC AAC TAT TTC ACT
 199 Met Glu Glu Pro Val Thr Leu Thr Phe Ala Cys Gln Tyr Leu Asn Tyr Phe Thr

 649 AAA GCT ACT TCT CTC AGC CCT CAG GTG CAG CTG TCG ATG TCA GCA GAC GTT CCT
 217 Lys Ala Thr Ser Leu Ser Pro Gln Val Gln Leu Ser Met Ser Ala Asp Val Pro

 703 TTG GTG GTG GAG TAC CGC ATC CCG GAC ATT GGC CAC ATC CGC TAC TAC CTG GCG
 235 Leu Val Val Glu Tyr Arg Ile Pro Asp Ile Gly His Ile Arg Tyr Tyr Leu Ala

 757 CCT AAG ATC GAG GAA GAA GAC AGC TGA ACTGAGACTGTGCCGTTTATTTCAATCCGTTCAAT
 253 Pro Lys Ile Glu Glu Glu Asp Ser ***
   TTAAGGATTCATTTTTTATTATCATGTAATATTCCTGCAATTTCTGTACACAGTGTGTATTCAGACAGAAT
   TGTGATAGTTATCTGATTAAGGCAATAAAATATTTTTCGTAATTAAAAAAAA
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Fig. 3. **Nucleotide sequence of *B. mori* PCNA cDNA and the deduced amino acid sequence.** The nucleotide and deduced amino acid sequences of *B. mori* PCNA cDNA. The open reading frame encodes a protein of 260 amino acid residues (shown below the nucleotide sequence).

indicate that the PCNA gene exists as a single copy per haploid genome.

The complete nucleotide sequences of the 1.1 kb cDNA fragment, pBmPCNA1A, and the 0.75 kb cDNA fragment, pBmPCNA9A, were determined (Fig. 3). The open reading frame was found to encode a polypeptide of 260 amino acids. The deduced amino acid sequence was 78.8% identical to that of *Drosophila* PCNA. In addition, the amino acid sequence of *B. mori* PCNA was 70.8, 70, 69.6, 60.4, and 35.1% identical to those of *Xenopus*, mouse, human, rice, and *Saccharomyces serevisiae* PCNAs, respectively.

The nucleotide sequence of the 3.3 kb *Eco*RI-*Sal*I fragment (Fig. 1), λgBmPCNA, was determined (Figs. 1 and 4). Comparison of the nucleotide sequences of the genomic DNA and the cDNA revealed the presence of a 185 bp intron in the non-coding region (Fig. 1). The nucleotide sequences at the splicing junctions were confirmed to agree with the consensus sequence for splicing (Fig. 4).

Determination of the Transcription Initiation Site and the Promoter Region of the PCNA Gene—To determine the transcription initiation site, a primer extension experiment was performed using a primer complementary to a region in the first exon. A single transcription initiation site was identified (Fig. 5) and defined as nucleotide position +1 (Fig. 4). The 5′ end of the isolated cDNA clone was at +12.

The 0.3 kb cDNA fragment was used as a probe for Northern blot hybridization analysis of RNA extracted from BmN4 cells. A single band was detected (Fig. 2B), indicating that transcription of the *B. mori* PCNA gene starts from one site.

To determine the promoter region, we constructed plasmids for the luciferase assay, and transfected them into BmN4 cells. Two deletion constructs from the 5′ end were made using the available restriction enzyme sites. A deletion from about position −2700 to position −466 did not result in any significant change in luciferase activity (data not shown), indicating that the limit of the 5′ end of the promoter is downstream of position −466. To determine the 3′ end of the promoter, two PCR primers were synthesized. One PCR product generated using primer BmPP-1 lacked the first intron, while the other generated using primer BmPP-2 contained the first intron. An about 70% reduction of the luciferase activity was observed with a deletion from +347 to +146 (Fig. 6). Because this deleted region contains an intron, some unknown element may play a role in activating the promoter of this gene. From these results, it is concluded that the promoter region resides within the region from −466 to +347.

At nucleotide positions −122 to −115, we found a sequence (5′-TATCGATT) similar to DRE (5′-TATCGATA) (Fig. 4). Since this sequence functions in activation of the promoter and in binding to the DREF, as described below, we named this site, BmDRE-P. In addition, we found a sequence identical to the E2F-binding site (5′-TTTCGCGC) at nucleotide positions −12 to −5.

Effects of Mutations at the BmDRE-P and E2F-Binding Sites on the Promoter of the B. mori PCNA Gene—Luciferase expression plasmids with mutations in the DRE or

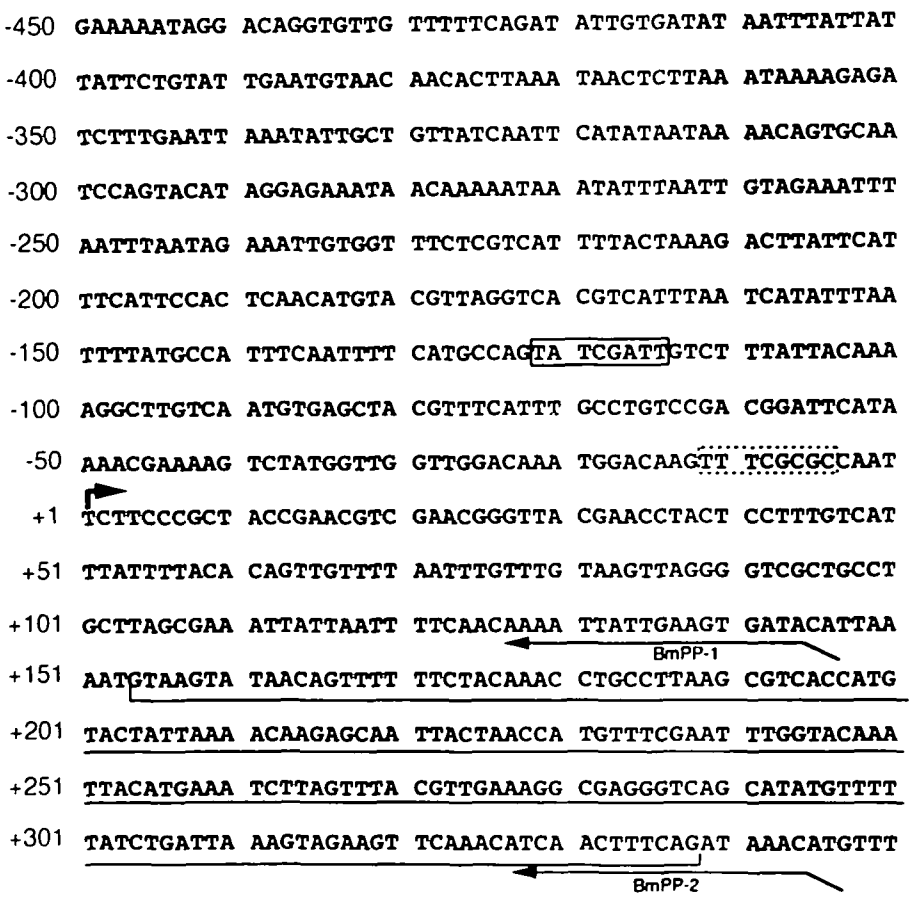

Fig. 4. **Nucleotide sequence of the 5′ upstream region of the *B. mori* PCNA gene.** The transcription initiation site is indicated by an arrow and is numbered +1. The DRE sequence and E2F-binding site are boxed by solid and broken lines, respectively. The first intron is indicated by the bracket. The positions of the primers (BmPP-1 and BmPP-2) used for the PCR are also indicated.

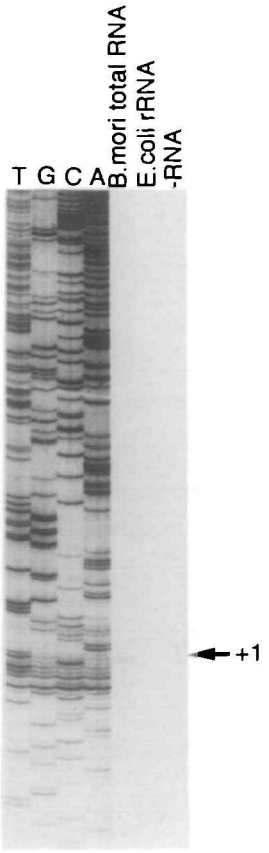

Fig. 5. **Mapping of the transcription initiation site by primer extension analysis.** The ^{32}P-labeled 34 mer primer (P.E. primer) complementary to the PCNA mRNA was hybridized with total RNA isolated from *B. mori* BmN4 cells or *E. coli* rRNA as indicated at the top. The primer was extended using reverse transcriptase as described under "MATERIALS AND METHODS." To align the extended products with the genomic DNA sequence, a parallel dideoxy sequencing reaction was carried out using the same 34 mer primer (lanes A, C, G, and T). The transcription initiation site was defined as +1.

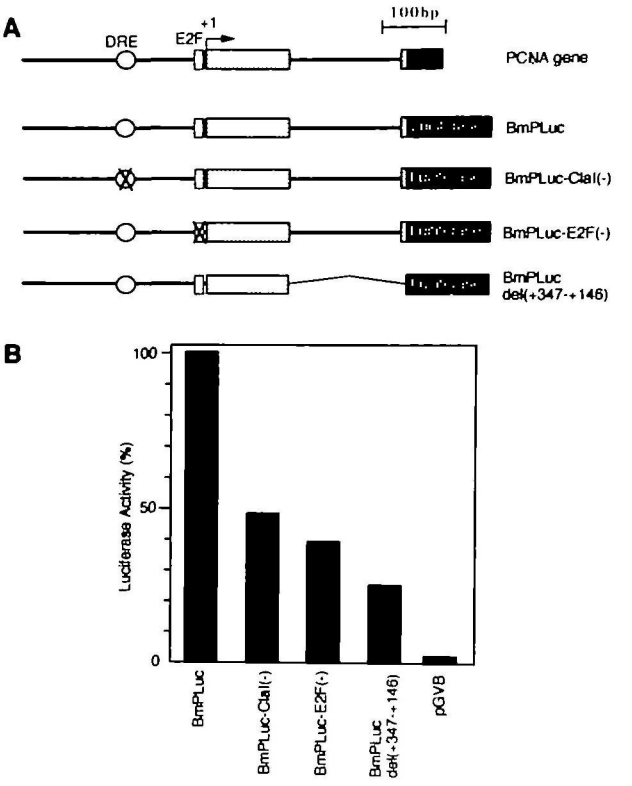

Fig. 6. **Effects of mutations on promoter activity of the PCNA gene.** (A) Features of the reporter luciferase plasmids are schematically illustrated. The DRE sequence is indicated by the open circle and the E2F-binding site by the open box. The shaded and solid boxes indicate the 5′-untranslated and coding sequences, respectively. The mutated sites are indicated by crosses. An internal deletion is also indicated. (B) These reporter luciferase plasmids (10 μg each) were transfected into BmN4 cells, and then the luciferase activities were determined. The activities are presented relative to that of the wild-type BmPLuc construct.

E2F-binding site were constructed (Fig. 6A), and transient expression assays on BmN4 cells were performed. The mutation in the DRE resulted in a 50% reduction of the luciferase activity, and that in the E2F-binding site in a 60% reduction (Fig. 6B), indicating that both are required for high promoter activity.

Detection of the B. mori DREF—A gel mobility shift assay was performed using a recombinant *Drosophila* DRE-binding factor (DREF) (*41*) to examine whether the DRE sequence of the *B. mori* PCNA gene is recognized by the *Drosophila* DREF. When the GST-DREF fusion protein was mixed with the ^{32}P-labeled BmDRE-P oligonucleotide, a shifted band was detected (Fig. 7A, lane c). The unlabeled oligonucleotide containing BmDRE-P as a competitor decreased the shifted band progressively (Fig. 7A, lanes d to g). In contrast, the shifted band was decreased to only a limited extent on the addition of a competitor with a mutation in the DRE sequence (Fig. 7A, lanes h to k). The results indicate that the DRE is specifically recognized by *Drosophila* DREF, and also suggest that the DRE/DREF transcriptional regulatory system functions to control *B. mori* PCNA gene expression.

To examine whether a DREF homolog might exist in *B. mori*, a gel mobility shift assay involving extracts of BmN4 cells was performed, with the ^{32}P-labeled BmDRE-P oligonucleotide as a probe. A shifted band was observed (Fig. 7B, lane b) which was competitively abolished by adding an excess amount of BmDRE-P or DcDRE-P (Fig. 7B, lanes c, d, g, and h). In contrast, BmDRE-PM or DcDRE-PM containing base-substitution mutations in DRE showed no competition (Fig. 7B, lanes e, f, i, and j). The results indicate that a factor(s) specifically binding to DRE exists in BmN4 cells.

When monoclonal antibodies against *Drosophila* DREF were added to the binding reactions, the specifically shifted band also disappeared (Fig. 7C, lanes c to h). However, a control monoclonal antibody against chick DNA polymerase α did not have any effect (Fig. 7C, lanes i to k). The DRE was, thus, specifically recognized by monoclonal antibodies against *Drosophila* DREF, further pointing to the existence of a DREF homolog in BmN4 cells.

To obtain further insight into this DREF homolog, immuno Western blot analysis was performed. When monoclonal antibodies against *Drosophila* DREF pretreated with either glutathione-Sepharose or glutathione-Sepharose bearing GST were used, 100 and 86 kDa polypeptides were

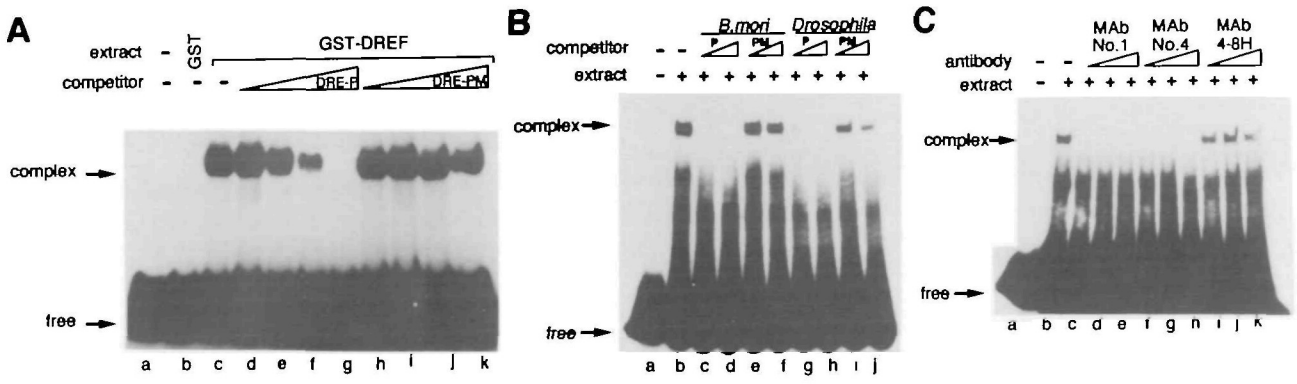

Fig. 7. **Gel mobility shift assay.** (A) The assay was carried out using the GST-DREF recombinant fusion protein and a ^{32}P-labeled DRE oligonucleotide derived from the *B. mori* PCNA gene as a probe. The oligonucleotide was incubated with GST-DREF (lanes c to k), GST (lane b), or with neither (lane a). Excess amounts of the unlabeled wild-type DRE oligonucleotide (lanes d to g) or mutant-type DRE oligonucleotide carrying a 3-bp mutation in the DRE sequence (lanes h to k) were added to the reaction mixtures as competitors. The amounts of competitors were as follows: 1 ng, lanes d and h; 5 ng, lanes e and i; 10 ng, lanes f and j; 50 ng, lanes g and k. (B) The ^{32}P-labeled *B. mori* DRE oligonucleotide was used as a probe and incubated with (lanes b to j) or without (lane a) a whole BmN4 cell extract. Excess amounts of the unlabeled *B. mori* wild-type DRE oligonucleotide (lanes c and d), the *B. mori* mutant-type DRE oligonucleotide (lanes e and f), the *Drosophila* wild-type DRE oligonucleotide (lanes g and h), or the *Drosophila* mutant-type DRE oligonucleotide (lanes i and j) were added to the reaction mixtures as competitors. The amounts of competitors were as follows: 4 ng, lanes c, e, g, and i; 16 ng, lanes d, f, h, and j. (C) The ^{32}P-labeled DRE oligonucleotide was used as a probe and incubated with (lanes b to k) or without (lane a) the whole BmN4 cell extract, which had been treated with anti-DREF monoclonal antibodies (No. 1) (lanes c to e), anti-DREF monoclonal antibodies (No. 4) (lanes f to h), anti-chick polymerase α monoclonal antibodies (4-8H) (lanes i to k), or no antibodies (lane a and b).

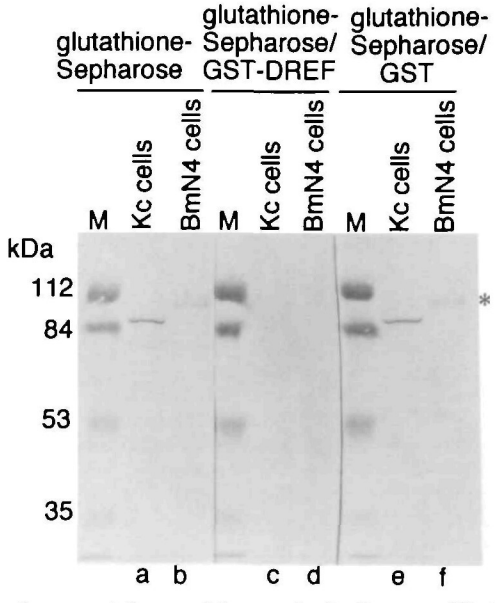

Fig. 8. **Immuno Western blot analysis.** Immuno Western blot analysis was performed as described under "MATERIALS AND METHODS." Whole cell extracts of *Drosophila* Kc cells (lanes a, c, and e) and *B. mori* BmN4 cells (lanes b, d, and f) were subjected to electrophoresis (50 μg of cellular protein per lane) in an 8% polyacrylamide gel containing 0.1% SDS, followed by protein transfer to a PVDF membrane (Bio-Rad). Anti-DREF antibodies (No. 1) were used as the primary antibodies after pretreatment with glutathione-Sepharose (lanes a and b), glutathione-Sepharose bearing GST-DREF (lanes c and d), or glutatione-Sepharose bearing GST (lanes e and f). M, prestained marker proteins: β-galactosidase (112 kDa), BSA (84 kDa), ovalbumin (53 kDa), carbonic anhydrase (36 kDa).

detected in extracts of *B. mori* BmN4 cells and *Drosophila* Kc cells, respectively (Fig. 8, lanes a, b, e, and f). These bands were not detected after pretreatment of the primary antibodies with glutathione-Sepharose bearing the GST-DREF fusion protein (Fig. 8, lanes c and d).

DISCUSSION

In our previous studies, promoters of the *Drosophila* genes encoding PCNA, the 180 and 73 kDa subunits of DNA polymerase α, and cyclin A were found to be positively regulated by the DRE in both cultured cell (*24-27*) and transgenic fly systems (*28*). The widespread presence of DRE sequences in the *Drosophila* DNA database suggests that this is a common regulatory element responsible for the coordinated expression of many proliferation-related genes (*42*). Furthermore, overexpression of the *zerknüllt* gene product (Zen) was found to result in repression of the promoter action for the PCNA and DNA polymerase α 180 kDa subunit genes by impeding the DREF binding (*43*). This could be overcome by overexpression of the DREF protein in Kc cells (*29*). These lines of evidence indicate that DREF is an important transcription regulatory factor involved in proliferation- and differentiation-related control. However, up to the present, this system has not been reported to exist in other organisms.

The silk gland cells of the mulberry silkworm, *B. mori*, contain large quantities of DNA as a result of endomitosis, and DNA polymerases isolated from this source have been extensively characterized (*44, 45*). However, the cloning of genes and cDNAs encoding DNA replication enzymes such as DNA polymerases and PCNA has not been reported to our knowledge. In the present sutudy, we isolated the *B. mori* PCNA gene and found a sequence similar to that of the *Drosophila* DRE in its promoter region. The introduction of a 2 bp insertional mutation into the DRE resulted in a reduction of the promoter activity. Furthermore, a putative *B. mori* DREF homolog which specifically binds to the

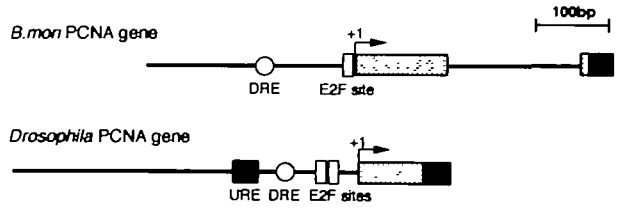

Fig. 9. **Schematic features of the promoter regions of the *B. mori* and *Drosophila* PCNA genes.** The major transcription initiation sites are indicated by arrows. The shaded and solid boxes indicate the 5′-untranslated and coding sequences, respectively. DREs are indicated by open circles and E2F-binding sites by open boxes. An upstream regulatory element (URE) which is required to activate the *Drosophila* PCNA gene promoter during larval stages (*50*) is indicated by a hatched box.

DRE and is recognized by anti-*Drosophila* DREF monoclonal antibodies was detected in *B. mori* cells. These results indicate that transcription of the *B. mori* PCNA gene is also regulated by the DRE/DREF system. Although promoter sequence information is not available for other *B. mori* genes involved in DNA replication, the possibility that the transcription of many *B. mori* DNA replication-related genes may be similarly controlled clearly warrants further investigation.

The E2F regulation system is evolutionarily conserved among various eukaryotes, and a *Drosophila* homolog of the mammalian E2F1 has been isolated (*46-48*). E2F-binding sites exist in the promoter regions of the DNA polymerase α 180 kDa subunit (*46*), DNA polymerase α 73 kDa subunit (*25*), and PCNA (*49*) genes, and appear to function in both cultured *Drosophila* cells and living flies (*25, 49*). The finding in the present study of a sequence carrying the E2F-binding site consensus in the promoter region of the *B. mori* PCNA gene is therefore also of interest. A three-base substitution mutation in the E2F-binding site again reduced the promoter activity, suggesting the *B. mori* PCNA gene is regulated by E2F, like the *Drosophila* DNA replication-related genes, although further analysis is necessary to clarify this point.

The organization of the transcriptional regulatory elements of *B. mori* and *Drosophila* PCNA genes is summarized in Fig. 9. The similarities regarding the DRE and E2F-binding sites most likely point to a common regulatory mechanism for the expression of both the *B. mori* and *Drosophila* PCNA genes.

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