PCF1 and PCF2 Specifically Bind to cis Elements in the Rice Proliferating Cell Nuclear Antigen Gene

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We have previously defined the promoter elements, sites Ila and Iib, in the rice proliferating cell nuclear antigen (PCNA) gene that are essential for meristematic tissue-specific expression. In this study, we isolated and characterized cDNAs encoding proteins that specifically bind to sites Ila and Iib. The two DNA binding proteins, designated PCF1 and PCF2, share >70% homology in common conserved sequences at the N-terminal regions. The conserved regions are responsible for DNA binding and homodimer and heterodimer formation, and they contain a putative basic helix-loop-helix (bHLH) motif. The structure and DNA binding specificity of the bHLH motif are distinguishable from those of other known bHLH proteins that bind to the E-box. The motif is >70% homologous to several expressed sequence tags from Arabidopsis and rice, suggesting that PCF1 and PCF2 are members of a novel family of proteins that are conserved in monocotyledons and dicotyledons. A supershift assay using an anti-PCF2 antibody showed the involvement of PCF2 in site Ila (site Iib) binding activities in rice nuclear extracts, particularly in meristematic tissues. PCF1 and PCF2 were also more likely to form heterodimers than homodimers. Our results suggest that PCF1 and PCF2 are involved in meristematic tissue-specific expression of the rice PCNA gene through binding to sites Ila and Iib and formation of homodimers or heterodimers.

INTRODUCTION

Transitions from the G1-to-S phase of the cell cycle in dividing cells and the G0-to-S phase in quiescent cells are important steps in the process of cell proliferation. Most of our knowledge about the control of the G1 (G0)-to-S phase transition is based on studies with yeast and mammalian cells. These studies have suggested that G1 cyclins and their associated kinases are key regulators of the G1 (G0)-to-S transition. Recent studies have identified the genes for G1 cyclins in plants (Dahl et al., 1995; Soni et al., 1995), suggesting that regulation by the G1 cyclins and their associated kinases seems to be commonly involved in the control of the G1 (G0)-to-S transition in eukaryotes. In addition, in eukaryotes, when quiescent cells are stimulated to enter into S phase, the expression of several genes involved in DNA synthesis, including the proliferating cell nuclear antigen (PCNA) gene, is induced at the G1 (G0)-to-S phase boundary—a final step of the G1 (G0)-to-S phase transition.

In mammalian cells, the transcription of these genes is regulated to a large extent by transcription factors E2F (Ogris et al., 1993; DeGregori et al., 1995; Tommasi and Pfeifer, 1995) and Sp1 (Karseder et al., 1996; Lin et al., 1996; Slansky and Farnham, 1996). On the other hand, in budding yeast, the MiU cell-cycle-box binding protein Dsc1 has been reported to regulate genes expressed at the G1 (G0)-to-S phase boundary (Lowndes et al., 1992a, 1992b). In plants, cell cycle progression, including the G1 (G0)-to-S phase transition, remains to be investigated. To date, the transcriptional factors regulating DNA synthesis-related genes have not been identified. In animals, the structures of E2F and Sp1 and their transcriptional regulatory mechanisms are quite different from those of Dsc1 in yeast. This suggests that the regulatory mechanisms in the G1 (G0)-to-S phase transition in plants may be different also.

The PCNA, an auxiliary protein of DNA polymerase δ (Bravo et al., 1987; Prelich et al., 1987b), participates in a variety of processes, such as DNA replication (Prelich et al., 1987a), DNA repair synthesis (Toschi and Bravo, 1988; Shivji et al., 1992), and cell cycle control through interactions with the CDK (cyclin-dependent kinase)–cyclin–CDI (cyclin-dependent kinase inhibitor) complex (Waga et al., 1994). The PCNA gene is induced at the G1 (G0)-to-S phase boundary (Almendral et al., 1987; Matsumoto et al., 1987; Jaskulski et al., 1988) and is well conserved in eukaryotes (Suzuka et al., 1991). In addition, the rice PCNA protein produced in bacteria has been found to stimulate DNA synthesis catalyzed by DNA polymerase δ from human cells (Matsumoto et al., 1994), suggesting the functional conservation of PCNA among eukaryotes.

Among the genes that are expressed in the G1 (G0)-to-S phase boundary in plants, the rice PCNA gene is one of the most characterized genes in studies examining transcriptional control. The expression of the rice PCNA gene is restricted exclusively to meristematic regions and is controlled at the transcriptional phase (Kosugi et al., 1991). The

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PCNA protein is also present in proliferating cells but absent from nondividing cells and terminally differentiated plant tissues (Daidoji et al., 1992). Loss-of-function analysis of the rice PCNA promoter using transgenic plants has demonstrated that two elements (sites Ila and Ilb) in the proximal region are essential for the proliferating cell-specific transcriptional activity (Kosugi et al., 1995). On the other hand, two repeated-site Ila sequences located upstream of the cauliflower mosaic virus 35S minimal promoter confer transcriptional activation in tobacco protoplasts (Kosugi et al., 1995). This suggests that sites Ila and Ilb most probably function as positive cis-acting elements in proliferating cells. In addition, results of electrophoretic mobility shift assays (EMSAs) have also shown that these sites bind to nuclear proteins with identical or similar DNA binding specificity in rice and tobacco (Kosugi et al., 1995). These data suggest that sites Ila and Ilb have a similar function in diversified species. Thus, it is imperative to understand the fine details and role of sites Ila and Ilb and nuclear factors binding to the sites in rice PCNA gene expression.

In this report, we describe the cloning and characterization of proteins PCFl and PCF2 that specifically bind to sites Ila and Ilb in the promoter region of the rice PCNA gene. These proteins have a deduced basic helix-loop-helix (bHLH) motif that is responsible for DNA binding and dimerization. We further demonstrate that PCFl and PCF2 are novel types of bHLH proteins and distinct from other known bHLH transcriptional factors. We also discuss the importance of these proteins in the regulation of the PCNA gene.

RESULTS

Cloning of the PCFl and PCF2 cDNAs

We have previously defined the promoter elements (site Ila and site Ilb) essential for meristematic tissue–specific expression of the rice PCNA gene (Kosugi et al., 1995). In this study, we isolated cDNAs for specific binding proteins for sites Ila and Ilb. Because the initial attempts to isolate cDNAs by in vitro screening were unsuccessful, we used the yeast one-hybrid system for cloning. This system has been developed for the isolation of sequence-specific DNA binding proteins (Li and Herskowitz, 1993; Wang and Reed, 1993). In this system, a hybrid expression library, which produces fusion proteins with a transcriptional activation domain such as Gal4p, is introduced into a yeast strain carrying a reporter gene. This leads to the activation of the reporter gene containing the binding site of interest within its promoter region through the binding of hybrid proteins to the binding site.

We originally constructed reporter genes consisting of an iso-1-cytochrome c CYC1 minimal promoter fused to the HIS3 coding region in which 12 copies of the site Ila or site Ilb element were inserted upstream of the CYC1 promoter. The pGAD424 cDNA library produced from rice suspension-cultured cells was introduced into a yeast strain carrying the reporter gene (site Ila)<sub>12</sub>-CYC1-HIS3. When ~5 × 10<sup>6</sup> Leu<sup>+</sup> yeast clones were plated on SD synthetic leucine- and histidine-free media, ~5 × 10<sup>3</sup> colonies that showed histidine auxotrophy appeared after 4 days. The plasmids in these colonies were rescreened, and two independent HIS<sup>+</sup> clones were isolated. To examine whether the HIS<sup>+</sup> phenotype of these clones was dependent on site Ila present in the reporter gene, we used two other reporter genes, (site Ilb)<sub>12</sub>-CYC1-HIS3 and CYC1-HIS3, containing 12 copies of site Ilb and no repeated sequence in their promoter regions, respectively. Results presented in Table 1 show that the (site Ilb)<sub>12</sub>-CYC1-HIS3 reporter gene was activated in these clones but that the CYC1–HIS3 control reporter gene was not. These results indicate that activation of the reporter gene in these HIS<sup>+</sup> clones was dependent on the sequences of sites Ila and Ilb that were inserted upstream of the HIS3 reporter genes and suggest that cDNAs in these clones encode DNA binding proteins specific for sites Ila and Ilb.

We then examined the specificities of DNA binding to PCFl and PCF2 proteins encoded by these cDNA clones. The cDNA inserts were cloned into pMAL-c2 and introduced into Escherichia coli strains. The recombinant fusion proteins maltose binding protein (MBP)-PCFl and MBP–PCF2, containing regions extending from residues 25 to 183 in the PCFl and from residues 1 to 265 in the PCF2 amino acid sequences, respectively, were purified by using an affinity column, and an EMSA was conducted. MBP–PCFl and MBP–PCF2 bound specifically to sites Ila and Ilb but not to a mutated site Ila sequence (mlla), even when 100 ng of proteins was added (Figure 1A, lanes 6 and 15). In addition, MBP itself had no ability to bind to these DNA probes (data not shown). These observations indicate that PCFl and PCF2 are DNA binding proteins specific for sites Ila and Ilb and that the DNA binding specificity and affinity of PCFl are similar to that of PCF2. Introduction of this mutated sequence in the rice PCNA promoter leads to a loss of the promoter activity (Kosugi et al., 1995).

We also examined the activities of site Ila binding in nuclear extracts from rice suspension-cultured cells using a competition assay. When nuclear extracts were subjected to an EMSA using the Ila probe, a broadly shifted band of a DNA–protein complex was observed (Figure 1B, lane 1). The

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<th>Reporter Gene</th>
<th>pGAD–PCFl</th>
<th>pGAD–PCF2</th>
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<tr>
<td>His&lt;sup&gt;a&lt;/sup&gt;</td>
<td>His&lt;sup&gt;a&lt;/sup&gt;</td>
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<sup>a</sup>HIS<sup>+</sup> or HIS<sup>+</sup> represents a phenotype of transformants on histidine-free medium.

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<th>Table 1. trans-Acting Effect of PCFl and PCF2 Clones on HIS3 Reporter Genes in Yeast</th>
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<tr>
<td>cDNA Clone in pGAD424</td>
<td>CYC1–HIS3</td>
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<td>pGAD–PCFl</td>
<td>His&lt;sup&gt;a&lt;/sup&gt;</td>
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<tr>
<td>pGAD–PCF2</td>
<td>His&lt;sup&gt;*&lt;/sup&gt;</td>
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addition of excess Ila or Ilib competitor DNA abolished the formation of this complex in a concentration-dependent manner (Figure 1B, lanes 2 to 4 and 8 to 10), although this was not observed when an mlla competitor was used (Figure 1B, lanes 5 to 7), indicating that nuclear proteins that bind to site Ila are specific for sites Ila and Ilib. These results demonstrate similar binding specificities between recombinant proteins and proteins in nuclear extracts.

**Binding Activity of Site Ila in Nuclear Extracts Contains PCF2**

To confirm that the binding activity of sites Ila or Ilib in rice nuclear extracts contains PCF2, we performed an EMSA using an antibody raised against recombinant MBP–PCF2C (a MBP fusion with the C-terminal region of PCF2). Immunoblot analysis using rice nuclear extracts showed that IgG fractions prepared from anti–MBP–PCF2 antiserum were specific for the PCF2 protein (Figure 2A) because the preimmune serum gave no significant signals (data not shown). In addition, PCF2 was detected in rice leaf nuclear extracts from both meristematic lower regions and nonmeristematic upper regions of leaves, suggesting constitutive accumulation of the PCF2 protein in rice plants (Figure 2A). The molecular mass of PCF2 detected by immunoblot analysis was ~35 kD, which is close to the calculated 37 kD based on the amino acid sequence of PCF2. To evaluate further the specificity of anti–MBP–PCF2 polyclonal antibody, we performed an EMSA using recombinant thioredoxin (Trx)-PCF2. The supershift assay showed that the anti–MBP–PCF2 IgG fraction specifically reacted with the recombinant protein to form a supershift band (Figure 2B, lane 5), but a control IgG (anti–MBP–OsGBF IgG) from an antiserum raised against an MBP fusion with a rice G-box binding factor did not (Figure 2B, lane 4). Using the anti-PCF2 antibody, we performed an EMSA using leaf nuclear extracts from meristematic or nonmeristematic regions. Proteins that bound to a site Ila-oligonucleotide probe were detected as broad bands in the middle of columns in both nuclear extracts, indicating that site Ila binding activity was nearly constitutive (Figure 2C). The addition of an anti–MBP–PCF2 IgG fraction produced supershift bands in both fractions (meristematic and nonmeristematic) of nuclear extracts (Figure 2C, lanes 6 and 7). However, the strength of the signal was considerably higher in meristematic compared with nonmeristematic tissue. A control antibody, anti–MBP–OsGBF IgG, failed to show supershift band formation (Figure 2C, lanes 4 and 5), and the anti–MBP–PCF2 IgG itself contained no activity for site Ila binding (Figure 2C, lane 1).

These findings indicate that the formation of the supershift band results from interaction between anti-PCF2 antibody
Figure 2. PCF2 Contained in Site Ila Binding Activities in Rice Nuclear Extracts.  
(A) Immunoblot analysis for PCF2 in rice nuclear extracts. Nuclear extracts (12 μg of protein per lane) prepared from the lower (M, meristematic) and upper (NM, nonmeristematic) regions of rice leaves were fractionated on an SDS-polyacrylamide gel and probed with anti--MBP--PCF2 polyclonal antibodies. The gel mobilities of the markers and their native molecular masses (in kilodaltons) are indicated at right.  
(B) Supershift assay with the recombinant PCF2 protein. An EMSA was performed, as given in Figure 1. The recombinant fusion protein Trx--PCF2 (10 ng) was mixed with the Ila probe labeled with phosphorus-32 (lanes 3 to 5). Subsequently, 1.3 μg of the anti--MBP--PCF2 (}±) lane 5) or the anti--MBP--OsGBF (}±) lane 4) IgG fraction, as a control, was added to the reaction. As an alternative control, 30 ng of the recombinant protein Trx was incubated with the Ila probe (lanes 1 and 2), and the anti--MBP--PCF2 IgG fraction was added (lane 2). In lanes 1 and 3, no serum (IgG fraction) was added to the EMSA reaction. An arrow marks a supershift band.  
(C) Supershift assay with rice nuclear extracts. Nuclear extracts (2.5 μg) from the lower (meristematic [M], lanes 2, 4, and 6) and upper (nonmeristematic [NM], lanes 3, 5, and 7) regions of rice leaves were mixed with the Ila probe labeled with phosphorus-32. Subsequently, 1.3 μg of the anti--MBP--PCF2 (}±) lanes 6 and 7) or the anti--MBP--OsGBF (}±) lanes 4 and 5) IgG fraction, as a control, was added to 10 μL of the reaction mixtures. In a second control, no serum (IgG) was added (lanes 2 and 3). The anti--MBP--PCF2 IgG (1.3 μg) was incubated with the DNA probe alone, as a third control (lane 1). An arrow marks supershift bands.

and site Ila binding proteins and that proteins responsible for site Ila binding activities in rice nuclear extracts contain PCF2. These results further suggest that the DNA binding activity of PCF2 is higher in actively dividing cells than in nondividing cells, although the protein level is the same in both types of cells.

Structure of PCF1 and PCF2

Sequence analysis of the PCF1 and PCF2 cDNAs showed that these inserts were in frame with the GAL4 activation domain. Because the lengths of their mRNAs estimated from RNA gel blot analysis were ~0.9 kb for PCF1 and 1.7 kb for PCF2, they appeared to have partial sequences. By using the plaque hybridization method, we isolated nearly full-length cDNA clones for PCF1 and PCF2 as probes of the originally isolated cDNAs, although the clones contained no in-frame stop codon upstream of the first ATG. The cDNA clones for PCF1 and PCF2 contained 840- and 1520-bp inserts, respectively, and these open reading frames encoded 183 and 373 amino acid residues, respectively (Figures 3A and 3B). Comparative analysis of PCF1 and PCF2 showed a conserved region consisting of 68 amino acids in their N-terminal regions. The conserved amino acid sequence showed 71% homology, whereas other regions showed no sequence similarities. We used the deduced PCF1 and PCF2 amino acid sequences to search the Swiss-Prot database,
Cloning of PCFl and PCF2 cDNA

A PCFl

but no protein with a similar sequence was found. Although we failed to find a characteristic motif for transcription factors in primary amino acid structures, the secondary structures predicted by computer analysis indicated that the conserved region would contain an HLH structure. Because a stretch of the basic amino acid-rich region locates upstream of the predicted HLH structure, it appears that the conserved regions contain a bHLH motif. A search for the conserved sequence, using GenBank and EMBL databases, indicated that translated amino acid sequences from seven Arabidopsis and one rice expressed sequence tags showed >70% homology with the conserved amino acid sequence (Figure 4). This finding suggests that PCFl and PCF2 are members of a novel family of proteins.

DNA Binding Domain in PCFl and PCF2

A DNA binding domain in PCFl was defined, and the role of the putative bHLH domain in the DNA binding was confirmed. To test the DNA binding abilities of PCFl mutants, we used the yeast one-hybrid system rather than an in vitro assay because only very small amounts of stably solubilized recombinant proteins were obtained from some of the mutants. Three deletion mutants of PCFl were generated and cloned into the pGAD424 vector to fuse with the GAL4 activation domain in the vector (Figure 5A). These constructs and the originally isolated pGAD-PCF1, with a region extending from residues 25 to 183 in the PCFl amino acid sequence, were introduced into a yeast strain carrying the site Ila,,-CYC7-HIS3 reporter gene. These constructs were engineered based on the assumption that if the PCFl mutants retain site Ila binding ability, the GAL4ad-PCFl fusions expressed in the yeast would activate the reporter gene through binding to the site Ila sequence. pGAD-PCF(b1-cl), containing the conserved region, including residues 51 to 120 of the PCFl amino acid sequence, activated the HIS3 reporter gene (Figure 5B). The number and growth rate of colonies appearing on the histidine-free medium were indistinguishable from those of the pGAD-PCF1-introduced colony. Deletion of the basic region (PCF1 \( \text{b2} \)) or C-terminal region of the conserved domain (CC region) preceded by the putative HLH region (PCF1 \( \text{c2} \)) completely failed to yield histidine-auxotrophic yeast, abolishing the DNA binding ability of PCF1. These results suggest that the conserved region

Figure 3. Nucleotide Sequences of PCFl and PCF2 cDNAs and Their Deduced Amino Acid Sequences.

(A) The nucleotide sequence of the PCFl cDNA and its deduced amino acid sequence. A bHLH region in a conserved domain with homology to PCF2 is underlined. A dashed line indicates an additional C-terminal region of the conserved domain (CC region).

(B) The nucleotide sequence of the PCF2 cDNA and its deduced amino acid sequence. The bHLH and CC regions and DNA termini of the originally isolated clone are marked as given in (A). The nucleotide sequences of the PCFl and PCF2 cDNAs have GenBank accession numbers DE7260 and D87261, respectively.

Arrowheads denote the 5' and 3' termini of the original cDNA clone obtained by the yeast one-hybrid screen.
containing a predicted bHLH region in PCF1 acts as a bHLH motif responsible for specific DNA binding. The corresponding conserved region of PCF2 is also likely to be necessary for DNA binding because this common domain is the only region conserved between PCF1 and PCF2.

**Dimerization of PCF1 and PCF2**

It is well documented that bHLH transcription factors bind their cognate DNA sequence as a homodimer or a heterodimer with other partners. Because of the likelihood of dimerization between PCF1 and PCF2 (containing a bHLH motif), we assayed the affinity of PCF1 and PCF2 proteins for homologous and heterologous interactions by using the yeast two-hybrid system. PCF1 chimeric proteins, each with a GAL4 DNA binding domain and activation domain, interacted with each other and activated GAL1-lacZ and GAL1-HIS3 reporter genes, indicating the formation of a homodimer of PCF1. Similar results were obtained for PCF2 fusions (Table 2). Moreover, when PCF1 and PCF2 proteins were coexpressed in yeast, compared with a homologous interaction between PCF1 or PCF2, at least a fivefold increase in activation of the reporter gene was observed. This suggests that PCF1 and PCF2 preferentially bind DNA as a heterodimer rather than as a homodimer. In addition, to examine the involvement of the CC region in dimer formation, a PCF1 deletion mutant PCF1(b1-h) containing a bHLH region but lacking the CC region was also generated. This mutant lost the ability to interact with wild-type PCF1 and PCF2. The data from this study clearly show that the CC region is essential for protein–protein interactions among PCFs. Together with the fact that deletion of the CC region resulted in the loss of DNA binding ability of PCF1, as shown in Figure 5B, these results strongly suggest that dimer formation is essential for DNA binding.

**Expression of mRNAs for PCF1 and PCF2**

Finally, we also performed RNA gel blot analysis to examine cell specificity for the accumulation of PCF1 and PCF2 transcripts. Total RNAs were extracted from leaves, seedlings,

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**Figure 4.** Comparison of the Conserved Regions of PCF1 and PCF2 with Sequences Translated from Arabidopsis and Rice Expressed Sequence Tags.

Sequences ATTS2702 to T13768 are from amino acid sequences translated from Arabidopsis expressed sequence tags. They show significant homology to the region containing a putative bHLH motif conserved between PCF1 and PCF2. Sequence R46844 is from a rice expressed sequence tag. Solid boxes indicate conserved amino acids; open boxes indicate amino acids similar to the conserved one. The bHLH region is indicated at top.

**Figure 5.** Localization of the DNA Binding Domain of PCF1.

(A) Schematic representation of PCF1 and truncated versions. The PCF1 cDNA in pgAD424, originally isolated by library screening, was used in this analysis. Truncated fragments of the PCF1 cDNA were generated by PCR and cloned into pgAD424 to produce the GAL4 activation domain fusions. Shaded and solid boxes represent basic and helix-loop-helix regions, respectively; hatched boxes represent the CC regions. (B) The trans-acting effect of truncated PCF1s fused with the GAL4 activation domain on (site Ila), b-CYC1-HIS3 reporter gene in yeast. The pgAD–PCF constructs were introduced into a yeast strain carrying the YIH2a reporter gene. His' and His- indicate phenotypes of transformants that are able and unable to grow on histidine-free medium, respectively.
Table 2. Yeast Two-Hybrid Assay for the Interaction between PCF1 and PCF2

<table>
<thead>
<tr>
<th>Fusion with GAL4bd&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Fusion with GAL4ad&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Reporter Gene</th>
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<tr>
<td>pGBT-PCF1</td>
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<td>pGAD424</td>
<td>His&lt;sup&gt;-&lt;/sup&gt; &lt;0.1</td>
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<td>His&lt;sup&gt;-&lt;/sup&gt; &gt;0.1</td>
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<td>pGBT-PCF1(b1-h)</td>
<td>pGAD-PCF1</td>
<td>His&lt;sup&gt;-&lt;/sup&gt; &gt;0.1</td>
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<sup>a</sup>GAL4bd, GAL4 DNA binding domain.
<sup>b</sup>GAL4ad, GAL4 activation domain.
<sup>c</sup>His<sup>-</sup> or His<sup>+</sup> represents a phenotype of transformed yeast on histidine-free medium.
<sup>d</sup>Values represent means of β-galactosidase activities from three independent colonies. Unit is defined as the amount that hydrolyzes 1 μmol of o-nitrophenyl β-D-galactopyranoside per minute.

and suspension-cultured cells. Leaves were separated into upper, middle, and lower regions, as illustrated in Figure 6, to compare differences in their transcripts in tissues or cells with different levels of cell proliferation. The lower region of leaves contained the greatest number of dividing cells compared with the upper and middle regions, as shown in the hybridized signal for rice PCNA gene transcripts (Figure 6). Seedlings and suspension-cultured cells also appeared to contain actively dividing cells, as shown by the expression of the PCNA gene (Figure 6). Both PCF1 and PCF2 transcripts were abundant in the lower region of leaves, whereas less transcript accumulation was detected in the upper region of leaves. The expression of the PCF1 and PCF2 genes as well as the PCNA gene appeared, in general, to be growth regulated.

**DISCUSSION**

**Cloning of the PCF1 and PCF2 cDNAs That Bind to Sites Ila and IIb**

cis-Acting elements located at positions -197 and -178 of the rice PCNA promoter were recognized by nuclear proteins that had near-identical binding preferences (Kosugi et al., 1995). In this study, we isolated two cDNA clones that encode DNA binding proteins specific for sites Ila and IIb. Our results show that in N-terminal regions, both PCF1 and PCF2 proteins contain a conserved region consisting of a putative bHLH domain and an adjacent C-terminal region (CC region). Our data strongly suggest that this conserved region is responsible for DNA binding and the ability to dimerize. Because this conserved sequence has been found in several expressed sequence tags of unknown function in Arabidopsis and rice, PCF1 and PCF2 could be classified as members of a novel family of transcription factors that is conserved in monocotyledons and dicotyledons. The observations that the rice PCNA promoter also functions in tobacco plants and that nuclear proteins binding to sites I la and IIb are present in rice and tobacco nuclear extracts further support this view (Kosugi et al., 1995). In addition, site I la– and/or IIb–protein complexes detected as broadly shifted bands in an EMSA with nuclear extracts (Figures 1 and 2) may represent multimembers of the PCF protein family.

**The Unique bHLH Motif in PCF1 and PCF2 Is Distinct from bHLH Proteins Binding to the E-Box**

In animals, the bHLH transcription factors, represented by MyoD, c-Myc, and the upstream stimulatory factor, bind to the E-box (CANNTG; N stands for any nucleotide) in promoters as homodimers or heterodimers and regulate transcription of a variety of genes involved in cell proliferation and differentiation (Blackwell and Weintraub, 1990; Blackwell et al., 1990). Plant bHLH proteins, B and R gene products from

**Figure 6.** PCF1 and PCF2 mRNA Levels in Rice Tissues and Cells. Rice leaf RNA was isolated from the lower, middle, and upper parts of leaves, as illustrated. RNAs were isolated from 7-day-old seedlings and rice suspension-cultured cells at mid-log phase. Total RNA (20 μg) was loaded, and equal loading of the RNA was confirmed by ethidium bromide (EtBr) staining. The full-length PCF1 and PCF2 cDNAs and rice PCNA cDNA were radiolabeled and used for hybridization.
maize, and the del gene product from Antirrhinum regulate genes involved in anthocyanin biosynthesis through binding to promoter sites corresponding to the E-box (Goff et al., 1990; Ludwig et al., 1990; Roth et al., 1991; Goodrich et al., 1992). Although both PCF1 and PCF2 share a bHLH motif predicted from their primary sequences, they do not bind to the E-box (data not shown) but to sites Ila (GTGGGCCCCG) and Ilb (ATGGTCCCA). No homology in primary amino acid sequence was detected between known bHLH proteins and PCFs.

It is interesting that E2F is the only known transcription factor that does not bind the E-box, although it contains a bHLH motif required for DNA binding. In mammals and Drosophila, E2F is involved in the control of a variety of growth- and cell cycle-regulated genes that are required for entry into S phase (DeGregori et al., 1995; Duronio et al., 1995). Jordan et al. (1994) have found that the center of the basic region in E2Fs (E2F1 to E2F4) contains a proline and glycine, which may prevent α-helix formation throughout the length of the basic region. In contrast, in the E-box binding bHLH proteins, basic regions form an α-helical structure. Replacement of the proline-glycine pair in the basic region of E2F1 with alanines resulted in a significant reduction in binding to the E2F site (Jordan et al., 1994). The basic regions in PCF1 and PCF2 also contain proline and glycine, which could prevent α-helix formation and distinguish the DNA binding property of PCF1 and PCF2 from that of other bHLH proteins that bind to the E-box.

In addition, E2F, Sp1, and Dsc1, which are transcription factors that play an important role in the G1 (G0)-to-S phase transition of the cell cycle in animals and yeast, have a common feature in their preferred binding sequences—GC-rich sequences. The GC core in the consensus E2F site (TTT[G/C][G/C][G/C]) is sufficient for binding of E2F1 (Jordan et al., 1994). In this regard, the promoter regions of housekeeping genes, including DNA synthesis-related genes, contain GC-rich regions, and the specific bindings of these transcription factors to their cognate sites in the GC-rich regions are expected to regulate DNA synthesis genes. Because the binding sequence of PCF1 and PCF2 is also rich in GC nucleotides, PCF1 and PCF2 might have some properties that are functionally analogous to Sp1, Dsc1, and especially E2F.

**Function of PCF1 and PCF2**

Mutations of sites Ila and Ilb, to which PCFs specifically bind, in the rice promoter lead to a considerable reduction in the transcriptional activity in actively dividing cells but do not result in deregulated expression in nondividing cells (Kosugi et al., 1995). This suggests that sites Ila and Ilb function as positive cis-acting elements in the rice promoter. Furthermore, gain-of-function analysis of site Ila with a heterologous promoter demonstrated that site Ila mediates transcriptional activation (Kosugi et al., 1995). The PCFs and nuclear proteins that bind to sites Ila and Ilb appear to have common preferred binding sequences. In addition, results of the EMSA using the anti-PCF2 antibody indicated that the binding activities of site Ila in nuclear extracts are at least in part from PCF2 and probably also from PCF1. Thus, it is likely that the PCF family is involved in a positive regulation of the rice PCNA gene in proliferating cells through binding to sites Ila and Ilb. In addition, the observation that the heterodimeric interaction between PCF1 and PCF2 was considerably stronger than their homodimeric interaction implies that members of the PCF family may form homodimers and heterodimers with other cell type-specific or temporal-specific members and regulate the transcription of DNA synthesis-related genes such as the PCNA gene.

PCF1 and PCF2 transcripts are abundant in actively dividing cells, whereas their proteins, as well as site Ila and/or site Ilb binding activities in nuclear extracts, appear to exist in both dividing and nondividing cells. The latter observation does not correlate with the expression of PCNA protein, which is not present in quiescent cells (Daidoji et al., 1992). Similar examples that accumulation of transcripts and proteins do not correlate have also been observed in many studies with histone genes. Accumulation of PCF1 and PCF2 transcripts may be high in actively dividing cells to maintain a constant level of PCF proteins in newly formed cells.

Binding activities of site Ila and/or site Ilb are present in nuclear extracts from dividing and nondividing cells. This is apparently inconsistent with the fact that sites Ila and Ilb function as positive cis-acting elements in the rice PCNA promoter. Hence, the binding activities of site Ila and/or site Ilb in dividing cells should be functionally different from those in nondividing cells. The supershift assay using the anti-PCF2 antibody showed that the DNA binding activity of PCF2 was higher in nuclear extracts from actively dividing cells than from nondividing cells. This observation suggests that some members of the PCF protein family may function as transcriptional activators in dividing cells, whereas other members act as transcriptional repressors in nondividing cells. In this view, PCF2 has the potential to act as a transcriptional activator of the PCNA gene.

Another possible mechanism by which PCFs could mediate activation of PCNA gene expression in dividing cells might be analogous to the mechanism in the animal system. Transcription factors E2F and Sp1 control growth-regulated genes in animals and are present throughout the cell cycle (Mudryj et al., 1990; Shirockar et al., 1992; Lin et al., 1996). However, certain regulatory mechanisms, including protein-protein interactions, prevent these factors from activating growth-regulated genes in quiescent cells. PCF1 and PCF2 may also control PCNA gene expression by interacting with other proteins. The results of an EMSA using nuclear extracts from tissues with a low competence for cell division showed the presence of site Ila-protein complexes with a slow mobility (Figure 2). It is possible that other proteins interact with PCFs in nondividing cells, resulting in slow mobility in EMSAs. However, it is also possible that some high molecular mass PCFs are present in nondividing cells or that degradation of PCFs is promoted in actively dividing cells.
It is conceivable that if a protein interacts with PCFs in nondividing cells, it might function as a transcriptional repressor like the E2F–retinoblastoma (RB) complex. In this regard, a member of the RB protein family has been identified recently in maize (Grafi et al., 1996; Xie et al., 1996). The pocket proteins, including RB, negatively regulate the G1 (G0)–to–S phase transition of the cell cycle interacting with E2F. Nagar et al. (1995) have found that the replication protein of a plant DNA geminivirus, tomato golden mosaic virus, induces PCNA in terminally differentiated cells of the host plant. In addition, the replication protein of a wheat dwarf geminivirus forms a stable complex with a member of the RB protein family (Xie et al., 1995). These proteins are analogous to the oncoproteins of animal DNA tumor viruses and suggest the presence of cell cycle–regulatory factors that are sequestered from a complex with a plant RB homolog by the replication protein of geminiviruses in plants, as occurs in the E2F–RB complex in animals. Together with the fact that PCFs have some features related to E2F as stated, it is also possible that in plants, PCFs interact with members of the RB protein family to regulate the cell cycle–associated genes, including the rice PCNA gene.

In summary, we analyzed the rice PCNA gene to understand the mechanisms controlling the G1 (G0)–to–S phase transition in the plant cell cycle. The cis–acting elements, sites Ila and IIb, regulating the rice PCNA gene seem to mediate transcriptional activation at the G1 (G0)–to–S phase boundary, as described previously. PCF1 and PCF2 that specifically bind to sites Ila and IIb may act as transcription factors to control DNA synthesis–related genes in plants. In particular, PCF2, with a high level of DNA binding activity in meristematic tissues, may act as a transcriptional activator of these genes.

METHODS

Construction of Plasmids

To construct reporter genes for the yeast one-hybrid screening, an iso-1-cytochrome c CYC1 minimal promoter extending from positions +49 to −178 and the HIS3 coding region were isolated by polymerase chain reaction (PCR) with specific primer pairs 5'-GGACTCCGGGGTCAAGAGGATCCGCGCTGATATAGCG-3' and 5'-GGACTCTAGCTAGGTGGTCTTATAGAAG-3' for CYC1 and 5'-CTACTAGGATCCAGAGGAAGAACCTATGG-3' and 5'-GTGACGAGATTCTACTACATAAGACACCTTTTGTTG-3' for HIS3 as a template for Saccharomyces cerevisiae genomic DNA. PCR was performed with Ex Taq polymerase (Takara, Tokyo, Japan), using standard conditions. The nucleotide sequences of the PCR products were verified by sequencing. The CYC1 promoter region was inserted into the SacI-XbaI sites of pBluescript SK+ (Stratagene, La Jolla, CA). The HIS3 coding sequence was inserted into the XbaI-EcoRI sites of the construct in which the CYC1 promoter was inserted to produce a CYC1–HIS3 construct. Fragments corresponding to 12-repeated site Ila (site Ila)-IIb and 12-repeated site IIb (site IIb)-Ila oligomers were generated by annealing and ligating oligonucleotides 5'-TCGAGATCCGGGCGGTAAAGTGGGGCCGTATGCA-3' (where underlining indicates two copies of the 12mers of site Ila) and 5'-TACGAGTCGAGGTGGCGGTGCCCACCTACGGGCCACC-3' for site IIb and 5'-ACTCTAGGATCCGGGGTCCACCGACGGTGGACATGCA-3' and 5'-AGTGGGGATCCAGGTGGACATGCA-3' for site Ila. In these reporter genes, the intention of using the 12 copies of site Ila and IIb is based on the idea that increasing copies of the binding sites would reinforce the binding of PCFs (PCNA gene–controlling element binding factors) to the sites and facilitate the interaction of PCFs with components of the basal transcription machinery.

After cloning these fragments in the Smal site of the pBluescript SK+ vector, each fragment excised with SacI and EcoRV was inserted into SacI and blunt-ended SalI sites of the CYC1–HIS3 construct. Fragments corresponding to (site Ila)-IIb–CYC1–HIS3 and (site IIb)-Ila–CYC1–HIS3 were excised with SacI, treated with T4 polymerase, and then digested with EcoRI. Fragments were then cloned in the EcoRI and blunt-ended AatII sites of a yeast integration vector, which was generated by deleting the EcoRI-AatII fragment from pG79 vector (Bartel et al., 1993a) (ClonTech, Palo Alto, CA). The resulting reporter constructs, YH12a and YH12b, are integration vectors harboring the HIS3 reporter gene directed by 12 copies of site Ila and site IIb, respectively. A control construct, YH1, containing the CYC1–HIS3 gene was generated from the construct given above by deleting the 12-repeated site Ila region.

For plasmid construction for maltose binding protein (MBP) fusion genes with PCF1 and PCF2, the cDNA inserts from the originally isolated pGAD-PCF1 and pGAD-PCF2 clones were inserted into the EcoRI and BamHI sites of the pMAL-c2 vector, respectively (New England Biolabs, Beverly, MA). For a thioredoxin (Trx) fusion, the full-length PCF2 cDNA was excised with EcoRI and SalI and inserted into the corresponding sites of pET32b (Novagen, Madison, WI). For constructs for the production of antiserum against a MBP–PCF2 fusion, the Sall–HindIII fragment corresponding to the C-terminal 210 amino acids of PCF2 was inserted in frame with the MBP-coding sequence of pMAL-c2 to generate pMAL-PCF2.

To generate a pGAD–PCF1 construct, the EcoRI–BamHI–restricted fragment of the original isolated pGAD–PCF1 clone was inserted into the corresponding sites of pG9T9. The full-length coding region of PCF2 was also inserted into pG4AD424 (Bartel et al., 1993a) (ClonTech) and pG79 vectors in frame to generate pGAD–PCF1 and pGAD–PCF2. Deletion mutants of PCF1 and pGAD–PCF1(b1–c1), pGAD–PCF2(b2), and pGAD–PCF1(c2), were generated by cloning PCF1 deletion fragments produced by PCR. PCF1 was inserted as a template of the pGAD–PCF1 plasmid with PCF1-specific primers b1 (5'-CACTCGAGAATTCCGAGAGGATTCCCGACTGTCGCAGAGGATCTTACCATGACACGGCATATGACGATG-3'), b2 (5'-CACTCGAGAATTCCGAGAGGATTCCCGACTGTCGCAGAGGATCTTACCATGACACGGCATATGACGATG-3'), and b3 (5'-CACTCGAGAATTCCGAGAGGATTCCCGACTGTCGCAGAGGATCTTACCATGACACGGCATATGACGATG-3') for HIS3 and the corresponding sites of pGAD424. Fragments amplified with primer pairs b1 and b2, and GAL4ad and c2 were digested with EcoRI and BamHI and cloned in pGAD424. Fragments amplified with primer pairs c1 and c2, and GAL4ad and c2 were digested with EcoRI and BamHI and cloned in pGAD424. The pGAD–PCF1(b1–c1) construct was generated by cloning a fragment extending from the basic domain to the C-terminal regions of the conserved domain of PCF1, respectively. GAL4ad and TADH1 primers were specific for GAL4 activation domain and alcohol dehydrogenase ADH1 terminator regions in pG4AD424. Fragments amplified with primer pairs c1 and c2, and TADH1, and GAL4ad and c2 were digested with EcoRI and BamHI and cloned in pG4AD424. The pGAD–PCF1(b1–c1) construct was generated by cloning a fragment extending from the basic domain to the latter helix region in the helix–loop–helix (HLH) domain of PCF1 into pG79. This fragment was produced by PCR with primer set b1 and c2 (5'-CACTCGAGAATTCCGAGAGGATTCCCGACTGTCGCAGAGGATCTTACCATGACACGGCATATGACGATG-3'). The integrity of the cloned sequences was verified by sequencing.
Yeast Transformation and Yeast Two-Hybrid Assay

Yeast was grown in YEPD or SD synthetic medium, as described in Rose et al. (1990). Transformation was performed by the lithium-polyethylene glycol method (Ito et al., 1983). A typical transformation yielded 2 to 5 × 10^5 transformants per μg of plasmid DNA. Integrative transformation was achieved as follows. The YH2a, YH2b, and YH constructs were linearized at the Muni site within the Trp1 coding region and targeted to a trp1 locus of a yeast strain (MATαura3-52 his3-201 ade2-101 trp1-163 his3-A200 leu2-3,11) obtained from the Institute for Fermentation, Osaka, Japan). Total DNA from several colonies showing growth on an SD synthetic tryptophan-free medium and no growth on histidine-free medium was extracted to confirm the integration of reporter genes into the yeast genome. Confirmation that the transformants carried no plasmid DNA and that entire regions of the reporter genes were retained on genomic DNA was established by PCR with two-combinatorial primer sets—TαOH1, the 3' specific CYC1 primer, and a primer (5'-GGGGTTATTGTCTAGCAGCG-3') corresponding to positions 4009 to 4029 on the pGBT9 vector.

The yeast two-hybrid assay was performed by simultaneous transformation with the pGAD424- and pGBT9-based plasmids. The yeast strains HF7c (Feilotter et al., 1994) and SFY526 (Bartel et al., 1993b) were used for assays with the HIS3 and lacZ reporter genes, respectively. The transformed HF7c was tested for growth on the synthetic medium without leucine, tryptophan, and histidine. The transformed SFY526 grown on leucine- and tryptophan-free medium was assayed for β-galactosidase activity. Quantitative β-galactosidase assay using o-nitrophenyl P-D-galactopyranoside was performed according to the method described previously by Yocum et al. (1984).

Yeast One-Hybrid Screening

The cDNA yeast expression library was produced as follows. Random primed cDNA was synthesized from poly(A)^+ RNA prepared from rice suspension-cultured cells (Oryza sativa cv Nipponbare) using a cDNA synthesis kit (Pharmacia). The cDNAs, to which EcoRI adapters were ligated, were inserted into a pGAD424 vector predigested with EcoRI followed by treatment with calf intestine alkaline phosphatase. The library was transformed into Escherichia coli DH5α by electroporation, and a total of 1.8 × 10^6 independent colonies were amplified for plasmid extraction. A 500-mL culture of the yeast strain carrying the YH2a reporter gene was transformed with a total of 50 μg of the cDNA library and 2.5 mg of denatured salmon sperm DNA as a carrier DNA. Approximately 5 × 10^6 Leu^+ transformants were incubated overnight in liquid SD synthetic leucine- and tryptophan-free medium for expression of the introduced genes. The yeast was then collected by centrifugation and plated on SD medium lacking leucine, tryptophan, and histidine for 4 days at 30°C. Approximately 5 × 10^5 colonies were pooled, and plasmid DNA was isolated from the transformants. The rescued plasmids were reintroduced into the yeast strain carrying YH2a for the second screening. More than 10^6 colonies per μg of plasmid appeared on the leucine- and histidine-free medium. About 30 colonies were randomly collected from the Leu^+ His^+ colonies, and plasmid DNA were extracted from the culture of each colony. For further elimination of false-positive clones, the plasmids rescued next were each introduced into a yeast strain carrying the YH2a or YH2b reporter gene, thus confirming whether the His^+ phenotype of the transformants depended on the site Ila or site Iib element in the reporter gene. Plasmids that activated both (site Ila)^+ and (site Iib)^+ CYC1-HIS3 reporter genes but not the CYC1-HIS3 gene were characterized further by restriction mapping and sequence analysis using a dye-terminator cycle sequencing kit (Applied Biosystems, Foster City, CA) and a DNA sequencer (model 373A; Applied Biosystems).

The full-length cDNAs for PCF1 and PCF2 were isolated from the oligo(dT)-primed cDNA library of rice leaves and suspension-cultured cells constructed in λZAP (Stratagene). Approximately 10^5 plaques were transferred onto Hybond N membranes (Amersham International) and hybridized at 68°C with 32P-labeled 550- and 900-bp probes corresponding to inserts of the original PCF1 and PCF2 clones isolated by the yeast one-hybrid screen, respectively. Two and five positive phages were isolated for PCF1 and PCF2, respectively, and the plasmids excised in vivo from these phages were sequenced.

Production of MBP and Trx Fusion Proteins

The pMAL-c2 and pET32 constructs carrying PCF1 or PCF2 cDNA were introduced into E. coli (XL1-Blue for pMAL-c2 and BL21(DE3) for the pET32 plasmid). E. coli was cultured in Terrific broth (Ausubel et al., 1987) until OD_{600} reached ~1.0. In the next step, isopropyl β-D-thiogalactopyranoside in a final concentration of 0.5 mM was added and incubated for 3 hr at 37°C. Proteins were extracted with a buffer consisting of 20 mM Hepes-NaOH, pH 7.8, 1 M NaCl, 1 mM EDTA, 2 mM DTT, and 0.1% Triton X-100. MBP fusion proteins from extracts were isolated by one-step purification with an amylose resin (New England Biolabs) column, according to the instructions provided by the manufacturer. The Trx fusion, containing a six-histidine tag, was purified with a nickel-chelate matrix, according to the instructions provided by the manufacturer (Novagen).

Production of Antiserums Raised against PCF2

The fusion protein of the C-terminal region of PCF2 with MBP was produced in E. coli transformed with the pMAL-PCF2C construct. The protein was purified with an amylose resin column, eluted in Freund’s complete or incomplete adjuvant, and injected into New Zealand white rabbits to produce the anti—MBP—PCF2 antibody. In a similar way, the anti—MBP—OsGBF antibody was produced using a recombinant MBP fusion with a rice G-box binding factor (OsGBF), whose cDNA has been recently isolated in our laboratory (S. Kosugi and Y. Ohashi, unpublished results). From the antiserums, each IgG fraction was prepared by precipitating IgG with a saturated ammonium sulfate solution (Ausubel et al., 1987).

Preparation of Nuclear Extracts

Nuclear extracts from leaves and suspension-cultured cells of rice (cv Nipponbare) were prepared as described previously by our laboratory (Kosugi et al., 1996). The leaf extracts were prepared from the upper and lower regions of leaves.

Electrophoretic Mobility Shift Assays

DNA probes were generated by annealing oligonucleotides spanning the regions of interest and by filling in the single-strand overhangs with α-[^32P]-dCTP using the Klenow fragment of DNA polymerase I. Binding reactions were performed using a volume of 10 μL contain-
ing 50 fmol of oligonucleotide probe, 0.1 to 1.0 µg poly(di-dC), 1 x binding buffer (20 mM Hepes-KOH, pH 7.8, 100 mM KCl, 1 mM EDTA, 1 mM DTT, 0.05% BSA, and 10% glycerol), and 5 to 100 ng of the recombinant protein or 2.0 to 2.5 pg of protein from the nuclear extracts. The mixtures were incubated for 30 min at room temperature and loaded on native 5% polyacrylamide (acrylamide-bisacrylamide, 29:1 [w/w]) gels. For electrophoretic mobility shift assays (EMSA) with specific antibodies, anti-IgG fractions were added to preincubated (15 min) reaction mixtures, and the mixtures were incubated for another 30 min at room temperature. Electrophoresis was conducted at 4 V/cm for 40 min with 0.5 x TBE (45 mM Tris-borate and 0.5 mM EDTA, pH 8.2) buffer at room temperature. Gels were dried and autoradiographed using intensifying screens.

Immunoblot Analysis

Nuclear extracts prepared from the upper and lower regions of rice leaves were electrophoresed on 12.5% SDS-polyacrylamide gels (12 µg of nuclear protein per lane) and transferred to an Immobilon membrane (Millipore, Bedford, MA). The membranes were blocked for 1 hr with a blocking buffer (PBS containing 0.2% casein and 0.1% Tween 20), incubated with a 1:500 (v/v) dilution (13 pg/mL) of the polyclonal anti-MBP-PCF2 IgG fraction in blocking buffer for 1 hr at 25°C, and then washed three times with blocking buffer. Antibodies bound to the filters were detected by incubation with 1:2000 (v/v) dilution of alkaline phosphatase-conjugated anti-rabbit IgG followed by visualization with a CSPD chemiluminescent substrate, according to the instructions provided by the manufacturer (Tropix, Bedford, MA).

RNA Gel Blot Analysis

Total RNA was isolated from different tissues and suspension-cultured cells, as described previously (Kirk and Kirk, 1985). RNA (20 µg) was fractionated in 1% formaldehyde–agarose gels and transferred to a nylon filter (Hybond N+; Amersham International), according to the method of Sambrook et al. (1989). Ethidium bromide (25 µg/mL) was added to each sample to allow visualization of RNA under UV light to confirm equal loading of each sample. Hybridization was performed at 68°C in a solution containing 0.2 M Na2HPO4, pH 7.2, 1 mM EDTA, 7% SDS, and 1% blocking reagent (Boehringer Mannheim). Filters were washed twice in a buffer containing 20 mM Na2HPO4, pH 7.2, and 1% SDS at room temperature followed by a single washing at 68°C. DNA probes were prepared from gel-purified fragments of the full-length PCF1, PCF2, and rice PCNA cDNAs and labeled with α-32P-dCTP, using a Redi-primed labeling kit (Amersham International).

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