Gene Arrangement and Sequence of the 5S rRNA in *Filobasidiella neoformans* (*Cryptococcus neoformans*) as a Phylogenetic Indicator

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We cloned the 5S rRNA gene and determined its organization in the four genes encoding rRNAs in a ribosomal DNA repeat unit of *Filobasidiella neoformans*, the teleomorph of *Cryptococcus neoformans*. The 5S rRNA gene contained 118 nucleotides and was located 1 kb upstream from the 18S rRNA gene within the 8.6-kb fragment of the ribosomal DNA repeat unit. The sequence of the 5S rRNA gene from *F. neoformans* was more similar to the sequence of the 5S rRNA gene from *Tremella mesenterica* than to the sequences of the 5S rRNA genes from *Filobasidium* species. The arrangement of the rRNA genes in *F. neoformans* closely resembles the arrangement of the rRNA genes in mushrooms such as *Schizophyllum commune*, *Agaricus bisporus*, and *Coprinus cinereus* in that the 5S rRNA-coding region not only is located within the repeat unit that encodes the other rRNAs but also is transcribed in the same direction as the other rRNA genes. This is the first description of the arrangement of rRNA genes in a species belonging to the Heterobasidiomycetes.

In 1989, the gene cluster encoding rRNAs in a strain of *Cryptococcus neoformans* was cloned by Restrepo and Barbour, who used a ribosomal DNA (rDNA) clone of *Saccharomyces cerevisiae* as a probe (21). The arrangement of rDNA subunits within this 8.6-kb cloned fragment was determined to be 18S-5.8S-25S, a universal pattern in all other eukaryotes that have been characterized (reviewed in reference 17). Restrepo and Barbour were unable to determine the position of the 5S rRNA gene, but they postulated that it was adjacent to the 5.8S rRNA gene. The 18S, 5.8S, and 25S rRNA genes are transcribed by RNA polymerase I as one primary transcript that is subsequently processed into the mature rRNAs prior to release from the nucleus. The 5S rRNA gene, on the other hand, is transcribed by RNA polymerase III without any subsequent processing (20, 36). In fungi, the location of the 5S rRNA gene varies, but this gene has never been shown to be in the same region as the 5.8S rRNA gene. For example, the 5S rRNA gene in *Neurospora crassa* (12, 23), *Aspergillus nidulans* (5), *Cochliobolus heterostrophus* (13), *Yarrowia lipolytica* (27), or *Schizosaccharomyces pombe* (25) is not part of the rDNA cluster and is distributed throughout the genome. In other fungi, the 5S rRNA gene is part of a tandemly repeated rDNA cluster (6, 8, 9, 11, 19, 30–32). In these cases, the 5S rRNA gene is located outside the 18S-5.8S-25S rRNA transcriptional unit and is transcribed either in the same direction as the other three rRNA genes or in the opposite direction (reviewed in reference 13).

We cloned the 5S rRNA gene from *Filobasidiella neoformans* by using an oligonucleotide probe which was constructed on the basis of conserved DNA sequences found in other fungi related to *Filobasidiella neoformans*. The map location of the 5S rRNA gene was identified, and its organization compared with the organization of the other three rRNA genes in the rDNA repeat unit was clearly defined.

MATERIALS AND METHODS

DNA extraction. The genomic DNA of *Cryptococcus neoformans* was extracted by using the method of Varma and Kwon-Chung (28), with slight modifications. The yeast cells were grown overnight on plates containing yeast nitrogen base agar (Difco Laboratories, Detroit, Mich.) supplemented with 2% glucose instead of in YEPD broth (2% glucose, 2% peptone, 1% yeast extract). The lysing solution contained 0.1 M EDTA instead of 0.45 M EDTA.

DNA probe and Southern blot analysis. A probe consisting of 47 nucleotides (TTAGTACCACGGTGGGGGACCA CGCGGGAATCCTAGGTGCTGTGGTT) was synthesized on the basis of the sequences in a highly conserved region of 5S rRNA that has been characterized in several heterobasidiomycetous yeasts, *Filobasidium floriforme*, *Filobasidium capsuligenum*, and *Tremella mesenterica* (34). The oligonucleotide sequences ranged from position 71 to position 118 of the 5S rRNA genes of the two *Filobasidium* species. Genomic DNA from a strain of *Filobasidiella neoformans*, strain ATCC 34873 (= B-3501), was digested with endonucleases and electrophoresed on a 1% agarose gel. The DNA was then transferred to a nylon membrane (Hybond-N; Amersham, Arlington Heights, Ill.). The 47-mer oligonucleotide was 5' end labeled by using T4 polynucleotide kinase and [γ - 32 P]ATP (Amersham). The 8.6-kb DNA fragment of *Cryptococcus neoformans* containing the rDNA repeat unit (pBIR6; obtained from B. I. Restrepo) was radiolabeled by using a random priming kit (Stratagene, La Jolla, Calif.) and [α - 32 P]dCTP (Amersham). The membranes were then hybridized, washed, and exposed to X-ray film as previously described (29).

Pulsed-field electrophoresis. Agarose plugs containing chromosomes from *Cryptococcus neoformans* isolates were prepared and electrophoresed with a contour-clamped homogeneous electric field (CHEF) gel system by using the procedure described previously (37). The separated chromosomes were transferred to a nylon membrane and hybridized with radiolabeled probes as described above.

DNA sequencing. DNA sequencing was performed by the dideoxy method by using a Sequenase version 2.0 kit obtained from U.S. Biochemicals, Cleveland, Ohio.

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FIG. 1. Chromosomal location of rRNAs in *Filobasidiella neoformans* as determined by separating chromosomes (by CHEF gel electrophoresis) and probing with plasmid pBIR6 containing the *Cryptococcus neoformans* rDNA cluster. Lanes 1, ethidium bromide-stained strain ATCC 34873 (= B-3501) chromosomal banding pattern; lanes 2, Southern blots of the CHEF gel hybridized with pBIR6 (A) and the 5S rRNA oligonucleotide probe (B).

Mapping of rRNA genes. To map the boundaries of all four rRNA genes within the 8.6-kb fragment, the complete sequences of 18S and 25S rDNAs were obtained from Arturo Casadeval. The sequence of 5.8S rRNA was obtained from GenBank (accession number M94516).

RESULTS

Location of the 5S rRNA gene. Southern hybridization was carried out to determine whether the 5S rRNA gene in *Filobasidiella neoformans* is randomly distributed, as is the case in *N. crassa* and several other ascomycetous fungi, or is located within the repeat unit that encodes the 18S, 5.8S, and 25S rRNAs. A CHEF blot was hybridized with plasmid pBIR6 (containing an 8.6-kb rDNA insert) and an oligonucleotide that represented the highly conserved 5S rRNA gene sequence in heterobasidiomycetous yeasts (34) closely related to *Filobasidiella neoformans*. Both probes hybridized to chromosome 2 (Fig. 1) (37). The Southern hybridization patterns of genomic DNA from strain B-3501 digested with *Eco*RI, *Bam*HI, and *Xho*I (Fig. 2) were similar to each other when the preparations were probed with pBIR6 or 5S rRNA oligonucleotides, indicating that the 5S rRNA gene is clustered with the genes on pBIR6. To determine the location of the 5S rRNA gene on the 8.6-kb rDNA insert, plasmid pBIR6 was digested with *Eco*RI, *Bam*HI, and *Bgl*II and analyzed by Southern blot hybridization

FIG. 2. Genomic DNA of strain ATCC 34873 digested with *Eco*RI (lanes R), *Xho*I (lanes X), and *Bam*HI (lanes Ba) and fractionated by gel electrophoresis. The Southern blot of the genomic DNA was hybridized with pBIR6, a *Cryptococcus neoformans* rDNA cluster probe (A), and with the 5S rRNA oligonucleotide probe (B).

by using oligonucleotides from the 5S rDNA sequences. The probe hybridized with a 1.8-kb *Eco*RI fragment and *Bam*HI fragment larger than 6 kb, as well as with *Bgl*II fragments (Fig. 3), which indicated that the 5S rRNA gene is located on the left side of the first *Bgl*II site on the map shown in Fig. 4A. To determine the exact site of the 5S rRNA gene, pBIR6 was digested with *Eco*RI as well as with *Eco*RI plus *Pvu*II and subjected to a Southern hybridization analysis performed with the 5S oligonucleotide probe. The 1.8-kb *Eco*RI fragment and the 1.4-kb *Eco*RI-*Pvu*II fragment hybridized with the 5S rRNA probe (Fig. 5), indicating that the 5S rRNA gene is located between the *Pvu*II site and the first *Eco*RI site on the 8.6-kb insert (Fig. 4A). The 1.4-kb *Eco*RI-*Pvu*II fragment was cloned into pBluescript II (Stratagene) and was sequenced to confirm the presence of the 5S rRNA gene.

Arrangement of rRNA genes in the 8.6-kb fragment. The restriction map of the 8.6-kb rDNA fragment published previously (21), together with the total sequence of the 5S rDNA (this study), 18S rDNA (7), 5.8S rDNA (18), and 25S rDNA (7), allowed us to determine the boundaries of the four rRNA genes in the *Cryptococcus neoformans* rDNA repeat. Figure 4 shows a comparison of our map with the previously published map (21). The 5S rRNA gene is located about 1 kb upstream from the 18S rRNA gene. The distance between the coding regions of the 18S and 5.8S rRNAs and the coding regions of the 5.8S and 25S rRNAs was less than 200 bases. The DNA sequence indicated that the direction of transcription of the 5S rRNA gene was the same as the direction of transcription of the 18S-5.8S-25S rRNA transcriptional unit.

Sequence comparisons of the 5S rRNA gene. The oligonu-

FIG. 3. To determine the arrangement of the 5S rRNA gene in the rDNA cluster, plasmid pBIR6 DNA was digested with *Eco*RI (lane R), *Bam*HI (lane B), and *Bgl*II (lane Bg), fractionated by gel electrophoresis, transferred to a nylon membrane, and hybridized with the 5S rRNA oligonucleotide probe. The numbers on the left denote molecular size (in kilobases).

FIG. 5. *Eco*RI-digested (lane 1) and *Eco*RI-*Pvu*II-digested (lane 2) plasmid pBIR6 DNAs hybridized with the 5S rRNA oligonucleotide probe to show the location of the 5S rRNA gene. The numbers on the left denote molecular size (in kilobases).

cleotide probe used in this study was constructed by using the highly conserved regions (positions 71 to 118) of the 5S rRNA genes of *Filobasidium capsuligenum* and *Filobasidium floriforme*, which are considered to be the organisms that are most closely related to *Filobasidiella neoformans* in the family Filobasidiaceae (16). The sequence in this region is identical in *Filobasidium capsuligenum* and *Filobasidium floriforme* (Fig. 6). As is the case for the two *Filobasidium* species, there were 118 nucleotides in the 5S rRNA gene of *Filobasidiella neoformans*. The difference between the genus *Filobasidiella* and the genus *Filobasidium* was reflected in the sequence of the gene since there were four nucleotide differences between the two genera within the conserved region from nucleotide position

71 to nucleotide position 118. There were only three nucleotide differences between the two *Filobasidium* species in the total 5S rRNA gene sequence, while there were 28 and 29 nucleotide differences between the two *Filobasidium* species and *Filobasidiella neoformans*.

DISCUSSION

Although the sequences of 5S rRNAs have been reported for numerous species of heterobasidiomycetous yeasts (1–3, 14, 33–35, 38), the location of the 5S rRNA gene has not been investigated in this group of fungi. The 5S rRNA gene of *Filobasidiella neoformans* is within the rDNA repeat unit, as is the case for *Mucor racemosus* (10), *Torulopsis utilis* (24), *Saccharomyces cerevisiae* (19, 26), several other ascomycetous

FIG. 4. (A) Restriction map showing the arrangement of *Cryptococcus neoformans* rRNA genes in pBIR6 as reported by Restrepo and Barbour (21). (B) Organization of rRNA genes determined in this study. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; C, *Cl*aI; E, *Eco*RI; H, *Hind*III; V, *Pvu*II.

FIG. 6. Sequence alignment for 5S rRNA genes from *Filobasidium capsuligenum*, *Filobasidium floriforme*, and *Tremella mesenterica*. The sequence of the *Filobasidium capsuligenum* 5S rRNA gene is in capital letters. The lowercase letters in the sequences indicate the nucleotides that were different from *Filobasidium capsuligenum* nucleotides. The GenBank accession number of the *Filobasidiella neoformans* 5S rRNA gene sequence is L14753.

yeasts (27, 30, 31), various Holobasidiomycetes that have been examined (6, 8, 9, 11, 32), and several species of Oomycetes (15, 22). In *Saccharomyces cerevisiae* and *Torulopsis utilis*, the direction of transcription of the 5S rRNA gene is opposite the direction of transcription of the 18S-5.8S-25S rRNA unit. The direction of transcription of the 5S gene in *M. racemosus* has not been determined. As in *Filobasidiella neoformans*, the 5S rRNA genes in the majority of the Holobasidiomycetes species that have been analyzed are transcribed in the same direction as the other rRNA subunits (6, 8, 9, 11, 32). One exception is *Coprinus comatus* (9), in which the 5S rRNA gene and the other rRNA genes are oriented in opposite directions. In various species of the genera *Phytophthora* and *Saprolegnia* (water molds) the 5S rRNA gene is transcribed in the same direction as the 18S-5.8S-25S rRNA transcriptional unit (15). It is evident, therefore, that the organization of the 5S rRNA gene exhibits divergence among the fungi, and the direction of the transcription does not appear to be uniform within each phylum. It is noteworthy, however, that all of the fungi studied so far and found to have the 5S rRNA genes scattered in the genome belong to the Ascomycota. The scattered pattern of the 5S rRNA genes, therefore, may be a useful genetic marker for ascomycetes.

Depending on the length of the spacer sequences between the 5S rRNA gene and the three other rRNA genes, the 5S rRNA gene can be closer to either the 18S rRNA gene or the 25S rRNA gene in the rDNA repeat unit. The 5S rRNA gene of *Filobasidiella neoformans* is located closer to the 18S rRNA subunit, while the 5S rRNA genes of all of the Holobasidiomycetes that have been tested thus far are located closer to the 25S rRNA subunit (6, 8, 9, 11, 32).

Restrepo and Barbour isolated RNA from *Cryptococcus neoformans*, electrophoresed it on 1% agarose, and subjected it to Northern (RNA) blot hybridization by using the 8.6-kb insert of plasmid pBIR6. The probe hybridized to the 25S and 18S rRNA subunits, but the 5S and 5.8S rRNAs were not detected on the blots (21). The 5S and 5.8S rRNAs are too small to be detected when they are electrophoresed on 1% agarose but can be detected on an acrylamide gel containing urea (12).

In recent years, rRNA genes have been isolated from fungi by using PCR techniques (15, 18). Primers have been designed to clone the 5.8S rRNA gene, as well as the small- and large-subunit rRNA genes of *Filobasidiella neoformans* (18). The 47-mer oligonucleotide which we used appeared to be the most useful probe for cloning not only the 5S rRNA gene of *Filobasidiella neoformans* but also the 5S rRNA genes of many species of Holobasidiomycetes. The 47-nucleotide sequences from position 71 to position 118 of the 5S rRNAs of four mushroom species, *Schizophyllum commune*, *Coprinus cinereus*, *Coprinus radiatus*, and *Bjerkandera adusta*, exhibited 98 to 100% similarity with the nucleotide sequences of *Filobasidium* species (34). It was surprising that there was a 4-nucleotide difference (92% similarity) between *Filobasidium* species and *Filobasidiella neoformans* in this region, while there was only a 2-nucleotide difference (96% similarity) between *Filobasidiella neoformans* and *Tremella mesenterica* (34). Substantial divergence between the genus *Filobasidium* and *Filobasidiella neoformans* has also been revealed by the 5.8 rRNA gene sequences (18). In their recent comprehensive review, Boekhout et al. treated the family Filobasidiaceae as a member of the order Tremellales (4). Although our data corroborate the classification system of Boekhout et al., more fungi in the family Filobasidiaceae will have to be analyzed for their 5S rRNA sequences before the phylogenetic significance of the

sequence divergence among the members of the family can be assessed.

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