NOTES

Isolation, Characterization, and Expression of the Gene Encoding the β Subunit of the Mitochondrial Processing Peptidase from Blastocladiella emersonii

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A 2.3-kb BamHI-KpnI fragment was isolated from a partial genomic library and shown by nucleotide sequence analysis to contain the entire coding region of the gene encoding the β subunit of the Blastocladiella mitochondrial processing peptidase (β-MPP). The predicted β-MPP protein has 465 amino acids and a calculated molecular mass of 50.8 kDa. S1 nuclease protection assays revealed an intron, 209 bp in size, interrupting the coding region between the putative signal sequence and the mature protein. Northern blot analysis showed that β-MPP mRNA levels decrease significantly during B. emersonii sporulation, reaching basal levels in the zoospore stage. The amount of β-MPP protein, determined in Western blots, unlike its mRNA, does not vary significantly throughout the fungal life cycle.

The general mitochondrial processing peptidase (MPP) is a protein complex responsible for the processing of matrix-targeting signals from nucleus-encoded precursor proteins that are imported by the mitochondria. The MPP has been purified from the mitochondria of different organisms: yeast (30), Neurospora (9), rat liver (10, 12, 13, 21), potato tuber (2), and spinach leaves (5). In fungi and mammals, the MPP consists of two nonidentical but structurally related subunits, α-MPP and β-MPP, both of which are necessary for the processing of precursor proteins. MPP is a metalloendoprotease that requires divalent cations for activity and is inhibited by the metal chelators EDTA and o-phenanthroline (26). The two subunits share certain amino acid motifs, including a putative metal-binding sequence, HFLEH in the β subunit and HFLEK in the α subunit (12). MPP acts on hundreds of unrelated precursor proteins yet removes the presequence in a single specific cleavage reaction (23). Although there are no specific sequence motifs in mitochondrial import-targeting signals, these peptides have certain characteristic features. They are hydrophilic, rich in basic and hydroxylated amino acids, generally lacking acidic residues, and able to fold into an amphiphilic α-helix or β-sheet (24). They are usually between 20 and 35 residues long, and an arginine is found at position −2 or −3 relative to the cleavage site in most mitochondrial precursors from different species (20, 23, 24, 27, 28).

The aquatic fungus Blastocladiella emersonii is characterized by an interesting developmental cycle with well-defined stages: germination, vegetative growth, and sporulation (for a review, see reference 15). The cycle begins with the zoospore, a motile, uninucleated nongrowing cell which germinates rapidly and synchronously in the presence of nutrients. The germination process leads to the formation of the germling cell, which undergoes vegetative growth. During this stage, nuclear division is not accompanied by cell division, and so multinucleated cells, the sporangia, are generated. At any time during exponential growth, nutrient starvation induces another transitional stage, sporulation, which culminates in the intracellular formation of zoospores, which are then released to the medium. The zoospores contain a single giant mitochondrion, which is fragmented into several normal-sized mitochondria during germination in a process which is independent of protein synthesis (3). During sporulation, these multiple individual mitochondria fuse, giving rise to the huge single mitochondrion found in the zoospore (14).

The purpose of this work was to study the expression of the β-MPP gene throughout the B. emersonii life cycle in order to investigate possible variations during the drastic morphological changes experienced by the mitochondria in this organism.

Construction of the partial genomic library. A partial cDNA, 0.6 kb in size, encoding the carboxy-terminal portion of the Blastocladiella β-MPP was fortuitously isolated from an Agt11 library, during the screening procedure used to clone the Blastocladiella hsp60 cDNA. To clone the entire β-MPP gene, B. emersonii genomic DNA was digested with BamHI and KpnI, size fractionated by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized to the β-MPP cDNA labeled with 32P by random primed synthesis (6). A single band of hybridization in the region corresponding to 2.3 kb was observed. The DNA fragments from 2 to 4 kb were then excised from a similar agarose gel, electroeluted, and ligated to BamHI-KpnI-digested Bluescribe. After transformation into Escherichia coli, the resulting partial library was screened by colony hybridization with the β-MPP cDNA as probe.

DNA sequence analysis. The genomic fragment containing the β-MPP gene was subjected to restriction endonuclease digestion, and the various fragments obtained were subcloned into M13mp18 and M13mp19 for nucleotide sequence analysis by the dideoxynucleotide chain termination method (25) with the Sequenase DNA sequencing kit (Amersham).

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Characterization of the β-MPP gene from *B. emersonii*. A 2.3-kb BamHI-KpnI genomic fragment, containing the entire coding region of the β-MPP gene plus sequences upstream and downstream of it, was isolated from a partial genomic library. Nucleotide sequence determination showed a single open reading frame, interrupted by a small intron of 209 bp (Fig. 1).

The predicted amino acid sequence of the *Blastocladiella* β-MPP, encompassing 465 amino acids, has 65% identity and 79% similarity to the sequence of the β-MPP from *Neurospora crassa* (9) and 60% identity to the corresponding proteins from yeast (29) and from rat liver (11, 22). The putative metal-binding sequence (HXXEH), essential for the catalytic activity of the MPP (12), is conserved in the *Blastocladiella* protein.

**FIG. 1.** Nucleotide sequence of the *B. emersonii* β-MPP gene and deduced amino acid sequence. Capital letters indicate deoxynucleotides in exons or sequences upstream and downstream of the coding region of the gene; lowercase letters show the deoxynucleotides in the intron. The deduced protein sequence is shown below the nucleotide sequence. Endonuclease restriction sites are shown in boldface type. Nucleotide 1 denotes the A of the ATG of the initiator methionine. Residues preceding it are indicated by negative numbers. Transcription initiation sites predicted by primer extension analysis (see Fig. 3) are indicated (●). The putative helix-loop-helix transcription factor-binding motifs (CANNTG) are boxed. The underlined sequences are complementary to the oligonucleotides used for primer extension and S1 protection experiments. The arrows indicate the putative signal sequence cleavage sites. The putative metal-binding sequence (HFLEH) is shown in boldface type. The putative polyadenylation signal is doubly underlined.
3' end of the intron was determined with a 5'-end-labeled probe, which was prepared by labeling an 18-residue synthetic oligonucleotide (C-4) complementary to nucleotides (nt) 1310 to 1327 of the \( b\)-MPP gene with \( g^{-32P}\)ATP and T4 polynucleotide kinase (New England Biolabs). The labeled 18-mer was then annealed to a single-stranded DNA from M13mp19 containing a 1.2-kb \( \text{BamHI-PstI} \) fragment (see Fig. 2) from the genomic clone (coding strand) and extended with the Klenow polymerase (Boehringer Mannheim). The probe (33106 cpm) was ethanol precipitated with 50 \( \mu \)g of total RNA isolated from \( B.\ emersonii \) zoospores, and the nucleic acid pellet was resuspended in 28 \( \mu \)l of formamide plus 7 \( \mu \)l 40 mM PIPES buffer (pH 6.4) containing 400 mM NaCl and 1 mM EDTA. The annealing reaction was carried out for 3 h at 52°C; then the samples were diluted with 350 \( \mu \)l of 30 mM sodium acetate (pH 4.6) containing 1000 mM NaCl, 1 mM ZnSO\(_4\) and 20 \( \mu \)g of salmon testis DNA per ml and digested at 37°C for 30 min with 50 \( \mu \)l of S1 nuclease (Amersham). After digestion, the nucleic acids were analyzed by electrophoresis in 7 M urea–7.5% polyacrylamide gels followed by autoradiography. Sizing of the protected fragments was carried out by comparison with a sequencing ladder generated by using the 18-mer C-4 as primer and M13mp19 containing the \( \text{BamHI-PstI} \) fragment (coding strand). Figure 2A shows the protected fragment obtained, of 76 nt, which localizes the 3' end of the intron to position 251. A splice acceptor site (gtagC) can be found at this position, which conforms to the consensus for \( B.\ emersonii \) introns (4).

S1 nuclease protection assays were also carried out to determine the 5' end of the intron. In this case, a uniformly labeled probe was obtained by 5'-end labeling of the 18-mer C-4, which was then annealed to a single-stranded DNA from M13mp19 containing a 1.2-kb \( \text{BamHI-PstI} \) fragment (see Fig. 2) from the genomic clone (coding strand [Fig. 2]) and extended with Klenow polymerase. The probe was then digested with \( \text{PstI} \) at the restriction site present in the M13mp19 polylinker and located upstream of the \( \text{SmaI} \) site where the blunt-ended \( \text{HaeIII} \) fragment was originally cloned. The labeled probe, isolated after denaturing polyacrylamide gel electrophoresis and electrophoresis, was ethanol precipitated with 50 \( \mu \)g of total RNA isolated from \( B.\ emersonii \) zoospores and processed as described above for the determination of the 3' end of the intron. As shown in Fig. 2B, two protected fragments were obtained. The 68-nt fragment indicated the position of the 5' end of the intron, which corresponds to position +43 where there is a splice donor site (Ggtacg), which also follows the consensus for \( B.\ emersonii \) introns (4). The 76-nt fragment also obtained confirms the 3' end of the intron.
phobic environments, as determined with the NNPREDICT program (18).

**Primer extension mapping of the transcription start sites.**

To determine the transcription start sites of the *Blastocladiella b*-MPP gene, primer extension experiments were performed. An 18-nt primer (C-5), complementary to positions 110 to 127 of the *b*-MPP gene coding region, was 5’-end labeled with \([\gamma-32P]ATP\) and hybridized with either total RNA from *Blastocladiella* cells at 2 h of sporulation or yeast tRNA as a control. The hybrids were then extended with reverse transcriptase, as previously described (1). Multiple bands were detected in *Blastocladiella* RNA, whereas no bands were detected in the control (Fig. 3). The extension products were distributed in four groups (positions 75/76, 66/67, 63/64, and 38) relative to the adenine of the initiator methionine codon. Consistent with the presence of multiple transcription start sites, the 5’ noncoding region of *Blastocladiella b*-MPP gene revealed no TATA box or CCAAT box sequences (Fig. 1). No other characteristic features could be observed in the 5’ regulatory region, except for two putative core sequences (CANNTG; positions −291 to −286 and −39 to −34) for binding of helix-loop-helix transcription factors (19).

**Expression of the b-MPP gene at the RNA and protein level.**

The relative amount of *b*-MPP mRNA was determined in cells at different stages of *Blastocladiella* development. Total RNA was isolated, as described by Maniatis et al. (16), from synchronized cells at different times during the fungal life cycle. The RNA was subjected to electrophoresis under denaturing conditions and then transferred to a Hybond N+ membrane (Amersham), as previously described (1). Northern blot analysis, with the *b*-MPP cDNA as probe, showed a single hybrid-
A single intron which interrupts the coding region between the putative signal sequence and the mature protein was identified. From an evolutionary point of view, an intron positioned between the signal sequence and the mature protein could be a very good example of a protein encoded in the mitochondrial genome whose gene was transferred to the nucleus sometime after the endosymbiotic event (8). There are two possible sites for the processing of the signal sequence, if we consider the need for an arginine residue at position −2 relative to the cleavage site (20, 23, 24, 27, 28). The first putative cleavage site, which is located in the region preceding the intron, between Asn-13 and Val-14, results in a signal sequence of 13 amino acids, whereas the second site, located immediately after the 3′ splicing sequence, between Ser-16 and Leu-17, results in a signal sequence with 16 amino acids. This second cleavage site would produce a mature protein with the amino-terminal sequence Leu-Ala-Thr, which is identical to that determined for the mature N. crassa β-MPP (9).

The drastic morphological changes occurring in the mitochondria during the B. emersonii life cycle, which include fragmentation during the germination of a giant single mitochondrion present in the zoospores into several normal-sized mitochondria and the fusion of these multiple mitochondria during sporulation, giving rise to the huge single mitochondrion of the zoospore (3), led us to investigate possible changes in β-MPP mRNA and protein levels, throughout the B. emersonii life cycle. However, even though some change in the amount of β-MPP mRNA was observed during sporulation, no significant variation was detected in the level of β-MPP protein during the fungal life cycle. These results indicate that despite the profound alterations in morphology, mitochondria are still capable of importing proteins during all stages of B. emersonii development.

Nucleotide sequence accession number. The nucleotide sequence of the B. emersonii β-MPP gene has been submitted to the GenBank/EMBL Data Bank and assigned accession no. U41300.

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