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Molecular Characterization of a Novel Repetitive Element from *Pneumocystis carinii* from Rats

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A repetitive DNA sequence, Rp-α, was isolated from rat-derived *Pneumocystis carinii*. The genome of rat-derived *P. carinii* contained 10 to 15 copies of Rp-α, which were located on most chromosomes, but no Rp-α could be detected in *P. carinii* derived from either humans or mice. Sequence analysis of two copies of the repeat showed them to be related but distinct. Each of them contained several copies of the 9-base sequence TAACCCTAA, arranged as direct repeats. Oligonucleotides consisting of multimers of this 9mer hybridized to the same set of chromosomes recognized by cloned copies of the Rp-α repeat. When used in DNA fingerprinting, the Rp-α repeat was capable of distinguishing between different isolates of rat-derived *P. carinii*.

Pneumocystis carinii causes pneumonia in AIDS patients and other immunocompromised individuals (14, 22). *P. carinii* also infects a wide variety of other vertebrate hosts (11). Although it does not have some of the characteristics typical of fungi, molecular genetic evidence indicates that the organism is a pathogenic fungus (4–8, 13, 15, 20, 24, 26). It has not been possible to establish clonal cultures in vitro (3), but organisms can be isolated in large numbers from the lungs of immunosuppressed laboratory rats (21). Physical analysis of rat-derived *P. carinii* chromosomes via pulsed-field gel electrophoresis has identified a number of distinct varieties of the organism (10, 12, 25). Typically, rat colonies that have been maintained in different locations harbor *P. carinii* with different electrophoretic karyotypes. To explore the significance of these karyotypic differences, we have been cloning DNA fragments from the genome of rat-derived *P. carinii*. In the analysis of one such clone, which contained an α-tubulin gene, we discovered a DNA sequence, Rp-α, which was less than 500 bp in length, present in the *P. carinii* genome 10 to 15 times, and distributed among most of the chromosomes. Sequence analysis of two cloned copies of the Rp-α repeat showed them to be nonidentical and to contain clusters of the 9mer TAACCCTAA arranged as direct repeats. All rat-derived *P. carinii* organisms analyzed had Rp-α, but none could be detected in *P. carinii* derived from either humans or mice. When an Rp-α was used as a hybridization probe in DNA fingerprinting assays, *P. carinii* from different rat colonies produced distinct band patterns.

MATERIALS AND METHODS

General materials and methods. Restriction enzymes and T4 DNA ligase were from Bethesda Research Laboratories (Bethesda, Md.). The "Prime-it" labeling kit and the plasmid BluescriptSKII+ were from Stratagene (La Jolla, Calif.). Radioisotopes were from New England Nuclear (Wilmington, Del.). *Taq* polymerase was from Promega (Madison, Wis.). Hybond N+ membrane was from (Amersham, Amersham, United Kingdom). The methods used for radiolabeling DNA, Southern blotting, library screening, nucleic acid hybridization, restriction enzyme digestion, and subcloning were described previously by Sambrook et al. (16). Polymerase chain reactions were performed under standard conditions recommended in the GeneAmp DNA Amplification Reagent Kit from Perkin-Elmer Cetus (Norwalk, Conn.). The reactions were repeated through 25 cycles of 1 min at 94°C, 2 min at 55°C, and 3 min at 72°C. DNA sequencing was performed by using a Sequenase kit from U.S. Biochemicals (Cleveland, Ohio).

***P. carinii* isolation.** Rat-derived *P. carinii* organisms were obtained from the lungs of individual immunosuppressed rats as previously described (10, 20). Several different rat strains from several vendors were used. These included the Sprague-Dawley strain from Zivic Miller, the Holtzman strain from Sasco, Inc., and from Harlan Industries, Fischer and Wistar strains from Hilltop Laboratory, and Lewis and Brown Norway strains from Charles River Laboratory. Human-derived *P. carinii* organisms were isolated from lung tissue taken at autopsy from a patient with histopathologically documented *P. carinii* pneumonia. The tissues were minced, homogenized, subjected to hypotonic conditions (to lyse erythrocytes), treated with DNase, and embedded in agarose as described previously for rat-derived organisms (10). Mouse-derived *P. carinii* organisms were isolated from the lungs of four *scid/scid* mice, obtained from Charles Sidman, College of Medicine, University of Cincinnati. The organisms from the four mice were pooled and processed as described for rat-derived *P. carinii*.

Agarose gel electrophoresis. For field inversion gel electrophoresis (FIGE), DNA samples were prepared as described previously (10). FIGE was performed in 1% agarose in 45 mM Tris hydrochloride–45 mM boric acid–1.25 mM EDTA (0.5× TBE) plus 0.1 M glycine for 48 h at 160 V and then an additional 120 V for 96 h. Field polarity was switched between 50 s forward and 25 s backward by using a PC 750 pulse controller (Hoefer Scientific Instruments, San Francisco, Calif.). For standard agarose gel electrophoresis, genomic DNA was prepared and digested in agarose gel plugs as previously described (18). Electrophoresis was in 1% agarose in 1× TBE at 24 V for 48 h. DNA was

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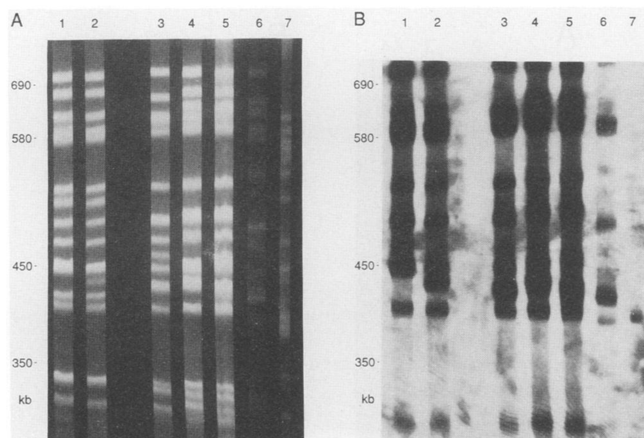

FIG. 1. A cloned DNA fragment containing the α-tubulin gene from rat-derived *P. carinii* hybridized to multiple chromosomes. (A) Ethidium bromide-stained *P. carinii* chromosomes separated by FIGE. Lanes 1 to 6 contained *P. carinii* from six individual rats. The rat strains used were as follows: Sprague Dawley (lanes 1 and 2), Holtzman (lane 3), Lewis (lanes 4 and 6), Brown Norway (lane 5). Lane 7 contained *P. carinii* from four *scid/scid* mice. (B) Autoradiogram produced after hybridization of a radioactive *Bgl*II-*Eco*RI fragment (Fig. 2) to the gel in panel A. For details on hybridization and washing, see the text.

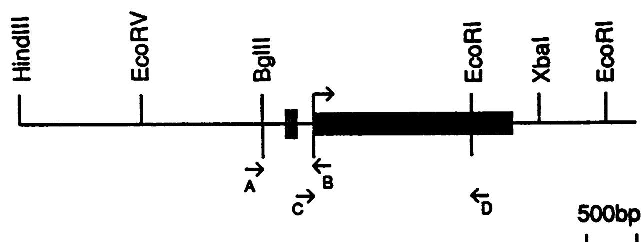

FIG. 2. Restriction map of TUB1. The filled bar indicates the α-tubulin gene. The gene is transcribed in the direction indicated by the arrow above the filled bar. The cross-hatched box shows where the Rp-α1 sequence was later found. The arrows marked A, B, C, and D indicate the locations of primers used for polymerase chain reactions.

transferred from gels to Hybond N+ membranes by the alkaline capillary blotting method recommended by Amersham.

Hybridization of a cloned DNA fragment containing the α-tubulin gene from rat-derived *P. carinii* was in a mixture containing 6× SSPE (1× SSPE is 0.18 M NaCl, 10 mM $NaPO_4$, and 1 mM EDTA [pH 7.7]), 0.1% sodium dodecyl sulfate (SDS), 50 μg of salmon sperm DNA per ml, and 10× Denhardt's solution (16) for 12 to 18 h at 65°C. After hybridization, washing was in 2× SSPE–0.4% SDS at 60°C twice for 30 min.

Hybridizations of Rp-α oligonucleotides to *P. carinii* from rats and humans and of Rp-α to *Eco*RI-digested genomic DNA from four rat-derived *P. carinii* isolates were both in 6× SSPE–1% SDS at 50°C overnight. After hybridization, washing was in 2× SSPE–0.4% SDS at 50°C twice for 30 min.

RESULTS

Isolation and identification of the alpha repeat. The first copy of the alpha repeat (Rp-α) was found 250 bp upstream of an α-tubulin gene cloned from rat-derived *P. carinii* (29). The presence of a repetitive sequence was initially suggested by the results of an experiment in which a 13.4-kb genomic clone, TUB1, which contained the α-tubulin gene, was used as a hybridization probe to map the gene to a *P. carinii* chromosome. The TUB1 clone hybridized to several chromosomal bands resolved by FIGE (Fig. 1). When the same FIGE gel blot was hybridized to a *P. carinii* α-tubulin cDNA, only one chromosome band was positive (29), indicating that hybridization of TUB1 to multiple chromosomes was probably not due to the presence of several α-tubulin genes.

The location of the alpha repeat within TUB1 was determined by excising various fragments of TUB1 with restriction enzymes and by using each restriction fragment as a hybridization probe on FIGE gel blots. These experiments mapped the repeat to a 1.6-kb *Bgl*II-*Eco*RI fragment, which included most of the α-tubulin gene (see Fig. 2). The DNA lying on either side of the 1.6-kb fragment was found to hybridize to the 425-kb FIGE band, indicating that the 13.4-kb DNA fragment contained in the TUB1 clone was derived from a single locus in the *P. carinii* genome (data not shown). The 1.6-kb fragment was sequenced, which allowed further mapping of the repeat to be done by generating DNA fragments via the polymerase chain reaction. These experiments localized the repeated sequence to the 500-bp region between primers A and B shown in Fig. 2.

Nucleotide sequence analysis of the repeat in TUB1 and one other Rp-α element. The sequence of the 500-bp region containing the repeat was determined. Part of this sequence is shown in Fig. 3A. The sequence contained multiple copies of a 9-bp sequence, TAACCCTAA, which is underlined in Fig. 3A. To determine if this simple sequence was repeated in the *P. carinii* genome, a 33-base oligonucleotide with the sequence TAACCCTAATGTAACCCTAATGTAACCC TAA was synthesized and used to probe a FIGE gel blot. The 33mer hybridized to the same set of chromosomal bands as did the 500-bp fragment (Fig. 4, lanes 3).

A second 27-base oligonucleotide $(TAACCCTAA)_3$ was used as a probe to screen a genomic library previously prepared from rat-derived *P. carinii* (10). A positive clone was isolated that showed a restriction enzyme digestion pattern different from that of TUB1. DNA fragments containing the repeat were identified, subcloned into a plasmid, and sequenced. Figure 3B shows the sequence of 413 bp from the clone (Rp-α2) compared with the last 427 bp of the Rp-α1 sequence. (The first 97 bp of Rp-α1 showed no significant identity with those of Rp-α2). As expected, the Rp-α2 sequence also contained direct repeats of the sequence TAACCCTAA, shown in boxes labeled 1 to 11 in Fig. 3B. However, identity was not limited to these distinctive 9mers. The largest region of high identity (between boxes 6 and 9) spanned 73 bp, 68 of which were shared by both sequences. While 36 of the 68 matched bp in this region were either perfect or degenerate TAACCCTAA repeats, there was also a region that lacked the TAACCCTAA motif in which 20 of 22 nucleotides matched (see large box between repeats 7 and 8). By introducing gaps in one sequence or the other, it was possible to align 77% of the first 259 bases of the Rp-α1 sequence with the first 272 bases of Rp-α2. In contrast, the last 168 bases of Rp-α1 were only 40% identical to the last 141 bases of Rp-α2.

Copy number and polymorphism of Rp-α elements. Rp-α probes hybridized to 9 or 10 chromosomal bands separated

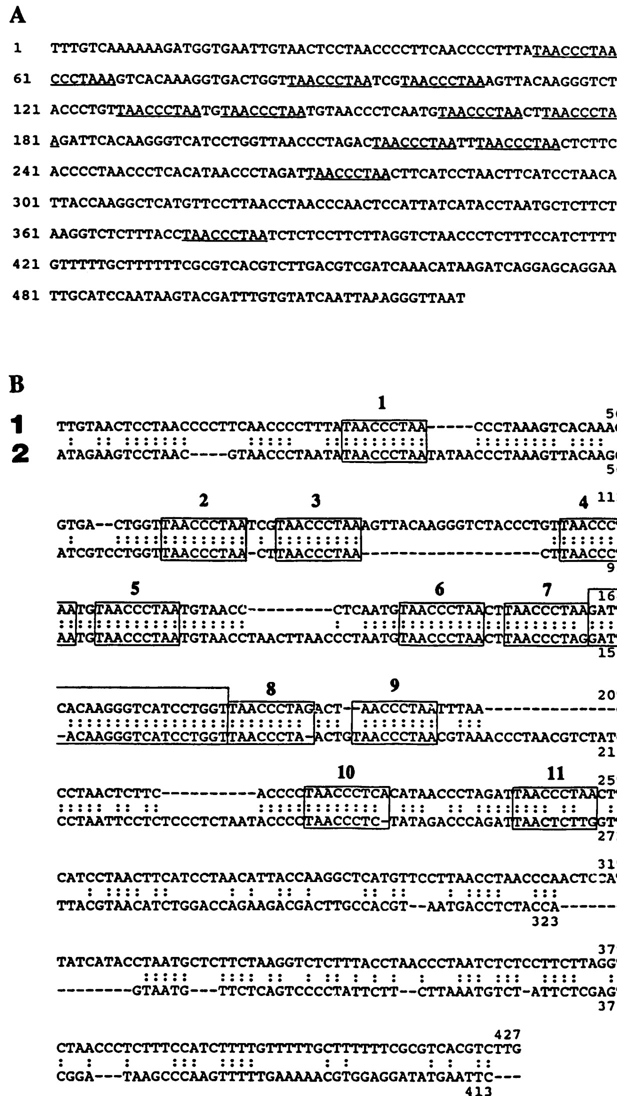

FIG. 3. Nucleotide sequence and alignment of two members of the Rp-α family. (A) Sequence of Rp-α1. (B) Alignment of Rp-α1 and Rp-α2. Dashed lines indicate gaps introduced to maximize alignment. Numbers at the ends of lines are nucleotide numbers. The 11 aligned TAACCCTAA sequences are boxed and numbered. Each sequence contains additional TAACCCTAA sequences and degenerate versions of this sequence.

by FIGE (depending on the isolate analyzed; Fig. 1 and 4), suggesting that there were at least that many copies of the repeat in the genome. Consistent with this, when a genomic library was probed with an Rp-α probe, about eight times as many positive plaques (203 plaques) were identified as when the library was hybridized to the single-copy thymidylate synthase gene (27 plaques). A third copy-number estimate was obtained by Southern blot analysis of the DNA fragments produced from the *P. carinii* genome after digestion with the restriction enzyme *Eco*RI. *P. carinii* organisms were isolated from four different rat colonies. The genomic DNA from each *P. carinii* isolate was fragmented by digestion with the restriction enzyme *Eco*RI, and the fragments were separated by agarose gel electrophoresis. The DNA was transferred from the gel to a nylon membrane by

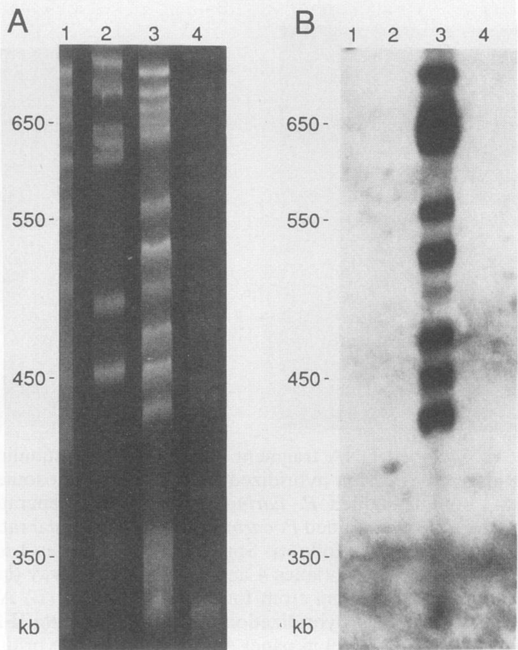

FIG. 4. Hybridization of Rp-α to *P. carinii* from rats and humans. (A) Ethidium bromide-stained gel of *P. carinii* chromosomes separated by FIGE. Lanes: 1, lambda concatenate ladder; 2, *S. cerevisiae*; 3, *P. carinii* from a Sasco, Inc., Holtzman rat; 4, human-derived *P. carinii* from postmortem lung tissue. (B) Autoradiogram produced after hybridization of a radioactive oligonucleotide, $(TAACCCTAATG)_3$, to the gel in panel A. For details on hybridization and washing, see the text.

capillary flow, and the membrane was subjected to hybridization to the radioactive 27mer described above. The band patterns produced from the four isolates of rat-derived *P. carinii* are shown in Fig. 5. The Rp-α probe hybridized to between 14 and 16 bands, depending on the isolate of rat-derived *P. carinii* analyzed. The band patterns produced by the samples in lanes 1 and 2 were clearly different from those in lanes 3 and 4. Samples in lanes 3 and 4 were indistinguishable from each other in this experiment. Samples in lanes 1 and 2 were very similar but not identical.

To determine whether any of the bands shown in Fig. 5A could have resulted from hybridization to rat DNA, DNA was prepared from cultured rat cells and subjected to the same DNA fingerprinting analysis as the *P. carinii* samples. Figure 5B shows that the Rp-α probe did hybridize to several bands in the *Eco*RI-digested rat DNA, but these bands did not correspond to those seen in the *P. carinii* samples. The Rp-α probe would be expected to hybridize to rat DNA because the sequence TAACCCTAA is found at the telomeres of mammalian chromosomes (2).

The DNA fingerprinting analysis shown in Fig. 5A can produce an overestimate of the copy number if the DNA is not fully digested with the restriction enzyme. To assess the completeness of the restriction enzyme digestion, the blot shown in Fig. 5A was stripped and hybridized to a probe containing 16 kb of *P. carinii* DNA containing the cluster of rRNA genes. The rRNA locus probe hybridized to the expected fragments, and there was no evidence of partial digestion (data not shown).

Distribution of Rp-α sequences among *P. carinii* from different host species. All of the experiments described above

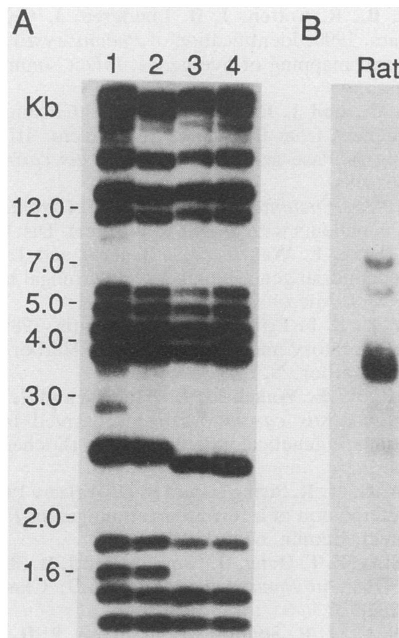

FIG. 5. Hybridization of Rp-α to *Eco*RI-digested genomic DNA from four rat-derived *P. carinii* isolates. (A) Lanes 1 to 4 contained *Eco*RI-digested *P. carinii* genomic DNA from each of four different rat colonies. Samples in lanes 1 and 2 were from Fischer 344 and Wistar strain rats from Hilltop Laboratories. Samples in lanes 3 and 4 were from Holtzman strain rats from Harlan Industries and Sasco, Inc. (B) *Eco*RI-digested rat DNA. Both panels were hybridized with a radiolabeled 1.6-kb *Bgl*II-*Eco*RI fragment from TUB1 (Fig. 2). For details on hybridization and washing, see the text.

were done using rat-derived *P. carinii*. To determine if Rp-α sequences were present in *P. carinii* derived from mice and humans, FIGE was used to separate the chromosomes of *P. carinii* isolates from several rat colonies along with one sample of organisms obtained from *scid/scid* mice and a sample of organisms obtained from a patient. The chromosomal bands resolved by FIGE were transferred from the gel to a nylon membrane by capillary flow, and the membrane was subjected to hybridization to a radioactive probe made from the 1.6-kb *Bgl*II-*Eco*RI fragment known to contain the Rp-α repeat (Fig. 2). Figure 1 shows that while the probe hybridized to the majority of bands present in all rat-derived *P. carinii* (lanes 1 to 6), this probe hybridized to only one band in an electrophoretic karyotype of mouse-derived *P. carinii* (Fig. 1, lanes 7). Subsequent experiments showed that this band also hybridized to a probe prepared from a cDNA copy of the rat-derived *P. carinii* α-tubulin gene and that it did not hybridize to the $(TAACCCTAAA)_3$ oligonucleotide probe (not shown), indicating that the band shown in lane 7 of Fig. 1B was due to cross-hybridization between α-tubulin gene sequences. Figure 4 shows that the 1.6-kb *Bgl*II-*Eco*RI probe did not produce a detectable signal in the lane containing *P. carinii* from humans or in the lane containing *Saccharomyces cerevisiae* (lane 2).

DISCUSSION

Rp-α appears to be a dispersed short repetitive element that is widespread among rat-derived *P. carinii*. It is polymorphic, as evidenced by the sequence divergence seen in the two copies analyzed, and can distinguish types of *P. carinii* in DNA fingerprint analysis. In contrast, this repetitive element was not in evidence in *P. carinii* from a SCID mouse colony nor was it detectable in a *P. carinii* sample isolated from a human patient.

Rp-α seems to be distinct from other repeated sequences previously described in *P. carinii*. Rp3-1 (19) was found to be dispersed but was much larger (10 kb) and more highly repeated (70 copies per genome) than Rp-α. As might be expected from these differences, Rp-α failed to hybridize to a cloned copy of Rp3-1 (data not shown). Another repeat has recently been described that resembles Rp3-1 in size and repetitiveness (23).

The TAACCCTAA sequence found in Rp-α is intriguing because it resembles the sequence found at the telomeres of several species, including species as distantly related as mammals (2), flagellates (1), and slime molds (9). In each of these species, telomeres consist of long runs consisting of a tandem repeated 6mer, CCCTAA. This raises the question of the possible relationship between Rp-α and telomeres of *P. carinii*. The telomere sequence of *P. carinii* chromosomes is not known, but a number of observations are inconsistent with the idea that either one of the sequenced copies of Rp-α was from a telomere of *P. carinii*. First, the genomic library was not constructed to allow the efficient capture of telomeric DNA fragments. As might be expected from this, neither of the two Rp-α elements analyzed were at the end of a cloned DNA fragment. In the case of the TUB1 clone, a comparison of the clone with genomic DNA showed that the clone was an authentic genomic fragment. Second, neither of the two cloned copies of Rp-α analyzed was a simple run of CCCTAA, which would be expected on the basis of the structure of telomeres in other species. Last, Rp-α probes did not hybridize to every chromosomal band in rat-derived *P. carinii* electrophoretic karyotypes and did not hybridize to any bands in electrophoretic karyotypes of *P. carinii* derived from mice and humans.

If it is not a telomere, could Rp-α be related to telomeres of *P. carinii*? Three possibilities come to mind. Rp-α could be an internal telomere-like sequence, which has been described in a number of species (28). Internal telomere-like sequences are thought to be produced when telomerase adds a string of nucleotides to an end generated by a chromosome break. Ligation of the modified break leaves telomeric repeats at the site of the healed break. Another possibility would be that Rp-α is part of the subtelomeric region. In *S. cerevisiae*, subtelomeric sequences are more complex than telomeric sequences but contain short stretches of sequence identical to the 6mer repeated at the telomere. A third possibility would be that Rp-α elements are genes that encode the guide RNAs that are incorporated into the telomerase (27). The TAACCCTAA sequence is identical to what would be expected to be used as the template sequence of the telomerase guide RNA in an organism with telomeres made up of CCCTAA repeats. However, the currently available evidence is inconsistent with any of these possibilities. If *P. carinii* telomeres were composed of TAACCC repeats, one would expect that oligonucleotides composed of TAACCCTAA would hybridize to all of the chromosomal bands in *P. carinii* electrophoretic karyotypes. However, this was not the case. This suggests either that CCCTAA is not the telomeric sequence of *P. carinii* or that a subset of chromosomes in rat-derived *P. carinii* contains too little CCCTAA to be detected. Other explanations of these hybridization data are also feasible, and the possible relationship between Rp-α elements and telomeres of *P. carinii* will not be resolved until a telomere is isolated from *P. carinii*.

Whatever the reason for its similarity to telomeres from other species, Rp-α elements will be useful for discerning variation among *P. carinii* organisms. In the analysis of rat-derived *P. carinii*, Rp-α can be of use in DNA fingerprint analyses. In contrast, this element was undetectable in samples of *P. carinii* obtained from four *scid/scid* mice and from a human patient. The absence of this repeat from these types of *P. carinii* provides a genetic marker in the nuclear genome that can distinguish the rat-derived organism from its relatives and provides evidence that the nuclear genomes of *P. carinii* from different hosts are significantly different, as has been shown for the mitochondrial genomes of *P. carinii* from rats and humans (17).

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