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Biochimica et Biophysica Acta 1475 (2000) 119–124

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

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A silent antifungal protein (AFP)-like gene lacking two introns in the mould *Trichoderma viride*

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Received 18 October 1999; received in revised form 6 March 2000; accepted 17 March 2000

Abstract

In cells of the mould *Trichoderma viride*, the existence of an antifungal protein (AFP)-like gene consisting of 285 bp was confirmed by Southern analysis that genomic DNA of *T. viride* could hybridize with the cDNA of mature AFP of *Aspergillus giganteus* MDH 18894. Except for the absence of two introns, the nucleotide sequence of the AFP-like gene was identical to that of the AFP gene of *A. giganteus* in positions 336–479, 568–649, and 706–765. The AFP-like gene could not be transcribed into its mRNA in *T. viride* cells as examined by RT-PCR using total RNAs of *T. viride* as template. Furthermore, AFP could not be detected either directly from the culture medium of *T. viride* or by Western analysis. However, the AFP-like gene could be actively expressed like the cDNA of AFP in *Escherichia coli* cell. Recombinant AFP exhibited similar antifungal activity as native AFP.

Keywords: AFP-like gene; Intronless; *Trichoderma viride*

1. Introduction

A common feature of many genes in higher organisms is that they are encoded in discontinuous bits of coding DNA separated by intervening noncoding sequences (introns) (for review see [1]). In contrast to mammalian introns, introns of filamentous fungi are usually short, on average less than 100 bp [2]. The occurrence and position of introns within corresponding genes of different organisms provide a basis for speculation as to the origin of these DNA elements and the relationship between fungal genera [2].

The requirement of the intron for efficient cytoplasmic accumulation of mRNA, i.e., intron-dependent gene expression, has been demonstrated for many genes including those encoding mouse thymidylate synthase [3], plant alcohol dehydrogenase-1 [4], immunoglobin μ [5], ribosomal protein L32 [6], β-globin [7], purine nucleoside phosphorylase [8], and triosephosphate isomerase [9]. It has been proposed that the presence of introns can protect pre-mRNAs from degradation in the nucleus [10], facilitate polyadenylation and excision of an adjacent intron [7,9] as well as target mRNAs for export to the cytoplasm [11].

The characterization of an antifungal protein (AFP) gene from the imperfect ascomycete *Aspergillus giganteus* MDH 18894 has been reported, and sequence analysis shows that its open reading frame is interrupted by two small introns (89 and 56 bp) with conserved splice sites [12]. In this paper we report that in *Trichoderma viride* there is a silencing AFP-like gene lacking two introns that are present in *A. giganteus*. However, the AFP-like gene can be actively expressed in *Escherichia coli* cells.

2. Materials and methods

2.1. Culture of fungi

T. viride and *A. giganteus* MDH 18894 were cultured

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0304-4165/00/$ – see front matter
PII: S0304-4165(00)00047-7

separately under the same conditions at 30°C as described [13] in 1-l flasks containing 250 ml of culture medium composed of 1.5% beef extract, 2.0% peptone, 2.0% corn starch and 0.5% NaCl (w/v). The aeration and agitation for this fermentation were provided by a rotary shaker. After 3 days, the medium was filtered through eight layers of cheesecloth. Mycelia were collected for the extraction of DNA or RNA, and the medium for detection of AFP.

2.2. Isolation and sequencing of the AFP-like gene

Genomic DNA was isolated from the fresh mycelia of *T. viride* or *A. giganteus* by the method of Wnendt et al. [12]. Three primers were used for isolating the AFP-like gene. Primer 1, 5′-G GGA TCC ATG AAG TTC GTT TCT CTC GC-3′; primer 2, 5′-C GCG CAT ATG GCC ACA TAC AAT GGC-3′ and primer 3, 5′-G GAG CTC CTA GCA GTA GCA CTT C-3′ were designed according to the published nucleotide sequence of AFP gene of *A. giganteus* MDH 18894 (see Fig. 2B). The underlined sequences indicate the positions of the *Bam*HI, *Nde*I and *Sac*I sites used in subsequent cloning steps. Primers 1 and 3 were designed for amplifying the AFP precursor sequence, primers 2 and 3 for mature AFP. DNA amplification was performed using DNA polymerase from Promega, in GeneAmp PCR system 2400 (Perkin Elmer), under the following conditions: 94°C for 5 min; 94°C for 30 s, 58.5°C for 30 s, and 72°C for 30 s for 30 cycles; and 72°C for 3 min. After digestion with *Bam*HI and *Sac*I, the DNA fragments amplified with P1 and P3 were cloned into vector pUC19. Nucleotide sequence analysis was done with the T7 Sequenase v2.0 kit (Amersham, UK).

2.3. Isolation of RNA and RT-PCR amplification

Total RNAs were isolated from the fresh mycelia of *T. viride* or *A. giganteus* using the RNeasy kit (Qiagen), and then used directly as template in the RT-PCR reaction according to the instruction for use of AMV Reverse Transcriptase (Promega). Synthesis of first-strand cDNA was primed with primer 3 and the subsequent PCR amplification was performed with primers 2 and 3 under the above-mentioned conditions.

2.4. Southern blot analysis

Approximately 20 μg of genomic DNA of *T. viride* or *A. giganteus* was digested with either *Eco*RI or *Hin*dIII at 37°C overnight. The digested DNA was subjected to electrophoresis on a 1.0% agarose gel and blotted onto a nitrocellulose membrane. Subsequently the Southern blot was probed with 153 bp of cDNA encoding mature AFP of *A. giganteus*. The probe was radiolabeled with [α-^{32}P]-dATP using a nick translation system kit (Promega).

2.5. Western blot analysis

Total proteins from cultures or purified AFP were run on a 15% SDS-polyacrylamide gel and transferred to BA83 nitrocellulose membranes (Schleicher and Schüll Co.), then subjected to Western analysis with anti-AFP rabbit antiserum. The immunoreactive proteins were detected with ECL Western Detection Kit (Amersham, UK). Anti-AFP antibodies were raised in three New Zealand White rabbits by the method described below. After subcutaneous injections of 0.2 mg of native AFP in complete Freund's adjuvant (Sigma), further subcutaneous injections were administered 4 weeks later as four times 0.1 mg native AFP in incomplete Freund's adjuvant with intervals of 2 weeks between successive doses.

2.6. Construction of expression vector and expression of AFP in E. coli DE3 cells

The *Nde*I–*Sac*I fragments, amplified from the pUC19 vector containing the AFP-like gene or the AFP gene by primers 2 and 3, were inserted into a derivative of plasmid pET 24a(+) (Novagen). These new plasmids containing the AFP-like gene or the AFP gene were named pET-AFL or pET-AFP. *E. coli* DE3 cells were transformed with pET-AFL and pET-AFP, respectively. The transformed *E. coli* DE3 cells were grown in 250 ml of LB medium at 37°C to an $OD_{600\,nm}$ of 0.6–0.8. Expression was induced by the addition of IPTG to a final concentration of 1 mM, and the cells were shaken for a further 4 h at 37°C. After harvesting, the cells were washed with lysis buffer (2 mM EDTA, 50 mM Tris–HCl, pH 8.8), and suspended in 25 ml lysis buffer containing 100 μg/ml of lysozyme and 0.1% Triton X-100, and then disrupted by sonication. Cell debris was removed by centrifugation (20 000 × *g*, 15 min, 4°C). Ammonium sulfate was added to the supernatant to 75% saturation. After centrifugation, the pellet which contained the bulk of the endogenous bacterial proteins was discarded. The supernatant was dialyzed against water at 4°C. The dialyzed pool, which was filtered through a 0.22 μm filter and adjusted to pH 8.8 with 1 M NaOH, was loaded onto a CM-cellulose 52 column (2×10 cm) equilibrated with Tris–HCl buffer (50 mM, pH 8.8). After passing all of the supernatant, the column was extensively washed with the Tris–HCl buffer until the absorbance of the effluent was lower than 0.05 at 280 nm. The recombinant AFP was eluted as a single peak using 1.5 M NaCl.

2.7. Assay for antifungal activity

Antifungal activity of AFP was assayed against the pathogen *Fusarium moniliforme*. The inhibition of hyphal extension was done as described by Xu and Reddy [14]. The results were recorded by photography.

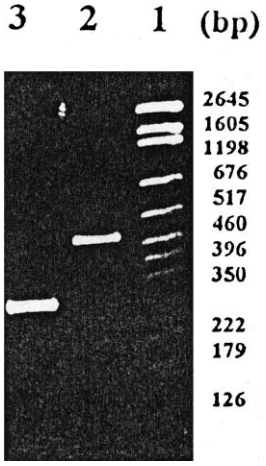

Fig. 1. Characterization of the AFP-like gene obtained by PCR through 5% polyacrylamide gel electrophoresis. Lane 1, pGEM markers; lane 2: template DNA from *A. giganteus*; lane 3: template DNA from *T. viride*.

3. Results and discussion

3.1. Isolation and characterization of the AFP-like gene of *T. viride*

A. giganteus and *T. viride* are imperfect ascomycetes, and belong to the same family Moniliaceae. The former produces an extracellular AFP (Fig. 1). We tried to seek the homologous AFP gene in *T. viride*. Unexpectedly, using the genomic DNA of *T. viride* as the template, an AFP-like gene was found by PCR with synthetic primers designed on the published nucleotide sequence of AFP gene (see Fig. 2B). Analysis of the AFP-like gene with 5% polyacrylamide gel showed that it was smaller than the AFP gene of *A. giganteus*. The nucleotide sequence of the AFP-like gene was determined by cloning it into vector pUC19. It was very interesting that, except for lacking two introns located in positions 480–568 and 650–705 in the AFP gene, the nucleotide sequence of the AFP-like gene was completely identical to positions 336–

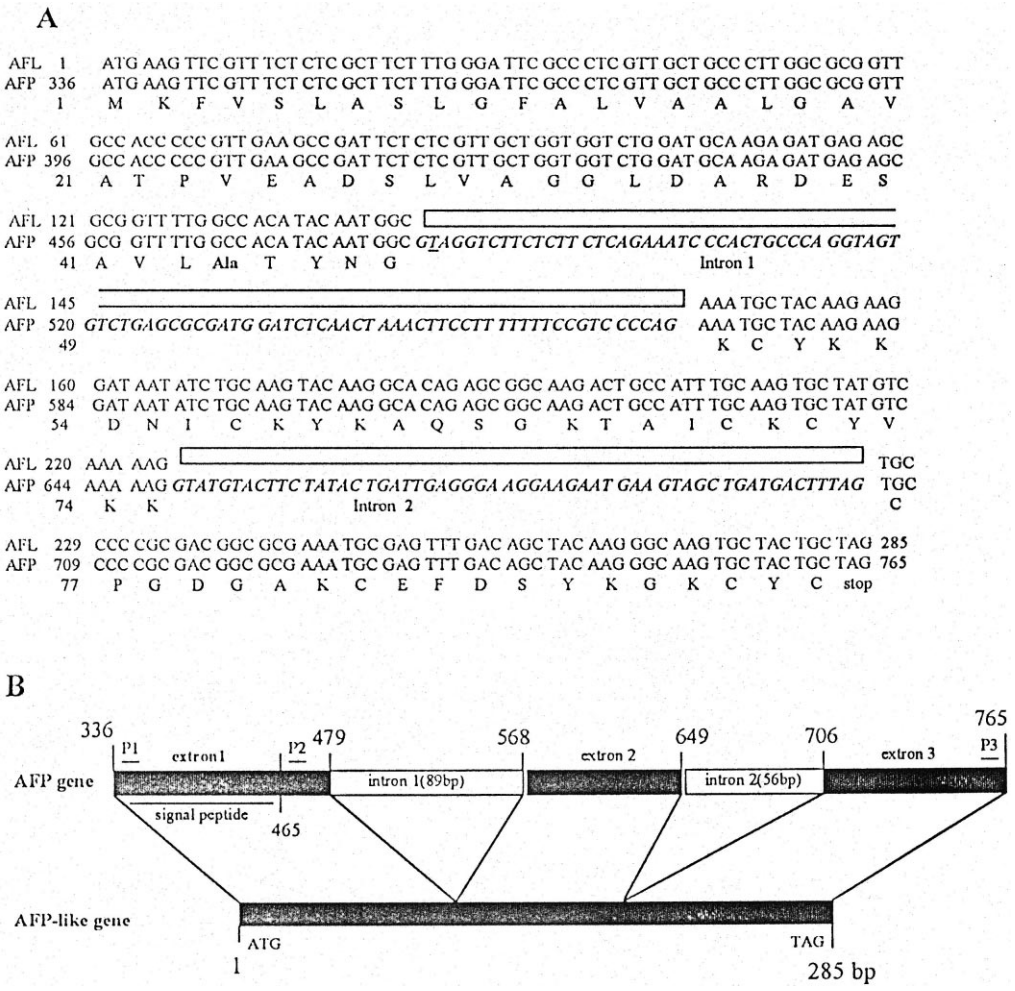

Fig. 2. (A) Alignment of the nucleotide sequence of the AFP-like (AFL) gene of *T. viride* and the AFP gene of *A. giganteus* [12]. The deduced amino acid sequence of the AFP precursor is listed below the corresponding nucleotide sequence. The N-terminal amino acid of the mature AFP is alanine (labeled with three letters, Ala). Italic letters represent nucleotide sequences of introns of *A. giganteus*. The rectangles demonstrate sequence gaps of the AFP-like gene. (B) Schematic structures of the AFP and AFP-like genes. P_1, P_2 and P_3 demonstrate primer 1, primer 2 and primer 3, respectively.

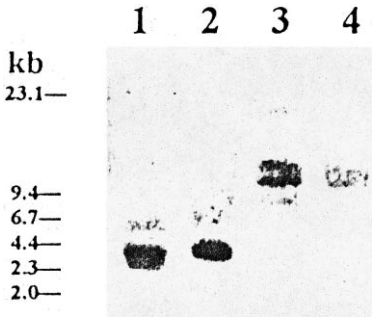

Fig. 3. Southern blot analysis of the AFP-like gene. Lanes 1 and 3: DNA of *A. giganteus* was digested with *Eco*RI and *Hin*dIII respectively; lanes 2 and 4: DNA of *T. viride* was digested with *Eco*RI and *Hin*dIII respectively. The DNA was separated on a 1% agarose gel. The numbers at the left margin indicate the position and size of fragments from *Hin*dIII-digested bacteriophage λ DNA.

479, 568–649, and 706–765 of the AFP gene (Fig. 2A). The nucleotide sequence of the AFP-like gene was virtually compatible with that of the cDNA encoding the precursor of AFP. Fig. 2B shows schematically the structural difference between the AFP gene and the AFP-like gene.

In order to further verify the result that the AFP-like gene exists in the genome of *T. viride*, a comparative Southern analysis was performed with genomic DNAs from the two moulds *T. viride* and *A. giganteus*. For this purpose, the cDNA encoding mature AFP obtained by RT-PCR from mRNA of *A. giganteus* was used as a probe. The hybridization results showed that this probe hybridized to an approximately 2.8 kb *Eco*RI fragment and a 9 kb *Hin*dIII fragment of *T. viride* (Fig. 3), indicating that *T. viride* really contained the AFP-like gene in its genome. In comparison, this probe also hybridized to a 2.7 kb *Eco*RI fragment and a 10 kb *Hin*dIII fragment of *A. giganteus* as reported [12].

3.2. The AFP-like gene did not transcribe into mRNA in *T. viride* cells

The question whether the AFP-like gene could be transcribed into mRNA in *T. viride* cells was examined by RT-PCR using total RNAs of *T. viride* as a template according to the method as described in Section 2. For comparison, the transcription of the AFP gene of *A. giganteus* was detected under the same conditions. The RT-PCR products were detected by 5% polyacrylamide gel electrophoresis stained with ethidium bromide. Fig. 4 shows that the AFP gene could be transcribed into its mRNA and the AFP-like gene could not. This result indicates that the AFP-like gene was silent in *T. viride* cells while the AFP gene was transcribed actively in *A. giganteus* cells.

3.3. No AFP was detected from culture medium of *T. viride*

When total proteins from the culture media of *A. giganteus* and *T. viride* were examined by electrophoresis on a 15% polyacrylamide-SDS gel, the extracellular mature

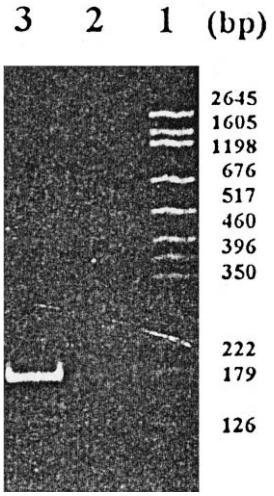

Fig. 4. Detection of mRNA by 5% PAGE of RT-PCR products. Lane 1: pGEM markers; lane 2: template RNA from *T. viride*; lane 3: template RNA from *A. giganteus*.

AFP from *A. giganteus* could be clearly seen on the gel while no related protein was detected from the culture medium of *T. viride* (Fig. 5A). Alternatively the proteins were transferred to BA83 nitrocellulose membranes for Western analysis by immunoblotting with anti-AFP serum. The result also showed that the AFP-like gene did not express in *T. viride* cells (Fig. 5B).

3.4. Expression of the AFP-like gene in *E. coli* cells

In order to test whether the AFP-like gene could be expressed like the cDNA of AFP in *E. coli* cells, the AFP-like gene encoding mature AFP was inserted between the *Nde*I and *Sac*I sites of the pET 24a(+) expression vector. For comparison, a control experiment in which the cDNA encoding mature AFP of *A. giganteus* was also inserted into the same site was carried out. Expression

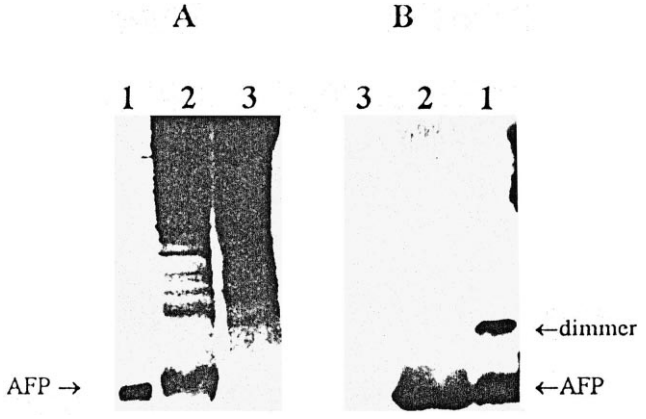

Fig. 5. Determination of AFP by 15% SDS–PAGE or Western blot analysis. (A) Silver-stained proteins. Lane 1: purified AFP; lane 2: proteins in culture medium of *A. giganteus*; lane 3: proteins in culture medium of *T. viride*. (B) Western blot of pattern (A) using polyclonal anti-AFP serum. Lanes 1–3 are the same as in (A).

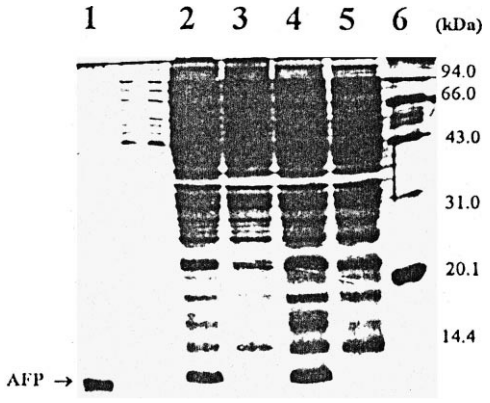

Fig. 6. Analysis of expression of the AFP-like gene in *E. coli* DE3 cells by 15% SDS–PAGE. The transformed bacterial cell was induced by adding 1 mM IPTG and incubated at 37°C for 3 h. Lane 1: pure AFP; lanes 2 and 3, the clarified crude extract before and after induction of transformed cells containing the AFP gene, respectively; lanes 4 and 5: the clarified crude extract before and after induction of transformed cells containing the AFP-like gene; lane 6: protein markers, the molecular masses are indicated in the right margin.

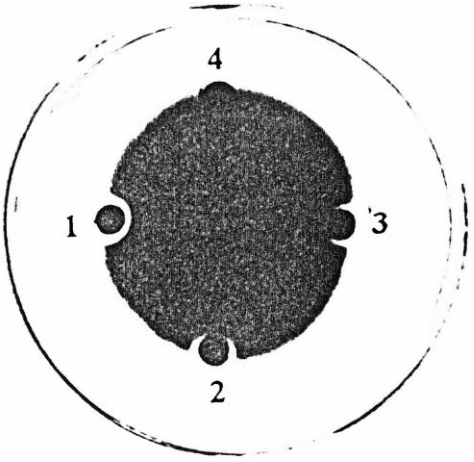

Fig. 8. Assay of antifungal activity of recombinant AFPs. Direct inhibition of the hyphal growth of the pathogen *F. moniliforme* by purified recombinant AFPs. Disk 1 contains 2 μg of pure AFP; disk 2 contains 2 μg of recombinant AFP of the AFP-like gene; disk 3 contains 2 μg of recombinant AFP; disk 4 is a water blank (control).

of mature AFP was monitored in the soluble fraction of *E. coli* cells by SDS–PAGE analysis. It was ascertained in this way that the recombinant AFPs were produced in the soluble protein (Fig. 6). Analyses of the purified recombinant AFPs by 15% SDS–PAGE and Western blotting indicated that the molecular mass and immunity activity of the recombinant AFPs were identical to that of native AFP (Fig. 7). The inhibitory activity of recombinant AFPs against the growth of the pathogen *F. moniliforme* was also similar to that of native AFP (Fig. 8). These results suggest that the AFP-like gene encodes active AFP.

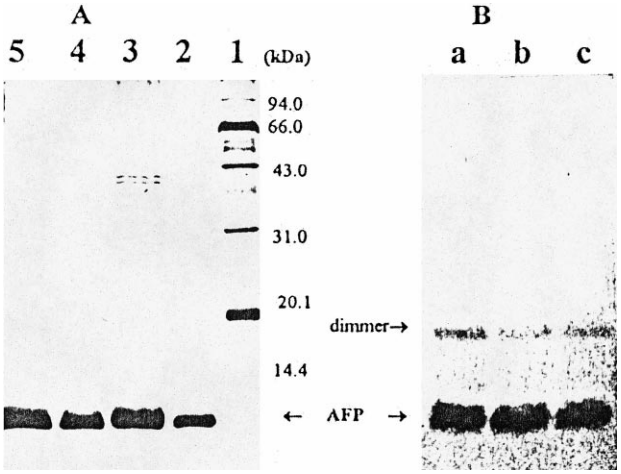

Fig. 7. Characterization of the purified recombinant AFPs by 15% SDS–PAGE or Western blot. (A) Pattern of silver-stained proteins. Lane 1: molecular weight markers; lane 2: pure AFP; lane 3: supernatant after ammonium sulfate precipitation; lane 4: purified recombinant AFP; lane 5: purified recombinant AFP of AFP-like gene from CM-cellulose 52 chromatography. (B) Western blot analysis of purified recombinant AFPs using polyclonal anti-AFP serum. Lane a: pure AFP; lane b: purified recombinant AFP; lane c: purified recombinant AFP of the AFP-like gene.

The experimental results show that in the genome of *T. viride* there does indeed exist a silent AFP-like gene lacking two introns that are present in the AFP gene of *A. giganteus*. However, the AFP-like gene could be expressed in *E. coli* cells producing the active AFP suggesting that the AFP-like gene has the character of an encoding gene. This interesting finding raised two questions that remain to be investigated. First, why did the AFP-like gene become silent in *T. viride* cells? Secondly, is the expression of the AFP-like gene intron-dependent? Most filamentous fungal genes possess introns while the equivalent genes of *Saccharomyces cerevisiae* do not. However, these intron-free yeast genes are still functional [2]. Besides introns, many factors such as the regulatory sequences of the 5′ upstream and 3′ downstream regions of the gene which have not been sequenced might probably cause AFP-like gene silencing.

Acknowledgements

The authors thank Dr. Norbert Ulbrich, Universitätsklinikum Charité, Medizinische Universitätsklinik III, AG Experimentelle Rheumatologie und Biochemie, Germany, for his gift of *Aspergillus giganteus* MDH 18894. This work is supported by two grants from The Natural Science Foundation of China and two grants from Academia Sinica.

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