# Isolation and Characterization of a Recombinant Antigen of *Pneumocystis carinii*

A. G. SMULIAN,[1,2]* J. R. STRINGER,[3] M. J. LINKE,[1,2] AND P. D. WALZER[1,2]

*Division of Infectious Diseases, Department of Medicine,[1] and Department of Molecular Genetics,
Biochemistry and Microbiology,[3] University of Cincinnati College of Medicine, and
Cincinnati Veterans Affairs Medical Center,[2] Cincinnati, Ohio 45267*

*Pneumocystis carinii* contains a major group of antigens which migrates as a broad band of 45 to 55 kDa and 35 to 45 kDa in organisms derived from rats and humans, respectively. This complex is among the most common *P. carinii* antigens found in the respiratory tract and is recognized by serum antibodies of infected individuals. We have isolated a cDNA clone encoding the 3' portion of a 45- to 55-kDa antigen of rat-derived *P. carinii*. The predicted protein encoded by this cDNA contains a distinctive domain composed of 10 copies of a 7-amino-acid sequence motif rich in glutamic acid residues. Affinity-purified antibodies to this peptide reacted with the 45- to 55-kDa band of rat-derived *P. carinii* and with the 35- to 45-kDa band of human-derived *P. carinii*, indicating shared epitopes. The fusion protein was recognized by serum antibodies from rats and humans with natural exposure to *P. carinii* and by human immunodeficiency virus patients with *P. carinii* pneumonia. The production of this recombinant protein should allow more detailed studies of the host-parasite relationship of this important opportunistic infection.

*Pneumocystis carinii* is an important pulmonary pathogen of compromised hosts, but the immunobiology and pathogenesis of infection caused by this organism are poorly understood. In recent years, several important *P. carinii* antigens have been identified by Western blot (immunoblot) studies (15, 16, 21, 23, 37). A glycoprotein antigen (gp120) that displays an apparent molecular size of 120 kDa by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions appears to reside on the surface of the organism. This prominent moiety has epitopes that are cross-reactive between *P. carinii* isolated from humans, rats, mice, rabbits, and ferrets as well as species-specific epitopes. Another major class of antigens found in rodent-derived *P. carinii* migrates as a broad band in the 45- to 55-kDa range. In human-derived *P. carinii*, a similar intensely staining band of reactivity migrates between 35 and 45 kDa. It is the most common antigen found in the lungs and bronchoalveolar fluid of humans with *P. carinii* pneumonia (32, 37).

Analysis of *P. carinii* antigens has been limited by the lack of a reliable culture system and other formidable technical problems. Infected lungs are the major source of organisms, but this material may be contaminated with host cells and other microbes. Biochemical purification has resulted in only small quantities of purified antigen (14, 25, 30). Recombinant DNA techniques have led to the identification of several *P. carinii* genes (9, 11), but until now no genes encoding *P. carinii* antigens had been isolated.

In the present study, we isolated a cDNA clone encoding a portion of an immunodominant 45- to 55-kDa antigen of rat-derived *P. carinii*. The predicted protein encoded by this cDNA contained a distinctive domain composed of 10 copies of a 7-amino-acid sequence motif rich in glutamic acid residues. The peptide produced by the expression of the cDNA in *Escherichia coli* was recognized by sera from immune rats and from patients with *P. carinii* pneumonia.

## MATERIALS AND METHODS

**Microorganisms.** *P. carinii* organisms were isolated from corticosteroid-treated Lewis rats by a method described previously (6, 7). Briefly, the lungs of the sacrificed animal were harvested and homogenized in a Stomacher 80 blender (Tekmar Inc., Cincinnati, Ohio) and then filtered through sterile gauze. After centrifugation at 1,000 × g for 10 min at 4°C, the pellet was treated with 0.85% ammonium chloride for 10 min to lyse erythrocytes. The pellet was washed twice with phosphate-buffered saline solution (PBS), resuspended in PBS, and filtered several times through a 10-μm-pore-size Mitex filter to remove contaminating host lung cells. Aliquots of the homogenate were examined with Diff-Quick stain (American Scientific Products, McGaw Park, Ill.) for quantitation of the organisms and were then spread on Mueller-Hinton and Sabouraud dextrose agar plates for detection of bacterial and fungal contaminants. Specimens with no detectable bacterial or fungal contamination were stored at −70°C for later use. Human-derived *P. carinii* organisms, used as a source of antigen, were isolated from an autopsy lung sample from an AIDS patient by similar techniques.

**Antibodies.** Hyperimmune rabbit serum raised to SDS-solubilized rat-derived *P. carinii* (37) was used for screening the cDNA library and for initial characterization of resultant clones. The rabbit serum was rendered specific for *P. carinii* by adsorption with normal rat lung and sequential passage over columns containing albumin and *E. coli* XL-1 Blue lysate proteins immobilized by linkage of their amino groups to a coupling gel (Aminolink; Pierce, Rockford, Ill.). Hyperimmune rabbit serum raised to SDS-solubilized human-derived *P. carinii* isolated from an AIDS patient (37) was used following adsorption to desiccated fragments of normal human lung.

Monoclonal antibodies to rat-derived and human-derived *P. carinii* were raised in BALB/c mice by standard techniques (7, 24). The antibodies were characterized by the apparent molecular weights of the antigens that they recognized and by the presence of cross-reactivity between rat-

* Corresponding author.

and human-derived antigens. The monoclonal antibodies demonstrated no reactivity to bacteria, fungi, or protozoa by Western blot or indirect fluorescent-antibody (IFA) assays.

Rat sera containing *P. carinii*-specific antibodies were obtained from three sources: (i) nonimmunosuppressed rats naturally exposed to *P. carinii*, (ii) immunosuppressed rats with *P. carinii* pneumonia, and (iii) rats that had developed pneumocystosis and had been allowed to recover following withdrawal of immunosuppression. Sera were also obtained from rats that were free of *P. carinii* and had no detectable *P. carinii*-specific antibodies (39, 40). Either these sera were used individually or specimens from five animals with antibodies to *P. carinii* were combined to form an "immune" rat serum pool. Human sera were obtained from normal healthy blood donors at the Hoxworth Blood Bank, Cincinnati, Ohio. Sera from human immunodeficiency virus (HIV)-infected individuals were obtained from the University of Cincinnati AIDS Related Disease Clinic. Random samples were obtained from CDC class II and III patients (five patients from each class) and from five CDC class IV patients who had not had any episodes of *P. carinii* pneumonia. Serum specimens from some patients were also combined to form pooled "immune" serum. In addition, multiple sequential samples of serum were examined from five additional CDC class IV patients, each with one or more episodes of *P. carinii* pneumonia (26).

Polyclonal antibodies against the fusion protein were produced in a rabbit by using Titermax (CytRx, Norcross, Ga.) as an adjuvant. The rabbit was then boosted 4 weeks later and bled the following week. Polyclonal rabbit anti-β-galactosidase antibodies were obtained from 5-Prime, 3-Prime, West Chester, Pa.

**SDS-PAGE, immunoblot, and IFA assays.** SDS-PAGE and immunoblotting were performed as previously described (23, 37). Preparative gels of partially purified fusion protein, β-galactosidase (Sigma, St. Louis, Mo.), and rat- and human-derived *P. carinii* were electrophoresed and transferred to nitrocellulose. Strips cut from these blots were reacted with individual sera overnight at 4°C, washed extensively, and probed with goat anti-rat, anti-human, or anti-mouse immunoglobulin G (heavy- and light-chain specific). IFA was performed on *P. carinii* organisms that were heat or acetone fixed to Teflon-coated glass slides, as previously described (38).

**Nucleic acid isolation from *P. carinii*.** Organisms from a single Lewis rat were isolated as described above. RNA was isolated from $10^{11}$ *P. carinii* organisms by the method of Chomczynski and Sacchi (4). The organisms were homogenized in 3 volumes of 5 M guanidinium thiocyanate–sodium acetate in acid phenol in a Dounce homogenizer. The homogenate was extracted with chloroform, and the RNA was precipitated from the aqueous phase by the addition of 3 volumes of isopropanol. The degree of rat RNA contamination was assessed by the relative intensity of ribosomal bands after agarose gel electrophoresis. The 26S ribosomal subunit of *P. carinii* appeared nine times more intense than the rat 28S ribosomal band, showing that approximately 90% of the intact RNA was of *P. carinii* origin. Polyadenylated RNA was purified from total RNA by chromatography through an oligo(dT) cellulose column (Stratagene, San Diego, Calif.). Five hundred micrograms of total RNA yielded 9 μg of polyadenylated RNA.

For pulsed-field gel electrophoresis (PFGE), *P. carinii* organisms were embedded in 0.6% low-gelling agarose (Sigma), and the embedded organisms were treated with proteinase K and sarcosyl to extract the DNA. PFGE was then

performed by using conditions previously described (19). *P. carinii* DNA and plasmid and phage DNA were extracted by standard methodologies (19, 31).

**cDNA library construction.** Hemimethylated double-stranded cDNA was prepared from 4 μg of *P. carinii* mRNA by the RNase H method (Uni-ZAP; Stratagene) according to the manufacturer's instructions (17). After the fill-in reaction, *Eco*RI linkers were added, followed by cleavage by *Xho*I, to facilitate directional cloning into lambda ZAPII. The cDNA was then ligated into the bacteriophage lambda ZAPII between the *Eco*RI and *Xho*I sites, packaged with Gigapack Gold Plus packaging extract (Stratagene), and plated on *E. coli* PLK-F' cells. The library was amplified and replated on *E. coli* XL-1 Blue cells for further screening.

*E. coli* strains XL-1, Sure, PLK-F', and HB101 were cultured in Luria-Bertani broth as needed.

**Library screening and characterization of immunoreactive clones.** By using standard procedures, the cDNA library was induced with isopropyl-β-D-thiogalactopyranoside (IPTG) and screened with polyclonal rabbit antiserum raised against rat-derived *P. carinii*. Immunoreactive clones were isolated, and phage DNA was prepared from each reactive clone. The insert for each of these clones was amplified by polymerase chain reaction (PCR) using primers within the polylinker of lambda ZAPII (primer 1, AGCTCCACCGCGGTGGCG; primer 2, TGGGTACCGGGCCCCCCC). Concurrently run controls included Bluescript plasmids with no insert or with an insert of known size. PCR products from the two classes of inserts identified were labeled with [α-$^{32}$P]dCTP (New England Nuclear, Boston, Mass.) by random priming. These probes were hybridized to dot blots of phage DNA of all the immunoreactive clones under stringent conditions. One isolate from each class of immunoreactive clones was excised from lambda ZAPII by coinfection with helper phage R408 to yield Bluescript SK II(−) plasmid clones.

**Plasmid subclones and expression of recombinant subclones in *E. coli*.** Bluescript clones pSK599 and pSK790 were the source of all subsequent constructs. To ascertain if the fusion protein produced by pSK599 was the immunoreactive protein, a frameshift was created that altered the reading frame and resulted in the production of a 59-amino-acid peptide. The frameshift mutation was created as follows: pSK599 was digested with *Xba*I, blunt ended with the Klenow fragment of DNA polymerase, religated, and transformed into XL-1 Blue cells. Colonies were picked and plasmids were screened for the loss of the *Xba*I site.

To determine if the repeated domain was immunoreactive, a plasmid lacking the portions of the cDNA 5' or 3' to the repeat was constructed. The construct pSK599Δ316 was made as follows: pSK599 (which lacks the 5' portion of the cDNA) was linearized by digestion with *Eco*O109 at a site 3' to the *Xho*I site, partially digested with *Ava*II (at a site 3' to the repeat within the cDNA [bp 316] and at two sites within the ampicillin gene of Bluescript), blunt ended with the Klenow fragment of DNA polymerase, religated, and transformed into XL-1 Blue cells. The structure of plasmid pSK599Δ316 was verified by double-strand sequencing across the junctions formed by the deletion.

A pUR/SK599 construct was made from ligating the *Bam*HI-*Xho*I fragment of pSK599 (following blunt ending of the *Xho*I site) into the *Bam*HI-modified *Hind*III sites of the pUR 278 vector. This construct was transformed into the appropriate *E. coli* strains. Recombinant clones were induced in Luria-Bertani broth in the presence of 25 μg of ampicillin per ml and 1 mM IPTG. Total protein lysates were prepared by resuspending the cells harvested from 1 ml of

the broth culture in SDS-PAGE sample buffer. Aliquots were then subjected to electrophoresis and immunoblotting. Larger-scale production and partial purification of the insoluble β-galactosidase fusion protein were performed by standard procedures (32). The pSK/SK599 fusion protein was then subjected to electrophoresis and immunoblotting. *E. coli* containing pUR was subjected to the same procedure to produce β-galactosidase, which was used as a control.

**Sequence analysis of the cDNA.** The cDNA inserts of pSK599 and pSK790 were sequenced by a modified double-stranded DNA sequencing technique (Sequenase 2.0; United States Biochemicals, Columbus, Ohio) with the T3 and T7 sequencing primers and three internal primers (P#223, TT TGTAGGTCTGCCCAA; P#456, GCCTACTGAAGAAGG GA; and P#462, GTTCTGTCCCTTCTT). The reaction products were visualized by electrophoresis on 8% polyacrylamide sequencing gels and autoradiography. Both strands of the molecule were sequenced, and the sequence was analyzed with DNANALYZE software (42).

**Purification of antibodies from recombinant protein.** Antibody bound to recombinant bacteriophage plaques or preparative immunoblots was eluted with a solution of 0.1 M glycine–0.1 M NaCl, pH 2.6. The eluted (affinity-purified) antibody was neutralized with 2 M Tris-HCl, pH 8.0, and 20% fetal calf serum was added (2).

**Nucleotide sequence accession number.** The nucleotide sequence reported in this paper has been submitted to the GenBank/EMBL/DDBJ data bank under accession number M77242.

## RESULTS

**Isolation of immunoreactive cDNAs.** Hyperimmune polyclonal rabbit antiserum raised to SDS-solubilized rat-derived *P. carinii* was used to screen 125,000 plaques of a high-titer lambda ZAPII cDNA library. Fifty-one immunoreactive clones were isolated, and phage DNA was extracted for PCR amplification and cross-hybridization studies. Two classes of inserts were found. Most phage (46 of 51) contained an insert of approximately 600 bp; the remaining five phage contained an insert of approximately 800 bp.

To ascertain if the phage within each group were related and not only of similar size, the insert of one representative phage from each class (pSK599 and pSK790) was labeled with [α-$^{32}$P]dCTP. These probes were then hybridized to plaques of all the remaining phages. Cross-hybridization was noted between all clones within each class and between the two classes of inserts. This suggested that all immunoreactive clones were derived from the same or closely related messages. A representative from each class of inserts was rescued from lambda ZAPII as Bluescript plasmids pSK599 and pSK790 by coinfection with helper phage R408.

**Sequence of cloned cDNA.** cDNA inserts pSK599 and pSK790 were both sequenced by modified double-strand sequencing (Fig. 1). pSK599 contained the 3′ portion of clone pSK790 from bases 191 to 790. The sequence displayed several features consistent with those expected of a *P. carinii* mRNA. A poly(A) tail was found that was related to a *Xho*I site in a manner consistent with the production of cDNA with a *Xho*I-poly(T) primer. A canonical polyadenylation signal (AAUAAA) was present 46 bp upstream of the poly(A) tail. The insert of pSK790 had a 695-nucleotide open reading frame (ORF) extending from the beginning of the insert to a stop codon at position 695. Eighty percent of the codons within the ORF had either A or T in the third position of the codon; a strong A or T bias has been noted in both of

the previously sequenced protein-encoding genes of *P. carinii* (9, 11). The ORF was predicted to encode a 225-amino-acid peptide with a molecular mass of 24,905 Da. The predicted peptide contained a repeated motif consisting of 10 copies of a 7-amino-acid sequence (Fig. 2). Each copy was initiated by a proline residue and was extremely rich in glutamic acid residues. Some variability was seen among the repeated segments, but proline in position 1, threonine in position 2, and glutamic acid in position 4 were almost invariant.

**Hybridization of cloned cDNA to *P. carinii* chromosomal and Northern (RNA) blots.** To show that the cloned cDNA was represented within the genome of *P. carinii*, labeled cDNA was hybridized to a PFGE chromosomal blot of *P. carinii*. Labeled insert cDNA from pSK790 hybridized strongly to a chromosome of 670 kb on a field inversion gel electrophoresis blot of rat-derived *P. carinii* DNA (Fig. 3). This probe showed no reactivity to rat DNA by dot blot analysis. To determine if the cloned cDNA was nearly full-length, labeled pSK790 was used to probe a Northern blot of *P. carinii* RNA. The cDNA hybridized to a single band of approximately 1.3 kb, indicating that the cDNA is a copy of the 3′ end of a 1.3-kb mRNA (not shown). An mRNA of this size could encode a protein as large as 43 kDa.

**Expression of fusion proteins.** A fusion protein encoded by pSK599 was analyzed by immunoblotting to verify reactivity with the rabbit polyclonal antiserum used to screen the library. To produce fusion protein, a broth culture of *E. coli* XL-1 containing pSK599 was induced with IPTG. Immunoblots of the total protein lysate were probed with rabbit anti-rat-derived *P. carinii* antiserum. An immunoreactive band of about 28,000 Da was detected (not shown). This size was similar to that predicted for the peptide that would be produced from pSK599. To verify that the 28-kDa band represented the fusion protein produced by pSK599, a frameshift mutation was created in pSK599 that altered the translational reading frame such that a fusion protein of only 59 amino acids, 39 of which were from plasmid vector codons, was produced. The immunoreactive band of 28,000 Da could not be detected when this frameshift mutant was induced. The 28-kDa protein was not present in induced *E. coli* XL-1, XL-1 with Bluescript plasmid, or uninduced pSK599.

To determine if the repeated domain was immunoreactive, plasmid pSK599Δ316 was used. Plasmid pSK599Δ316 contained a truncated cDNA which would be predicted to produce a 20-kDa fusion protein that included residues 65 to 169 (Fig. 1). Two broth cultures of *E. coli* XL-1 Blue containing pSK599Δ316 were grown to log phase, at which time one was induced with IPTG. Total protein lysates were separated by SDS-PAGE, immunoblotted, and probed with rabbit anti-rat-derived *P. carinii* antiserum. An inducible immunoreactive band of approximately 20 kDa was detected (data not shown).

Induction of pSK599 resulted in the production of only small quantities of fusion protein. To obtain fusion protein in large amounts, the cDNA was excised from pSK599 and inserted into the pUR vector. Cloning into pUR 278 resulted in the fusion of the 164-amino-acid *P. carinii* peptide to the end of β-galactosidase from *E. coli*. Large quantities of stable fusion protein were obtained from the induction of the pUR/SK599 construct. Total protein lysates of induced *E. coli* contained an immunoreactive protein of about 150,000 Da (β-Gal/599 peptide) as well as multiple reactive lower-molecular-weight bands. No reactivity was detected in induced *E. coli* containing pUR vector alone, pUR with an

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                             1            .                    .                         .
        -13   G AAT TCC GAC GAG AAA GTG CAG GAT AAG TGT TTG GGT GCA GGT CTT CCT GGT
          1                 Lys Val Gln Asp Lys Cys Leu Gly Ala Gly Leu Pro Gly


                      .     50               .               .                 .               .
         40   CCT CAT CCT TCT CCT CCT CCT GAA CAT TCT CCT GCG GAA CTA CCA GTT CAA GCC
         14   Pro His Pro Ser Pro Pro Pro Glu His Ser Pro Ala Glu Leu Pro Val Gln Ala


                      .    100               .              .                  .               .
         94   GAT TTT ACT AAG ACA CAT ACT ATA CTT TCT GTA TCC ACG GTA CTA GTA ACA GAA
         32   Asp Phe Thr Lys Thr His Thr Ile Leu Ser Val Ser Thr Val Leu Val Thr Glu


              150              .               .                 .               .           200
        148   CTG. GCG CCG GAA ACG CAT ACT TAT ACA AAA ACA TAT ATT AAG ACA AAA ACA TGT
         50   Leu Ala Pro Glu Thr His Thr Tyr Thr Lys Thr Tyr Ile Lys Thr Lys Thr Cys


                          .               .                .                .            250
        202   TGT TTG GGC AGA CCT ACA AAA ACA GAA GAA GAA ACA CTT GAA CCA GAA CCT ACT
         68   Cys Leu Gly Arg Pro Thr Lys Thr Glu Glu Glu Thr Leu Glu Pro Glu Pro Thr


                      .               .                .                .           300
        256   GTA GAA GAG GAA GAA CCT ACT GAA GAA GAG GAA GAA CCT ACT GAA GAA GAG GAA
         86   Val Glu Glu Glu Glu Pro Thr Glu Glu Glu Glu Glu Pro Thr Glu Glu Glu Glu


                  .               .               .                 .           350
        310   AAG CCT GCT GAA GAA GAG GAA AAG CCT ACT GAA GAA GAG GAA AAG CCT ACT GAA
        104   Lys Pro Ala Glu Glu Glu Glu Lys Pro Thr Glu Glu Glu Glu Lys Pro Thr Glu


                  .               .                .           400                   .
        364   GAA GAG GAA AAG CCT ACT GAT GAA AAG GAA AAG CCT ACT GTA GAA GGG GGA GAA
        122   Glu Glu Glu Lys Pro Thr Asp Glu Lys Glu Lys Pro Thr Val Glu Gly Gly Glu


                  .               .                .           450                   .               .
        418   CCT ACT GAA GAA GAG GAA GAG CCT ACT GAA GAA GGG ACA GAA AAT CAT GAT GAT
        140   Pro Thr Glu Glu Glu Glu Glu Pro Thr Glu Glu Gly Thr Glu His Thr Asp Asp


                      .               .           500                   .               .
        472   GAT GGA CCT GAT TCA GAT AAT ACG CAT GGG GAA GAG GAA ACT GAA GAT CAA TGT
        158   Asp Gly Pro Asp Ser Asp Asn Thr His Gly Glu Glu Glu Thr Glu Asp Gln Cys


                      .               .           550                   .               .
        526   TCA ATT ACG GAA ACA GTG ACA GTG ACA GTG ACC CCC GAA AAA ACC CTT GAA GCA
        176   Ser Ile Thr Glu Thr Val Thr Val Thr Val Thr Pro Glu Lys Thr Leu Glu Ala


                  .               .           600                   .               .               .
        580   GCT GAA AAA GGT CAT GGT GTT CGT GTA GAA GGA TTT CAG AAG GAA ATG TTA ATT
        194   Ala Glu Lys Gly His Gly Val Arg Val Glu Gly Phe Gln Lys Glu Met Leu Ile


                          .           650                   .               .               .
        634   TGT GCA ATT ATA GGA ATG ACA TTA GGA GTA TGG ATA ATT CTT TAA AAT TTG AAA
        212   Cys Ala Ile Ile Gly Met Thr Leu Gly Val Trp Ile Ile Ile *


              .           700                   .               .               .               .
        688   ATA TTT TGT AAT AAT AAG TAT GTA AAA TTA TCA AAT AAA GAT ATG TTT GTT TTT


              750                   .                  .               .               .
        742   CTC TTG TTA AAT ATA ATT AAT ATA CAT TTG CAAAAAAAAAAAAAAAAAAAA
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FIG. 1. Sequence of cloned cDNA. Clone pSK790 was sequenced in both directions by the dideoxy-chain termination method. The predicted amino acid sequence is derived from the insert DNA sequence in frame with β-galactosidase of the vector. Nucleotides and amino acids are numbered at the left from the first base within the cDNA (*Eco*RI linker not included). The region encoding 10 copies of the glutamic acid-rich repetitive motif is in boldface. The putative polyadenylation signal at position 721 is underlined. Clone pSK599 stretches from nucleotide 191 to the 3' end of pSK790.

irrelevant insert, or uninduced pUR/SK599. Fusion protein β-Gal/599, partially purified from inclusion granules within induced *E. coli*, was recognized by the rabbit anti-rat-derived *P. carinii* antiserum used to detect the reactive cDNA clone but not by rabbit antiserum to human-derived *P. carinii* (Fig. 4, lanes 1 and 2). However, the fusion protein was recognized by pooled immune serum specimens from both rats and humans (Fig. 4, lanes 3 and 4). These sera demonstrated no reactivity to β-galactosidase immunoblotted under identical conditions. Sera from rats with no exposure to *P. carinii* and no demonstrable anti-*P. carinii* antibodies did not react with the β-Gal/599 fusion protein. Monoclonal antibodies to the *P. carinii* gp120 antigen also did not detect the β-Gal/599 fusion protein.

**Affinity-purified antibodies react with a 45- to 55-kDa**

antigen of rat-derived *P. carinii*. To identify the *P. carinii* protein encoded by the cloned cDNA, cDNA-encoded proteins produced in *E. coli* were used to immunopurify antibodies specific for the *P. carinii* epitopes. Antibodies were captured by the incubation of polyclonal antiserum raised against rat-derived *P. carinii* with either filters containing 10,000 plaques of the lambda ZAPII bacteriophage clone expressing the recombinant protein or filters containing the β-Gal/599 fusion protein. Bound antibodies were eluted from the filters and were then tested for reactivity with *P. carinii* by immunoblotting. As seen in Fig. 5, lane 2, the eluted antibodies reacted with a band of 45 to 55 kDa from rat-derived *P. carinii*. This reactivity could be significantly blocked by prior incubation of the antibodies with the fusion protein (Fig. 5, lane 3). The eluted antibodies also recog-

| 84. | Pro | Thr | Val | Glu | Glu | Glu | Glu |
|-----|-----|-----|-----|-----|-----|-----|-----|
| 91 | Pro | Thr | Glu | Glu | Glu | Glu | Glu |
| 98 | Pro | Thr | Glu | Glu | Glu | Glu | Lys |
| 105 | Pro | Ala | Glu | Glu | Glu | Glu | Lys |
| 112 | Pro | Thr | Glu | Glu | Glu | Glu | Lys |
| 119 | Pro | Thr | Glu | Glu | Glu | Glu | Lys |
| 126 | Pro | Thr | Asp | Glu | Lys | Glu | Lys |
| 133 | Pro | Thr | Val | Glu | Gly | Gly | Glu |
| 140 | Pro | Thr | Glu | Glu | Glu | Glu | Glu |
| 147 | Pro | Thr | Glu | Glu | Gly | Thr | Glu |
| | | | | | | | |
| Consensus | Pro | Thr | Glu | Glu | Glu | Glu | Glu/Lys |
| | 10 | 9 | 7 | 10 | 7 | 8 | 5/5 |

FIG. 2. Repetitive motif of pSK790. Ten copies of a 7-amino-acid repetitive motif were encoded in amino acid residues 84 to 154. Variability between the repeated elements in some positions was noted. A consensus sequence, and the frequency with which this consensus sequence is met, is shown below the individual repeats.
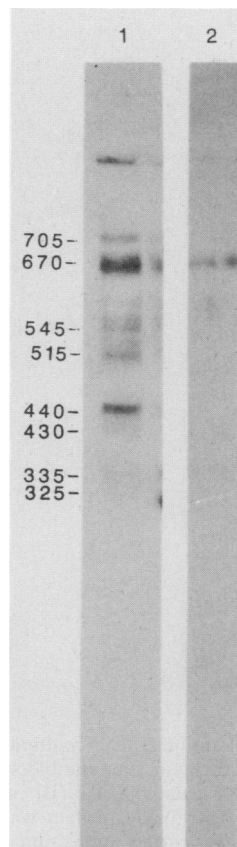


FIG. 3. Mapping of cloned cDNA to a *P. carinii* chromosome. Rat-derived *P. carinii* chromosomes were separated by field inversion gel electrophoresis and transferred to a Nytran filter. For chromosome markers, lane 1 was probed with $\alpha$-$^{32}$P-labeled repeated DNA sequence RP 3-1, which hybridizes to all chromosomal bands (34). An identical lane, lane 2, was probed with $\alpha$-$^{32}$P-labeled pSK790. Sizes of the chromosomes (in kilobases) are indicated to the left. The cloned cDNA maps to a chromosome of approximately 670 kb.

nized a band of approximately 35 kDa on immunoblots of human-derived *P. carinii* (Fig. 5, lane 5). Polyclonal antibodies raised to the fusion protein β-Gal/599 reacted with bands similar to those recognized by the eluted antibodies on immunoblots of both human- and rat-derived *P. carinii*.

To determine the location of the antigen on *P. carinii*, the eluted antibodies were reacted with whole *P. carinii* organisms by the IFA technique. No fluorescent staining occurred, suggesting either that the antigen is not a component of the outer cell wall or that its epitopes are not accessible.

**Recognition of a β-Gal/599 fusion protein by a panel of rat and human sera.** To determine the frequency with which epitopes within the β-Gal/599 fusion protein were recognized by rat and human antibodies, preparative SDS-polyacrylamide gels containing β-Gal/599 fusion protein, β-galactosidase, or *P. carinii* lysate were immunoblotted to nitrocellulose, which was then cut into strips and reacted with the sera of interest. These results are summarized in Table 1.

In the rat study, sera from 20 rats with antibodies to the 120-kDa and 45- to 55-kDa antigens of rat-derived *P. carinii* were screened for reactivity against the β-Gal/599 fusion protein and β-galactosidase. Serum from 18 of the 20 rats reacted avidly with the β-Gal/599 fusion protein but did not react with β-galactosidase. Some reactivity was seen to lower-molecular-weight bands with both the β-Gal/599 fusion protein and β-galactosidase preparations, but no correlation was noted between these bands and the reactivity with the intact fusion protein. Sera from four rats were sampled when the rats arrived at our facility, at which time the sera exhibited no anti-*P. carinii* antibodies, and again after the rats had recovered from an episode of *P. carinii* pneumonia induced by immunosuppression (39, 40). These animals all developed antibody responses to rat-derived *P. carinii* and to the β-Gal/599 fusion protein, but they demonstrated no change in their antibody response to β-galactosidase (Fig. 6).

In the human study, sera from 20 healthy blood donors were tested in parallel against human-derived *P. carinii*, rat-derived *P. carinii*, β-Gal/599 fusion protein, and β-galac-

tosidase by Western blotting. Sera from 18 of 20 individuals contained antibodies to human-derived *P. carinii*, while 13 of these 18 recognized rat-derived material. Of these 13, 10 recognized the 45- to 55-kDa antigen of rat-derived *P. carinii*, while the remainder recognized only higher-molecular-weight antigens. Antibodies reacting with the β-Gal/599 fusion protein were found in 6 of the 10 serum specimens that reacted with the 45- to 55-kDa antigen of rat-derived *P. carinii* but in none of the sera from other individuals. None of these sera demonstrated reactivity with intact β-galactosidase.

Sera from randomly selected CDC class II, class III, or class IV HIV-infected patients were also studied (Table 1). Of 10 class II or III patients, 7 demonstrated reactivity to rat-derived *P. carinii* and 6 had antibodies to human-derived organisms. None of these patients demonstrated reactivity to either the β-Gal/599 fusion protein or β-galactosidase. Ten CDC class IV HIV-infected patients were studied. Of five patients with no prior *P. carinii* pneumonia, three had antibodies to rat- and human-derived *P. carinii*. Reactivity with the β-Gal/599 fusion protein was detected in two of these three individuals. Five of the class IV patients had suffered multiple episodes of pneumocystosis. These pa-
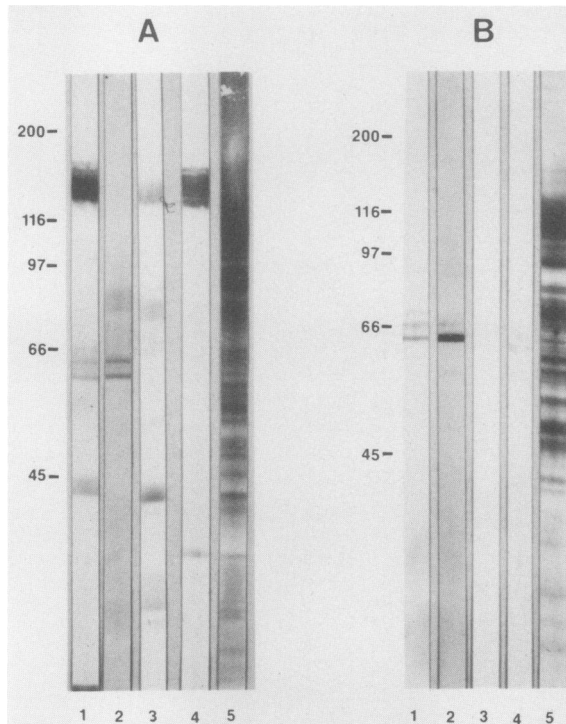
FIG. 4. Reactivity of antibody from multiple sources of antibody to the β-Gal/599 fusion protein. Immunoblots of partially purified fusion protein (A) and β-galactosidase (B) reacted with different sources of antibodies. The fusion protein was recognized by the rabbit anti-rat-derived *P. carinii* antiserum used to detect the reactive cDNA clone (lane 1), pooled immune rat serum (lane 3), and pooled immune human serum (lane 4). Polyclonal rabbit antiserum to human-derived *P. carinii* did not detect the fusion protein (lane 2). An anti-β-galactosidase antibody reacted with the fusion protein, intact β-galactosidase, and multiple lower-molecular-weight bands (lane 5). No reactivity to intact β-galactosidase was noted on immunoblots probed with the specific anti-*P. carinii* sera (panel B, lanes 1 to 4). Numbers at left indicate molecular masses (in kilodaltons).
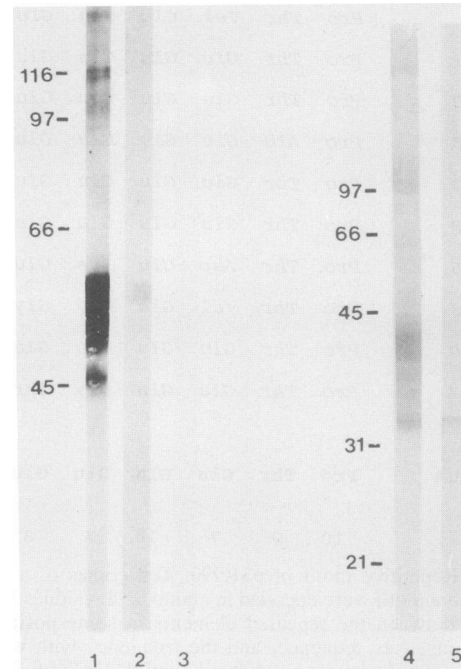
FIG. 5. Detection of *P. carinii* antigen corresponding to β-Gal/599 fusion protein. Preparative immunoblots of the recombinant fusion protein were probed with rabbit polyclonal anti-rat-derived *P. carinii* antiserum. Monospecific polyclonal antibodies eluted off these blots were used to probe immunoblots of rat-derived *P. carinii* (lane 2) and human-derived *P. carinii* (lane 5). Reactivity could be blocked by prior incubation of the eluted antibodies with partially purified fusion protein (lane 3). Rat- and human-derived *P. carinii* are shown probed with rabbit polyclonal anti-rat-derived *P. carinii* antiserum in lanes 1 and 4, respectively. Numbers at left indicate molecular masses (in kilodaltons).

tients all had detectable antibodies to both human- and rat-derived *P. carinii*. Three of these patients demonstrated reactivity to the β-Gal/599 fusion protein. None of the class IV patients had antibodies to β-galactosidase.

Among the CDC class IV patients with multiple episodes of pneumocystosis, the presence of antibodies to the β-Gal/599 fusion protein varied with time (Fig. 7). For one patient, antibodies were detected in all specimens and did not appear to change with episodes of *P. carinii* pneumonia. A second patient developed antibodies to the fusion protein associated with a third episode of *P. carinii* pneumonia. During this same period, the patient had antibodies against human-derived *P. carinii* in all serum specimens but had developed antibodies to rat-derived *P. carinii* only at the time of the third episode of pneumonia. Another patient had antibodies to both human- and rat-derived *P. carinii* and to the fusion protein; these antibodies diminished with the progression of his disease despite repeated episodes of *P. carinii* pneumonia.

## DISCUSSION

The immune response to *P. carinii* has been studied extensively (15, 16, 18, 21, 23, 25, 37, 39, 40), but no single

*P. carinii* antigen has yet been purified and structurally characterized. Most studies of *P. carinii* antigens have focused on a high-molecular-weight complex (gp120) which has been found in organisms obtained from rats, mice, ferrets, rabbits, and humans (15, 16, 21, 23, 37). The gp120 antigen, which migrates as a prominent discrete band on SDS-polyacrylamide gels and immunoblots, resides on the surface of *P. carinii* and is highly susceptible to proteolytic

TABLE 1. Serum antibody responses of rats and humans to the β-Gal/599 fusion protein, β-galactosidase, and rat- and human-derived *P. carinii*

| Source of antibody (n) | Proportion reactive to following antigen | | | |
|---|---|---|---|---|
| | β-Gal/599 protein | β-Galacto-sidase[a] | Rat *P. carinii* | Human *P. carinii* |
| Immune rats (20) | 18/20 | 0/20 | 20/20 | ND[b] |
| Healthy human controls (20) | 6/20[c] | 0/20 | 13/20 | 18/20 |
| HIV-infected humans | | | | |
| CDC class II/III (10) | 0/10 | 0/10 | 7/10 | 6/10 |
| CDC class IV (10) | 5/10 | 0/10 | 8/10 | 8/10 |

[a] No detection of reactivity to intact β-galactosidase was noted, but there was some reactivity to lower-molecular-weight bands.
[b] ND, not determined.
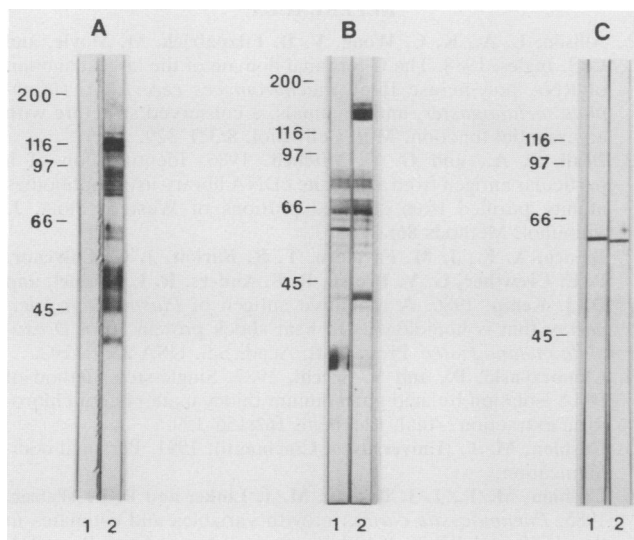[c] These 6 individuals are a subset of the 13 with antibodies reactive to rat-derived *P. carinii*.

FIG. 6. Reactivity of rat serum with β-Gal/599 fusion protein. Sera from four naive rats were sampled on arrival at our facility and again after recovery from *P. carinii* pneumonia induced by immunosuppression. A representative example is shown in which sera were reacted with rat-derived *P. carinii* (A), fusion protein (B), and β-galactosidase (C) immunoblots. Serum sampled on arrival was used in lane 1, while serum sampled after recovery from pneumocystosis was used in lane 2 of each panel. Molecular masses (in kilodaltons) are marked to the left of each panel.



FIG. 7. Sequential antibody responses of AIDS patients to the recombinant protein β-Gal/599. Sequential antibody responses to the recombinant fusion protein are shown for two AIDS patients with multiple episodes of pneumocystosis. Each episode of *P. carinii* pneumonia is indicated by an arrow. Numbers below each lane represent weeks after the first episode. The serum antibody responses of these patients to *P. carinii* antigens have previously been published (27). Numbers at left are molecular mass markers (in kilodaltons).

degradation with agents such as trypsin. This moiety is thought to have a role in adherence to host tissue through fibronectin and mannose receptors (13, 28, 29).

Another major class of antigens appears as a broad band of 45 to 55 kDa in rat-derived *P. carinii* and of 35 to 45 kDa in human-derived *P. carinii*. Although prominent on immunoblots, these antigens stain poorly with protein stains on SDS-polyacrylamide gels, suggesting the presence of a high carbohydrate or lipid content. The relationship of the 45- to 55-kDa antigen of rat-derived *P. carinii* to the 35- to 45-kDa *P. carinii* antigen, or that of these two antigens to gp120, is poorly understood. The 45- to 55-kDa band is the earliest antigen and among the most prominent of those found in the respiratory tract of infected rats (20), and the 35- to 45-kDa band is the most common antigen found in the lungs and bronchoalveolar lavage fluid of patients with pneumocystosis (32, 37).

We used expression cloning to isolate the 3' portion of a cDNA encoding an antigen of rat-derived *P. carinii* which appears to be from the 45- to 55-kDa class. Antibodies affinity purified by absorption to a β-Gal/*P. carinii* fusion protein bound exclusively to material in the 45- to 55-kDa region of rat-derived *P. carinii* on an immunoblot, and the reaction could be blocked by prior incubation of the antibodies with the β-Gal/599 fusion protein. These same antibodies reacted with the 35- to 45-kDa band of human-derived *P. carinii*, thus providing the first definitive evidence that these antigens share epitopes. These results were confirmed by polyclonal antibodies raised to the β-Gal/599 fusion protein. The cloning of this antigen will allow detailed analysis of its structure and epitope specificity and will be very helpful in characterizing the host immune responses.

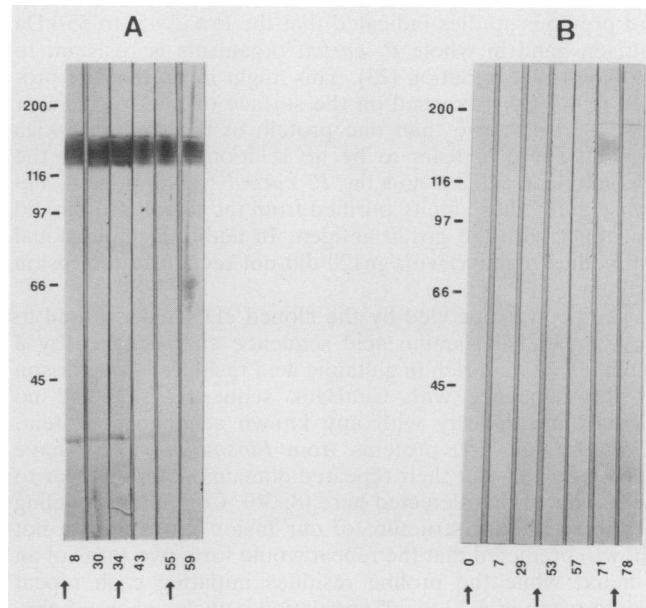In the rat model of pneumocystosis, serum antibodies to the 45- to 55-kDa band appear earlier than those to gp120 and persist for a longer period of time (5, 40). The present study has shown that 90% of rats with serum antibodies to *P. carinii* recognize the β-Gal/599 fusion protein. Although detailed analysis was not performed, the temporal relationship of antibody development to the β-Gal/599 fusion protein was similar to that of the 45- to 55-kDa antigen. The reactivity to the β-Gal/599 fusion protein was not due to the presence of antibodies directed against the β-galactosidase moiety. Antibodies to intact β-galactosidase were not detected, and reactivity to lower-molecular-weight bands remained constant in sequentially studied sera whereas changes in reactivity to the fusion protein were evident. The incidence of anti-β-galactosidase antibodies has previously been found to be highly variable, and the low incidence detected in this study is similar to results of earlier reports (8, 27).

Seroepidemiological surveys have shown that the 35- to 45-kDa human-derived *P. carinii* antigen is the most frequently recognized antigen among normal and immunocompromised humans (26). Given the evidence that epitopes on the 45- to 55-kDa antigen of rat-derived *P. carinii* are present on the 35- to 45-kDa antigen of human-derived *P. carinii*, human sera might be expected to recognize the β-Gal/599 fusion. Antibodies to the β-Gal/599 fusion protein were detected in a number of human serum specimens from both healthy and HIV-infected individuals. The frequency and pattern of human antibodies to the β-Gal/599 peptide were variable. The significance of the variability is not known and will require further study to resolve. Nevertheless, the data clearly showed that humans mount antibody responses to β-Gal/599.

From its amino acid sequence, the recombinant antigen was predicted to have multiple proteolytic cleavage sites,

but previous studies indicated that the broad 45- to 55-kDa antigen band in whole *P. carinii* organisms is resistant to proteolytic degradation (23). This might mean that the protein is not fully exposed on the surface of the organism or that there is more than one protein in the 45- to 55-kDa region. There appears to be no relationship between the recombinant antigen and the *P. carinii* gp120 antigen, because antibodies affinity purified from the fusion protein did not react with the gp120 antigen. In addition, monoclonal antibodies reactive with gp120 did not recognize the fusion protein.

The peptide encoded by the cloned cDNA was found to have an unusual amino acid sequence characterized by a repeated domain rich in glutamic acid residues. Comparison of this sequence with GenBank sequences detected no significant similarity with any known sequenced protein. Glutamic acid-rich proteins from *Plasmodium* spp. have been reported, but their repeated domains do not appear to be similar to that detected here (8, 36). Computer modeling of the secondary structure of our fusion protein (data not shown) predicted that the repeat would form two turns of an α-helix, while the proline residues initiating each repeat would result in a 20 to 30° angulation between each α-helix. A short hydrophobic domain at the carboxyl terminus of the peptide might represent a membrane anchor.

In many organisms, amino acid repeats are found within the products of structural genes (e.g., the merozoite surface antigen of *Plasmodium falciparum*), within enzymes (e.g., RNA polymerase II), and within housekeeping proteins (e.g., *Plasmodium falciparum* heat shock protein 70) (1, 3, 22, 33). These repeated domains do not disrupt the function of these molecules. To our knowledge, similar repeated domains within proteins of fungal origin have rarely been reported (1). This is of note, given the recent evidence that *P. carinii* may be a fungus (10, 35, 41). The recombinant fusion protein with this repeated domain is highly immunoreactive. In plasmodia, highly variable, immunodominant repeated regions of antigens are thought to divert the host immune response from critical antigenic targets (12).

The isolation of this cDNA and the resulting production of a recombinant *P. carinii* antigen in pure form has allowed more detailed study of a *P. carinii* antigen and the host immune response in *P. carinii* infection. An important outcome of this work was the demonstration of epitopes shared by rat-derived and human-derived *P. carinii*. It should be possible to use the rat-derived *P. carinii* cDNA as a probe to clone the human analog of this gene and, through analysis of the gene sequence, to determine the basis for the difference in rat- and human-derived *P. carinii* forms of the antigen. The recombinant protein will serve as a pure antigen for the development of more specific humoral and cellular immune response assays and for the production of monoclonal and monospecific polyclonal antisera. Such reagents should be useful in refining the serology of *P. carinii* for application in studies on the epidemiology, diagnosis, and treatment of pneumocystosis.

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