Nucleic Acids Research, Vol. 20, No. 14 3763–3772

Sequence and variability of the 5.8S and 26S rRNA genes of *Pneumocystis carinii*

Yong Liu, Marissa Rocourt, Sueihua Pan[1], Chientzu Liu[1] and Michael J.Leibowitz*
Department of Molecular Genetics and Microbiology and [1]Department of Pathology, UMDNJ – Robert Wood Johnson Medical School, 675 Hoes Lane, Piscataway, NJ 08854-5635, USA

Received January 29, 1992; Revised and Accepted June 23, 1992 GenBank accession nos M86759 – M86761 (incl.)

ABSTRACT

The sequence of the coding region of the rRNA operon of rat-derived *Pneumocystis carinii* has been completed, including the genes for 5.8S and 26S rRNA. These genes show homology to the rRNA genes of yeast, and an apparent group I self-splicing intron is present in the 26S rRNA gene. Like a similar intron in the 16S rRNA gene, this intron is in a phylogenetically conserved region. Variation in the 26S rRNA sequence was noted between *P.carinii* organisms isolated from two different sources.

INTRODUCTION

Pneumocystis carinii is a ubiquitous eukaryotic microorganism, causing asymptomatic infections in most humans early in childhood (1), but causing life-threatening pneumonia in immunosuppressed hosts, including patients with AIDS (2). Although morphologically *P.carinii* has properties associated with both protozoa and yeasts, the 16S rRNA coding sequence of *P.carinii* grown in immunosuppressed rats most resembled that of the yeast *Saccharomyces cerevisiae* (3). This sequence also included a 390 base pair insertion resembling a Group I intron, located 31 nucleotides from the 3′ end of the rRNA gene (3). Absence of this sequence from mature 16S rRNA (4) and demonstration of its ability to spontaneously excise from transcripts of cloned fragments of the gene (5) confirmed its identity as a self-splicing intron (6–7). The sequence of the 5S rRNA of *P.carinii* grown in nude rats showed closer similarity to 5S rRNA of Amoeba and Myxomycota than to that of Ascomycetes such as Saccharomyces (8). However, the validity of sequence analysis of such relatively short rRNA genes as a taxonomic tool has been questioned (9–10). In *S.cerevisiae*, the 5S rRNA is encoded in the same genomic repeated element encoding 16S, 5.8S and 26S rRNAs, but on the opposite strand (reviewed in 11), although most eukaryotes studied do not have the gene for 5S rRNA linked to those for the other rRNA species. Hybridization of chromosomal DNA separated by pulsed field electrophoresis with 16S rRNA-derived probes has localized the 16S rRNA gene of Pneumocystis to one or two 500 kbp. chromosomal DNAs, with the gene for 5S rRNA apparently located elsewhere (12–13).

In order to better understand the molecular genetics of Pneumocystis, we have determined the sequence of the portion of the major rRNA-encoding operon (encoding the 16S, 5.8S and 26S rRNA molecules) from organisms derived from the lungs of immunosuppressed rats, including the genes for 5.8S and 26S rRNAs. These results indicate that these two genes also show similarity to the homologous genes of *S.cerevisiae*, with the gene for 26S rRNA also containing an apparent Group I self-splicing intron.

The relatedness of different Pneumocystis isolates has been difficult to determine in the absence of a long-term culture method for this organism. The 5S rRNA gene amplified by polymerase chain reaction (PCR) from multiple infected humans and rats had the identical sequences (14). However, rat and human-derived organisms showed sequence differences in their mitochondrial DNA (15). When portions of the 26S rRNA gene from two different sources were sequenced, we found that phylogenetically variable regions of the gene differed between these two organisms. This marked sequence difference between 26S rRNA gene sequences may represent differences between clones of the same species or may indicate the existence of more than one species within the genus Pneumocystis. In either case, such differences might provide a mechanism of recognizing the relationships between different individual Pneumocystis isolates for epidemiological studies. The existence of such differences between small subunit rRNA sequences from different isolates was previously suggested based on a single nucleotide difference obtained by two different laboratories (4).

METHODS

Growth and purification of *Pneumocystis carinii*

Sprague-Dawley rats from Sasco, Inc. (Omaha, NE) were maintained in isolation cages with protective filters (Lab Products, Maplewood, NJ) were immunosuppressed by addition of dexamethasone (1 mg/ml) and tetracycline (0.5 mg/ml) to their drinking water. Water and autoclaved 8% protein diet (ICN) were provided *ad libitum*. Hooded rats (Harlan-Sprague-Dawley, Indianapolis, IN), provided by R.Heikkila (deceased) of this institution were treated in the same way but not isolated. Rats were sacrificed after 8–12 weeks of immunosuppression or when

* To whom correspondence should be addressed

Table I. Oligonucleotides Used

Number	Sequence	5′ Coordinate	Ref.
228A	AACAGCTATGACCATGAT	pUC polylinker	
229	TTCCCAGTCACGACGTTG	pUC polylinker	
230	TGTAAAACGACGGCCAGT	pUC polylinker	
1138	AGGGATTGGTTGGCCTGGTCCTCCGAA	637(+), 16S	3
1887	CTTTCCAGTAATAGGCTTATCG	1726(−), 16S	3
2892	GCTATCCTGAGGGAAACTTCGG	964(−), 26S	
2893	CCCGTCTTGAAACACGGACCAAGG	635(+), 26S	
2894	CCCGCGATCAGCAAAAGCTAATCTGG	1374(−), 16S	3
2917	CCATACAGAAGACCATTCTTTATCCC	507(−), DHFR	19
2918	GGCCGATCAAACTCTCTTCC	58(+), DHFR	19
2919	GGGAAAAGGTCGTGGGGAGCG	977(−), TS	18
2920	GGGGAAGACCGCCCTGATAGG	58(+), TS	18
2982	GAGCCAATCCTTATCCCGAAGTTACG	1933(−), 26S	
2983	GTCTAAACCCAGCTCACGTTCCC	2933(−), 26S	
3175	GGGTGGTGGTGCATGGCCG	1262(+), 16S	3
3176	CCTTCCGCAGGTTCACCTACGG	1796(−), 16S	3
3243	CCGCAGCAGGTCTCCAAG	1833(+), 26S	
3425	CGAAAGAGAGGAGGTAGCACC	368(+), intron, 16S	5
3426	GGTCCGTGTTTCAAGACGGG	654(−), 26S	
3427	GGGAACGTGAGCTGGGTTTAG	2911(+), 26S	
4016	GGTTTGGCAGGCCAACATCGG	485(+), 26S	
4138	CCATGAAAGTGTGGCCTATCG	2715(+), 26S	
4139	GCCTGGTCAGACAACCGC	3049(−), 26S	
4169	GGATTATGGCTGAACGCC	3074(+), 26S	
4170	GGCTTAATCTCAGCAGATCG	3328(−), 26S	
4358	GACGAGGCATTTGGCTACC	2267(−), 26S	
4443	GTACACACCGCCCGTCGC	1631(+), 16S	3
4743	TTTAGCTCTTGATTGTAG	556(+), 26S, Pc2	
4744	CGCATATTTTATATTATG	3234(−), 26S, Pc2	
4746	GTTAGCTCTTGGCTTCTG	556(+), 26S, Pc1	

Table I lists all primers used for PCR amplifications and sequencing. The underlined G in 3243 was predicted for the 26S rRNA gene sequence based on sequences from other organisms, but was A in the actual 26S rRNA sequence of *P.carinii*. The underlined C in 4169 was present in the 26S rRNA gene of *P.carinii* from Hooded rats (Pc2) but was A in the homologous location in organisms from Sprague-Dawley rats (Pc1), as described in the text. The underlined C in 3425 is from the published intron sequence (5) but was T in a clone of the intron amplified using flanking exon-derived primers 4434 and 3176 (data not shown). TS indicates the thymidylate synthase (18) and DHFR the dihydrofolate reductase (19) genes of *P.carinii*.

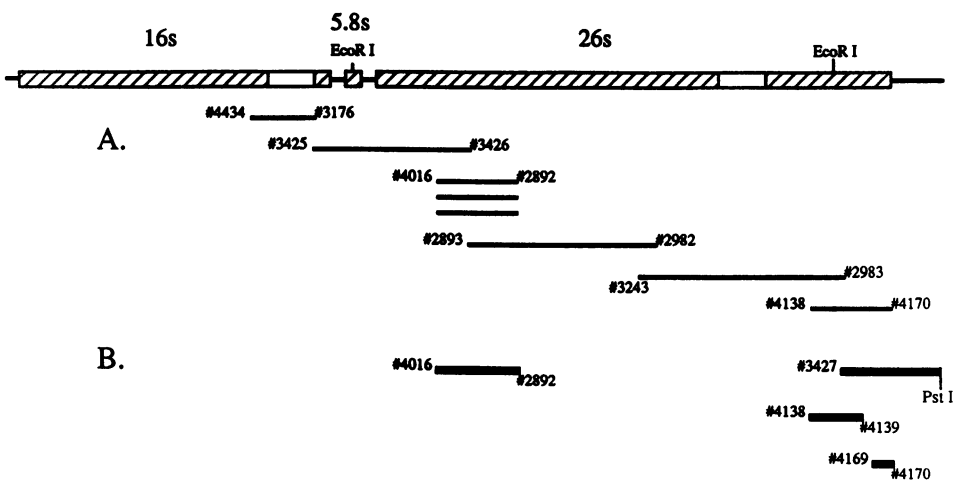


Figure 1. The top line represents the DNA sequence of a portion of the rRNA-encoding gene(s) of *P.carinii* isolated from immunosuppressed Sprague-Dawley rats (Sasco), and the horizontal lines below represent PCR amplifications, which were subsequently cloned and sequenced as described in the text. Thin lines (A) indicate PCR products from Sprague-Dawley rats (Sasco) and heavy lines (B) indicate PCR products from Hooded rats. Numbers indicate oligonucleotide primers (Table I) used in each PCR reaction.

signs of respiratory distress were observed. All subsequent procedures were done at 4°C. Each pair of lungs was removed, minced with a scissors and the homogenate was suspended in 25 ml of Dulbecco's Modified Eagle's Medium (DMEM) and centrifuged for 10 min at 200×*g* to remove tissue debris and lung cells. The supernatant was then transferred to a fresh tube, cells were collected at 1,600×*g* and resuspended in 3 ml of phosphate buffered saline (PBS). Suspended cells were loaded

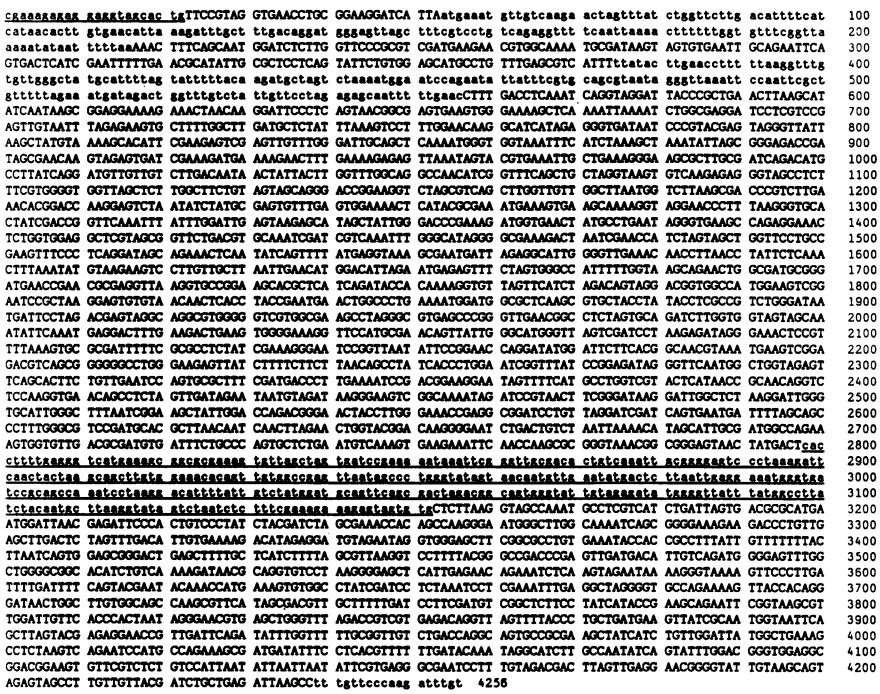


Figure 2. The total contiguous sequence determined for *P. carinii* from immunosuppressed Sprague-Dawley rats (Sasco) by the strategy shown in Figure 1A is shown. All sequences were determined at least twice on each strand from the clones described in Figure 1A, except for the last 18 nucleotides (indicated in lower case) which were determined from DNA amplified from Hooded rats as described in the text. Except for this region, capital letters indicate rRNA coding sequences (positive strand), lower case letters indicate spacers, and underlined lower case letters indicate Group I introns. The initial 22 nucleotides are from the 3′-terminal portion of the Group I intron in 16S rRNA. Nucleotides 23−53 are the second exon of 16S rRNA, 54−216 are internal transcribed spacer 1 (ITS1), 217−374 the gene for 5.8S rRNA (identified by similarity to other 5.8S rRNA sequences), 375−556 ITS2, and 557−4256 are the gene for 26S rRNA, with a Group I intron sequence in lower case underlined. This sequence has been deposited at EMBL/GenBank under accession No. M86760.

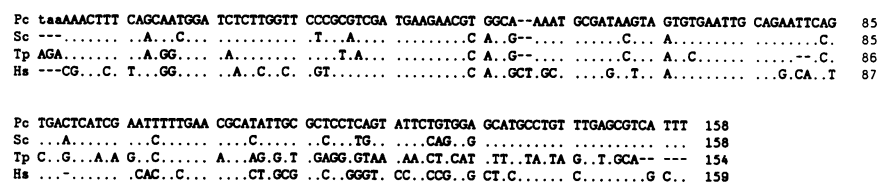


Figure 3 indicates comparison of the sequence of the 5.8S rRNA gene of *P. carinii* shown in Figure 2 with the homologous sequences from *Saccharomyces cerevisiae* (24) indicated as Sc, *Tetrahymena pyriformis* (25) indicated as Tp, and *Homo sapiens* (26) indicated as Hs. Since the actual 5.8S rRNA sequence was not determined, the termini of the *P. carinii* gene have been chosen based on the known sequence of the homologous gene of *S. cerevisiae*, to which it appears to be closely related. The three nucleotides 5′ to the proposed rRNA 5′ terminus are indicated here in lower case letters.

on discontinuous Percoll gradients (10−40% in 10% steps) and after centrifugation at 1,600×*g* for 30 min, trophozoites were found at the 10−20% interface, cysts with some trophozoites and a few mammalian cells at the 20−30% interface, and predominantly mammalian cells with some cysts at the 30−40% interface.

For *in vitro* cultivation of *P. carinii*, mink lung cells of line ATCC CCL64 (16) grown to 80% confluence in 10 cm petri dishes in DMEM supplemented with 10% fetal calf serum were used as feeder cells. Percoll gradient purified cysts (5×10^5) were added to each plate in the presence of penicillin, streptomycin, gentamicin and fungizone, followed by incubation at 37°C in a humidified 5% CO_2 incubator. After 1−3 days in culture, the plates were gently agitated and the Pneumocystis-containing medium was collected and centrifuged at 100×*g* for 5 min to pellet contaminating detached mammalian cells. Only a few mammalian cells detached during the culture period and these were efficiently removed by the centrifugation.

Microscopic techniques

Pneumocystis trophozoites were quantitated in 5 μl samples air dried on microscope slides and stained with Diff-Quik (Baxter Healthcare Co., Miami, FL). Cysts were identified by toluidine blue O stain (17). All quantitation was done by counting three 5 μl samples for a total of 30 oil immersion fields for each sample. All cultures and purified Pneumocystis preparations were negative for fungal and bacterial contamination by microscopy and culture, and for Mycoplasma contamination by MycoTect kit (Gibco BRL).

Extraction of nucleic acids from Trophozoites

P.carinii cells from mink lung cell cultures were harvested by centrifugation at 3,000 rpm for 30 minutes at 4°C in a Sorvall SS-34 rotor, and were washed with chilled PBS. Cells were resuspended in 50 mM Tris-HCl, 50 mM Na-EDTA, pH 8.0, and were lysed by incubation at 65°C for 30 minutes in the presence of 1% SDS. Proteins were removed by precipitation on ice in the presence of 1.25 M potassium acetate followed by centrifugation at room temperature. Total nucleic acids were then concentrated by precipitation in an equal volume of absolute ethanol on ice.

Oligonucleotides

DNA oligonucleotides were synthesized by beta-cyanoethyl phosphoramidite chemistry on automated DNA synthesizers (Cyclone, Milligen and 380B, Applied Biosystems), and were purified by chromatography on NENsorb-Prep cartridges (NEN-DuPont). Oligonucleotides used are listed in Table I.

Amplification and cloning of DNA

Pneumocystis carinii DNA was amplified by means of PCR performed in a DNA Thermal Cycler (Perkin Elmer Cetus) using thermostable DNA polymerase from *Thermus aquaticus* (AmpliTaq, Perkin Elmer Cetus). Reactions were run in the presence of 0.2 mM of each dNTP, 0.4 μM of each of the indicated primers, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, gelatin (0.001% w/v), and 5 units of AmpliTaq DNA polymerase in 100 μl total volume. Amplifications of over 1 kb. segments were performed by incubation at 95°C for 2 minutes followed by 30 cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes, followed by a 7 minute incubation at 72°C. Amplifications of fragments of less than 1 kb. were performed by 2 cycles of 94°C for 2 minutes, 58°C for 1 minute, and 72°C for 45 seconds, followed by 30 cycles of 94°C for 1 minute, 58°C for 1 minute, and 72°C for 1 minute, followed by incubation at 72°C for 1 minute. For some PCR reactions the thermostable DNA polymerase from *Thermus thermophilus* (Hot Tub, Amersham) was used, under reaction conditions recommended by the manufacturer using 1.5 units of polymerase in a 100 μl reaction, using 2 cycles of 94°C for 2 minutes, 58°C for 1 minute, and 70°C for 2 minutes, followed by 30 cycles of 94°C for 1 minute, 59°C for 1 minute, and 70°C for 3 minutes, followed by incubation at 70°C for 10 minutes. After PCR reaction, products were purified by agarose gel electrophoresis, treated with T4 DNA polymerase (BRL) to generate blunt ends, phosphorylated with T4 polynucleotide kinase (Pharmacia), ligated under blunt end ligation conditions to *Sma*I-cut pUC18 DNA (plasmid provided by J. Dougherty of this department), and transformed into *E. coli* DH5α competent cells (BRL, Bethesda, MD) as described (20). Cells were grown in LB medium and plasmid DNA was extracted and purified as described (20).

DNA Sequence Determination

DNA sequence determination was performed on the Genesis 2,000 Automated DNA Sequencer (DuPont) according to the manufacturer's instructions for sequencing reactions run on covalently closed superhelical DNA templates, using DNA polymerase from bacteriophage T7 (Sequenase version 1.0, U.S. Biochemicals). Primers used included oligonucleotides 228A, 229, and 230 (Table I), which base pair with regions flanking the pUC18 polylinker, and others listed in Table I. For inserts of over 300 nucleotides without convenient internal primer binding sites, nested deletions were generated as described (20), which were then sequenced using the standard primers. All sequences reported were determined at least twice for each DNA strand.

RESULTS

Sequence of the rRNA operon of *P.carinii*

Prior to use for these experiments, nucleic acids from *P.carinii* were shown to be from that source by confirmation of previously published sequences using PCR methods. Primers 2920 and 2919 used in a PCR reaction yielded a single 920 bp. product (based on agarose gel electrophoresis), the size predicted for the thymidylate synthase gene with its 4 intervening sequences (18). A PCR utilizing primers 2918 and 2917 amplified a single 493 bp. product, as predicted for the dihydrofolate reductase gene with a 43 bp. intervening sequence (19). The *P.carinii*-specific primers for 16S rRNA, 1138 and 2894, yielded a single PCR product of the predicted 738 bp. size (3). The 'universal' 16S rRNA primers, 3175 and 3176, generated two PCR products: one was 925 bp. in length, the size predicted for the 16S rRNA gene with its Group I intron (3, 5), and the other was 535 bp. in length. This smaller fragment had a sequence identical to the corresponding region of human 18S rRNA (21), and presumably represents amplification of contaminating mink lung cell ribosomal DNA rather than amplified reverse transcript (22) of *P.carinii* rRNA (data not shown).

Figure 1 shows the amplifications used for subsequent cloning and sequence determination. Each PCR product, produced using primers listed in Table I, was cloned into pUC18 and both strands were sequenced at least twice. All overlapping segments yielded the same sequence, indicating an error rate of Taq polymerase-catalyzed PCR (23) of less than one per 500 nucleotides. We cannot rule out rare misincorporation events in the regions which

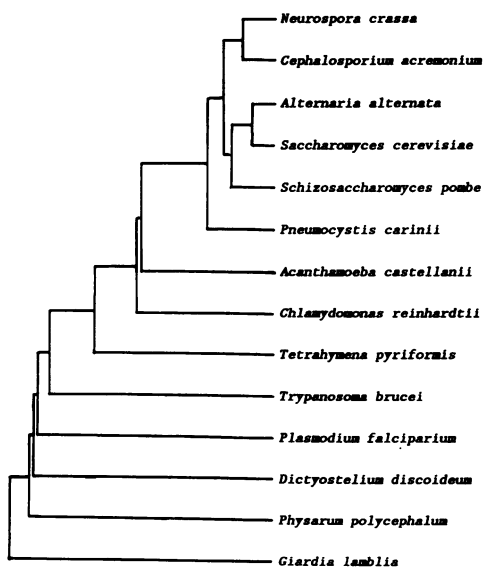


Figure 4 is a dendrogram generated by the 'pileup' program of the Wisconsin-GCG package indicating sequence similarity (but not necessarily evolutionary relationships) among the 5.8S rRNAs compared in Table II.

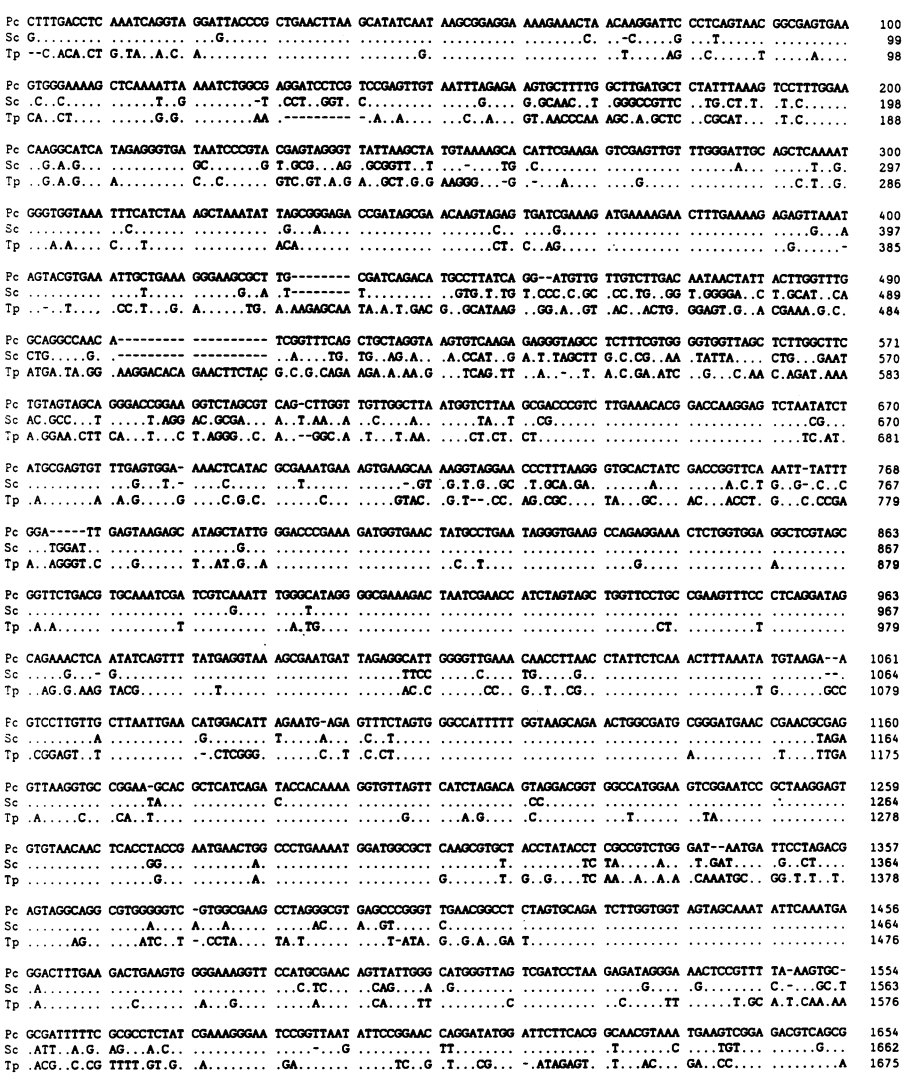
were only amplified once. The sequence determined for the amplified portion of the rRNA operon of *P.carinii* is shown in Figure 2. The sequence of the final exon of the 16S rRNA gene agrees with that previously reported (3), although the third base from the 3′ end of the intron (C) previously reported (5) is absent in our sequence. This sequence has been confirmed in an additional amplified fragment (amplified with primers 4434 and 3176) including the entire intron sequence (data not shown).

The sequence of the 5.8S rRNA gene indicated in Figure 2 was recognized by similarity to the 5.8S rRNA sequences from other eukaryotes (24−26), with which it is compared in Figure 3. The 5.8S rRNA sequence is 87% identical with the homologous rRNA of *S.cerevisiae*, which was also the species to which *P.carinii* showed closest relatedness of its 16S rRNA gene (3). In contrast, the 5.8S rRNA sequence was 67% and 69% identical with the homologous genes of *T.pyriformis* and *H.sapiens*, respectively. Table II shows a similarity matrix of fungal and protozoan 5.8S rRNA sequences; these data are expressed as a dendrogram in Figure 4. The *P.carinii* 5.8S rRNA sequence is most similar to five fungal sequences, and is more distantly related to sequences from various protoza, algae and slime molds. However, this dendrogram may not be identical to one based on evolutionary distances. Also, taxonomic classification based on sequences of relatively short genes with conserved regions may not be valid, as has been suggested from genes for 5S rRNA (9−10).

Figure 5 shows the sequence of the 26S rRNA gene from Figure 2 compared to homologous genes from *S.cerevisiae* (27) and *T.pyriformis* (28). The indicated *P.carinii* sequence has an apparent Group I self-splicing intron sequence (see below) omitted after nucleotide 2241, and the *T. pyriformis* sequence has an intron of the same type omitted from a location four nucleotides 3′ to the homologous site in the *P.carinii* gene (28). Thus the 26S rRNA genes of both *P.carinii* and *T. pyriformis* have Group I self-splicing introns inserted into the same relatively conserved region. Comparison of the three sequences shown in Figure 5 indicates the relative conservation of some regions of the 26S rRNA genes, and the greater phylogenetic variability of other regions. Comparison of the coding regions of the four completely sequenced 26S rRNA genes of microbial eukaryotes are shown in Table III; *P.carinii* is again more similar to *S.cerevisiae* than to *T. pyriformis*.

Group I self-splicing introns of rRNA genes

As was indicated in Figure 2, an apparent Group I self-splicing intron interrupts the 26S rRNA gene sequence in *P.carinii*. This intron is recognizable by the presence of the conserved P, Q, R, and S segments (boldface in Figure 6A) present in all introns



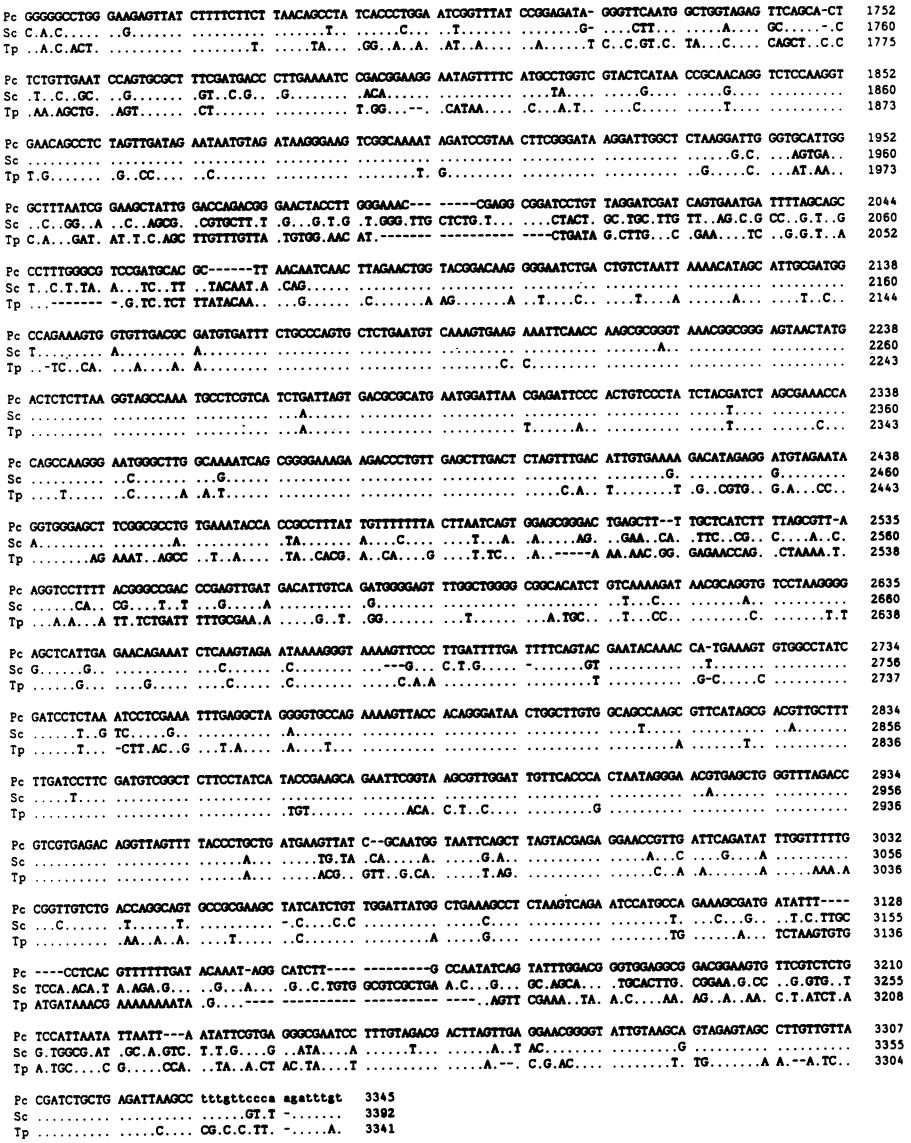


Figure 5 shows a comparison of the sequence of the 26S rRNA genes of *P.carinii* (Pc) from Figure 2, with homologous sequences from *S.cerevisiae* (Sc), and *T. pyriformis* (Tp). As indicated in the text, the Group I self-splicing introns in the *P.carinii* and *T. pyriformis* genes have been omitted. The final 18 nucleotides of the *P.carinii* sequence were determined from organisms from immunosuppressed Hooded rats as indicated in Figure 2.

of this class, as previously reviewed (6–7). There is 74% identity between the sequence of the putative Group I intron in the 26S rRNA gene and that previously reported (5) in the 16S rRNA gene. Our sequence for the intron in the 16S rRNA gene is identical to that reported (5) except for the absence of the third nucleotide from the 3′ end of the intron (C); we have confirmed the sequence of the 16S rRNA gene (3) 3′ to nucleotide 1649. Comparison of the sequences of the introns in the genes encoding 26S (Figure 6A) and 16S rRNA (5, Figure 2) reveals nearly complete identity in a 'core' region of the intron structure. Except for G89 of the 26S rRNA intron which corresponds to A in the 16S intron, all nucleotides beween sites 78 and 251 of the 26S intron are conserved in the 16S intron sequence. The remainder of the sequences of the two introns show less similarity, but secondary structure of the two introns is highly conserved.

Figure 6A indicates that the 26S rRNA gene intron can be folded into a structure similar to that reported for other Group I self-splicing introns (6–7), including that in the gene encoding 16S rRNA in *P.carinii* (5). This structure could not be reproduced by the Wisconsin-GCG 'fold' program (29), but is most consistent with the consensus folding proposed for Group I introns (7). The structure in Figure 6A contains the conserved P1 double-helix made up of a pairing of the 5′ exon-intron junction with an internal guiding intron sequence (IGS). It also contains an unusually long P8 helix with a bulge-loop on its 5′ side; although the previously proposed structure for the 16S intron (5) does not have such an elongated P8 helix, its structure also can be drawn in this way (Figure 6B).

We have utilized PCR primers pairing to the exons on either side of the 26S rRNA gene intron, including a 5′ primer with a 17-nucleotide 5′ extension consisting of a bacteriophage SP6 promoter (30), to generate a DNA product consisting of the intron sequence with portions of both flanking exons with an SP6 promoter at the 5′ end of the positive strand. Transcription of this DNA by bacteriophage SP6 RNA polymerase (Promega) results in production of RNA catalyzing self-splicing under similar

Table II. Sequence similarity of 5.8S rRNAs of simple Eukaryotes

	Nc	Ca	Aa	Sc	Sp	Pc	Ac	Cr	Tp	Tb	Pf	Dd	Pp	Gl
Nc	1.0000	.9299	.9236	.9172	.8599	.8854	.7771	.7308	.6883	.6624	.5159	.5414	.5097	.4483
Ca		1.0000	.8924	.8797	.8544	.8418	.7215	.7244	.6688	.6519	.4873	.5506	.4968	.4828
Aa			1.0000	.9494	.8987	.8671	.7722	.7436	.6883	.6582	.5380	.5506	.5161	.4483
Sc				1.0000	.9114	.8734	.7848	.7564	.7143	.6392	.5316	.5696	.5161	.4483
Sp					1.0000	.8165	.7407	.7500	.7143	.5879	.5273	.5432	.5290	.4759
Pc						1.0000	.7468	.7051	.6753	.6519	.5063	.5443	.5032	.4207
Ac							1.000	.7500	.6818	.5679	.5185	.5000	.5032	.4828
Cr								1.0000	.6429	.5641	.5513	.4744	.4516	.4552
Tp									1.0000	.5844	.5714	.5130	.5000	.4414
Tb										1.0000	.4702	.4691	.5161	.4138
Pf											1.0000	.4753	.4452	.3793
Dd												1.0000	.4065	.3862
Pp													1.0000	.4483
Gl														1.0000

Table II indicates the extent of genetic identity as indicated by the Wisconsin-GCG 'Distances' program. Sequences are from GenBank with the following accession numbers: *Neurosopora crassa*, Nc X02447; *Cephalosporium acremonium*, Ca X06574; *Alternaria alternata*, Aa X17454; *Saccharomyces cerevisiae*, Sc K01051; *Schizosaccharomyces pombe*, Sp J01359; *Pneumocystis carinii*, Pc; *Acanthamoeba castellani*, Ac K00471; *Chlamydomonas reinhardtii*, Cr M35013; *Tetrahymena pyriformis*, Tp M10752; *Trypanosoma brucei*, Tb X05682; *Plasmodium falciparum*, Pf J04683; *Dictyostelium discoideum*, Dd V00192; *Phyarum polycephalum*, Pp M13612; and *Giardia lamblia*, Gl M35013.

conditions to those reported (5) for self-splicing of the intron in the 16S rRNA gene (Y.Liu and M.J.Leibowitz, unpublished). Thus the three rRNA genes encoding 16S, 5.8S and 26S rRNA of *P.carinii* closely resemble their fungal homologues in sequence. However, they contain Group I self-splicing introns in the 16S and 26S rRNA genes, unlike known fungi but like some protozoa (28).

Sequence Variation between *P.carinii* Isolates

In the course of studies to confirm the sequence shown in Figure 2, various regions of the rRNA operon of *P.carinii* were repeatedly amplified and sequenced. Organisms obtained from the lungs of Sprague-Dawley rats (Sasco) immunosuppressed in isolation chambers yielded the same sequences for duplicate or overlapping amplifications, as summarized in Figure 1. When portions of the 26S rDNA were amplified, cloned and sequenced from *P.carinii* obtained from Hooded rats immunosuppressed without isolation, they were found to differ in sequence from the same regions obtained from organisms from Sprague-Dawley rats from Sasco (Figures 7 and 8). Figure 7 shows the sequence of a region of the 26S rRNA gene which was determined for five independent PCR products (summarized in Figure 1) using three different sets of primers from *P.carinii* from Sprague-Dawley rats, for the region of nucleotides 485–964 as shown in Figure 5. This sequence is denoted Pc1 in Figure 7, and was identical in three full-length and two partial clones of this region,

Table III. Sequence similarity of 26S rRNAs of simple Eukaryotes

	Pc	Sc	Tp	Pp
Pc	–	0.833	0.739	0.623
Sc		–	0.734	0.602
Tp			–	0.605

Table III indicates the extent of genetic identity of 26S rRNA gene sequences, calculated as in Table II. Abbreviations are as in Table II; sequences from GenBank include Sc, J01355; Tp, X54004; and Pp, V01159.

as indicated in the legend of Figure 7. When the pair of primers indicated in Figure 7 was used to amplify DNA from *P.carinii* from Hooded rats, the sequence indicated as Pc2 was obtained. Comparison of these sequences with those of *S.cerevisiae* and *T. pyriformis* 26S rRNA sequences demonstrates that the DNA sequences of the two *P.carinii* isolates differ from each other at multiple positions, with the differences occurring mostly in phylogenetically variable regions of the rRNA sequence. However, the two *P.carinii* sequences are clearly more similar to each other than to the sequence of the *S.cerevisiae* gene, indicating the phylogenetic relatedness of these two isolates. Similarly, as is shown in Figure 8, the 3'-terminal region of the 26S rRNA gene of *P.carinii* from these two sources differed from each other, with most of the differences in phylogenetically non-

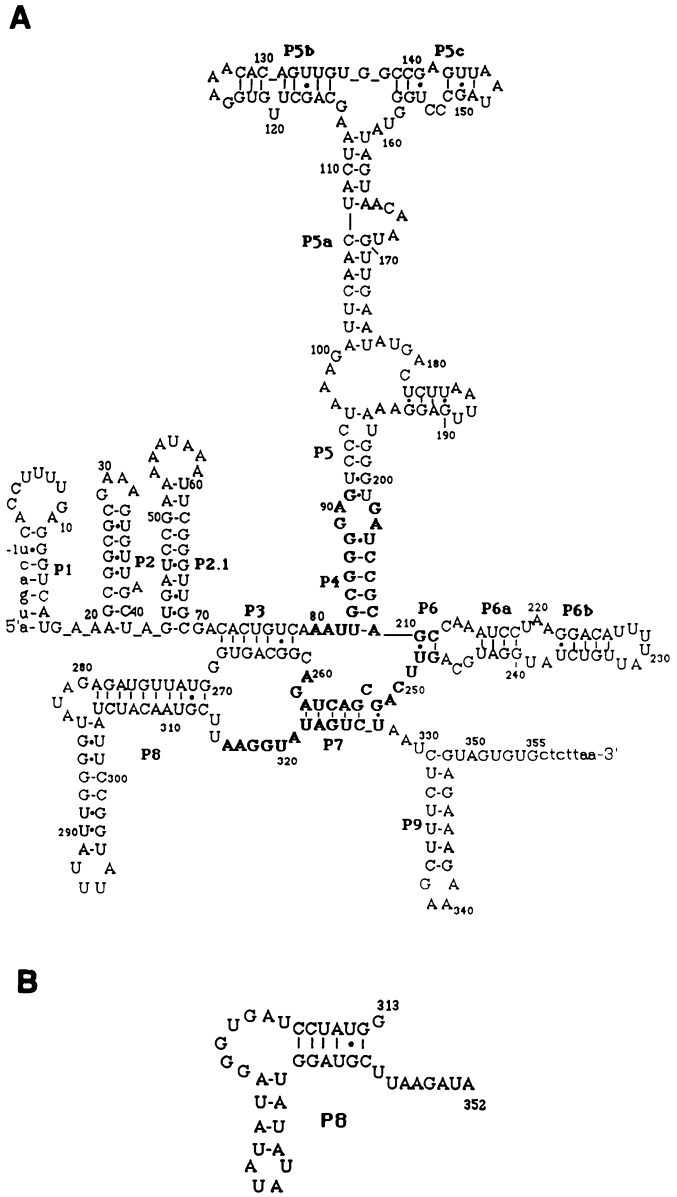


Figure 6 (panel A) shows the secondary structure into which the apparent Group I intron in the gene for 26S rRNA of *P. carinii* can be folded. The helices P1-P9 are conserved among Group I introns (6–7). The bases in the intron are numbered 1 through 355, and the flanking exon regions are indicated in lower case letters. The consensus sequences P (nucleotides 80–91), Q (nucleotides 202–211), R (nucleotides 247–260) and S (nucleotides 316–327) are indicated in boldface. Panel B shows an alternative folding for the P8 helix of the intron (5) in the 16S rRNA gene.

conserved regions; again the two *P. carinii* genes showed greater similarity to each other than to the genes from other species.

When Pc1 DNA template was amplified by PCR using the primer pair 4358 (universal) and 4746 (Pc1-specific), the expected 2,067 bp product was produced; in contrast, no product was generated from Pc2 template with these same primers (Figure 9). Similarly, primers 4743 (Pc2-specific) and 4744 (Pc2-specific) amplified an approximately 3.0 kbp product from Pc2 template; no similar product was seen with Pc1 template (Figure 9). Note that in some reaction a barely detectable band of the same size seen with Pc2 template was seen with Pc1 template using the latter primer pair. These data are consistent with Pc1 and Pc2

```
Pc1  GGTTTGGCAG GCCAACA--- ---------- ------TCGG TTTCAGCTGC TAGGTAAGTG   525
Pc2  .......... .......--- ---------- ------.... .......... .......AGA
Sc   AT..CACTG. ....G..--- ---------- ------..A. ...TG.TG.. AG.A...A.C
Tp   A.G.C.ATGA .TA.GG.AAG GACACAGAAC TTCTACG.C. G.CAGAAGA. A.AA.G...T

Pc1  TCAAGAGAGG GTAGCCTCTT TCGTGGGGTG GTTAGCTCTT GGCTTCTGTA GTAGCAGGGA   585
Pc2  ........A. .......... .TT..... T......... .AT.GTA... .C...T....
Sc   CAT..GA.T. TAGCTTG.C. CG..AA.TAT TA....CTG. ..GAATAC.G CC...T....
Tp   CAG.TT..A. .-..T.A.C. GA.ATC..G. ..C.AAC.AG AT.AAAA.GG AA.CTTCA..

Pc1  CCGGAAGGTC TAGCGTC--- -AG-CTTGGT TGTTGGCTTA ATGGTCTTAA GCGACCCGTC   640
Pc2  .......... ....AAAATA T..-...... .......... .......... ..........
Sc   .T.AGGAC.G CGA...A--- -..T.AA..A ..C....A.. .....TA..T ..CG......
Tp   .T...CT.AG GG..C.A--- -..--GGC.A .T...T.AA. ....CT.CT. CT........

Pc1  TTGAAACACG GACCAAGGAG TCTAATATCT ATGCGAGTGT TTGAGTGGA- AAACTCATAC   699
Pc2  .......... .......... .......... ...G.....- ....C...G.
Sc   .......... .......... .....CG... .......... ...G...T.- ....C.....
Tp   .......... .......... ....TC.AT. .A.......A .A.G.....G ....C.G.C.

Pc1  GCGAAATGAA AGTGAAGCAA AAGGTAGGAA CCCTTTAAGG GTGCACTATC GACCGGTTCA   759
Pc2  .......... .......... .-...G.... .....C-G.. ......C... ..........
Sc   ...T...... ......-.GT .G.T.G..GC .T.GCA.GA. ......A... .....A.C.T
Tp   ......C... .....GTAC. .-...GCC.. G..G-C.... TA...GC... AC...ACCT.

Pc1  AATT-TATTT GGA-----TT GAGTAAGAGC ATAGCTATTG GGACCCGAAA GATGGTGAAC   813
Pc2  ....-..... ...-----.C .....G.... .......... .......... ..........
Sc   G..G-.C..C ...TGGAT.. .......... ......G... .......... ..........
Tp   G...C.CCGA A..AGGGT.C ...G...... T..AT.G..A .......... ..........

Pc1  TATGCCTGAA TAGGGTGAAG CCAGAGGAAA CTCTGGTGGA GGCTCGTAGC GGTTCTGACG   873
Pc2  .......... .......... .......... .......... .......... ..........
Sc   .......... .......... .......... .......... .......... ..........
Tp   ..C..T.... .......... ....G..... .......... A......... .A.A......

Pc1  TGCAAATCGA TCGTCAAATT TGGGCATAGG GGCGAAAGAC TAATCGAACC ATCTAGTAGC   933
Pc2  .......... .......... .......... .......... .......... ..........
Sc   .......... .....G.... ....T..... .......... .......... ..........
Tp   .........T .......... ..A.TG.... .......... .......... ..........

Pc1  TGGTTCCTGC CGAAGTTTCC CTCAGGATAG C  964
Pc2  .......... .......... .......... .
Sc   .......... .......... .......... .
Tp   .......CT. .........T .......... .
```

Figure 7 shows the sequence of the region from nucleotides 485 through 964 of the 26S rRNA gene from *P. carinii* from Sprague-Dawley rats, as indicated in Figure 5 (Pc1). This sequence was determined for three PCR products made using oligonucleotides 4016 and 2892 as primers and for PCR products made using the oligonucleotide pair 3425 and 3426, and the pair 2893 and 2982, each resulting in products partially overlapping this region. This entire sequence was thus determined on four or five isolates, with four separate sequence determinations made for each PCR product. The sequence of DNA amplified using the same primers (4016 and 2892) from *P. carinii/l/* from Hooded rats is indicated as Pc2. The homologous regions of genes from *S. cerevisiae* (Sc) and *T. pyriformis* (Tp) are also indicated. The numbering is according to the 26S rRNA sequence of Pc1 as in Figure 5. The sequence denoted Pc2 has been deposited at EMBL/GenBank under accession No. M86761.

each containing predominantly genes encoding single distinct major 26S rRNA sequences.

External transcribed spacer sequence

The sequence of the 26S rRNA gene shown in Figure 3 contains a phylogenetically conserved *EcoR*I site at position 2875, which is located in a highly conserved region of the sequence. DNA isolated from *P. carinii* from Hooded rats was restricted with pairs of restriction enzymes, including *EcoR*I and various other '6-cutters,' and the resulting fragments were then ligated into pUC18 cut with the same pairs of restriction enzymes. The product of each of the ligation reactions was then subjected to PCR amplification, with thermostable DNA polymerase from *Thermus thermophilus* (Hot Tub, Amersham) using the primer pair: oligonucleotide 3427, which pairs on the positive strand at positions 2911–2931, and oligonucleotide 230, which pairs with a pUC18 region 3′ to the polylinker (on the negative strand). When such PCR reactions were analyzed by agarose gel electrophoresis with visualization of bands by ultraviolet light-induced fluorescence in the presence of ethidium bromide, only the pair of restriction enzymes *EcoR*I and *Pst*I generated a visible DNA band. When this band was cloned and sequenced, its 5′ region had the sequence indicated as Pc2 in Figure 8, followed by the final 18 nucleotides of the 26S rRNA gene as indicated in Figure 5 and 381 nucleotides of the following spacer region

shown in Figure 10, which would correspond to the external transcribed spacer region in the homologous operon of most eukaryotes (reviewed in 31). When the same ligation-dependent PCR procedure was followed using the DNA from *P.carinii* from Sprague-Dawley rats, no visible band of DNA was detected. This presumably indicates that the *Pst*I site in the spacer of the DNA denoted Pc2 is absent in Pc1 DNA, and the next one is presumably too distant to support ligation-dependent PCR.

DISCUSSION

The rRNA operon of *P.carinii*

Although the exact phylogenetic relationship of *P.carinii* to other species remains unknown, we have confirmed that the 5.8S and 26S rRNA genes, like that for 16S rRNA (3), are similar in primary sequence to the homologous genes of *S.cerevisiae*. This finding contrasts with the report that the 5S rRNA gene most resembles the sequence of the homologous genes of Amoeba or Myxomycota rather than those of the Ascomycetes (8). The organization of the major rRNA operon of *P.carinii* differs from that of *S.cerevisiae* in that for the former there is no evidence

```
Pc1 GGGAACGTGA GCTGGGTTTA GACCGTCGTG AGACAGGTTA GTTTTACCCT GCTGATGAAG   2970
Pc2 .......... .......... .......... .......... .......... ..........
Sc  ......A... .......... .......... .......... .......... A........T
Tp  .......... .......... .......... .......... .......... A........A

Pc1 TTATC--GCA ATGGTAATTC AGCTTAGTAC GAGAGGAACC GTTGATTCAG ATATTTGGTT   3028
Pc2 .....--... .......... .A........ .......... .......... ..........
Sc  G.TA.CA... ..A......G .A........ .........A ...C....G. ...A......
Tp  CG..GTT..G .CA......T .AG....... .......... C..A.A.... ...A.....A

Pc1 TTTGCGGTTG TCTGACGAGG GAGTGCCGCG AAGCTATCAT CTGTTGGATT ATGGCTGAAA   3088
Pc2 .......... .......... .......... .......... .......... .........C
Sc  .......C.. .....T.... ..T....... ....-.C... .C.C...... .........C
Tp  AA.A...... .....AA..A ..A.....T. ......C... .......... ...A.....G

Pc1 GCCTCTAAGT CAGAATCCAT GCCAGAAAGC GATGAT--AT TTCCTCAC-G TTTTTTGATA   3145
Pc2 .......... .......... .......... ......AA.. ........AG A.........
Sc  .......... .......... ..T....C.. .G....TTC. ..G...-.AC ACAA.AT.G.
Tp  .......... .......... ..TG...... A..------- --T..A.GT. .GA.GAT.A.

Pc1 CAAATAGGCA TCTTGC---- ---------- ---CAATATC AG--TATTTG GACGGGTGGA   3186
Pc2 T.G....... .T....---- ---------- ---..G.... ..TA...... .........G
Sc  TGG...C.A. .AAG..GTCC TTGTGGCGTC GCTG..CCAT ..CAGGC.A. C.AC....C.
Tp  .G..A.AAA. .AA------- ---------- ---G..AT.A ..TTCGAAA. .TA.A.C...

Pc1 GGCGGACGGA AGTGTTCGTC TCTGTCCATT A--ATATTAA --TTAATATT CGTGAGGGCG   3242
Pc2 ....A..... .......... .......... .AG...A..T --AA.....G ........T.
Sc  CTT..CG.A. ..GCC.T.GG .GCT.G.TGG CGA..TGC.. TG.C.T.T.G ....G..ATA
Tp  .AA.AG..A. .AA.C.T.-A ...TAA.TGC TAATCG.A.T TCGA...TA. .A.GTAC.TA

Pc1 AATCCTTTGT AGACGACTTA GTTGAGGAAC GGGGTATTGT AAGCAGTAGA GTAGCCTTGT   3302
Pc2 .......... .......... .......... ......C... .......... ..........
Sc  ....A..... .T........ .A..TAC... .......... ....G..... ..........
Tp  ....T..... .......... A.--.C.G.A C......... ...T.TG... ....AA.--A

Pc1 TGTTACGATC TGCTGAGATT AAGCC   3327
Pc2 .......... .......... .....
Sc  .......... .......... .....
Tp  .TC....... .......... C....
```

Figure 8 shows a comparison of the sequences of the region from nucleotides 2911 through 3327 of the 26S rRNA gene of *P.carinii* (Pc1) from Sprague-Dawley rats (Figure 5) with the homologous regions from *P.carinii* from Hooded rats (Pc2) and from *S.cerevisiae* (Sc) and *T. pyriformis* (Tp). The fragment denoted Pc1 was amplified using primers 4138 and 4170; part of this sequence was confirmed for a clone of DNA amplified using primers 3243 and 2983. The sequence shown for Pc2 was determined based on amplifications using primer pair 4138 and 4139 and pair 4169 and 4170, and ligation-dependent PCR amplification of a fragment extending from oligonucleotide 3427 through a *Pst*I site 381 nucleotides past the 3′ end of the 26S rRNA gene (details in text).

that the 5S rRNA and 16S-5.8S-26S rRNA operon genes are part of the same repeated DNA unit, based on pulsed field electrophoresis studies (12–13). We have also failed to show linkage of the 5S rRNA gene to genes encoding 16S rRNA or 26S rRNA by the PCR techniques used in this paper (Y. Liu and M.J.Leibowitz, unpublished results). The amount of DNA obtained from *P.carinii* was limited, and so classical Southern analysis was not attempted.

The presence of Group I self-splicing introns in the 16S and 26S rRNA genes of *P.carinii* distinguishes this organism from *S.cerevisiae* and from its mammalian hosts. Since various compounds can specifically inhibit the splicing of Group I introns *in vitro* (32), Group I intron splicing from transcripts of nuclear genes might provide a specific target for development of new therapeutic agents against *P.carinii*.

Taxonomy of *P.carinii*

The exact taxonomic relationships of *P.carinii* remain uncertain, in part due to the limited number of eukaryotic microorganisms whose rRNA sequences are known. It is possible that once more organisms of this type are studied, the taxonomic relations within and between the Fungi and Protozoa may require some redefinition. This has already proven to be the case for the Microsporidia, which have been placed in a group distinct from all other eukaryotic microorganisms on the basis of their rRNA sequences (33).

In the absence of a long-term culture method or other tools for comparison of different *P.carinii* organisms, the number of species within the genus Pneumocystis is undefined. Antigenic differences between *P.carinii* obtained from different mammalian host species have been demonstrated (34–37), although their genetic basis is not proven. Although the 5S rRNA gene sequences of multiple human and rat isolates of *P.carinii* are identical (14), such isolates differ in the sequence of their

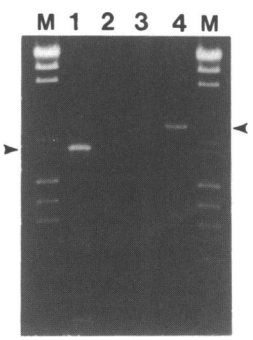


Figure 9 shows the results of PCR amplification confirming the sequence differences between Pc1 and Pc2 shown in Figures 7 and 8. Primers 4358 and 4746 were used to amplify Pc1 (lane 1) or Pc2 (lane2) DNA templates. Primers 4743 and 4744 were used to amplify Pc1 (lane 3) or Pc2 (lane 4) DNA. Lanes M contain a mixture of *Hind*III digested bacteriophage lambda DNA and *Hae*III digested replicative form DNA of bacteriophage ϕX174 (BRL).

```
TCAAAAAGAA CATTTCTTCT GAGTGGTGAG GGGTCCGTTA GAGCACACTC GCTCCTTGGA AGAGATGTTT TTTTTGATAT TAGGAACCAA TAGAATATTT   100
AGAATTTAAT TTAGATTAAA TTATAGAAGG GTATCTGTAG CGATAAGTTT CCATTTCAAA TTTTTCTGAT GCAGTAGTAT GTTCTTTTCT AAAATAAAAT   200
GATAGTTTAT TAATGATTAA ACTAATTATT ATCCTTTGGC CATCTTTTTC TACATTTTCC AGAAACAGAT CTAATTACGT TTTTGCTATC TATAATTATT   300
AAAAATAATC ATATATCTTT AAAGTTGACC TCAACGTCTT AAAATGTTTA GTTTTTTAAT TAACCCTAAA CCCTAGAACA C                       381
```

Figure 10 shows the sequence of the spacer region 3′ to the 26S rRNA gene of *P.carinii* from Hooded rats (Figure 8), which was determined by ligation-dependent PCR as described in the text. The sequences shown in Figures 8 and 10 have been deposited at EMBL/GenBank under accession No. M86759.

mitochondrial DNA (15). DNA hybridization methods with a cloned DNA fragment have also suggested the non-identity of human and rat-derived *P.carinii*, with differences noted among different human, but not rat, isolates (38). Based on these results, it has been suggested that subspecies of *P.carinii* might be designated based on the hosts from which they are isolated (39).

The data presented in this paper indicate that multiple differences exist between the predominant 26S rRNA gene sequences of *P.carinii* from Sprague-Dawley rats from Sasco which were immunosuppressed in isolation (and therefore presumably infected at some other location prior to their arrival here) and Hooded rats which were immunosuppressed here without isolation (and therefore presumably infected in this building or at some geographic location distinct from the site at which the Sprague-Dawley rats were infected). Since multiple independent PCR amplifications of portions of the 26S rRNA gene prepared from templates derived from different individual rats of the same type yielded identical sequences, there is no evidence that the differences observed between the two sources represent PCR artefacts or sequencing errors. However, since we did observe faint and variable PCR bands corresponding to the Pc1 sequence in some DNA preparations from *P.carinii* from hooded rats, we cannot exclude possible mixed infections with multiple strains, as previously claimed to occur (40), or heterogeneity of rRNA sequences within an individual cell, as has been reported in Plasmodium species (41). The variation between different *P.carinii* isolates resembles that seen between different individual humans, which also occurs in regions of the 26S rRNA gene which are phylogenetically non-conserved (42). Sequence differences in rRNA genes have been suggested as defining species differences within the genus Giardia (43).

Comparisons of the sequences of multiple *P.carinii* rRNA gene regions should determine the extent of variability present. If different human isolates of this organism vary as much as do different rat isolates, then these sequences could be useful as epidemiological markers for identifying strains of *P.carinii* and studying the spread of the organism and the relative roles of new infection versus reactivation of earlier asymptomatic colonization in the development of *P.carinii* pneumonitis in immunosuppressed humans, including patients with AIDS. Since different species of Tetrahymena differ more in their intron sequences than in the sequences of adjacent conserved regions encoding rRNA (28), such regions might prove to be even more variable between different *P.carinii* organisms. Further studies will be needed to determine the variability within and between species of the internal transcribed spacers (between the 16S and 5.8S rRNA and 5.8S and 26S rRNA genes) and external transcribed spacers (flanking the rRNA coding regions). If these spacers contain regions with specific functions in rRNA transcription or processing (31), such regions might show sequence conservation.

ACKNOWLEDGEMENTS

Support by the New Jersey Center for Advanced Biotechnology and Medicine is gratefully acknowledged. Y.L. was supported by a fellowship from the Core Curriculum in Molecular and Cell Biology of Rutgers University and UMDNJ. Oligonucleotides were provided by L.Flores of this department. We thank W.T.Hughes, Jr., of St. Jude's Children's Hospital for providing *P.carinii* cysts and helpful advice early in the course of these studies and D.Y.Thomas of the National Research Council of Canada for discussions of PCR methods.

REFERENCES

1. Pifer,L.L., Hughes,W.T., Stagno,S., and Woods,D. (1978) *Pediatrics*, **61**, 35–41.
2. Hughes,W.T. (1991) *Annu. Rev. Med.*, **42**, 287–295.
3. Edman,J.C., Kovacs,J.A., Masur,H., Santi,D.V., Elwood,H.J., and Sogin,M.L. (1988) *Nature*, **334**, 519–522.
4. Stringer,S.L., Stringer,J.R., Blase,M.A., Walzer,P.D., and Cushion,M.T. (1989) *Exptal. Parasitol.*, **68**, 450–461.
5. Sogin,M.L., and Edman,J.C. (1989) *Nucleic Acids Res.*, **17**, 5349–5359.
6. Cech,T.R. (1990) *Annu. Rev. Biochem.*, **59**, 543–568.
7. Cech,T.R. (1988) *Gene*, **73**, 259–271.
8. Watanabe,J., Hori,H., Tanabe,K., and Nakamura,Y. (1989) *Mol. Biochem. Parasitol.*, **32**, 163–168.
9. Halanych,K.M. (1991) *Mol. Biol. Evol.*, **8**, 249–253.
10. Steele,K.P., Holsinger,K.E., Jansen,R.K., and Taylor,D.W. (1991) *Mol. Biol. Evol.*, **8**, 240–248.
11. Warner,J. (1989) *Microbiol. Rev.*, **53**, 256–271.
12. Yonagathan,T., Lin,H., and Buck,G.A. (1989). *Molec. Microbiol.*, **3**, 1473–1480.
13. Lundgren,B., Cotton,R., Lundgren,J.D., Edman,J.C., and Kovacs,J.A. (1990) *Infect. Immun.*, **58**, 1705–1710.
14. Kitada,K., Oka,S., Kimura,S., Shimada,K., Serikawa,T., Yamada,J., Tsunoo,H., Egawa,K., and Nakamura,Y. (1991) *J. Clin. Microbiol.*, **29**, 1985–1990.
15. Sinclair,K., Wakefield,A.E., Banerji,S., and Hopkin,J.M. (1991) *Mol. Biochem. Parasitol.*, **45**, 183–184.
16. Radding,J.A., Armstrong,M.Y.K., Ullu,E., and Richards,F.F. (1989) *Infect. Immun.*, **57**, 2149–2157.
17. Witebsky,F.G., Andrews,J.W.B., Gill,V.J., and MacLowry,J.D. (1988) *J. Clin. Microbiol.*, **26**, 774–775.
18. Edman,U., Edman,J.C., Lundgren,B., and Santi,D.V. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 6503–6507.
19. Edman,J.C., Edman,U., Cao,M., Lundgren,B., Kovacs,J.A., and Santi,D.V. (1989) *Proc. Natl. Acad. Sci. USA*, **86**, 8625–8629.
20. Sambrook,J., Fritsch,E.F., and Maniatis,T. (1989) *Molecular Cloning: A Laboratory Manual*, Second Edition. Cold Spring Harbor Laboratory Press. Cold Spring Harbor.
21. Torczynski,R.M., Fuke,M., and Bollon,A.P. (1985) *DNA*, **4**, 282–291.
22. Jones,M.D., and Foulkes,N.S. (1989) *Nucleic Acids Res.*, **17**, 8387–8388.
23. Zhou,Y., Zhang,X., and Ebright,R.H. (1991) *Nucleic Acids Res.*, **19**, 6052.
24. Bell,G.I., Degennaro,L.J., Gelfand,D.H., Bishop,R.J., Valenzuela,P., and Rutter,W.J. (1977) *J. Biol. Chem.*, **252**, 8118–8125.
25. Fujiwara,H., and Ishikawa,H. (1982) *Nucleic Acids Res.*, **10**, 5173–5182.
26. Nazar,R.N., Sitz,T.O., and Busch,H. (1976) *Biochemistry*, **15**, 505–508.
27. Georgiev,O.I., Nikolaev,N., and Hadjiolov,A.A. (1981) *Nucleic Acids Res.*, **9**, 6953–6958.
28. Nielsen,H., and Engberg,J. (1985) *Nucleic Acids Res.*, **13**, 7445–7455.
29. Freir,S.M., Kierzek,R., Jaeger,J.A., Sugimoto,N., Caruthers,M.H., Neilson,T., and Turner,D.H. (1986) *Proc. Natl. Acad. Sci. USA*, **83**, 9373–9377.
30. Nam,S.-C., and Kang,C. (1988) *J. Biol. Chem.*, **263**, 18123–18127.
31. Musters,W., Planta,R.J., van Heerikhuizen,H., and Raué (1990) in Hill,W.E., Dahlberg,A., Garrett,R.A., Moore,P.B., Schlessinger,D., and Warner,J.R. (eds.), *The Ribosome*, Amer. Soc. Microbiol., New York, pp. 435–442.
32. van Ahsen,U., Davies,J., and Schroeder,R. (1991) *Nature*, **353**, 368–370.
33. Vossbrinck,C.R., Maddox,J.V., Friedman,S., Debrunner-Vossbrinck,P.A., and Woese,C.R. (1987) *Nature*, **326**, 411–414.
34. Kim,H.K., Hughes,W.T., and Feldman,S. (1972) *Proc. Soc. Exptal. Biol. Med.*, **142**, 304–309.
35. Walzer,P.D., and Rutledge,M.E. (1980) *J. Infect. Dis.*, **142**, 449.
36. Gigliotti,F., Stokes,D.C., Cheatham,A.B., Davis,D.S., and Hughes,W.T. (1986) *J. Infect. Dis.*, **154**, 315–322.
37. Link,M.J., Cushion,M.T., and Walzer,P.D. (1989) *Infect. Immun.*, **57**, 1547–1555.
38. Tanabe,K., Fuchimoto,M., Egawa,K., and Nakamura,Y. (1988) *J. Infect. Dis.*, **157**, 593–596.
39. Hughes,W.T., and Gigliotti,F. (1988) *J. Infect. Dis.*, **157**, 432–433.
40. Hong,S.-T., Steele,P.E., Cushion,M.T., Walzer,P.D., Stringer,S.L., and Stringer,J.R. (1990) *J. Clin. Microbiol.* **28**, 1785–1795.
41. Gunderson,J.J., Sogin,M.L., Wollett,G., Hollingdale,M., de la Cruz,V.F., Waters,A.P., and McCutchan,T.F. (1987) *Science*, **238**, 933–937.
42. Gonzalez,I.L., Gorski,J.L., Campen,T.J., Dorney,D.J., Erickson,J.M., Sylvester,J.E., and Schmickel,R.D. (1985) *Proc. Natl. Acad. Sci. USA*, **82**, 7666–7670.
43. van Keulen,H., Campbell,S.L., Erlandsen,S.L., and Jarroll,E.L. (1991) *Mol. Biochem. Parasitol.*, **46**, 275–284.