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Determination of the DNA-Binding Sequences of the Zn(II)2Cys6 Zinc-Cluster-Containing PRIB Protein, Derived from the Basidiomycete *Lentinus edodes* Gene[1]

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The 565 amino-acid PRIB protein with a Zn(II)2Cys6 zinc-cluster DNA-binding motif is the expression product of the *priB* gene, which is most actively transcribed in an early stage of fruiting-body formation by the basidiomycete, *Lentinus edodes*. PRIB produced in *Escherichia coli* using the bacteriophage T7 expression system was purified by ion-exchange chromatographies and then subjected to random binding-site selection analysis using a pool of random 24-bp oligonucleotides with 13-bp PCR primer sites at each end. The oligonucleotides (50 bp) selected for PRIB binding were cloned into pUC19. A total of 303 cloned DNA fragments were picked randomly and sequenced. The PRIB binding sites could be grouped into 25 individual sequences, suggesting a consensus sequence of 16 bp, 5′ GGGGGGGACAGGANCC 3′. Gel mobility-shift assaying of 10 randomly selected sequences all revealed a reasonable band shift. DNase I footprinting analysis of the 50-bp DNA fragment containing the sequence most similar to the consensus sequence showed that PRIB protects the entire 16-bp sequence from digestion by DNase I.

Key words: basidiomycete, binding-site selection, DNase I footprinting, gel mobility-shift, zinc-cluster.

DNA-binding zinc proteins so far reported are classified into several groups, such as the zinc finger, zinc twist, zinc cluster, and ring finger ones (*1, 2*). Zinc cluster DNA-binding domain Zn(II)2Cys6, in which two zinc atoms are bound to six cysteine residues, has been found in *Saccharomyces cerevisiae* GAL4 (*3*), *Neurospora crassa* QA1F (*4*), and other yeast and fungal transcription factors (*1*). During the course of studies on the molecular mechanism of fruiting-body formation by the basidiomycete, *Lentinus edodes*, we isolated a novel cDNA, *priBc*, encoding a 565 amino-acid DNA-binding protein (M_r 64,055) with a Zn(II)2Cys6 domain (aa residues 20-50) from a primordial cDNA library of the fungus (*5*). Just after the zinc cluster, there is a possible leucine zipper domain (aa residues 65-79; *6*) with a preceding short basic aa sequence (aa residues 54-60) like in other Zn(II)2Cys6 transcription factors. The *priB* transcript was abundant in primordia and small immature fruiting bodies. Mature fruiting bodies contained a lower level of the transcript, while vegetatively growing cells and preprimordial mycelia contained a negligible amount of the transcript, suggesting that the *priB* gene may play a role in the formation of fruiting bodies. To verify the DNA-binding activity of PRIB and to determine the target sequences of the protein, we performed random binding-site selection assaying (*7*). In this paper, we report that PRIB binds to unique 16-bp sequence which is different from those in the case of GAL4, QA1F, and other yeast and fungal transcription factors.

The PRIB protein was produced in *Escherichia coli* using expression vector pET-3a (*8*) as a fusion protein (M_r 65,492) linked to the first eleven aa of the T7 gene-10 protein sequence (s10 aa sequence) plus four aa specified by the linker sequence (*5*). The apparent size of 83 kDa found on SDS-PAGE was significantly larger than that inferred from the nt sequence. This migration anomaly may be possibly related to the highly basic isoelectric point (predicted pI of 8.5) of PRIB (*9*). The T7s10-PRIB fusion protein was purified by ion-exchange column chromatographies on phospho-cellulose and Resource-Q (Pharmacia) to the level of a single band on SDS-PAGE (see Fig. 1).

The random binding-site selection method (*7*) was used for the isolation of DNA sequences specifically recognized by the PRIB protein (Fig. 2). The oligonucleotide used comprised a random 24 base sequence flanked by two 13-nt PCR primers containing *Bam*HI and *Eco*RI restriction sites to facilitate the cloning of binding sequences after selection

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Abbreviations: aa, amino acid(s); *An, Aspergillus nidulans*; bp, base pair(s); cDNA, DNA complementary to RNA; kb, kilobase(s) or 1,000 bp; *Kl, Kluyveromyces lactis; Le, Lentinus edodes; Nc, Neurospora crassa;* nt, nucleotide(s); PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; PRIB, protein encoded by *priBc; priBc*, cDNA clone of *priB; Sc, Saccharomyces cerevisiae*; SDS, sodium dodecyl sulfate.

(*10*). The design of this oligonucleotide was based on the observation that the binding sites of the Zn(II)2Cys6 motif-containing yeast and fungal transcription factors are 8-23 bp in length (see Table I). Double-stranded randomized oligonucleotides were prepared by PCR and then incubated with the purified PRIB protein. The DNA-PRIB complex was immunoprecipitated by the addition of anti-PRIB antiserum and then protein-A (immunoprecipitin,

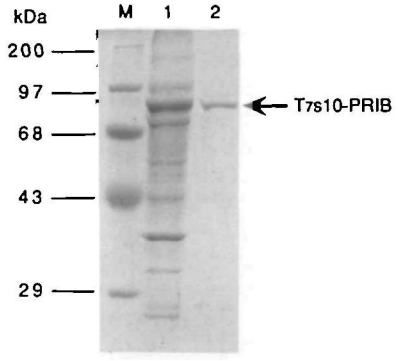

Fig. 1. **SDS-PAGE revealing the purity of the PRIB protein.** A crude protein extract (20 μg) of *E. coli* producing the T7s10-PRIB fusion protein (lane 1), 1 μg of the purified T7s10-PRIB protein (lane 2), and molecular size marker (lane M) were resolved by 0.1% SDS-10% PAGE and subsequently stained with Coomassie Brilliant Blue. *E. coli* strain BL21 containing the recombinant plasmid, pET-3a-priB (*5*), was cultured in NZCYM medium at 37°C for 2 h, and then the culture was induced with 0.2 mM isopropyl-β-D-thiogalactopyranoside (IPTG) at 25°C for 2 h according to the procedure of Sambrook *et al.* (*12*). The cells were harvested by centrifugation, and then resuspended in phosphate-buffered saline (130 mM NaCl, 10 mM Na_2HPO_4, 10 mM NaH_2PO_4, pH 7.2) and disrupted by sonication. After centrifugation, a half aliquot of the aqueous phase was saved as the crude protein extract. The other half aliquot was subjected to ion-exchange column chromatographies on phospho-cellulose and Resource-Q (Pharmacia), which resulted in a homogeneous T7s10-PRIB protein.

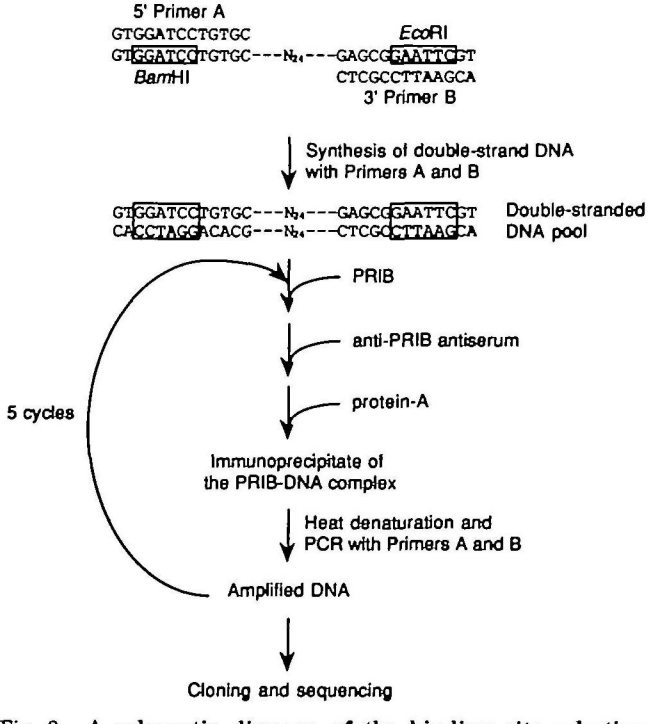

Fig. 2. **A schematic diagram of the binding site selection assay.**

TABLE I. **Alignment of recognition sequences of PRIB and other yeast and fungal transcription factors containing the Zn(II)2Cys6 zinc-cluster DNA-binding motif.**

Protein	Length (bp)	Binding sequence	Reference
Le PRIB	16	GGGGGGGACAGGANCC	This paper
An AFLR	10	TTAGGCCTAA	(*13*)
An ALCR	5	CCGCA	(*14*)
An NIRA	8	CTCCGA/C/TGG	(*15*)
An UaY	14	TCGGN$_6$CCGA	(*16*)
Nc QA1F	16	GGA/GTAAA/GC/TA/GC/TTTATCC	(*4*)
Sc GAL4	17	CGGAG/CGACA/TCAGG/CAGGC	(*17*)
Sc HAP1	23	CCCTCGCTATTATCGCTATTAGC TGGCCGGGGTTTACGGACGATGA	(*18*)
Sc LEU3	12	CGGCCAAGGCCG	(*19*)
Sc MAL63	15	C/AGCN$_5$C/AGC/G	(*20*)
Sc PPR1	17	TTCGGNA/GNNTC/TNCCGAA	(*21*)
Kl LAC9		Identical to that of GAL4	(*22*)

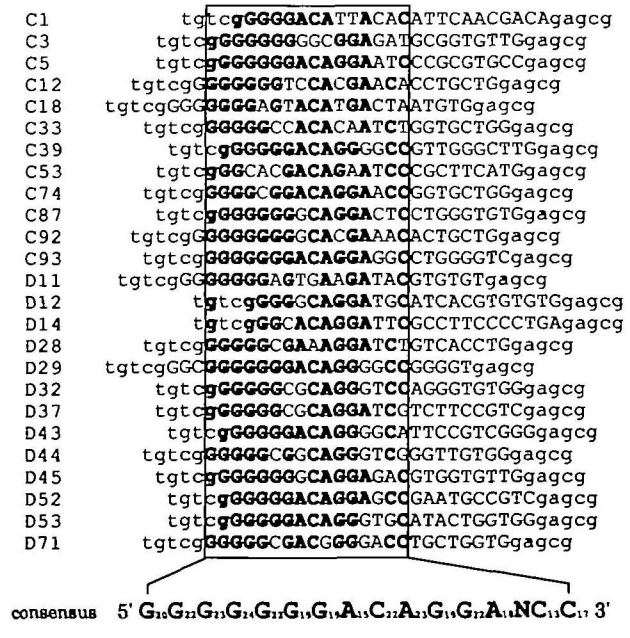

Fig. 3. **Selected binding sites for PRIB.** The sequences of 25 individual PRIB-binding sites derived through five rounds of selection (see Fig. 2) are shown and aligned as to similar 16-base sequences. PCR primer sequences are shown in lowercase letters and they contribute as binding sites in several cases. The consensus sequence, 5′ GGGGGGGACAGGANCC 3′, was determined based on the alignment of the 25 individual 16-base sequences so as to optimize matches. The highlighted bases in the 25 individual 16-base sequences are those identical to ones in the consensus sequence. The numerals on the right of the bases in the consensus sequence indicate how many bases out of 25 in the cloned binding sites are identical in the consensus sequence. The amplified DNA fragments derived through five rounds of selection were digested with both *Bam*HI and *Eco*RI, and then cloned into pUC19, followed by transformation of *E. coli* DH5α. A total of 303 clones were picked randomly and the DNA inserts were sequenced. These sequences could be grouped into 25 individual sequences; all the fragments were isolated more than ten times.

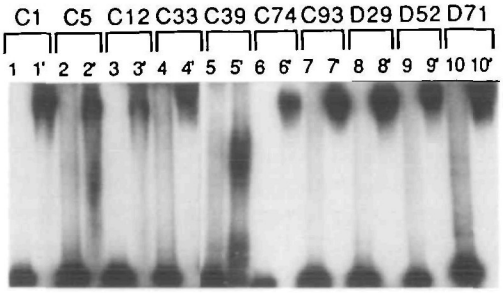

Fig. 4. **Gel mobility-shift analysis of 10 randomly selected PRIB-binding fragments.** Lanes 1-10, without PRIB; 1′-10′ with PRIB. DNA fragments containing *Bam*HI and *Eco*RI cohesive ends were end-labeled with [α-^{32}P]dCTP and Klenow fragment (TOYOBO). About 1 ng of each of the ^{32}P-labeled DNA fragments was incubated with approx. 10 ng of PRIB in 50 μl of the binding buffer [25 mM HEPES-KOH (pH 7.9), 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM $ZnCl_2$, 1 mM EDTA (pH 8.0), 300 mg/ml BSA, 100 μg/ml salmon sperm DNA] at room temperature for 15 min. The reaction mixtures were electrophoresed in 8% polyacrylamide gels using 0.5×TBE buffer. The gels were dried and then autoradiographed.

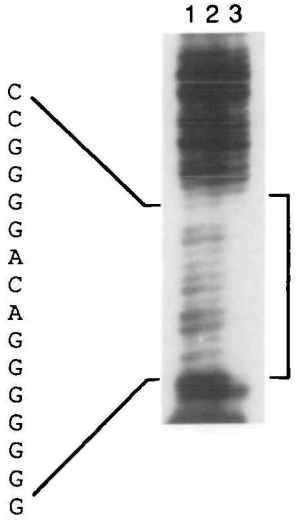

Fig. 5. **DNase I footprints of the PRIB protein on cloned fragment D29.** ^{32}P-end-labeled fragment D29 (approx. 1 ng) was incubated without PRIB (lane 1), or with 25 ng (lane 2) or 100 ng (lane 3) of PRIB under the conditions given in the legend to Fig. 3. To the reaction mixtures (50 μl each), DNase I, $CaCl_2$, and $MgCl_2$ were added to give final conc. of 1 μg/ml, 5 mM, and 5 mM, respectively, followed by further incubation on ice for 1 min. The DNase I reaction was stopped by the addition of 2 volumes of a buffer consisting of 100 mM Tris-Cl (pH 8.0), 100 mM NaCl, 10 mM EDTA (pH 8.0), 1% sodium lauroyl sarcosine, and 25 mg/ml sonicated salmon sperm DNA. The solutions were extracted with phenol/chloroform/isoamyl alcohol (25 : 24 : 1) and then precipitated with ethanol. The pellets were dissolved in loading dye [10 mM EDTA (pH 8.0), 0.25 mg/ml bromo-phenol-blue, 0.25 mg/ml xylene cyanol in formamide] and then loaded onto 8% denaturing polyacrylamide gels. After electrophoresis, the gels were dried and then autoradiographed.

GIBCO), followed by centrifugion. The precipitate was washed with Tris-buffered saline [25 mM Tris-HCl (pH 7.4), 137 mM NaCl, 5 mM KCl, 0.6 mM Na_2HPO_4] three times and then suspended in TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). This solution was boiled to release DNA from the complex. The DNA fragments recovered were amplified by PCR and then a half aliquot was subjected to another round of the selection procedure. After five enrichment cycles, the selected amplified DNA fragments were cloned into pUC19. A total of 303 cloned DNA fragments were subjected to sequence analysis.

The sequences of the 303 cloned DNA fragments could be grouped into 25 individual sequences, as shown in Fig. 3. The consensus sequence derived from these sequences comprised 16 bp, *i.e.* 5′ GGGGGGGACAGGANCC 3′, which is different from those of *An* AFLR, *An* ALCR, *An* NIRA, *An* UaY, *Nc* QA1F, *Sc* GAL4, *Sc* HAP1, *Sc* LEU3, *Sc* MAL63, *Sc* PPR1, and *Kl* LAC9 (see Table I). Among these binding sequences, the sequence of *Nc* QA1F seems to be slightly similar to that of *Le* PRIB.

To check that each of the selected sites was indeed a PRIB-binding site, 10 randomly selected individual sites were examined for complex formation in the presence of carrier DNA by means of a gel mobility-shift assay. The results shown in Fig. 4 indicate that PRIB binds to all the randomly selected fragments. To verify that PRIB actually recognizes and binds to the 16-bp sequence, we carried out DNase I footprinting analysis (*11*) of fragment D29, which contains the 16-bp sequence most similar to the consensus sequence. As shown in Fig. 5, PRIB protected the entire 16-bp sequence from digestion by DNase I.

In this study, we determined that the DNA-binding sequences of the Zn(II)2Cys6 zinc-cluster-containing PRIB protein (565 aa), which may play a role during fruiting-body development in the basidiomycetous mushroom, *L. edodes*, by the random binding-site selection method, the consensus sequence for the binding of PRIB being considered to comprise 16 bp, 5′ GGGGGGGACAGGANCC 3′, which is different from those of other yeast and fungal transcription factors.

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