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Genomic Organization of *ancop* Gene for α-COP Homolog from *Aspergillus nidulans*

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We have cloned a α-COP homolog, *ancop*, from *Aspergillus nidulans* by colony hybridization of chromosome specific library using α-COP homologous fragment as a probe. The probe DNA was amplified with degenerated primers designed by comparison of conserved region of the amino acid sequences of *Saccharomyces cerevisiae* α-COP, *Homo sapiens* HEP-COP, and *Drosophila melanogaster* α-COP. Full length cDNA clone was also amplified by RT- PCR. Comparison of genomic DNA sequence with cDNA sequence obtained by RT-PCR revealed 7 introns. Amino acid sequence similarity search of the anCop with other α-COPs gave an overall identity of 52% with *S. cerevisiae*, 47% with human and bovine, 45% with *Drosophila* and *Arabidopsis*. In upstream region from the transcription start site, a putative TATA and CAAT motif were also identified.

KEYWORDS: *Aspergillus nidulans*, α-COP, Genomic structure, Sequence homology

Eukaryotic intracellular transport between membrane-bound compartments of the secretory pathway is essential for cell growth and maintenance (Kreis and Pepperkok, 1994). This mechanism is involved in the formation of coated vesicles that must efficiently capture and package cargo molecules, and then target, dock, and fuse with appropriate acceptor compartment (Rothman and Orci, 1992; for reviews see Pryer *et al.*, 1992).

Two types of coated vesicle systems, clathrin coated vesicles and non-clathrin coated vesicles, are relatively well known. The clathrin coated vesicles are known to be derived from the plasma membrane and from the trans-Golgi network (Pearse and Robinson, 1990). The non-clathrin coated vesicles are known to regulate protein transport between the intermediate compartment and Golgi apparatus (Pepperkok *et al.*, 1993), and in intra-Golgi transport (Ostermann *et al.*, 1993). To date, two types of non-clathrin coats, COPI and COPII, have been identified (Schekman and Orci, 1996). COPI is composed of a small GTPase, ARF, and a multimeric complex known as coatomer, a conserved cytosolic 700 kDa protein complex consisting of seven subunits: α-, β-, β'-, γ-, δ-, ε-, and ζ-COP (reviewed in Rothman, 1996). COPI vesicles have been proposed to mediate intra-Golgi transport as well as ER-to-Golgi transport (Rothman and Orci, 1992; Pepperkok *et al.*, 1993). A second type of coat, COPII, is comprised of the Sec23p/Sec24p and Sec13p complexes, the small GTP-binding protein Sar1p and participates in transport of cargo molecules from ER to Golgi (Barlowe, 1995).

The major component of the fungal cell walls are mannoprotein, β-glucan, and chitin. Cell walls are shaped during morphogenesis and many proteins involved in wall biogenesis play a decisive role in morphogenesis of the fungi. For example, *Aspergillus nidulans* mutants that are defective in the synthesis of chitin exhibit excessive swelling and lysis of conidia (Borgia and Dodge, 1992). Recently, we have found that mutation in *Saccharomyces cerevisiae* α-COP homologous gene, *SOO1* is responsible for defect in cell wall biogenesis caused by decrease in β-1,3-glucan synthase activity (Lee *et al.*, 1999) and that Soo1p may contribute for maintenance of cell wall integrity by modulating the intracellular translocation and glycosylation of cell wall proteins (Lee, 2000). If the α-COPs in *S. cerevisiae* and *A. nidulans* were functionally conserved like other essential proteins in eukaryotes, we can speculate that α-COP may also play a role in cell wall biogenesis in *A. nidulans*.

The involvement of *α-cop*(*sod*^{VI}C) in germination and hyphal elongation in *A. nidulans* have been reported (Whittaker *et al.*, 1999), however genomic organization and function of the *α-cop* in *A. nidulans* have not been fully elucidated. Although the cDNA sequence of *sod*^{VI}C (GenBank accession No. AF053883) was reported by Whittaker *et al.* (1999), they did not clone a full length cDNA but cloned 5'-truncated forms of cDNA. In addition, the complete cDNA sequence of *sod*^{VI}C registered in GenBank was determined by the sequence comparison between previously known eukaryotic α-COP sequences and 5'-truncated cDNAs for Sod^{VI}C. The authors claimed that they have cloned genomic DNA for Sod^{VI}C, nevertheless no direct evidences for genomic structure of α-COP gene including introns and 5'-flanking region were presented. Therefore informations on the genomic organization of *α-cop* and its product will let us understand *in vivo* function of anCop during morphogenesis in *A. nidulans*. In this study, we cloned the genomic DNA and cDNA of *α-cop* gene of *A. nidulans* (*ancop*) by colony hybridization and RT-PCR, respectively, and per-

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formed sequence analysis and phylogenetic analysis of the *ancop*.

Materials and Methods

Strains and culture conditions

Chromosome-specific recombinant DNA libraries from *Aspergillus nidulans* (Brody *et al.*, 1991), and wild type strain of *A. nidulans* FGSC4 (Glasgow) were obtained from Fungal Genetic Stock Center (University of Kansas Medical Center, Kansas City, Kansas, USA). *E. coli* DH5α was used as a host for plasmid propagation.

E. coli strains were grown in a standard LB medium (0.5% yeast extract, 1% bacto-tryptone, 1% NaCl) at 37°C, supplemented with ampicillin (50 μg/m*l*) if neccesary (Sambrook *et al.*, 1989). X-gal plates were prepared by addition of IPTG (0.1 mM) and 5-bromo-4-chloro-3-β-D-galactoside (X-gal, 0.002%) to LB containing ampicillin. *A. nidulans* FGSC4 was grown at 30°C on CM (0.15% yeast extract, 0.15% casamino acid, 1% glucose, 20 m*l* minimal salt stock solution, 1 m*l* vitamin solution).

Nucleic acids manipulation and library screening

All the recombinant DNA techniques were as described by Sambrook *et al.* (1989). Plasmid DNA was prepared by using Wizard Plus SV Miniprep Kit (Promega). *A. nidulans* genomic DNA was isolated as described by Yelton *et al.* (1984). For RNA purification, *A. nidulans* culture was harvested by filtration through Miracloth (Calbiochem) and frozen rapidly with liquid N_2 followed by being ground to a powder with mortar and pestle. The broken cell mass was used for the purification of RNA and then the purified RNA was used for the cDNA synthesis by the standard methods (Sambrook *et al.*, 1989).

PCR was performed in a total volume of 50 μl, containing 100 ng template DNA, 1 μM of each primer, 2 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3) and 5 units of Taq DNA Polymerase (Takara). Thirty five amplification cycles were carried out and each cycle consists of 1 min at 94°C, 1 min at 50°C, 1.5 min at 72°C. The products were separated on a 0.8% agarose gel. Appropriate DNA band was purified and cloned into the pGEM-T vector.

For library screening, colonies of chromosome specific library were picked and cultured on the LB ampicillin media. Colonies were blotted onto on a Hybond N+ membrane and hybridized for 4 h with probe. The DNA probe for Southern blot analysis and library screening was labelled by digoxigenin using DIG system (Boehringer Mannheim) and detection was carried out with chemiluminescent immununoassay.

Nucleotide sequence analysis

Nucleotide sequences of both strands were determined by dideoxynucelotide chain termination method (Sanger *et al.*, 1977) using the Sequenase 2.0 DNA sequence kit (United States Biochemical Corp.) and an automatic DNA sequencer (AB137, ABI, USA). The analysis of DNA sequence databases was performed using the BLAST algorithm (Altschul *et al.*, 1990).

Phylogenetic analysis of anCop

For amino acid sequence comparison, the α-COPs from the GenBank were included. Amino acid sequence similarity was determined by BLAST program through NCBI database. Multiple alignment of amino acid sequences was performed by the CLUSTAL X (Thompson, *et al.*, 1997). The phylogenetic tree was generated by the Neighbor-Joining method using the PHYLIP phylogenetic inference package (Felsenstein, 1996). The resulting tree topologies were evaluated by a bootstrap method and bootstraps were performed for 1000 times.

Results and Discussion

Cloning of *α-cop* gene from the genomic library

PCR was performed with *Aspergillus nidulans* genomic DNA and degenerated primers Acop1 (5'-CARCTNTGGGAYTAYCGNATG-3': R:A/G, N:A/T/G/C, Y:C/T) and Acop4 (5'-RCANGTRTCNACYTCCCANGC-3') complementary to

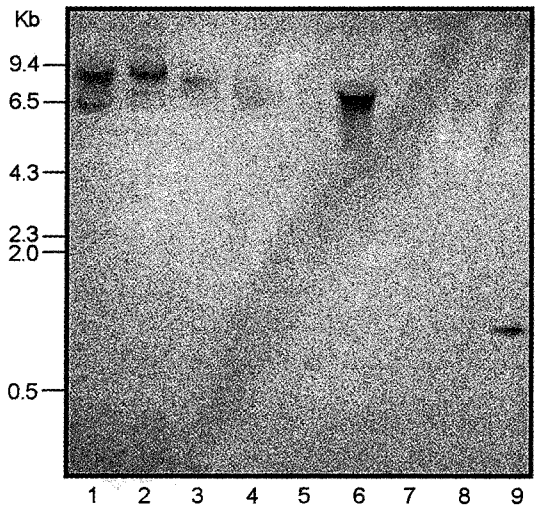


Fig. 1. Southern hybridization analysis of 8 pools of *A. nidulans* genomic library, each pool consisting of approximately 300 cosmids. DNAs of each pools were digested with *EcoR*I and separated by electrophoresis on a 0.8% agarose. Following transfer to a nylon membrane, the blot was hybridized with digoxigenin labelled 0.7 kb *EcoR*I-*Pst*I fragment in pAN700 as a probe. Lanes 1-8; each lane corresponds to a genomic library pool grouped by chromosome number I, II, III, IV, V, VI, VII, and VIII, respectively. Lane 9; the *EcoR*I-*Pst*I fragment from pAN7OO was used as a control.

the conserved amino acid sequences of α-COP in other eukaryotes. PCR product with the size of approximately 0.7 kb was cloned in the pGEM-T vector, yielding pAN700. After sequence determination, the *Eco*RI-*Pst*I fragment in pAN700 was used as a hybridization probe. Eight of chromosome specific library were made by genomic library purchased from the FGSC, each consisting of approximately 300 cosmids. Southern blot analysis of 8 pools of *A. nidulans* genomic library showed strong hybridization signal with the genomic library specific to chromosome VI and weak signal with from chromosome I, II, III, and IV (Fig. 1). The pools which showed weak signal might contain other components of COPI. This postulation can be supported by relatively high level of similarity, more than 40%, between *A. nidulans* α-COP and β-COPs from other organisms, such as *Saccharomyces cerevisiae* (GenBank Accession No. U11237), *Homo sapiens* (GenBank Accession No. X70476), *Drosophila melanogater* (GenBank Accession No. O62621), and *Arabidopsis thaliana* (GenBank Accession No. AB012247) (data not shown). When the genomic library for chromosome VI of *A. nidulans* was screened by colony hybridization, four positive clones (62D8, 62D12, 63F2, 63G3) were identified (Fig. 2). Among them, 62D12 clone was used for further analysis. Southern blot analysis with the 62D12 clone showed about 6.0 kb *Eco*RI fragment, 11 kb *Hind* III fragment, and 6.5 kb *Bgl*II fragments hybridized to the probe. The 6.0 kb *Eco*RI fragment was subcloned into the pBluescript II SK(+), yielding pE32 which was subjected to restriction enzyme mapping (Fig. 3) and nucleotide sequence determination. Comparison of nucleotide sequence of the DNA insert in the pE32 with that of *A. nidulans sod-*^{VI}*C* gene (Whittaker *et al.*, 1999) revealed that the *α-cop* gene fragment in the pE32 had 3'-truncation. Therefore, another set of primers ncopA (5'-ATGCTCACCAAGTTTGAGTC-GAA-3') and ncopB (5'-TTAGAGTTGGCTAGGGACAT-3') were designed for PCR. Full length of *α-cop* amplified by PCR was cloned in the pGEM T- vector, yielding pNcop10.

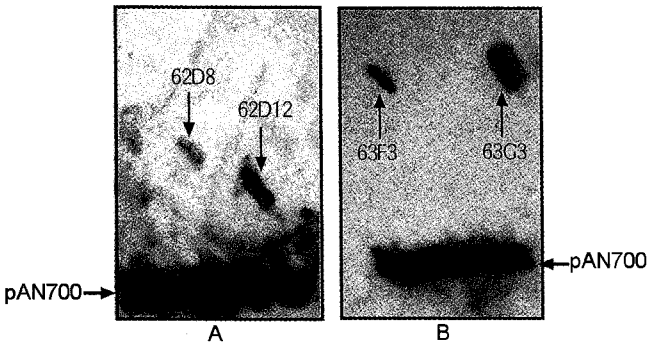


Fig. 2. Colony hybridization of *A. nidulans* chromosome specific genomic library with the *Eco*RI-*Pst*I fragment from pAN7OO as a probe. pAN700 is a positive control.

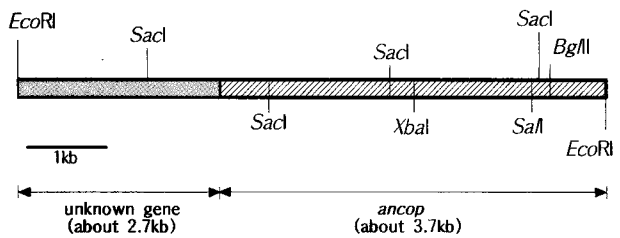


Fig. 3. Restriction map of pE32. It was digested with various restriction enzymes and analysed by Southern hybridization using digoxigenin labelled 0.7 kb *Eco*RI-*Pst*I fragment in pAN700 as a probe.

RT-PCR cloning of cDNA

Since only 1.7 kb of 5'-truncated form of cDNAs for Sod-^{VI}C, instead of full length cDNA, was available from Dr. Assinder's group (University of Wales, UK), we performed RT-PCR to obtain N-terminal part of the *α-cop* cDNA using new set of primers, ncopA and crt (5'-GCCTTGTAGGTG-GCAACAAT-3') and total RNA of *A. nidulans* as a template. Resulting a 2.5 kb RT-PCR product was cloned into the pGEM T-vector, named pA2 and nucleotide sequence of the RT-PCR product was confirmed. Two kb of *Not*I-*Bst*EII fragment in the pA2 was ligated with the 5'-truncated form of *α-cop* cDNA in pBluescript obtained form Dr. Assinder's group, yielding pNCCOP2.

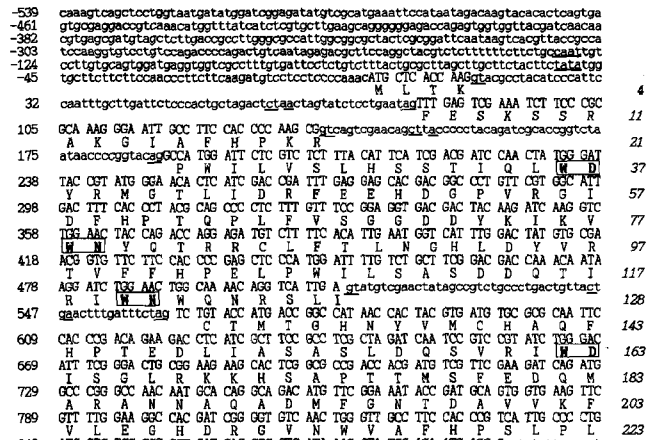
Genomic organization of α-COP gene

Sequence determination and analysis of genomic DNA of the *ancop* gene revealed the presence of a single open reading frame (ORF) of 3,615 bp encoding a protein of 1,205 amino acid with calculated molecular weight of 135 kDa as previously reported (Fig. 4). Sequence comparison of full length cDNA with genomic DNA localized 7 introns of which existence was previously mentioned (Whittaker *et al.*, 1999). The length of introns ranged from 53 to 71 bp and each intron contained a 5' splice site, GT, a 3' acceptor site, AG, and a internal lariat consensus sequences, CTNA (Fig. 4). A putative TATA and CAAT motif were identified upstream of putative transcriptional start site.

Amino acid sequence analysis of anCop

The comparison of the amino acid sequence of anCop with *S. cerevisiae* α-COP, *Drosophila* α-COP, human HEP-COP, and *A. thaliana* α-COP revealed significant identity, 52% with *S. cerevisiae*, 47% with human (*Home sapiens*) and bovine (*Bos primigenius*), 45% with fruit fly (*Drosophila melanogaster*) and *Arabidopsis thaliana* (Fig. 6).

The degree of amino acid homology with other α-COPs is highest in the N-terminal region of anCop containing six copies of WD40 repeat (van der Voorn and Ploegh, 1992), which is conserved sequence motif and is predicted to fold into a structure composed of β-strands and turns (Neer and



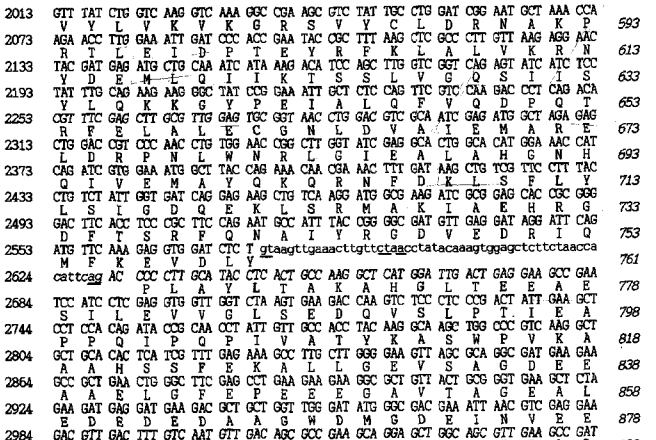


Fig. 4. The nucleotide sequence of *ancop* and its deduced amino acid sequence. Putative TATA and CAAT boxes in the 5'-non coding region and 5' and 3' splicing site and internal intron consensus sequences are underlined. The boxes in amino acid sequence represent the WD40 motif.

Smith, 2000). It has been also proposed that the WD40 motif interacts with proteins containing tetratricopeptide repeats, some of which are involved in protein transport (Harrison-Lavoie *et al.*, 1993). So, this repeated motif may be involved in protein interaction, e.g. acting as binding sites for contact with other proteins and membrane. The N terminal domain of *S. cerevisiae* α-Cop (scACop) consists of six WD40 repeats and is implicated in interaction with KKXX motif (Letourneur *et al.*, 1994). The comparison of N-terminal region of scACop and anCop revealed almost complete identity (Fig. 5).

Recently, we found that an amino acid substitution in the fifth WD40 repeat of the scACop is responsible for the temperature-dependent osmofragile phenotype of the *S. cerevisiae* mutant LP0353 caused by the defect in cell wall biogenesis at non-permissive temperature, 37°C (Lee *et al.*, 1999; Lee, 2000). Interestingly enough our recent unpublished results also showed that temperature-sensitive phenotype of *A. nidulans sod*^{vi}*C* mutant at 42°C could be rescued by the addition of osmotic stabilizer, such as 1.0 M sorbitol, to the medium. Although a definitive answer on this issue

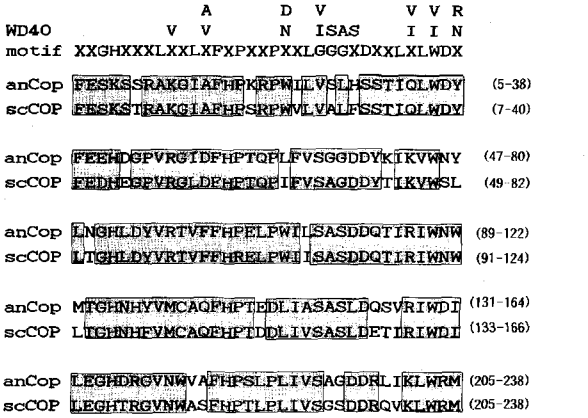


Fig. 5. Sequence alignment of anCop and *S. cerevisiae* α-COP with a consensus sequence of the WD40 motif. scCop: *S. cerevisiae* α-COP.

will need further experimental evidences such as analysis of cell wall components and activity assay of the enzymes for

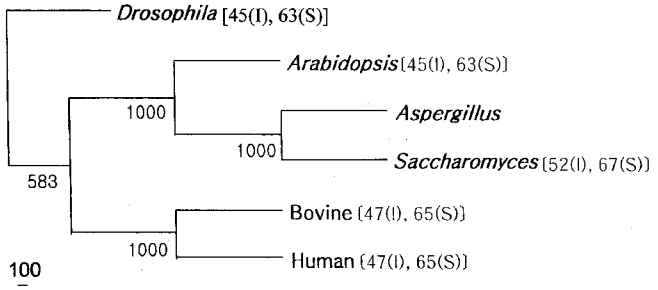


Fig. 6. Phylogenetic relationship among the α-COP proteins in various organisms. Numbers below branches are bootstrap percentages on the basis of 1000 replicates. Values in parenthesis indicate amino acid identity (I) and similarity (S) of various α-COPs with anCop. GenBank Accession Numbers for *Arabidopsis thaliana*, *Aspergillus nidulans*, bovine (*Bos primigenius*) *Drosophila melnogaster*, Human (*Homo sapiens*), and *Saccharomyces cerecvisiae* are AC006841, AF053883, X96768, AJ011114, U24105, and Z46617, respectively.

cell wall synthesis, our recent observations strongly suggest the possible involvement of anCop in cell wall boigenesis of *A. nidulans* as the case of *S. cerevisiae*.

Result form the phylogenetic analysis of anCop with other α-COPs (Fig. 6) reveled high level of similarity between scAcop and anCop. This result may also support the assumption that these proteins are related and fulfil analogous function in the cell. We will examine whether expression of the *ancop* of *A. nidulans* and the *SOO1*/α-*COP* of *S. cerevisiae* could suppress the temperature-dependent osmofragility of *S. cerevisiae soo1-1* mutant and of *A. nidulans* sod^{VI}C mutant, respectively.

In summary, we reported here the genomic organization of the gene for *A. nidulans* α-COP homolog and amino acid sequence analysis of its product, anCop. Further experiments such as nucleotide sequence analysis of $sod^{VI}C$ mutant allele, analysis of cell wall composition, and activity assay of cell wall synthesizing enzymes will give us valuable informations on the function of anCop in cell wall biogenesis in *A. nidulans*.

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