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Fungus-Specific Translation Elongation Factor 3 Gene Present in *Pneumocystis carinii*

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Historically, *Pneumocystis carinii* pneumonia has been the most frequent cause of morbidity and mortality in patients with AIDS. Antiprotozoan drugs are effective in the treatment and prophylaxis of *P. carinii* pneumonia, which lends credence to the widely held view that *P. carinii* is a protozoan. However, recent genetic evidence suggests that *P. carinii* should be classified as a fungus. Translation elongation factor 3 (EF-3) is an essential, soluble translation component which is unique to fungal protein synthesis and is not required for protein synthesis in other eukaryotes. We have identified and isolated a gene for EF-3 from *P. carinii*, adding more evidence for this organism's assignment as a fungus.

The severely immunocompromised status of patients with AIDS as well as of patients receiving immunosuppressive treatment allows opportunistic infection with a number of organisms. Historically, *Pneumocystis carinii* pneumonia (PCP) has been the most frequent cause of morbidity and mortality in patients with AIDS. Up to 80% of adult and 40% of pediatric patients with AIDS have contracted PCP at least once (1, 23). After successful treatment of PCP, a prophylactic treatment regimen is usually required because of the high probability of recurrent infection. *P. carinii* is a eukaryotic organism that primarily infects the lungs of a range of mammalian hosts, including humans, rats, and mice. Two morphological forms of *P. carinii*, the trophozoite and cyst, have been observed in infected lungs. The trophozoite often attaches to the pneumocytes, contains a single nucleus, and is irregularly shaped. The trophozoite appears to encapsulate into the thick-walled cyst, which contains up to eight singly nucleated intracystic bodies. The cysts are invariably round and remain unattached in the air sacs. It is not clear whether the trophozoite, the cyst, or another, unidentified form of the organism is infectious (5).

The taxonomic position of *P. carinii* within the eukaryotes has been unclear, and for years the literature has contained arguments for including *P. carinii* either among the fungi or among the protozoa (11). Classification has relied primarily on morphogical and ultrastructural data, as detailed examination of the biochemistry and genetics of *P. carinii* was severely hampered by the inability to propagate any form of the organism in vitro (3). *P. carinii* has been included among the protozoa because of morphological similarities to various protozoa (2, 30, 31). The microtubule arrangement, ultrastructure (28), and DNA content per cell (10) of *P. carinii* have been reported to be closest to those of various protozoans. In addition, *Pneumocystis* infections respond to antiprotozoan drugs, including trimethoprim-sulfamethoxazole and pentamidine, but not to currently used antifungal drugs.

However, several features of *P. carinii* have been interpreted as being characteristic of fungi (14, 16). *P. carinii* has staining properties consistent with a fungus, and a component of the cyst wall is β-1,3-glucan, a major component of fungal cell walls (15). Chitin, a macromolecule present in but not uniquely specific to fungi, has been detected in both the cyst and trophozoite (29). Moreover, the process of cyst formation resembles the meiotic process of the ascomycetes (32). Edman et al. (8) compared the sequence of the *Pneumocystis* 16S rRNA gene with those of the 16S rRNA genes of a variety of organisms (including protozoans) and found that this sequence was most similar to that of the yeast *Saccharomyces cerevisiae*. Subsequent analysis of the *Pneumocystis* thymidylate synthase (TS) gene similarly demonstrated that the deduced amino acid sequence is most similar to that of TS from *S. cerevisiae* and showed 65% identity between the proteins (9). In addition, the *Pneumocystis* TS and dihydrofolate reductase are separate proteins (7, 13), in contrast to protozoan TS and dihydrofolate reductase activities, which reside within a single polypeptide. A comparative sequence analysis of several *Pneumocystis* mitochondrial DNA genes has been reported and suggests that *P. carinii* is a fungus (20). Partial sequence analysis of the *Pneumocystis* β-tubulin gene also showed that it was most similar to the corresponding gene in dimorphic fungi (6).

In order to more clearly classify *P. carinii*, we investigated whether the organism contained the gene for a fungus-specific protein, translation elongation factor 3 (EF-3). Polypeptide elongation during protein synthesis in eukaryotic cells requires elongation factor 1 (EF-1) and EF-2. However, elongation in fungi requires a third, soluble component, EF-3 (25, 26). While EF-1 and EF-2 of fungi and higher eukaryotes are functionally interchangeable (22), EF-3 is unique to fungi. EF-3 was initially identified in *S. cerevisiae* cell extracts and consists of a 125-kDa monomeric protein (4). While the exact role of EF-3 in translation is unresolved, EF-3 exhibits ribosome-dependent GTPase and ATPase activities (4, 27). Biochemical and Western (immunoblot) analyses have shown that EF-3 is present in many yeast species and in filamentous fungi, such as *Neurospora*, *Aspergillus*, and *Mucor* spp. However, such analyses have failed to detect an EF-3 analog in *Tetrahymena* spp., *Chlamydomonas* spp., brine shrimp, rat liver, or HeLa cells (17, 21, 24). Considering that the configuration of the translational apparatus is different in protozoan and fungal systems, the identification of EF-3 in *P. carinii* would further clarify the affiliation of *P. carinii* with the fungi. This report

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documents the identification of an EF-3 gene in *P. carinii* and supports a fungal affiliation for this organism.

MATERIALS AND METHODS

Bacterial strains and media. *Escherichia coli* LE392 and XL1-Blue (Stratagene) were used as bacteriophage lambda and plasmid hosts, respectively. The strains were grown on LB supplemented with ampicillin (50 μg/ml) and/or tetracycline (12.5 μg/ml) as necessary.

***P. carinii* DNA library.** The *P. carinii* genomic DNA library was constructed from DNA extracted from trophozoites isolated from rat lung and was a generous gift of J. C. Edman (8). The DNA was randomly sheared, ligated to *Eco*RI linkers, and inserted into lambda ZAP-I (Stratagene).

Isolation of *P. carinii* DNA. Genomic DNA was prepared from rat-derived trophozoites kindly provided by the laboratory of Sherry Queener. The organisms were cultured on a human embryonic lung cell line and confirmed by that laboratory to be free of fungal contamination. The organisms were collected and stored frozen. The organisms were washed with buffer A (1 M sorbitol, 10 mM EDTA, 10 mM dithiothreitol [pH 7.5]) and incubated for 30 min at 37°C with 0.2 mg of Zymolyase 20000T per ml dissolved in buffer A. The organisms were collected, resuspended in buffer B (50 mM Tris, 20 mM EDTA, 0.1% sodium dodecyl sulfate [SDS] [pH 7.4]), and incubated at 65°C for 30 min. Potassium acetate was added to 1.5 M, and the sample was refrigerated overnight. The sample was centrifuged, and *P. carinii* DNA was precipitated from the supernatant by adding an equal volume of isopropanol.

Screening of the *P. carinii* DNA library. Approximately 60,000 phage from the DNA library were screened by plaquing on LE392 cells and subsequent transfer to nitrocellulose filters (19). The hybridization and wash conditions are described below. Several hybridizing plaques were identified with the *S. cerevisiae* EF-3 probe. Phagemids were rescued by coinfection of *E. coli* XL1-Blue with the appropriate lambda phage and R408 helper phage, as recommended by Stratagene. Isolate pPCEF-3-6.5 contained a 6.5-kb insert. Subsequent mapping of pPCEF-3-6.5 showed that an internal 3.5-kb region of the insert hybridized specifically to the *S. cerevisiae* probe. The internal 3.5-kb region was mapped with several restriction enzymes. Two *Xho*I fragments (1,550 and 1,507 bp, Fig. 1A) contained the majority of the hybridizing sequences and were subcloned into the Bluescript SK+ vector (Stratagene), yielding pEFX1 and pEFX2, respectively. In order to see whether the *S. cerevisiae* probe was hybridizing to other genes in the lambda library (in addition to the putative EF-3 gene), DNA from two additional phagemids was analyzed. Southern analysis showed that these phagemids contained a subset of the sequence in pPCEF-3-6.5 (data not shown), and only the putative EF-3 gene was hybridizing to the heterologous probe.

Probe preparation and hybridization conditions. A 3.5-kb *Xba*I DNA fragment derived from pYEF-3 (21), a plasmid containing the *S. cerevisiae* EF-3 gene, was used to screen the genomic library. The 3.5-kb *Xba*I fragment contains almost the entire coding region of the EF-3 gene (21). This fragment was isolated by agarose gel electrophoresis, purified by the GeneClean method, and labeled with [α-^{32}P] dGTP by using the USB Random-Primer labeling kit. Five probes (Fig. 1A) were derived from pPCEF-3-6.5, a plasmid containing the entire *P. carinii* EF-3 gene. The 1,507-bp and 1,550-bp *Xho*I DNA fragments, 1,289-bp *Xho*I-*Eco*RV DNA fragment, and 835-bp and 672-bp *Hin*dIII-*Xho*I DNA fragments were purified and labeled as described above.

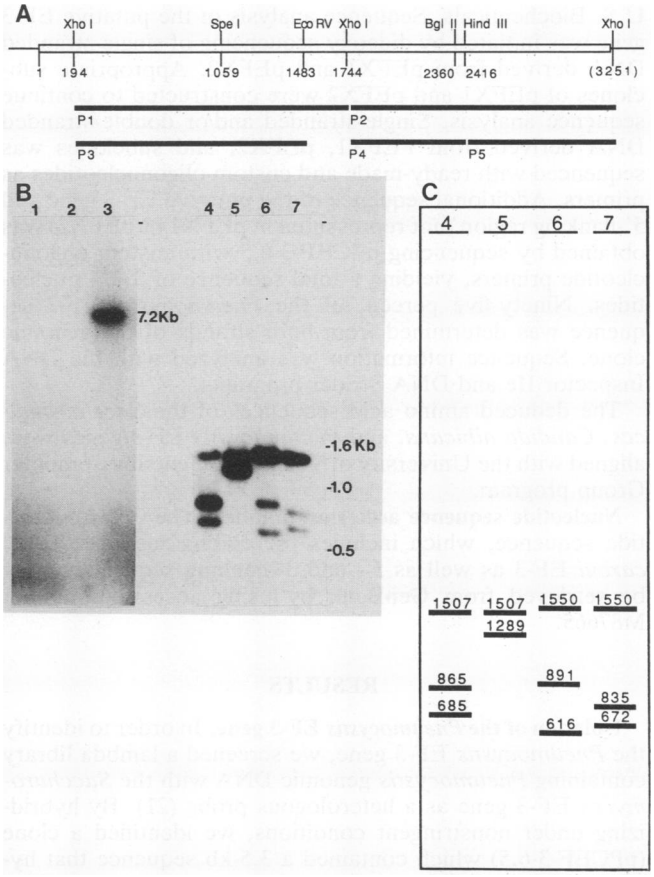

FIG. 1. Southern analysis of *Pneumocystis* genomic DNA shows a restriction site pattern consistent with the pattern predicted from the sequence of the cloned EF-3 gene. (A) Relevant restriction sites of the putative *Pneumocystis* EF-3 gene are shown. (B) Southern analysis of *Pneumocystis* genomic DNA was performed under stringent conditions with pPCEF-3-6.5-derived probes P1 through P5, illustrated in panel A. The genomic DNA was prepared from trophozoites cultured on a human embryonic lung cell line. Lanes 1 and 2, 1 μg of DNA isolated from two asymptomatic rats and digested with *Eco*RI. Lanes 3 to 7, 1 μg of *Pneumocystis* DNA digested with (lane 3) *Eco*RI, (lane 4) *Xho*I and *Spe*I, (lane 5) *Xho*I and *Eco*RV, (lane 6) *Xho*I and *Bgl*II, and (lane 7) *Xho*I and *Hin*dIII. Lanes 1 to 3 were probed with P1 and P2. Lanes 4 to 7 were probed with P3, P4, and P5. (C) Restriction fragments (sizes in base pairs) predicted from the sequence. Lanes 4 to 7 in panel C correlate with lanes 4 to 7 in panel B.

The filters containing plaque lifts of the library DNA were hybridized overnight in 15% formamide–5× SSPE (84 mM NaCl, 5.0 mM EDTA, 50 mM Na_2PO_4 [pH 7.4])–5× Denhardt's solution–0.125% SDS–0.1 mg of salmon sperm DNA per ml at 37°C. The filters were washed at 37°C with 1× SSC (0.15 M NaCl, 0.015 M sodium citrate)–0.1% SDS for 30 min and then with 0.2× SSC–0.1% SDS for 30 min. Southern analysis of *Pneumocystis* genomic DNA was performed as described above except that hybridization was carried out with 50% formamide and the hybridization and washes were performed at 42°C.

Nucleotide sequence determination and sequence analysis. All sequencing was performed with the Sequenase kit from

U.S. Biochemicals. Sequence analysis of the putative EF-3 gene was initiated by dideoxy sequencing of single-stranded DNA derived from pEFX1 and pEFX2. Appropriate subclones of pEFX1 and pEFX2 were constructed to continue sequence analysis. Single-stranded and/or double-stranded DNA derived from PEFX1, pEFX2, and subclones was sequenced with ready-made and custom oligonucleotides as primers. Additional sequence of the putative EF-3 gene and 5′-flanking region (not represented in pEFX1 or pEFX2) was obtained by sequencing pPCEF-3-6.5 with custom oligonucleotide primers, yielding a total sequence of 3,274 nucleotides. Ninety-five percent of the *Pneumocystis* EF-3 sequence was determined from both strands of the genomic clone. Sequence information was analyzed with the DNA Inspector IIe and DNA Strider programs.

The deduced amino acid sequences of the *Saccharomyces*, *Candida albicans*, and *Pneumocystis* EF-3 genes were aligned with the University of Wisconsin Genetics Computer Group program.

Nucleotide sequence accession number. The 3,274-nucleotide sequence, which includes the coding sequence of *P. carinii* EF-3 as well as 5′- and 3′-flanking sequences, may be retrieved from GenBank by using accession number M87665.

RESULTS

Isolation of the *Pneumocystis* EF-3 gene. In order to identify the *Pneumocystis* EF-3 gene, we screened a lambda library containing *Pneumocystis* genomic DNA with the *Saccharomyces* EF-3 gene as a heterologous probe (21). By hybridizing under nonstringent conditions, we identified a clone (pPCEF-3-6.5) which contained a 3.5-kb sequence that hybridized specifically to the probe. Sequencing of the pPCEF-3-6.5 phagemid and its subclones showed a single open reading frame of 3,126 nucleotides, coding for a deduced protein of 1,042 amino acids.

Comparative Southern analysis of genomic and cloned *Pneumocystis* DNA. The genomic lambda ZAPI library was constructed with DNA extracted from organisms which were partially purified from infected lung tissue. In order to confirm that the putative EF-3 sequence we had isolated from the library was indeed from *P. carinii* and not from contaminating fungi, we compared the restriction pattern of the cloned putative EF-3 sequence with that of genomic DNA isolated from *P. carinii* cultured on a human embryonic lung cell line. The in vitro-derived organisms were free of bacterial and fungal contamination. The genomic *Pneumocystis* DNA was digested with a several restriction enzymes and probed with pPCEF-3-6.5-derived sequences (Fig. 1B). Because the *Pneumocystis* EF-3 gene does not contain an *Eco*RI site, the 7.2-kb fragment shown in lane 3 contains the entire gene and flanking sequences. Lanes 4 to 7 show the restriction patterns generated by digesting the genomic DNA with selected restriction enzymes. Figure 1C shows the patterns predicted from the sequence data for the cloned EF-3 gene. As the restriction patterns generated from the genomic DNA are identical to those predicted from the cloned gene, we conclude that the EF-3 gene which we cloned originated from *P. carinii*. Despite the high degree of similarity between the deduced amino acid sequences of the *P. carinii*, *S. cerevisiae*, and *C. albicans* EF-3's, codon usage varies, so that restriction sites are not conserved between these genes and the *P. carinii* restriction patterns are unique.

Alignment of the *Pneumocystis*, *Saccharomyces*, and *Candida* EF-3 deduced amino acid sequences. Alignment of the deduced amino acid sequences for the *P. carinii* and *S. cerevisiae* EF-3's showed 57% identity between the proteins (Fig. 2). If conservative replacements are factored in, the *Pneumocystis* protein is 72% similar to *S. cerevisiae* EF-3. The high degree of similarity between the *Pneumocystis* and *Saccharomyces* EF-3 deduced amino acid sequences demonstrated that we had isolated the *Pneumocystis* EF-3 gene. While the *Candida* EF-3 sequence showed 78% identity to that of the *S. cerevisiae* protein, the *Pneumocystis* and *Candida* EF-3 sequences showed 57% identity.

Detailed comparison of the three EF-3 sequences showed several regions of identity from residue 425 to the carboxy termini of the proteins. In particular, the *Pneumocystis* EF-3 sequence includes two short amino acid sequence motifs (underlined and double-underlined in Fig. 2) which appear in duplicate within the protein. These motifs were observed in the *Saccharomyces* and *Candida* EF-3 sequences (19, 21, 22) and show a high degree of similarity to nucleotide-binding sites in a diverse group of proteins which bind nucleotides. The type A consensus sequence GGXXGXGKS/T has been found in some proteins, including bovine ATPase and adenylate kinase (21). The type B consensus sequence (four nonpolar residues followed by an aspartic acid; e.g., ILLLD) has also been observed in these enzymes as well as a number of other nucleotide-binding proteins.

The *Pneumocystis*, *Saccharomyces*, and *Candida* proteins contain highly basic carboxy-terminal sequences which include several lysine and lysine-arginine tracts. In contrast, the amino-terminal sequences show limited similarity.

DISCUSSION

We have isolated the *Pneumocystis* EF-3 gene by screening a lambda library containing *Pneumocystis* genomic DNA with the *Saccharomyces* EF-3 gene as a heterologous probe. One phage isolate contained a 6.5-kb insert, of which an internal 3.2-kb region hybridized to the *Saccharomyces* gene probe. Sequencing of this region showed a single open reading frame of 3,126 nucleotides, coding for a deduced protein of 1,042 amino acids. Alignment of the deduced amino acid sequence of *Pneumocystis* EF-3 with that of *Saccharomyces* EF-3 showed 57% identity between the proteins and confirmed that we had isolated an EF-3 gene, presumably from *P. carinii*. In order to verify that the EF-3 sequence we had isolated was indeed from *P. carinii* and not from another fungus present in the infected lung tissue used for library construction, we compared the restriction pattern of the cloned EF-3 sequences with that of genomic DNA isolated from *P. carinii* cultured in vitro. The restriction pattern of the genomic DNA was completely consistent with the pattern predicted from the sequence data for the cloned EF-3 gene and confirmed that the cloned gene originated from *P. carinii*.

Alignment of the *Saccharomyces*, *Candida*, and *Pneumocystis* EF-3 deduced amino acid sequences shows that all coding regions are nearly identical in length. Neither the *Saccharomyces* nor *Candida* gene contains introns (19, 21), and colinear alignment of the three deduced amino acid sequences suggests that the *Pneumocystis* gene does not contain an intron(s). However, several *Pneumocystis* genes have short introns (7, 9), and sequencing of a cDNA of the EF-3 gene is necessary to confirm the lack of introns. Comparison of the *Pneumocystis*, *Saccharomyces*, and *Candida* EF-3 deduced amino acid sequences highlights several

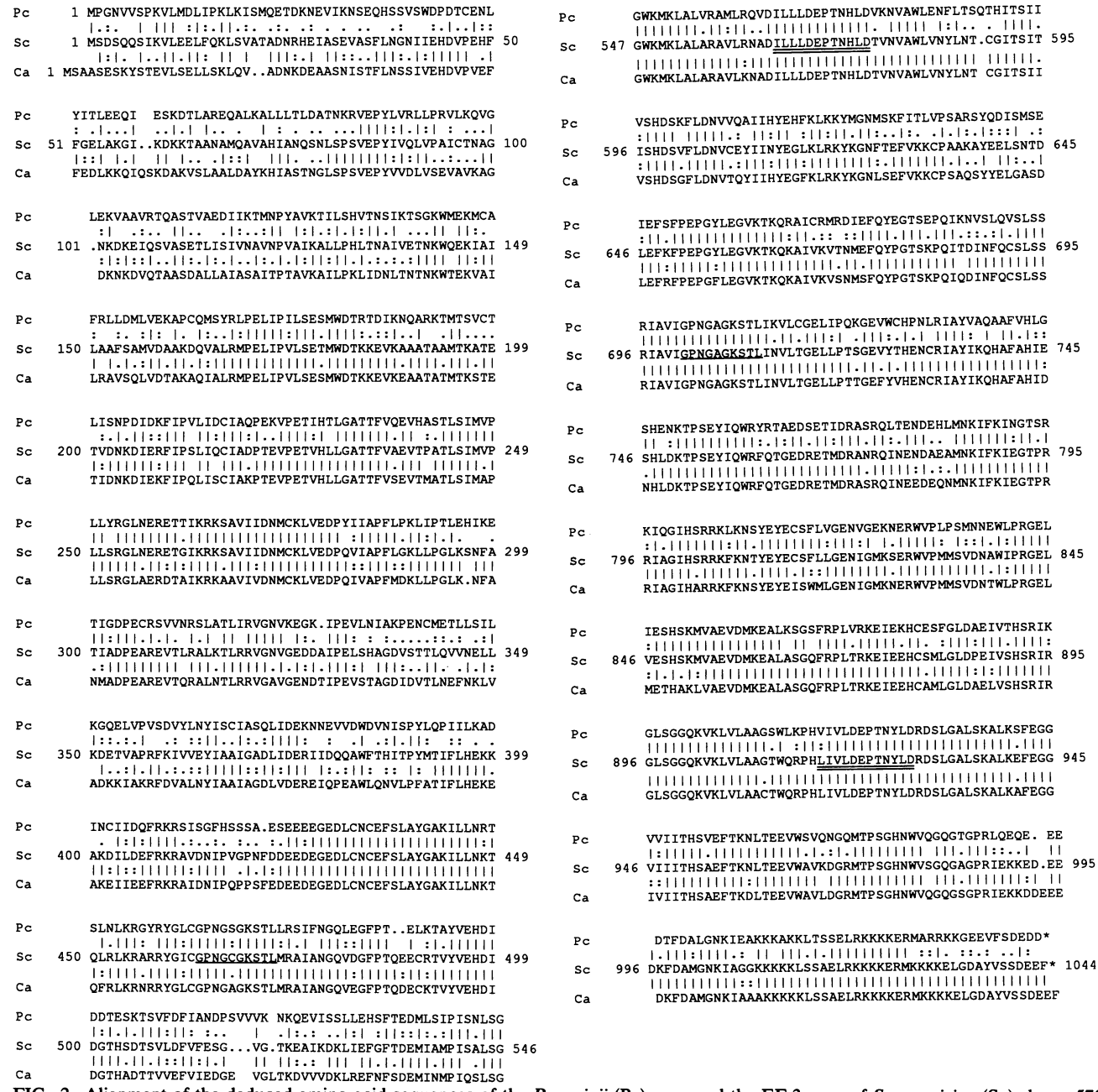

FIG. 2. Alignment of the deduced amino acid sequences of the *P. carinii* (Pc) gene and the EF-3 gene of *S. cerevisiae* (Sc) shows 57% identity. If conservative replacements are factored in, the predicted proteins are 72% similar. The *Saccharomyces* and *C. albicans* (Ca) genes show 78% identity, while the *Candida* and *Pneumocystis* genes show 57% identity. Two short amino acid motifs have been proposed as nucleotide-binding domains. Motif A (underlined) and motif B (double-underlined) both appear in duplicate and are conserved among the three EF-3 proteins.

regions of identity from residues 425 to the carboxy termini of the proteins. In particular, the *Pneumocystis* EF-3 sequence includes two amino acid sequence motifs, each of which appears in duplicate within the protein (Fig. 2). These motifs are almost identical to the analogous motifs first observed in *Saccharomyces* EF-3 by Qin et al. (21), as well as in the EF-3 of *C. albicans* (19). Qin et al. (21) stated that both motifs have been found in many different proteins that bind nucleotides. As EF-3 exhibits ribosome-dependent GTPase and ATPase activities (4, 27) and these motifs are highly conserved among three highly different organisms, it is feasible that these residues are important in binding GTP and/or ATP during protein synthesis.

The *Saccharomyces*, *Pneumocystis*, and *Candida* EF-3 proteins all contain highly basic carboxy-terminal sequences. Qin et al. have suggested that the carboxy-terminal sequence of EF-3 may interact with nucleic acid (21). While the interaction of EF-3 with the translation apparatus is

unclear, it is possible that the carboxy-terminal sequences of EF-3 interact either with rRNA or perhaps with tRNA. Significantly, the deduced amino-terminal sequences of the *Saccharomyces*, *Pneumocystis*, and *Candida* EF-3 proteins show limited similarity.

The identification of the EF-3 gene in *P. carinii* is the first documentation of a fungus-specific gene in the *Pneumocystis* genome and suggests that *P. carinii* elongates proteins in a fungus-specific manner. Surprisingly, Jackson et al. (12) have recently reported that Western blot analysis with polyclonal antibody raised against *Saccharomyces* EF-3 failed to detect EF-3 in lysates of partially purified *P. carinii*. There are several possible scenarios which would reconcile these seemingly conflicting results. It is possible that the *Pneumocystis* EF-3 gene is vestigial and is not expressed. However, the identity between specific regions of the *Saccharomyces, Candida*, and *Pneumocystis* proteins suggests that there has been selection against mutations creating changes in functionally or structurally important regions of *Pneumocystis* EF-3 and implies EF-3 expression. It is also possible that the organisms, which were primarily noncystic, were not metabolically active and were not expressing EF-3 at appreciable levels. The alignment data also show marked heterogeneity in the first 425 residues between the *Pneumocystis* and *Saccharomyces* proteins. If EF-3 was not expressed at high levels and the heterogeneity between the EF-3 proteins was significant enough to compromise the reactivity of the antiserum, it is possible that *Pneumocystis* EF-3 would not be detected.

The translational apparatus is the site of action of many useful antibiotics. If a unique translation elongation factor, such as EF-3, is expressed in *P. carinii*, it may provide a suitable target for an inhibitor which is free of toxic side effects. The current drug protocols used to treat PCP are moderately successful in some cases, but these drugs were not originally designed for this organism and do not reflect any knowledge of its metabolism or structure. Detailed analysis of EF-3 could provide information on translation-specific target sites for anti-*P. carinii* drug therapy.

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