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Function of mORF1 Protein as a Terminal Recognition Factor for the Linear Mitochondrial Plasmid pMLP1 from *Pleurotus ostreatus*

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The mitochondrial plasmid pMLP1 from a white-rot fungus, *Pleurotus ostreatus*, is a double-stranded DNA containing 381 bp terminal inverted repeat (TIR) whose 5'-ends are covalently bound by terminal proteins. The plasmid contains two major open reading frames (ORFs), encoding putative DNA and RNA polymerases, and a minor ORF encoding a small, highly basic protein. To identify the DNA binding activity that recognizes the TIR region of pMLP1, gel retardation assays were performed with mitochondrial extracts. A specific protein binding to a region between 123 and 248 nt within TIR was observed. We examined whether the gene product of mORF1 binds to this region specifically. *E. coli* cell extract which contains an overproduced mORF1 protein formed a complex specific to the region between 123 and 248 nt. Inclusion of mORF1 protein in the specific complex formed between *P. ostreatus* mitochondrial extract and TIR was confirmed by a supershift assay using polyclonal antibodies against the mORF1 protein. Our result suggests that the product of mORF1 may function as a terminal region recognition factor (TRF), recognizing an internal region in TIR.

Key words: *Pleurotus ostreatus*, linear mitochondrial plasmid, pMLP1, terminal inverted repeat (TIR), terminal protein, terminal recognition factor

Fungal linear mitochondrial plasmids have been reported to contain characteristic structures including terminal inverted repeats (TIRs), terminal proteins covalently linked to the 5' ends of DNA, and two major ORFs corresponding to RNA and DNA polymerases (6). The presence of TIR, terminal proteins, and plasmid-encoded DNA polymerase suggests a protein-primed mechanism of DNA replication (11). The general model for protein-primed replication mechanism as derived from studies of adenoviruses and bacteriophage 29 proposes that replication begins from either end of the linear genome, utilizing viral-encoded DNA polymerase (1, 7, 10, 12). The polymerase catalyzes the addition of the first deoxynucleotide into the terminal protein, which serves as the primer for elongation.

In addition to the terminal proteins and plasmid-encoded DNA polymerase, double-stranded DNA binding proteins that recognize the terminal regions play important roles in the initiation of replication (10). In adenovirus 2, two host-encoded nuclear transcription factors, NFI and NFIII, are required for efficient initiation of replication (8, 13). NFI, a 47 kDa protein, binds to a specific sequence within the TIR (8) and stimulates replication initiation by protein-protein interaction with the adenovirus-encoded DNA polymerase as well as by possible structural alteration of the origin (13). NFIII, a 90~95 kDa protein, equivalent to the Oct-1 transcription factor, also stimulates initiation of replication by possibly altering the structure at the origin (13). The p6 protein of bacteriophage 29 is required for efficient initiation of replication in *Bacillus subtilis* (9). The p6 protein recognizes the terminal fragment of the linear genome and binds cooperatively along the DNA, forming a nucleoprotein complex (9). In addition, the binding of p6 to relaxed circular DNA induced positive supercoiling, indicating a topological change in DNA (9).

The presence of the terminal recognition factor (TRF) in the fungal system has been suggested for plasmids in the killer yeast, *Kluyveromyces lactis*, due to its ability to bind terminal DNA fragments. In *K. lactis*, TRF1 was identified as a DNA binding protein that recognizes the terminal sequences of cytoplasmic plasmids, pGKL1 and pGKL2. Deletion analyses and DNase I protection experiments demonstrated that the activity recognizes a region

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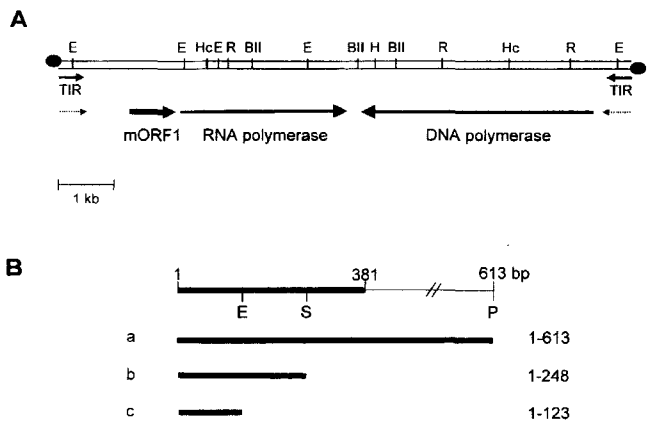

Fig. 1. Restriction map of pMLP1 plasmid (A) and the probes used in gel retardation assay (B). The location and orientation of putative ORFs are noted by arrows. The terminal protein is indicated by a solid dot. The terminal inverted repeats (TIRs) determined by nucleotide sequencing were shown by arrows at both DNA ends. E: *Eco*RI, Hc: *Hinc*II, R: *Eco*RV, BII: *Bgl*II, H: *Hin*dIII. The fragments used for gel retardation assay (B) contain nucleotides from the 5' end of TIR to 613 (a), 248 (b), or 123 (c) nt position.

between 107 and 183 nt within the TIR of pGKL1, and between 126 and 179 nt within the TIR of pGKL2. TRF1 of 16 kDa has been partially purified from yeast cytoplasmic extracts and found to be the product of ORF10 of pGKL2 (5). The amino acid sequence of the ORF10 gene product indicates that TRF1 is a highly basic protein. Comparison of TRF1 with other DNA-binding proteins known to recognize the terminal regions of linear DNAs, such as NF1 and NFIII for adenovirus DNA replication and p6 for ϕ29 DNA replication, suggests that TRF1 has a distinct mode of binding.

We previously isolated the linear mitochondrial plasmid pMLP1 from an edible mushroom, *Pleurotus ostreatus* (2, 3). The plasmid contains two major ORFs corresponding to RNA and DNA polymerases, and one minor ORF (mORF1) encoding a highly basic protein with no sequence match through database search (Fig. 1A). In this study we examined the possible function of the mORF1 protein as a terminal recognition factor.

Materials and Methods

Pleurotus ostreatus strains and culture condition

P. ostreatus NFFA2 was obtained from the National Federation of Forestry Association in Korea. Homogenized mycelium was grown in malt media (1% malt extract, 1% glucose, 0.5% yeast extract, 0.5% peptone) for 4~5 days at 30°C.

Bacterial strains, plasmids and culture media

Escherichia coli DH5α was used as a host for recombinant DNAs and grown in Luria-Bertani (LB: 1% NaCl, 1% tryptone, 0.5% yeast extract) medium containing 100 μg/ml ampicillin at 37°C. *E. coli* BL21 (DE3) pLysS was used to overproduce the recombinant proteins and grown in LB medium containing 34 μg/ml of chloramphenicol. The *E. coli* vector used for subcloning was pGEM3Zf(+) or 7Zf(+) (Promega). Expression vector pRSETc (Invitrogen) was used to overproduce the mORF1 protein in *E. coli*.

Preparation of mitochondria from *P. ostreatus*

Mitochondria were obtained as described by Kim *et al.* (2). Mycelia cultured in malt media were harvested and disrupted by an Omni-mixer at 5,000 rpm for 1 min. Cell debris were precipitated by centrifugation at 1,770 $\times g$ for 15 min. The supernatant was centrifuged at 21,000 $\times$ *g* for 25 min to obtain the mitochondrial pellet. The pellet was dissolved in TNE buffer (10 mM Tris-HCl, pH 8.0, 100 mM NaCl, 1 mM EDTA, pH 8.0) and stored at -20°C.

Gel retardation assay

Gel retardation assay was performed essentially as described by McNeel and Tamanoi (5). Extracts from *P. ostreatus* mitochondria or *E. coli* were prepared in TET (10 mM Tris-HCl pH 8.0, 1mM EDTA pH 8.0, 0.5% Triton X-100) buffer. The DNA fragments used as labeled probes (Fig. 1B) were the 613 bp right terminal fragment (fragment a, from end to *Pst*I site), the 248 bp terminal fragment (fragment b, from end to *Sna*BI site), and the 123 bp terminal fragment (fragment c, from end to *Eco*RI site). To label DNA ends, each fragment was subcloned into pGEM3Zf and digested with *Bam*HI/*Hin*dIII (fragments a and b) or *Eco*RI (fragment c). The eluted DNA fragments were labeled with [α-^{32}P]dATP by Klenow enzyme. The ^{32}P-labeled DNA probes (10 fmol) were incubated in the binding buffer (50 mM Tris-HCl pH 7.6, 5 mM $MgCl_2$, 1 mM DTT, 1 μg poly[dI-dC], 200 μg/ml BSA, and 10% glycerol) with various amounts of mitochondrial extracts from *P. ostreatus* for 20 min at 30°C in a total volume of 25 μl. When using *E. coli* extracts, 5 mM EDTA (pH 8.0) was added in the binding buffer. The reaction mixture was then resolved on a 5% polyacrylamide gel at 4°C in 0.5 $\times$ TBE buffer. The specificity of the retarded complex was demonstrated by competition using unlabeled pGEM3Zf DNA fragments digested with *Hpa*II as nonspecific competitors, or terminal fragments a, b, and c as specific competitors. In competition assay, the mitochondrial extracts were incubated with 50-fold molar excess of competing probes for 20 min at 30°C prior to the addition of the labeled probe.

Construction of pRSET-mORF1

A PCR product of 720 bp containing the full mORF1 was generated by a pair of mutagenic primers mlpN-1 (5' GTAAAGAAGAACATATGAAATATA 3', *Nde*I site underlined) and mlpC-3 (5' GATATGGATCCAATAAA-TTTATC 3', *Bam*HI site underlined). The resulting product digested with *Nde*I and *Bam*HI was subcloned into pRSETc (Invitrogen) to generate pRSETc-mORF1.

Preparation of antibody against mORF1 protein

E. coli BL21 (DE3) pLysS cells transformed with pRSETc-mORF1 were induced by adding 1 mM isopropyl-β-D-thiogalactoside (IPTG) for 3 h before harvest. After sonication, the insoluble fraction containing the expressed protein was purified according to the manufacturer's instruction (Novagen). Three mice (ICR) were immunized with about 20~30 μg of solubilized mORF1 protein, and were further injected at 2 week intervals for 6 weeks.

Supershift assay

To detect the presence of mORF1 protein in the complex, supershift assay was performed using antibody against mORF1. One μl of polyclonal antibody against mORF1 protein or 2 μl of control antibody against HRP (Horse-Radish Peroxidase) was incubated with 40 or 80 μg of mitochondrial extracts for 20 min at room temperature before adding the labeled probe of fragment b. The same binding conditions were used as described for gel retardation assays.

Results

Gel retardation assay with mitochondrial extracts from *P. ostreatus*

Gel retardation assay was performed using various lengths of terminal DNA fragments (Fig. 1B) and mitochondrial extracts. Both fragment which contains the entire TIR sequence and fragment b which contains TIR from 1 to 248 nt formed a retarded complexes (Fig. 2A, 2B). However, fragment c which contains TIR from 1 to 123 nt did not form a retarded complex (Fig. 2C). These results suggest that terminal recognition factor(s) may bind to a region between 124 and 248 nt in TIR. The specificity of the complex was examined by a competition assay using various competitors (Fig. 3). The retarded complex was competed specifically with either fragment a (lane 6) or b (lane 5), whereas it did not compete with *Hpa*II digested pGEM 3Zf (lane 3) or fragment c (lane 4). Therefore the bound complex is specific to the region between 124 and 248 nt in TIR.

Gel retardation assay with *E. coli* extract containing overproduced mORF1

The minor ORF1 from pMLP1 did not have any sequence match by a database search. The predicted product has a molecular mass of 30 kDa, abundant in basic residues. This property of the protein predicts its DNA-binding ability. The gene product of mORF1 was overproduced in *E. coli* using a pRSETc vector, following IPTG induction. The cell extract was incubated with terminal fragment b, and analyzed by gel

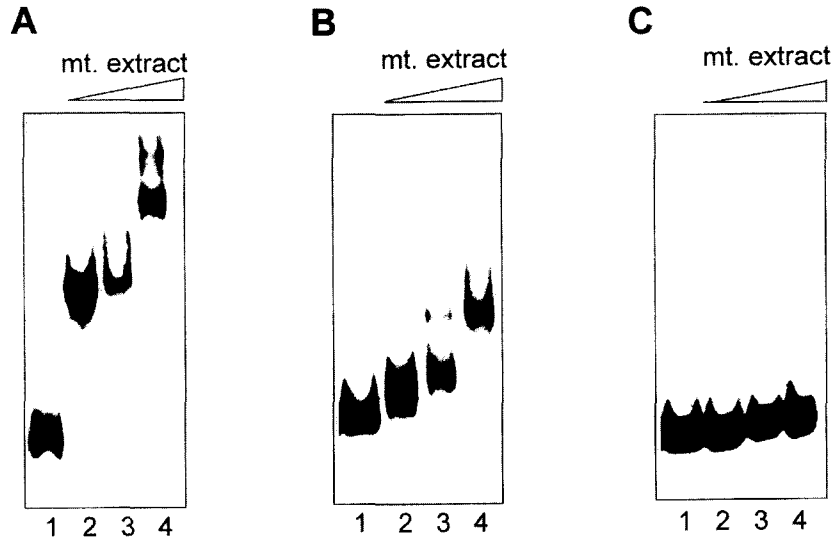

Fig. 2. Gel retardation assay of terminal fragments with *P. ostreatus* mitochondrial extracts. Ten fmol of ^{32}P-labeled fragment a (panel A), b (panel B), or c (panel C) was incubated with mitochondrial extracts (mt. extract) at 30°C for 20 min. The reaction mixture was electrophoresed on a 5% polyacrylamide gel at 4°C, followed by autoradiography. Mitochondrial extract containing 15 (lane 2), 30 (lane 3), or 60 (lane 4) μg of proteins was used in each binding reaction. The free probe was run in lane 1.

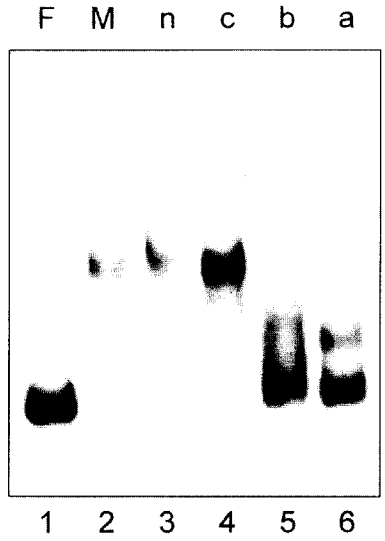

Fig. 3. Specificity of binding. Ten fmol of the labeled fragment b and 60 μg of mitochondrial extracts were used in each reaction. Before loading on PAG, competition was carried out with 50-fold molar excess of a nonspecific competitor (*Hpa*II digested pGEM3Zf (lane 3), fragment c (lane 4), fragment b (lane 5), or fragment a (lane 6). No competitor was included for the sample in lane 2. The free probe was shown in lane 1.

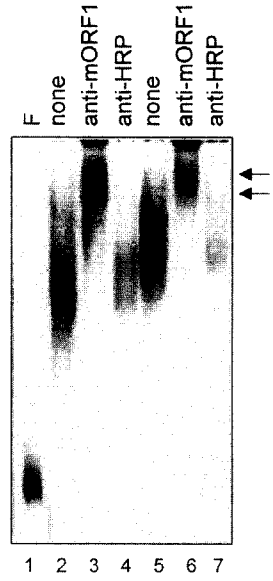

Fig. 5. The supershift assay using antibody against mORF1 protein. Mitochondrial extracts, 40 (lanes 2-4) or 80 μg (lanes 5-7), were incubated with probe b in the presence of antibodies against mORF1 (lanes 3 and 6) or horseradish peroxidase (HRP, lanes 4 and 7). Free probes (lane 1) or DNA binding complexes without antibody treatment (lanes 2 and 5) were shown. Supershifted complex bands are marked by arrows.

retardation assay (Fig. 4A). Ten μg of *E. coli* extract containing the mORF1 protein produced a retarded band with probe b (lane 6) whereas the control did not form any such complex (Fig. 4A). Fragment c did not form any mORF1-specific complex (Fig. 4B). This result indicates that the mORF1 protein binds specifically to the TIR region as observed for mitochondrial extracts.

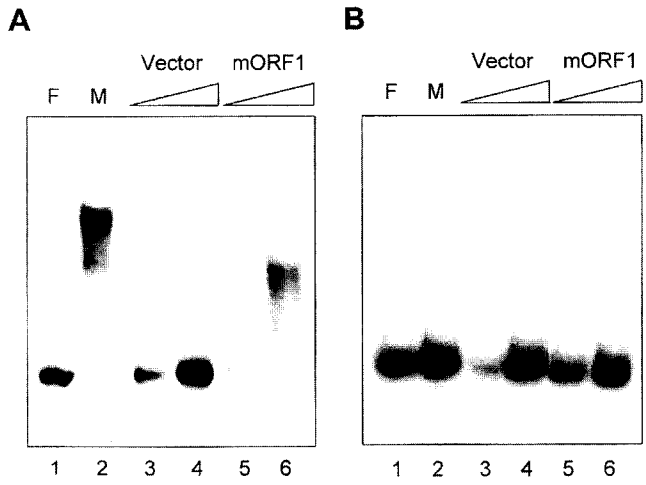

Fig. 4. Gel retardation assay of terminal fragments b (A) and c (B) with *E. coli* extracts containing overproduced mORF1 protein. Ten fmol of labeled fragments b or c were incubated with 60 μg of mitochondrial extracts (lane 2). Extracts from *E. coli* transformed with the control vector pRSETc (lanes 3 and 4) or the recombinant pRSET-mORF1 (lanes 5 and 6) were examined for retardation of ^{32}P-labeled fragment b (panel A) or c (panel B). The amounts of cell extracts used were 5 (lanes 3 and 5) and 10 μg (lanes 4 and 6), respectively. The free probe was shown in lane 1.

Supershift assay using antibody against mORF1 protein

To confirm the presence of the mORF1 protein in the retarded complex formed between mitochondrial extract and fragment b, we performed a supershift assay using an antibody against the mORF1 protein (Fig. 5). The supershifted bands were detected by antibody against the mORF1 protein (lanes 3 and 6), whereas a control antibody against horseradish peroxidase (HRP) did not further retard the complex (lanes 4 and 7).

Discussion

In killer yeast *K. lactis*, a factor that recognizes the termini of both pGKL1 and pGKL2, called the terminal region recognition factor 1 (TRF1), was identified (5). The presence of T-tracts within these two regions, but otherwise different dissimilar nucleotide sequences, suggests that TRF1 recognizes a common structural feature within the TIRs of the two plasmids. Comparison of the DNA sequences of the two protected regions of pGKL plasmids and pMLP1

revealed that there is a prevalence of T-stretches, although the actual DNA sequences are quite different. As judged from deletion analysis and DNase I protection assay, the TRF1 of pGKL recognized a region between 107 and 183 nt within TIR. In the case of pMLP1, at least a region between 124 and 248 nt within TIR was specifically recognized by a TRF-like factor(s) present in mitochondria. McNeel and Tamanoi (5) reported that ORF10 of pGKL1 encoded the TRF1 protein. The amino acid sequence of TRF1, deduced from the DNA sequence of pGKL1 ORF10, is highly polar and basic. The mORF1 protein of pMLP1 also contains highly basic residues (E.K.Kim, unpublished result). TRF1 was classified as a separate family of small, basic, DNA-binding proteins. In this respect the mORF1 protein seems to be a member of this family as TRF1 of pGKL1. Although NFI, NFIII, p6 and TRF1 are likely to play similar roles in DNA replication, the mode of their action may be different. In contrast to NFI and NFIII, TRF1 is a small protein that does not exhibit sequence-specific DNA binding. In contrast to p6, TRF1 is a basic protein and does not form nucleoprotein complexes characterized by a repeat pattern of protection and hypersensitivity to DNase I cleavage (9). Our result suggests that the mORF1 protein from *P. ostreatus* functions similarly to the TRF1 protein from *K. lactis*, as a small basic protein recognizing an internal region in TIR.

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