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Typing of *Pneumocystis carinii* Strains That Infect Humans Based on Nucleotide Sequence Variations of Internal Transcribed Spacers of rRNA Genes

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Small portions of the 18S and the 26S rRNA genes, the entire 5.8S rRNA gene, and internal transcribed spacers ITS1 and ITS2 (located between the 18S and 5.8S rRNA genes and between the 5.8S and 26S rRNA genes, respectively) of *Pneumocystis carinii* that infect humans were cloned and sequenced. The nucleotide sequences of the 18S, 5.8S, and 26S rRNA genes determined in the study were approximately 90% homologous to those of *P. carinii* that infect rats, while the sequences of ITS1 and ITS2 of *P. carinii* from the two different hosts were only 60% homologous. The 18S, 5.8S, and 26S rRNA gene sequences of *P. carinii* from 15 patient specimens were determined and were found to be identical to each other, whereas the ITS sequences were found to be variable. With the observed sequence variation, it was possible to classify the ITS1 sequences into two types and the ITS2 sequences into three types. *P. carinii* strains that had the same type of ITS1 sequence could have a different type of ITS2 sequence. On the basis of the sequence types of the two ITS regions, *P. carinii* from the 15 patients were classified into four groups. *P. carinii* from three patient specimens were found to contain two different ITS sequence patterns. More surprisingly, one additional specimen was found to have one ITS sequence typical of *P. carinii* isolates that infect humans and another typical of *P. carinii* isolates that infect rats. The studies indicate that it is possible to type *P. carinii* strains on the basis of their ITS sequences and that more than one ITS sequence pattern may be demonstrated in *P. carinii* from one patient, suggesting that coinfection with more than one strain of *P. carinii* may occur in the same patient.

Pneumocystis carinii is a major cause of pneumonia in immunocompromised patients, especially those with AIDS. Although there is evidence of variation (21, 23, 29, 30, 32), diversity among strains of *P. carinii* that infect humans has not been clearly defined. The existence of multiple strains of *P. carinii* that infect humans (hereafter referred to as human *P. carinii* for simplicity) was first demonstrated by examining the restriction fragment length polymorphisms of *P. carinii* genomes from three patients (32). However, restriction fragment length polymorphism analyses are not readily applicable to human clinical specimens because they require enormous numbers of organisms, and there is no culture system in which human *P. carinii* can be grown. In contrast, *P. carinii* isolates that infect rats (hereafter referred to as rat *P. carinii*) can be grown in tissue culture or passaged in rats (3, 5, 7, 9, 20, 27, 28); therefore, it has been possible to analyze the rat *P. carinii* genome more extensively. Up to five types of rat *P. carinii* strains have been described by molecular karyotyping on the basis of the numbers and the sizes of chromosomes separated by pulsed-field gel electrophoresis (6, 17, 35). Like restriction fragment length polymorphism analysis, pulsed-field gel electrophoresis also requires an enormous number of organisms, and clinical specimens, such as bronchoalveolar lavage (BAL) fluids, are inadequate for analysis of the genomes of human *P. carinii*.

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The characterization of *P. carinii* rRNA genes has allowed the development of another approach to the typing of strains of this organism. The entire rRNA gene cluster of rat *P. carinii* has been cloned and sequenced (10, 11, 25, 31), and its transcripts have been characterized (22, 24). As with other organisms, *P. carinii* has three species of nuclear rRNA transcripts derived from a single precursor: 16S, 5.8S, and 26S. The 16S rRNA of *P. carinii* has also been referred to as 18S rRNA (11); in this report it is referred to as 18S rRNA since it has been shown to be similar in size to the 18S rRNA of *Sacchromyces cerevisiae* (22). Both the 18S and the 26S rRNA genes of rat *P. carinii* have been shown to contain a group I self-splicing intron (10, 22, 24, 25), although rat *P. carinii* strains lacking the 18S rRNA gene group I self-splicing intron exist (22, 24). Portions of the rRNA genes of human *P. carinii* have also been examined, and a group I self-splicing intron was found to be present in the 26S but not in the 18S rRNA gene (24). On the basis of the presence and absence of the group I self-splicing intron, *P. carinii* strains have been classified into three types: two rat (Pc1 and Pc2) and one human (Pc3) (24).

Recently, PCR was used to amplify a portion of the large subunit mitochondrial rRNA gene of human *P. carinii* strains directly from biopsy specimens or BAL fluids (34). The amplified products were sequenced, and nucleotide sequence variations were detected (21). This approach has been extended to examine the internal transcribed spacer (ITS) regions of the rRNA genes of human *P. carinii* in the present study. Analysis of the nucleotide sequences obtained revealed that sequence variations also exist in the ITS regions and that

TABLE 1. Characteristics of specimens

Patient no. or DNA specimen	Group	ITS type	Date of specimen collection (mo/day/yr)	Underlying disease	Sex[a]	Age (yr)
Patient no.						
6	I	Ac	5/22/92	AIDS	M	31
7	II	Bb	3/30/93	AIDS	M	38
12	III and rat	Ba and Rat	11/07/83	ALL[b]	F	4
13	II	Bb	2/09/93	AIDS	F	25
21	III	Ba	3/24/92	AIDS	M	26
22	II	Bb	5/18/90	AIDS	M	44
29	I	Ac	4/22/93	AIDS	M	37
34	I and II	Ac and Bb	6/29/92	AIDS	M	40
35	II and IV	Bb and Bc	10/01/93	AIDS	M	33
37	I and III	Ac and Ba	5/01/92	BMT[c]	F	48
38	I	Ac	5/18/92	AIDS	M	30
DNA specimen						
A	II	Bb	1/06/86	AIDS	M	48
W	III	Ba	?	?	F	?
Z	II	Bb	9/?/85	AIDS	M	32
114	III	Ba	1/30/91	AIDS	M	29

[a] M, male; F, female.
[b] ALL, acute lymphocytic leukemia.
[c] BMT, bone marrow transplant recipient.

this sequence variation can be used to type human strains of *P. carinii*.

MATERIALS AND METHODS

Specimens. The majority of specimens used in the present study were obtained from the Clinical Microbiology Laboratory, Indiana University Medical Center (IUMC), and were remnants of BAL fluid or biopsy specimens after all diagnostic tests had been completed. One specimen was collected in 1983; the others were obtained during the period 1990 through 1993 (Table 1). All specimens were stored frozen at −70°C until use. Patients from IUMC included one individual diagnosed with acute lymphocytic leukemia, one bone marrow transplant recipient, and nine individuals with AIDS. Specimens were numbered according to the order in which they were processed for the study. Four DNA specimens (designated A, W, Z, and 114) isolated from infected humans were provided by Joseph A. Kovacs (National Institutes of Health) and have been described previously (24).

Processing of specimens for PCR and PCR conditions. Specimens collected at IUMC were processed for PCR by the procedures described by Lee et al. (21), and DNA specimens A, W, Z, and 114 were prepared as described by Ortiz-Rivera et al. (26). The PCR mixture contained template DNA, PCR buffer (21), 0.2 μM (each) PCR primer, 0.2 μM (each) deoxynucleoside triphosphates, and 2.5 U of *Taq* DNA polymerase (obtained from Perkin-Elmer, Norwalk, Conn.) in a total volume of 100 μl and was overlaid with 10 μl of mineral oil prior to amplification. For PCR with the primer set 1724F-3454R, the initial stage was a 10-min denaturation at 94°C; the second stage was 35 cycles of 94°C for 1 min, 47°C for 1 min, and 72°C for 3 min; and the final stage was a 10-min extension at 72°C. When the ITS1F-ITS2R1 primer set was used, the initial stage was a 10-min denaturation at 94°C; the second-stage PCR was 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 2 min; and the final stage was a 10-min extension at 72°C. For DNA specimens A, W, Z, and 114, amplification was performed by a two-step PCR method with the PCR mixture described above, except that primers were used at 0.4 μM. For the primary PCR, primers 7056 (5′-GGTAATCTT GTGAAACTCTG-3′; the positive strand of the 18S rRNA gene; 5′ coordinate 1514) and 7057 (5′-GTAATCCTACCT GATTTG-3′; the negative strand of the 26S rRNA gene; 5′ coordinate 27) were used. For the secondary PCR, which was performed with the gel-purified product of the primary PCR as the template, primers 4443 (25) and 7057 were used; coordinate numbers are based on the sequence of type Pc1 genomic DNA (25). Primary PCR was performed by two cycles of 94°C for 2 min, 58°C for 1 min, and 72°C for 1 min; this was followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1.5 min, with a final extension at 72°C for 5 min. Secondary PCR was done by two cycles of 94°C for 2 min, 51°C for 1 min, and 72°C for 1 min; this was followed by 30 cycles of 94°C for 1 min, 52°C for 1 min, and 72°C for 1 min, with a final extension of 72°C for 5 min.

Purification of PCR products. The PCR products were electrophoresed in a low-melting-point agarose gel, and the band representing the desired fragment was removed. The DNA fragment was isolated by melting the gel slice at 70°C in a 1.5-ml tube and was then purified by using the Magic PCR Preps kit (Promega, Madison, Wis.) according to the manufacturer's instructions. PCR products derived from DNA specimens A, W, Z, and 114 were purified as described previously (25).

Cloning and sequencing of PCR products. The purified PCR products were ligated into the TA-cloning vector pCRII (Invitrogen, San Diego, Calif.). The ligated products were introduced into *Escherichia coli* INVαF′ [F′ *endA1 recA1 hsdR17* (r_k^-, m_k^+) λ^- *supE44 thi-1 gyrA96 relA1* φ80 Δ*lac*ΔM15 Δ(*lacZYA-argF*)*U169 deoR*] by transformation. The cloned PCR products were sequenced with the Sequenase 2.0 DNA sequencing kit (U.S. Biochemical Corp., Cleveland, Ohio) and appropriate primers. At least three clones of each PCR product were sequenced to confirm the insert sequence. Purified PCR products derived from DNA specimens A, W, Z, and 114 were blunt-ended and ligated into the *Sma*I site of plasmid pUC18 as described previously (25). Cloned PCR products were sequenced at least twice on each strand by using appropriate primers on a Genesis 2,000 Automated DNA Sequencer (DuPont, Wilmington, Del.).

RESULTS

Since the ITS nucleotide sequences of rRNA genes in other organisms were shown to be variable (2, 19, 33, 36) and this region showed the greatest variation between rat-derived *P. carinii* type Pc1 and Pc2 (26), primers were designed to amplify the area containing both the ITS1 and ITS2 regions of *P. carinii*. The area between nucleotides 1724 and 3454 (nucleotide numbers of rat *P. carinii* type Pc1 rRNA genes) was first amplified with primers 1724F and 3454R. These two primer sequences were chosen because they were found to be more conserved among all rat *P. carinii* strains that have been sequenced (10, 25, 31). *P. carinii* in only 2 of the 11 BAL specimens from patient (Pt) 21 and Pt 29 that we examined generated PCR products of the expected size, suggesting that the rRNA gene sequences of rat and human *P. carinii* are not identical. The PCR products of *P. carinii* from Pt 21 and Pt 29 were cloned into pCRII and then sequenced.

The nucleotide sequence of *P. carinii* obtained from Pt 21 was compared with that of rat *P. carinii* (Fig. 1). The 18S rRNA gene of *P. carinii* from Pt 21 lacked the intron sequence that was shown to be present in some rat *P. carinii* isolates (10, 24, 31), denoted type Pc1 (24, 25). This finding was consistent with that described previously (24). The ITS1 sequence of *P. carinii* from Pt 21 was found to be 156 bp in length, which was 7 bp

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Rat PC          AAGTTGATCA AATTTGGTCA TTTAGAGGAA GTAAAAGTCG TAACAAGGT<    50
Hu PC (Pt 21)   ********** ********** ********** ********** *********<
                                                  18S←|   |→ITS1
Rat PC          <intron>TT CCGTAGGTGA ACCTGCGGAA GGATCATTA< <ATGAAA-TG   100
Hu PC (Pt 21)   <------>** ********** ********** *********< <--****A*-

Rat PC          TTGTCAAG-- -AA------- -CTAGTTTAT CTGGTTCTT- GACATTTTCA   150
Hu PC (Pt 21)   ---***-*CT T**ACACTTC C*****---- ---***-**A *-***C**--

Rat PC          TCATAACA-C TTGTGAACAT TAAAGAT--- TTGCTTTGAC -AGGA--TGG   200
Hu PC (Pt 21)   ***-****T* *-******--* *---TT*TTT ***-****G* G****GC***

Rat PC          GAG-----TA AGCTTTCGTC CT-GTC--AG AGGT-TTTCA ATT---AAAA   250
Hu PC (Pt 21)   ---CTTTT*T -*****--*-* **C*-*CA*- ****G***-- ***TTT****
                                                        ITS1←|   |→5.8S
Rat PC          CTTTTT---T GGTG--TTTC -GGTTAAAAA TAT--AATTT TT-AA>>>AA   300
Hu PC (Pt 21)   --****AAA* --**AA**** A*-**----- *-*AG***** **T**>>>**

Rat PC          ACTTTCAGCA ATGGATCTCT TGGTTCCCGC GTCGATGAAG AACGTGGCAA   350
Hu PC (Pt 21)   *******A** ********** ***C**T*** ********** **********

Rat PC          AATGCGATAA GTAGTGTGAA TTGCAGAATT CAGTGACTCA TCGAATTTTT   400
Hu PC (Pt 21)   ********** ********** ********** T*****A*** **********

Rat PC          GAACGCATAT TGCGCTCCTC AGTATTCTGT GGAGCATGCC TGTTTGAGCG   450
Hu PC (Pt 21)   ********C* **GC*****T ********AG ********** **********
                5.8S←|     |→ITS2
Rat PC          TCATTT<<<< TTATACTTGA ACCTTTTT-- ---------- AAGGTTTGTG   500
Hu PC (Pt 21)   *T*****<<<< ***-*G**-- -*******TC AAGCAGAAAA ****---*GA

Rat PC          TTGGGCTATG CA--T-T--T TAGTA-T--- --TTTTACAA G-ATGCTAGT   550
Hu PC (Pt 21)   *******T** **AA*A*AA* ***A*-*AAA ATA****T*T *C********

Rat PC          CTAAAATGGA ATCCAGAATA ---TTATTTC GT-GCAGCGT AATAGGGT--   600
Hu PC (Pt 21)   **G****--- -**A*A*G** GCT**T**** T*T**C---- --***T**CG

Rat PC          TAAATTCCAA TTCGCTGTTT TTAGAAATGA TAGACTGGTT TGTCTATTGT   650
Hu PC (Pt 21)   *****----** *********-- --G****-** -**GAAAAAA GC*------*
                                  ITS2←| |→26S
Rat PC          TCCTAG---- AGAGCAATTT TTGAAC>CTT TGACCTCAAA TCAGGTAGGA   700
Hu PC (Pt 21)   *T--*TAGAT *C*AG***** ------>--- *A*T****** *****C****

Rat PC          TTACCCGCTG AACTTAAGCA TATCAATAAG CGGAGGAAAA GAAACTAACA   750
Hu PC (Pt 21)   *C******** ********** ********** ********** **********

Rat PC          AGGATTCCCT CAGTAACGGC GA                                772
Hu PC (Pt 21)   ********** T********* **
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FIG. 1. Comparison of nucleotide sequences of portions of the rRNA genes and the ITS regions of *P. carinii* from rat and human hosts. The sequence of *P. carinii* from Pt 21 [Hu PC (Pt 21)] is compared with that of rat *P. carinii* type Pc1 (Rat PC) published by Liu et al. (25). Identical sequences are represented with asterisks, missing bases are shown with dashes, and different bases are indicated. These same symbols are also used for Fig. 2, 4, and 5. The numbers shown here and in Figures 2, 4, and 5 indicate position, not nucleotide numbers. Different regions of the rRNA genes are bracketed and are indicated by the arrows above the sequence.

shorter than that of rat *P. carinii* type Pc1 (25). The 5.8S rRNA gene was found to be composed of 158 nucleotides, the same length as that of rat *P. carinii*. The ITS2 sequence of *P. carinii* from Pt 21 was 173 bp long, which was 9 bp shorter than that of rat *P. carinii* type Pc1 (25). A close analysis of the sequences revealed that the 18S, 5.8S, and 26S rRNA genes were quite conserved between rat (type Pc1) and human *P. carinii* (100, 93.7, and 91.6% identities, respectively), but both ITS1 and ITS2 sequences differed significantly (56.3 and 69.4% identities, respectively). The GC content of the 18S, 5.8S, and 26S rRNA genes of both human and rat *P. carinii* was approximately 40%, whereas it was 30% in the ITS regions.

The same homology patterns were found between the sequences of *P. carinii* from Pt 21 and Pt 29 (Fig. 2). The nucleotide sequences of the 18S, 5.8S, and 26S rRNA genes that we determined from *P. carinii* from Pt 21 and Pt 29 were identical. However, six nucleotides in ITS1 and 11 nucleotides in ITS2 were different. In order to determine whether the sequence variations found in the ITS regions could be used to differentiate between different types of human *P. carinii*, additional patient specimens were examined. Since the primer set 1724F-3454R was designed on the basis of the rat *P. carinii* sequence and did not amplify all human *P. carinii* sequences, a new set of primers was developed on the basis of the human *P. carinii* sequences (from Pt 21 and Pt 29) that we had determined. This primer set (ITS1F-ITS2R1) amplified the region between nucleotide positions 53 and 602 (Fig. 2), including the last 28 bp of the 18S rRNA gene, the entire ITS1, 5.8S, and

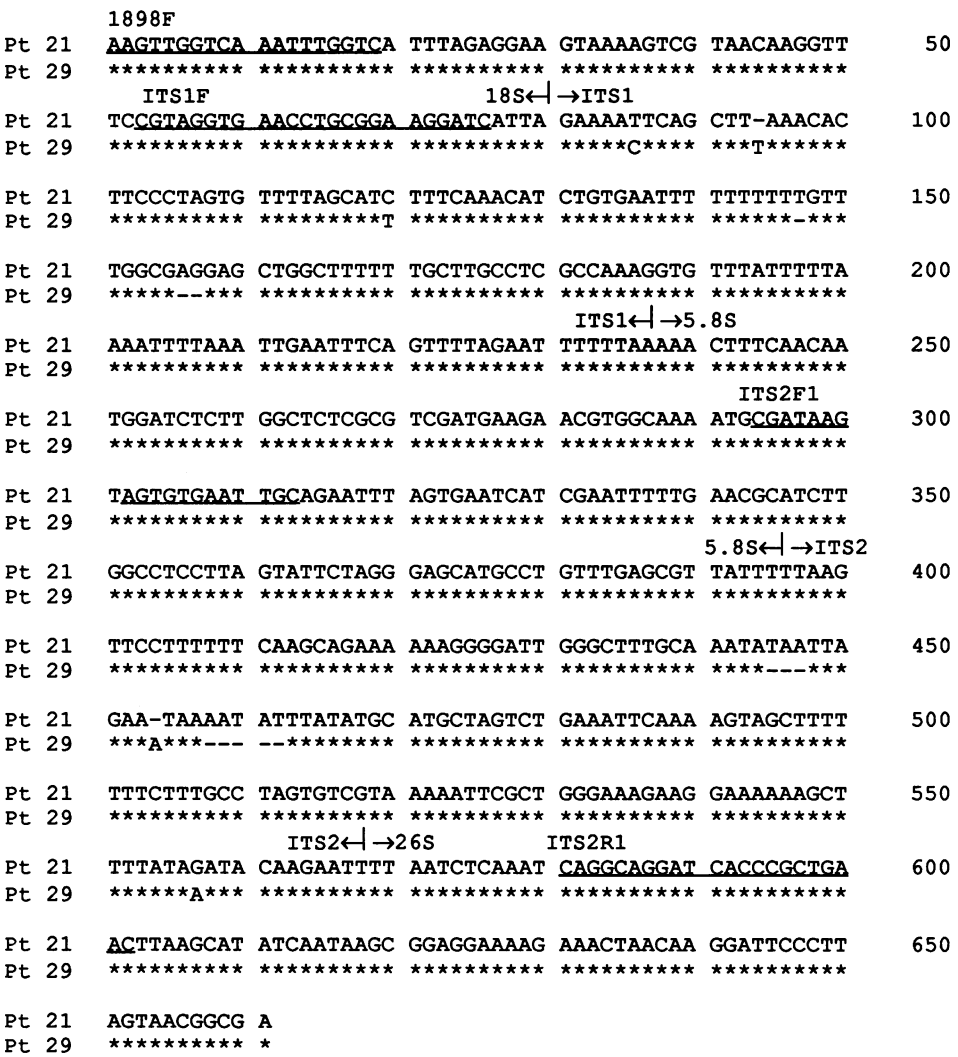

FIG. 2. Comparison of sequences of *P. carinii* from Pt 21 and Pt 29. The sequences shown include the last 80 bp of the 18S rRNA gene, the first 93 bp of the 26S rRNA gene, the entire 5.8S rRNA gene, ITS1, and ITS2. The underlined sequences were used to design the PCR primers for the present study. Representations of symbols and numbers are as described in the legend to Fig. 1. Regions of the rRNA genes are indicated above the sequence.

ITS2 regions, and the first 34 bp of the 26S rRNA gene. Another primer, ITS2F1, was also developed; in combination with primer ITS2R1, ITS2F1 amplified mainly the ITS2 region (Fig. 2 and 3). As indicated above, different primers were used to amplify the ITS regions from DNA specimens A, W, Z, and 114.

P. carinii isolates in BAL specimens collected from nine additional patients at IUMC and the four DNA specimens obtained from the National Institutes of Health were examined. The region spanning ITS1 and ITS2 was amplified with the primer set ITS1F-ITS2R1 (Fig. 3). The resulting 550-bp PCR products were cloned into the TA-cloning vector pCRII and were then sequenced. The ITS1 sequences of *P. carinii* from all 15 specimens (17 sequences) were aligned as shown in Fig. 4. The sequence obtained from Pt 6 was chosen as the prototype in Fig. 4 because 6 was the lowest patient number in the present study, and all other sequences were compared with this sequence. This comparison revealed that human *P. carinii* ITS1 sequences could be classified into two major types. These two types, designated A and B, differed in sequence at positions 6, 14, 76, and 77. Type A had a C residue at position 6 and a T residue at position 14, and there was a 2-bp deletion at positions 76 and 77. Type B had a T residue at position 6, an A residue at position 76, and G residue at position 77 and was missing a base at position 14. According to this typing system, 5 sequences were classified as type A and 12 were classified as type B. There were some sporadic variations within type B. Sequences of *P. carinii* from Pt 12 and DNA specimen 114 had an A residue at position 21, whereas *P. carinii* sequences from all other patients had a T residue at this position. The sequences of *P. carinii* from Pt 21 and Pt 37-b had a C residue instead of a T residue at position 40. The sequences of *P. carinii* from Pt 12, 21, and 37-b and DNA specimens A, W, Z, and 114 had an extra T residue at position 67, which might represent another type of ITS1 sequence. Of particular importance was that specimens from Pt 34 and Pt 37 contained both type A and type B ITS1 sequences. These sequences were designated Pt 34-a, Pt 34-b, Pt 37-a, and Pt 37-b, respectively (Fig. 4). Surprisingly, the specimen from Pt 12 contained an ITS1 sequence identical to that of rat *P. carinii* type Pc1 (24),

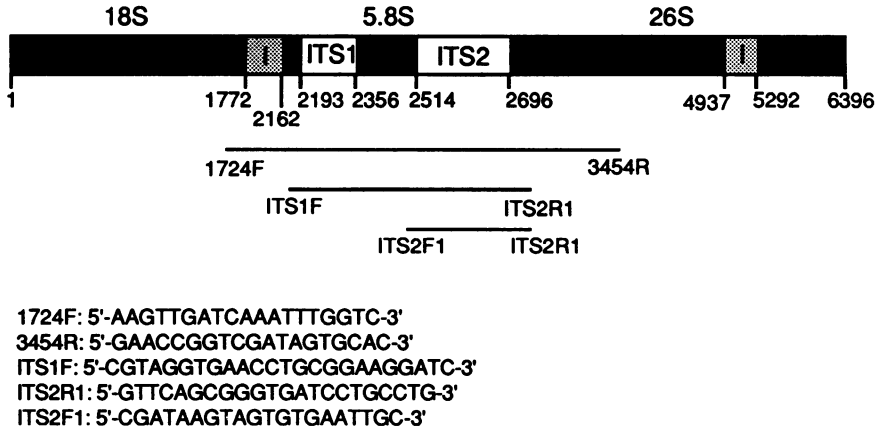

FIG. 3. rRNA genes of *P. carinii* and PCR primers used in the study. The organization of the rRNA genes of rat *P. carinii* type Pc1 is shown as a reference. The 18S, 5.8S, and 26S rRNA genes are represented by black boxes. The group I intron that is present in both the 18S and the 26S rRNA genes is indicated with shaded boxes. The ITS regions are illustrated as open boxes. The numbers below the diagram are nucleotide numbers of rat *P. carinii* rRNA genes. The 18S rRNA gene nucleotide numbers were derived from Edman et al. (10) and those of the remaining rRNA genes were from Liu et al. (25). The areas that were amplified from *P. carinii* present in human specimens and the primers used for PCR are indicated. The primer sequences are shown in the lower portion of the figure.

in addition to the one mentioned above. These findings suggested that Pt 34 and Pt 37 were simultaneously infected with two different strains of *P. carinii*.

The ITS2 sequences obtained from all 15 specimens were also aligned (Fig. 5). In this case, the sequence obtained from Pt 21 was used as the prototype since it had fewer missing bases. This alignment revealed three types of ITS2 sequences, designated a, b, and c. The type b sequence differed from the type a sequence by an insertion of 2 bp (AT) at positions 160 and 161 which were absent from the type a and c sequences. The type c sequence displayed more extensive variation from the sequences of types a and b; the sequence of type c lacked nucleotides at positions 50 to 52 and 63 to 67 and had an A residue instead of a G residue at position 165. Similar to the ITS1 sequences, there were also sporadic sequence variations among different *P. carinii* strains. The DNA specimen W sequence had an A residue instead of a T at residue position 67, the sequences of *P. carinii* from Pt 6 and Pt 38 had a G residue instead of a T residue at position 90, the sequence of *P. carinii* from Pt 22 and the DNA specimen A sequence had an extra C residue at position 116, the sequence of *P. carinii* from Pt 22 and the DNA specimen Z sequence lacked a T residue at position 156, the sequence of *P. carinii* from Pt 37-a had a C residue instead of an A residue at position 163, and DNA specimen W had an A residue at position 165, thus differing from other type a sequences but resembling the type c sequence at this position. The sequences of DNA specimens W and 114 also lacked an A residue at position 162. Since these sequence variations did not show any specific pattern, they were not considered to have any typing utility in the present study. As in the case of ITS1, a sequence from *P. carinii* from Pt 12 was found to be identical to that of ITS2 from rat *P. carinii* type Pc1 (25).

According to the types of ITS1 and ITS2, we designated the human *P. carinii* sequences mentioned above by a two-letter code, e.g., Ac, in which A represents the ITS1 type and c represents the ITS2 type (Table 1). Five sequences (of *P. carinii* from Pt 6, Pt 29, Pt 34-a, Pt 37-a, and Pt 38) belonged to combination type Ac. Seven sequences (of *P. carinii* from Pt 7, Pt 13, Pt 22, Pt 34-b, and Pt 35 and DNA specimens A and Z) were found to be Bb. Five sequences (of *P. carinii* from Pt 12, Pt 21, and Pt 37-b and DNA specimens W and 114) were classified as Ba. In addition to Bb, Bc DNA was also found in Pt 35, which was the only one Bc organism identified in the study. Pt 35 might represent another incidence of coinfection with two different strains of *P. carinii*. Overall, four groups of *P. carinii* strains were identified in the study. We arbitrarily designated them groups I (Ac), II (Bb), III (Ba), and IV (Bc).

Nucleotide sequence accession numbers. The nucleotide sequences of these four groups have been deposited in the GenBank with accession numbers U07220 (group I), U07226 (group II), U07221 (group III), and U07222 (group IV).

DISCUSSION

In this paper we described the development of a method for typing *P. carinii* strains that infect humans on the basis of the nucleotide sequences of ITS regions of rRNA genes. PCR was performed to amplify a chromosomal fragment between the 3′ end of the 18S rRNA gene and the 5′ end of the 26S rRNA gene. The nucleotide sequences of portions of the 18S and the 26S rRNA genes and the entire 5.8S rRNA gene that we have determined were found to be greater than 90% homologous to those of rat *P. carinii* sequences (10, 25, 31). This finding indicates that the sequences that we obtained are from human *P. carinii*. It is interesting that the ITS sequences of human *P. carinii* are only approximately 60% homologous with those of rat *P. carinii* type Pc1 (25).

The sequences of the 18S, 5.8S, and 26S rRNA genes of *P. carinii* from the 15 patients that we examined were identical to each other, whereas those of ITS1 and ITS2 were found to be variable between different specimens. Although these sequence variations were not extensive, they exhibited definite patterns (Fig. 4 and 5). The ITS1 sequences could be classified into two types, and the ITS2 sequences could be classified into three types. We also found that *P. carinii* isolates that had the same type of ITS1 might have different types of ITS2. Therefore, human *P. carinii* strains may have six different ITS sequence types. In the present study, we identified four different types from 15 patients. Nucleotide sequence variation in the ITS regions of rat *P. carinii* Pc1 and Pc2 has also been observed (26).

We have also identified three specimens that contained two different types of ITS sequences. Whether this represents

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Type A   Pt  6   GAAAACTCAG CTTTAAACAC TTCCCTAGTG TTTTAGCATT TTTCAAACAT CTGTGAATTT   60
         Pt 29   ********** ********** ********** ********** ********** **********
         Pt 34-a ********** ********** ********** ********** ********** **********
         Pt 37-a ********** ********** ********** ********** ********** **********
         Pt 38   ********** ********** ********** ********** ********** **********
Type B   Pt 13   *****T**** ***-****** ********** ********** ********** **********
         Pt 22   *****T**** ***-****** ********** ********** ********** **********
         Pt 34-b *****T**** ***-****** ********** ********** ********** **********
         Pt 35   *****T**** ***-****** ********** ********** ********** **********
         Pt  7   *****T**** ***-****** ********** ********** ********** **********
         Pt 12   *****T**** ***-****** A********* ********** ********** **********
         Pt 21   *****T**** ***-****** ********** *********C ********** **********
         Pt 37-b *****T**** ***-****** ********** *********C ********** **********
         A       *****T**** ***-****** ********** ********** ********** **********
         W       *****T**** ***-****** ********** ********** ********** **********
         Z       *****T**** ***-****** ********** ********** ********** **********
         114     *****T**** ***-****** A********* ********** ********** **********

Type A   Pt  6   TTTTTT-GTT TGGCG--GAG CTGGCTTTTT TGCTTGCCTC GCCAAAGGTG TTTATTTTTA   120
         Pt 29   ******-*** *****--*** ********** ********** ********** **********
         Pt 34-a ******-*** *****--*** ********** ********** ********** **********
         Pt 37-a ******-*** *****--*** ********** ********** ********** **********
         Pt 38   ******-*** *****--*** ********** ********** ********** **********
Type B   Pt 13   ******-*** *****AG*** ********** ********** ********** **********
         Pt 22   ******-*** *****AG*** ********** ********** ********** **********
         Pt 34-b ******-*** *****AG*** ********** ********** ********** **********
         Pt 35   ******-*** *****AG*** ********** ********** ********** **********
         Pt  7   ******-*** *****AG*** ********** ********** ********** **********
         Pt 12   ******T*** *****AG*** ********** ********** ********** **********
         Pt 21   ******T*** *****AG*** ********** ********** ********** **********
         Pt 37-b ******T*** *****AG*** ********** ********** ********** **********
         A       ******T*** *****AG*** ********** ********** ********** **********
         W       ******T*** *****AG*** ********** ********** ********** **********
         Z       ******T*** *****AG*** ********** ********** ********** **********
         114     ******T*** *****AG*** ********** ********** ********** **********

Type A   Pt  6   AAATTTTAAA TTGAATTTCA GTTTTAGAAT TTTTTAA   157
         Pt 29   ********** ********** ********** *******
         Pt 34-a ********** ********** ********** *******
         Pt 37-a ********** ********** ********** *******
         Pt 38   ********** ********** ********** *******
Type B   Pt 13   ********** ********** ********** *******
         Pt 22   ********** ********** ********** *******
         Pt 34-b ********** ********** ********** *******
         Pt 35   ********** ********** ********** *******
         Pt  7   ********** ********** ********** *******
         Pt 12   ********** ********** ********** *******
         Pt 21   ********** ********** ********** *******
         Pt 37-b ********** ********** ********** *******
         A       ********** ********** ********** *******
         W       ********** ********** ********** *******
         Z       ********** ********** ********** *******
         114     ********** ********** ********** *******
```

FIG. 4. Alignment of ITS1 sequences from all specimens examined. The sequence obtained from *P. carinii* from Pt 6 is shown, and the sequences of *P. carinii* from other patients are compared with this sequence. Similar sequences are arranged together to reveal types. On the basis of the alignment shown here, the sequences were classified into type A or type B.

coinfection with two different strains of *P. carinii* in the same patient or whether they are derived from different copies of rRNA genes remains to be investigated. It is not known whether the human *P. carinii* genome contains more than one copy of the rRNA genes. The observation that 80% (12 of 15) of the specimens that we have examined contained only one type of *P. carinii* ITS sequence indicates that the nucleotide sequences of different copies of rRNA genes (if multiple copies of rRNA genes exist) of human *P. carinii* had homogenized and that each strain of human *P. carinii* has a unique ITS sequence. This nucleotide sequence homogenization is known as concerted evolution (1, 4, 14–16) and has been observed in almost all organisms whose rRNA gene sequences have been examined. It is quite likely that different types of ITS se-

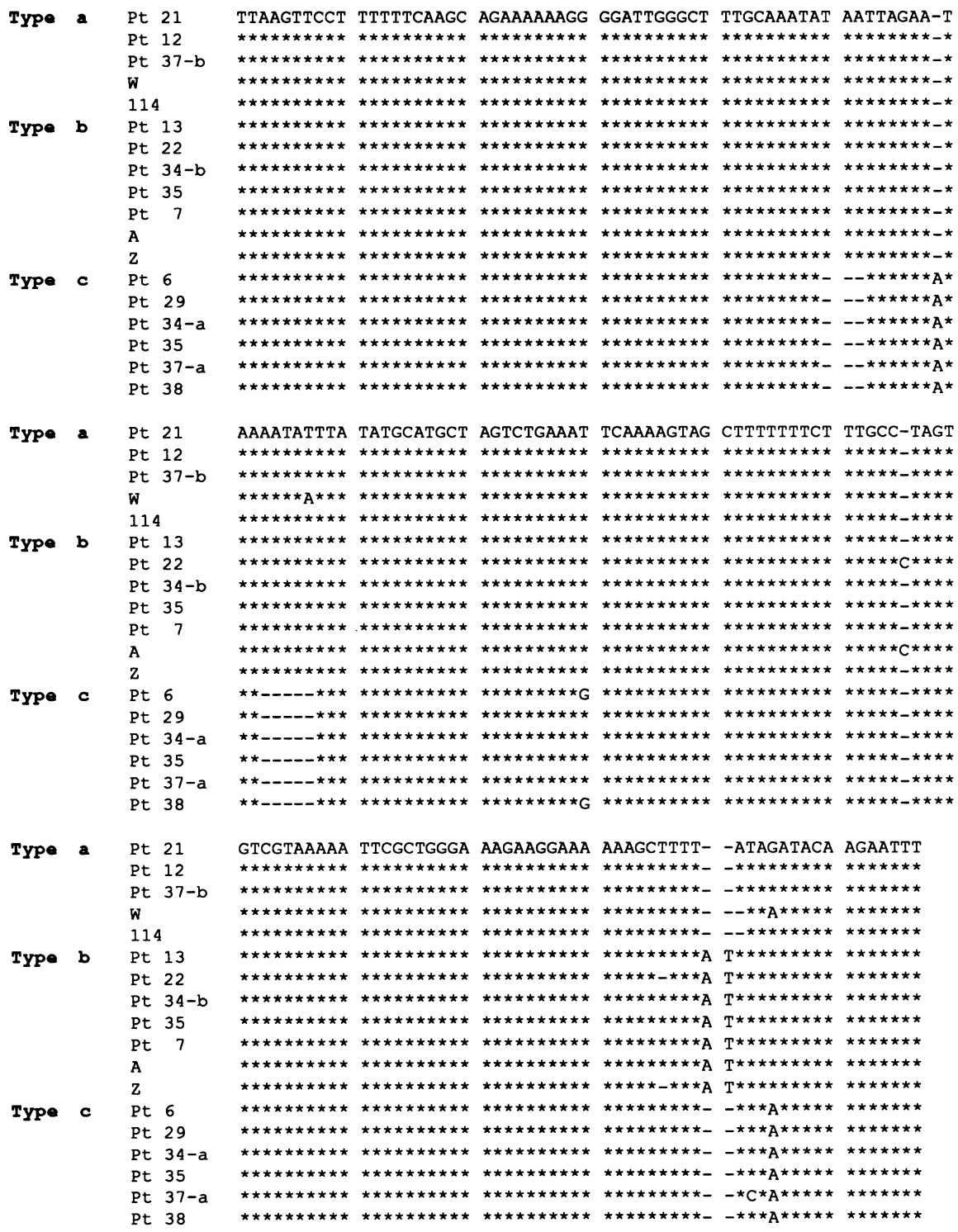

FIG. 5. Alignment of ITS2 sequences from all specimens examined. The sequences of *P. carinii* from Pt 21 are shown, and the sequences of *P. carinii* from all other patients are compared with this sequence. As in Fig. 4, similar sequences are arranged together. The ITS2 sequences were classified into three types: a, b, and c.

quences represent different strains of *P. carinii*. Coinfection with two different strains of *P. carinii* in the same host has been observed in rats by Cushion et al. (8) by molecular karyotyping techniques.

On the basis of the presence or absence of the group I intron and sequence differences in the 18S and the 26S rRNA genes, strains of *P. carinii* have been classified into three sequevars (for sequence variants), denoted Pc1, Pc2, and Pc3 (24, 25). We have shown in the present study that multiple subtypes of sequevar Pc3 can be demonstrated on the basis of sequence

variation within ITS1 and ITS2. Additional DNA specimens will need to be sequenced to determine if more sequevars and subtypes exist, including presently unknown subtypes within Pc1 and Pc2.

We have previously examined sequences within the mitochondrial rRNA gene from human *P. carinii* (21) and found one patient infected with a strain that appeared to be a hybrid of rat and human *P. carinii*. In the present study we obtained data which further substantiate this possibility. The specimen from this same patient (Pt 12) was found to contain *P. carinii* with two different rRNA gene sequences. One of the sequences was a typical human *P. carinii* sequence, while the other was identical to that of rat *P. carinii*. It is unlikely that the specimen from Pt 12 was contaminated with rat *P. carinii* since this specimen was analyzed in a laboratory where no *P. carinii*-infected rats were ever present. In addition, the three major PCR steps, specimen processing (preparation of template DNA), PCR setup, and analysis of PCR products, were done in three separate rooms to prevent contamination of template DNA by PCR products. The coexistence of human and rat *P. carinii* DNAs in the specimen from Pt 12 suggests that this patient was coinfected with human and rat strains of *P. carinii* and that a portion of the genomes recombined, giving rise to a hybrid sequence. If this possibility is proven, the term "rat *P. carinii*" and the proposal that *P. carinii* be classified on the basis of its host (12, 18) would be inappropriate. It appears that the strain which commonly infects rats, termed type Pc1 (24, 25), can also infect humans, although Gigliotti et al. (13) have shown that *P. carinii* from one mammalian host does not infect other animal species.

Although sequence variation was also found in the mitochondrial rRNA gene of *P. carinii*, this variation was irregular (21); therefore, it was not useful for typing human *P. carinii* strains. The use of ITS sequence variation to type human *P. carinii* will make it possible to explore the question of whether different strains of *P. carinii* differ in prevalence and virulence by conducting more extensive epidemiologic studies. Additional patient specimens should be examined to expand the data summarized in Table 1 to determine whether a specific strain of *P. carinii* is associated with a unique underlying disease. The methods developed in the present study will also enable us to determine whether a second episode of *P. carinii* infection in the same patient represents a relapse because of the failure of chemotherapy or a reinfection by the acquisition of a different strain of *P. carinii*. If it can be shown that the *P. carinii* strains isolated from patients with a second infection differ significantly from the strains isolated initially, it would suggest that immunosuppressed patients require protection from environmental exposure.

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