JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1998, p. 2491–2494 Vol. 36, No. 9
0095-1137/98/$04.00+0

Determination of Copy Number of rRNA Genes in *Pneumocystis carinii* f. sp. *hominis*

XING TANG,[1] MARILYN S. BARTLETT,[1] JAMES W. SMITH,[1] JANG-JIH LU,[2] AND CHAO-HUNG LEE[1]*

Departments of Pathology and Laboratory Medicine, Indiana University School of Medicine, Indianapolis, Indiana 46202,[1] and Department of Pathology, Tri-Service General Hospital and National Defense Medical Center, Taipei, Taiwan[2]

Received 2 March 1998/Returned for modification 29 April 1998/Accepted 13 June 1998

Differential PCR was performed to determine the copy number of rRNA genes in *Pneumocystis carinii* f. sp. *hominis*. Two different reference genes, thymidylate synthase (TS) and beta-tubulin (BTU) genes, were used. Primers for the internal transcribed spacer (ITS) region of nuclear rRNA genes and either the TS or BTU gene were mixed together to perform PCR on seven different bronchoalveolar lavage specimens from patients with *P. carinii* pneumonia. The radioactivity derived from the incorporated radioactive nucleotides of each PCR product band was then used to calculate the copy number of the ITS relative to that of the TS or BTU gene. The copy number ratio between the ITS and the TS gene was determined to be 0.8, and that between the ITS and the BTU gene was also 0.8. These results suggest that the ITS has the same copy number as the TS or BTU gene. Since the copy number of the TS or BTU gene is presumed to be 1, the results also suggest that *P. carinii* f. sp. *hominis* has only one copy of the ITS and thus one copy of the nuclear rRNA genes. Therefore, two types of ITS sequences derived from a specimen would indicate that the patient is infected by two types of *P. carinii* f. sp. *hominis*.

Pneumocystis carinii f. sp. *hominis* causes pneumonia in immunocompromised humans. It is analogous to the form of *P. carinii* that was first found in rats. *P. carinii* is a eukaryotic organism with an uncertain taxonomical classification. It was first identified as a form of trypanosome by Chagas (5) but was later reclassified as a different organism by Delanoe and Delanoe (6, 7). *P. carinii* was traditionally thought to be a protozoan because it has structures common to protozoa and is susceptible to antiprotozoan agents such as pentamidine and trimethoprim-sulfamethoxazole. Currently, *P. carinii* is considered to be a fungus, based on its ultrastructure, certain elements of its cellular biology, and nucleotide sequences of certain genetic loci. The ultrastructure of the cyst wall of *P. carinii* resembles that of fungi (30). Furthermore, *P. carinii* and fungal cell walls share a common epitope which has been identified by a monoclonal antibody (29). *P. carinii* also lacks some of the characteristic protozoan organelles, such as rhoptries, subpellicular tubules, and conoids (39). The nucleotide sequence of the 18S rRNA gene of *P. carinii* is more like that of fungi than that of protozoa (11, 35). The gene for elongation factor 3 (EF-3), which is found exclusively in fungi, has been found in *P. carinii* (40). In addition, thymidylate synthase (TS) and dihydrofolate reductase are two distinct enzymes in *P. carinii* (12, 14), whereas in protozoa, those activities are contained within a single bifunctional protein (36).

The entire nuclear rRNA gene cluster of *P. carinii* from rats has been cloned and sequenced (11, 13, 25), and its transcripts have been characterized (24). Like other organisms, *P. carinii* has three species of nuclear rRNA transcripts: 18S, 5.8S, and 26S. The regions located between 18S and 5.8S and between 5.8S and 26S are referred to as internal transcribed spacer (ITS) regions. The region located between the 18S and 5.8S rRNA genes is called ITS1, and the other is called ITS2. ITS sequences of *P. carinii* f. sp. *hominis* have been found to vary among different isolates. This nucleotide sequence variation has been used for typing, and approximately 60 different ITS sequences have been found (23). Some specimens were found to contain *P. carinii* f. sp. *hominis* with more than one type of ITS sequence (19–21, 23, 26, 37). It is not clear whether these different ITS sequence types represent different strains of *P. carinii* f. sp. *hominis* or whether they are derived from different copies of the rRNA genes of the same organism. In order to answer this question, we have determined the copy number of ITS in *P. carinii* f. sp. *hominis*.

MATERIALS AND METHODS

Specimens. Bronchoalveolar lavage (BAL) fluids from seven different patients with *P. carinii* pneumonia were obtained from the Clinical Microbiology Laboratory, Indiana University Hospital, after routine diagnostic procedures had been completed. These specimens were collected during the year 1997 and were processed for PCR as described previously (22).

PCR. All PCRs for this study were performed under the same conditions. The reaction volume was 20 μl, and the reaction mixture contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM $MgCl_2$, 40 μM each deoxynucleoside triphosphate, 5 pmol of each PCR primer, 10 μCi of α-^{35}S-dCTP, 1.2 units of *Taq* polymerase, and 50 ng of template DNA isolated from BAL specimens. Temperature cycles for each PCR included 1 cycle of 94°C for 2 min; 5 cycles of 94°C for 1 min and 68°C for 2 min; 28 cycles of 94°C for 40 s, 65°C for 40 s, and 68°C for 40 s; and 1 cycle of 72°C for 2 min.

Quantitation of PCR products. The amplified products were electrophoresed on an 8% polyacrylamide gel. A photograph of the gel was taken. The gel was dried and then exposed to X-ray film in order to obtain an autoradiogram. The gel was also exposed to a Storage Phospho Screen (Kodak, Rochester, N.Y.), and the radioactivity in each PCR product band captured by the screen was determined with a PhosphorImager (Storm 840; Molecular Dynamics, Sunnyvale, Calif.).

RESULTS

Since it was difficult to obtain a sufficient amount of *P. carinii* f. sp. *hominis* DNA to perform Southern blot hybridization, we decided to use differential PCR to determine the copy number of the ITS of *P. carinii* f. sp. *hominis*. A portion of the ITS

* Corresponding author. Mailing address: Department of Pathology and Laboratory Medicine, Indiana University School of Medicine, 1120 South Dr., FH 419, Indianapolis, IN 46202-5113. Phone: (317) 274-2596. Fax: (317) 278-0643. E-mail: chlee@iupui.edu.

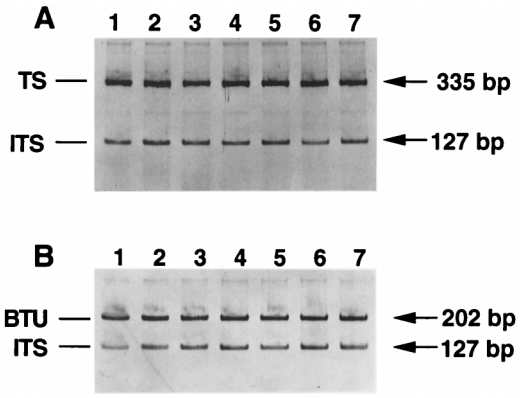

FIG. 1. Differential PCR for determination of the ITS copy number, using the TS gene (A) or the BTU gene (B) as the reference gene. Seven different BAL specimens were examined. The sizes of PCR products are given on the right.

regions is amplified simultaneously with a single-copy reference gene in a multiplex PCR. The copy number of the ITS is then determined based on the quantity of the ITS PCR product compared to that of the reference gene. Differential PCR is usually performed with single-step PCR. However, there was no single-step PCR capable of amplifying ITS regions at the time when this study was initiated. We speculated that the inability to amplify the ITS regions by single-step PCR with primers that have been described (23, 26–28) was due to the excessive length of the intended target. Therefore, we decided to develop another PCR method which amplifies a smaller fragment of the target. Upon closer examination of the ITS sequences of different types of *P. carinii* f. sp. *hominis*, two areas located at nucleotide positions 16 to 39 and 118 to 142 in the ITS2 region were found to be conserved among all 14 types of ITS2 (23). A pair of primers, FT4 (5′-CAAGCAGAAAAA AGGGGATTGGGC-3′) and RT4 (5′-CTTTCCCAGCGAAT TTTTACGACAC-3′), was designed based on the conserved sequences. The FT4 sequence is located at ITS2 nucleotide positions 16 to 39, and the RT4 sequence is located at positions 118 to 142. This pair of primers would amplify a fragment of 127 bp. After many trials of different PCR conditions, the conditions described in Materials and Methods were found to be optimal for amplification.

To perform differential PCR for determination of the ITS copy number, a single-copy reference gene is required. Since the sequence of the TS gene of *P. carinii* f. sp. *hominis* is available (14, 31) and this gene is not known to have more than one copy, it was chosen as a single-copy reference gene. Many different pairs of PCR primers were tried, but only one pair, TS-F (5′-CAGGTCAAGGAGTTGACCAACTAG-3′, nucleotide positions 440 to 463) and TS-R (5′-TTAAAGGGAAC ACCTAGCCCCATG-3′, nucleotide positions 751 to 774), was found to be able to amplify the TS gene of *P. carinii* f. sp. *hominis* under the PCR conditions which were determined to be optimal for the single-step ITS PCR. This pair of primers amplifies a fragment of 335 bp.

The differential PCR with the ITS as the target and the TS gene as the reference was then applied to seven BAL specimens that were confirmed microscopically to contain *P. carinii* f. sp. *hominis*. All seven specimens produced both TS (335 bp) and ITS (127 bp) bands in this differential PCR. Each specimen was assayed three times. A representative photograph of PCR product bands is shown in Fig. 1. The radioactivity derived from incorporated α-^{35}S-dCTP in each band was determined and recorded (Table 1). The copy number of the ITS relative to that of the TS gene was then calculated based on the radioactivity counts of these bands. Since the number of cytosine residues in the amplified TS fragment is 2.8 times that in the ITS (115 versus 41), the ITS copy number was determined by calculating (mean ITS counts $\times$ 2.8)/mean TS counts; thus, (50,823 $\times$ 2.8)/178,648 (Table 1). In this calculation, the sums of the means of three repeats for all seven TS and ITS bands were used and a value of 0.8 was obtained. This result suggests that the copy number of the ITS is 0.8 times that of the TS gene. The copy number of the TS gene is presumed to be 1, since it is not known to have more than one copy. Therefore, the copy number of the ITS would be 0.8. Because the minimum copy number of a gene is 1, this result implies that *P. carinii* f. sp. *hominis* has one copy of the ITS and thus one copy of nuclear rRNA genes.

To confirm this result, another differential PCR, using a different reference gene, the beta-tubulin (BTU) gene, was performed (9, 10). The same approaches were used for this PCR as for that with the TS gene. Many different pairs of primers were tried, and primers Tu-F (5′-TGGTTCCCTCAC CAAAAGTTTCCG-3′, nucleotide positions 1585 to 1608) and Tu-R (5′-GGATCCGGCAATTTCAATGTACGC-3′, nucleotide positions 1763 to 1786) were found to be the most effective in amplifying the intended target under the conditions that were found to be optimal for ITS. Differential PCR with both ITS and BTU gene primers was then performed on all seven BAL specimens. Each specimen was again assayed three times. The BTU gene PCR amplifies a fragment of 202 bp. As

TABLE 1. Radioactivity counts of PCR products from seven different BAL specimens

Specimen no.	Radioactivity count (mean ± SD)[a] of product from:			
	TS-ITS differential PCR		BTU-ITS differential PCR	
	TS	ITS	BTU	ITS
1	26,236 ± 575	6,346 ± 163	31,320 ± 10,061	10,036 ± 2,412
2	24,328 ± 167	8,629 ± 339	33,771 ± 929	13,782 ± 958
3	21,060 ± 3,092	9,047 ± 1,250	31,032 ± 194	16,963 ± 1,004
4	27,174 ± 904	6,274 ± 162	33,589 ± 2,112	17,240 ± 1,822
5	26,121 ± 1,651	7,778 ± 318	33,922 ± 722	14,056 ± 290
6	28,569 ± 652	4,820 ± 188	32,898 ± 5,696	19,682 ± 1,237
7	25,160 ± 674	7,929 ± 204	35,736 ± 4,650	16,357 ± 1,476
Sum[b]	178,648 ± 2,393	50,823 ± 1,502	232,268 ± 1,621	108,116 ± 3,116

[a] Means are averages of counts from three repeats.
[b] Sum of radioactivity of PCR products of all seven BAL specimens.

in the TS-ITS differential PCR, the radioactivity in each PCR product band was determined and used to calculate the ITS copy number (Table 1). In this system, the number of cytosine residues in the amplified BTU gene fragment is 1.76 times that in the ITS (72 versus 41). A value of 0.8 was obtained when the calculation (mean ITS counts × 1.76)/mean TS counts was performed; thus, (10,816 × 1.76)/232,268 (Table 1).

DISCUSSION

In this study, experiments were performed to determine the copy number of the ITS of *P. carinii* f. sp. *hominis*. Since ITS regions are parts of the nuclear rRNA genes in *P. carinii* f. sp. *hominis*, the copy number of the ITS is also the copy number of rRNA genes. With the TS-ITS differential PCR, the copy number of the ITS was determined to be 0.8 times that of the TS gene. The copy number of the TS gene is presumed to be 1, since it is not known to have more than one copy. Therefore, the copy number of the ITS would be 0.8. Because the minimum copy number of a gene is 1, this result implies that *P. carinii* f. sp. *hominis* has one copy of the ITS and thus one copy of nuclear rRNA genes. Using the BTU gene as a reference, we obtained the same result. The copy number ratio between the ITS and the BTU gene was also determined to be 0.8. Since the same results were obtained from two different methods, it is quite certain that the data are reliable.

Copy number determination is normally achieved by Southern blot hybridization. Unfortunately, we were unable to obtain any clinical specimens that contained sufficient numbers of *P. carinii* f. sp. *hominis* organisms for Southern blot analysis. Therefore, we used differential PCR, which has been used to determine the copy number of many different genes, including C-*myc* (1, 32), N-*myc* (3, 17), *erbB* (4, 8, 16, 33), HER-2/neu (38), EGFR and MDM-2 (18), and a parathyroid hormone-related peptide gene (34). Since many factors can affect the efficiency of PCR, each specimen was examined under the same conditions three different times, and the mean of radioactivity counts of PCR products derived from incorporated radioactive nucleotides in the three reactions was used to calculate the copy number. We did observe variations in PCR efficiency between runs. For example, the standard deviations of radioactivity counts for the TS PCR of the TS-ITS differential PCR range from 167 to 3,092 (Table 1). Similar degrees of variation were also seen in other PCRs. To minimize the effect of these variations in copy number determination, we used the sum of the mean counts of all seven specimens for the calculation and obtained the same result from the two different differential PCRs.

The determination of the copy number of *P. carinii* f. sp. *hominis* rRNA genes has an important implication in typing. As described previously, nucleotide sequence variations in the ITS region can be used to type *P. carinii* f. sp. *hominis* isolates. In these typing studies, a number of specimens were found to contain more than one ITS type. It was uncertain whether those specimens actually contained more than one type of *P. carinii* f. sp. *hominis* or whether multiple types of ITS represent multiple copies of rRNA genes in the same *P. carinii* f. sp. *hominis* isolate. Since the copy number of *P. carinii* f. sp. *hominis* rRNA genes has been determined to be 1, multiple types of ITS sequences would represent multiple types of *P. carinii* f. sp. *hominis* in a specimen.

The copy number of rRNA genes in rat *P. carinii* has been determined to be less than 2 (15). In this study, we showed that *P. carinii* f. sp. *hominis* has only one copy of nuclear rRNA genes. Both studies reveal an unusual property of *P. carinii*, since most organisms have more than one copy of nuclear rRNA genes (2). Although we have determined that *P. carinii* f. sp. *hominis* has only one copy of nuclear rRNA genes, this result awaits confirmation by sequencing of the entire *P. carinii* f. sp. *hominis* genome.

ACKNOWLEDGMENT

This study was supported by NIH grant RO1 AI 34304.

REFERENCES

1. **Abou-Elella, A., T. Gramlich, C. Fritsch, and T. Gansler.** 1996. c-myc amplification in hepatocellular carcinoma predicts unfavorable prognosis. 1996. Mod. Pathol. **9:**95–98.
2. **Birnstiel, M. L., M. Chipchase, and J. Speirs.** 1971. The ribosomal RNA cistrons. Prog. Nucleic Acid Res. Mol. Biol. **11:**351–389.
3. **Boerner, S., J. Squire, P. Thorner, G. McKenna, and M. Zielenska.** 1994. Assessment of MYCN amplification in neuroblastoma biopsies by differential polymerase chain reaction. Pediatr. Pathol. **14:**823–832.
4. **Brandt, B., U. Vogt, F. Harms, U. Bosse, K. S. Zanker, and G. Assmann.** 1995. Double-differential PCR for gene dosage estimation of erbB oncogenes in benign and cancer tissues and comparison to cellular DNA content. Gene **159:**29–34.
5. **Chagas, C.** 1909. Nova tripanozomiaza humana. Mem. Inst. Oswaldo Cruz **1:**159–218.
6. **Delanoe, P., and M. Delanoe.** 1912. Sur les rapports des kystos de carinii le Trypanosoma lewis. C. R. Acad. Sci. **155:**658–660.
7. **Delanoe, P., and M. Delanoe.** 1914. De la rareté de *Pneumocystis carinii* chez cobayes de la région de Paris; absence de kysts chez d'autres animaux lapin, grenouille, zanguilles. Bull. Soc. Pathol. Exot. **7:**271–274.
8. **Deng, G., M. Yu, L. C. Chen, D. Moore, W. Kurisu, A. Kallioniemi, F. M. Waldman, C. Collins, and H. S. Smith.** 1996. Amplifications of oncogene erbB-2 and chromosome 20q in breast cancer determined by differentially competitive polymerase chain reaction. Breast Cancer Res. Treat. **40:**271–281.
9. **Dyer, M., F. Volpe, C. J. Delves, N. Somia, S. Burns, and J. G. Scaife.** 1992. Cloning and sequence of a beta-tubulin cDNA from *Pneumocystis carinii*: possible implications for drug therapy. Mol. Microbiol. **6:**991–1001.
10. **Edlind, T. D., M. S. Bartlett, G. A. Weinberg, G. N. Prah, and J. W. Smith.** 1992. The beta-tubulin gene from rat and human isolates of *Pneumocystis carinii*. Mol. Microbiol. **6:**3365–3373.
11. **Edman, J. C., J. A. Kovacs, H. Masur, D. V. Santi, H. J. Elwood, and M. L. Sogin.** 1988. Ribosomal RNA sequence shows *Pneumocystis carinii* to be a member of the fungi. Nature **334:**519–522.
12. **Edman, J. C., U. Edman, M. Cao, B. Lundgren, J. A. Kovacs, and D. V. Santi.** 1989. Isolation and expression of the *Pneumocystis carinii* dihydrofolate reductase gene. Proc. Natl. Acad. Sci. USA **86:**8625–8629.
13. **Edman, J. C., J. A. Kovacs, H. Masur, D. V. Santi, H. J. Elwood, and M. L. Sogin.** 1989. Ribosomal RNA genes of *Pneumocystis carinii*. J. Protozool. **36:**18S–20S.
14. **Edman, U., J. C. Edman, B. Lundgren, and D. V. Santi.** 1989. Isolation and expression of *Pneumocystis carinii* thymidylate synthase gene. Proc. Natl. Acad. Sci. USA **86:**6503–6507.
15. **Giuntoli, D., S. L. Stringer, and J. R. Stringer.** 1994. Extraordinarily low number of ribosomal RNA genes in *P. carinii*. J. Eukaryot. Microbiol. **41:**88S.
16. **Gramlich, T. L., C. Cohen, C. Fritsch, P. B. DeRose, and T. Gansler.** 1994. Evaluation of c-erbB-2 amplification in breast carcinoma by differential polymerase chain reaction. Am. J. Clin. Pathol. **101:**493–499.
17. **Huddart, S. N., J. R. Mann, A. G. McGukin, and R. Corbett.** 1993. MYCN amplification by differential PCR. Pediatr. Hematol. Oncol. **10:**31–34.
18. **Hunter, S. B., K. Abbott, V. A. Varma, J. J. Olson, D. W. Barnett, and C. D. James.** 1995. Reliability of differential PCR for the detection of EGFR and MDM2 gene amplification in DNA extracted from FFPE glioma tissue. J. Neuropathol. Exp. Neurol. **54:**57–64.
19. **Keely, S. P., and J. R. Stringer.** 1997. Sequences of *Pneumocystis carinii* f. sp. *hominis* strains associated with recurrent pneumonia vary at multiple loci. J. Clin. Microbiol. **35:**2745–2747.
20. **Latouche, S., E. Ortona, E. Mazars, P. Margutti, E. Tamburrini, A. Siracusano, K. Guyot, M. Nigou, and P. Roux.** 1997. Biodiversity of *Pneumocystis carinii hominis*: typing with different DNA regions. J. Clin. Microbiol. **35:**383–387.
21. **Latouche, S., J.-L. Poirot, C. Bernard, and P. Roux.** 1997. Study of internal transcribed spacer and mitochondrial large-subunit genes of *Pneumocystis carinii hominis* isolated by repeated bronchoalveolar lavage from human immunodeficiency virus-infected patients during one or several episodes of pneumonia. J. Clin. Microbiol. **35:**1687–1690.
22. **Lee, C. H., J. J. Lu, M. S. Bartlett, M. M. Durkin, T. H. Liu, J. Wang, B. Jiang, and J. W. Smith.** 1993. Nucleotide sequence variation in *Pneumocystis carinii* strains that infect humans. J. Clin. Microbiol. **31:**754–757.
23. **Lee, C. H., J. Helweg-Larsen, B. Lundgren, J. D. Lundgren, X. Tang, S. Jin, B. Li, M. S. Bartlett, J. J. Lu, M. Olsson, S. B. Luvas, P. Roux, A. Cargnel,**

C. Atzori, O. Matos, and J. W. Smith. 1998. Update on *Pneumocystis carinii* f. sp. *hominis* typing based on nucleotide sequence variations in the internal transcribed spacer regions of rRNA Genes. J. Clin. Microbiol. **36:**734–741.
24. **Lin, H., M. T. Niu, T. Yoganathan, and G. A. Buck.** 1992. Characterization of the rRNA-encoding genes and transcripts, and a group-I self-splicing intron in *Pneumocystis carinii*. Gene **119:**163–173.
25. **Liu, Y., M. Rocourt, S. Pan, C. Liu, and M. J. Leibowitz.** 1992. Sequence and variability of the 5.8S and 26S rRNA genes of *Pneumocystis carinii*. Nucleic Acids Res. **20:**3763–3772.
26. **Lu, J. J., M. S. Bartlett, M. M. Shaw, S. F. Queener, J. W. Smith, M. Ortiz-Rivera, M. J. Leibowitz, and C. H. Lee.** 1994. Typing of *Pneumocystis carinii* strains that infect humans based on nucleotide sequence variations of internal transcribed spacers of rRNA genes. J. Clin. Microbiol. **32:**2904–2912.
27. **Lu, J. J., M. S. Bartlett, J. W. Smith, and C. H. Lee.** 1995. Typing of *Pneumocystis carinii* strains with type-specific oligonucleotide probes derived from nucleotide sequences of internal transcribed spacers of rRNA genes. J. Clin. Microbiol. **33:**2973–2977.
28. **Lu, J. J., C. H. Chen, M. S. Bartlett, J. W. Smith, and C. H. Lee.** 1995. Comparison of six different PCR methods for the detection of *Pneumocystis carinii*. J. Clin. Microbiol. **33:**2785–2788.
29. **Lundgren, B., J. A. Kovacs, N. N. Nelson, F. Stock, A. Martinez, and V. J. Gill.** 1992. *Pneumocystis carinii* and specific fungi have a common epitope, identified by a monoclonal antibody. J. Clin. Microbiol. **30:**391–395.
30. **Matsumoto, Y., S. Matsuda, and T. Tegoshi.** 1989. Yeast glucan in the cyst wall of *Pneumocystis carinii*. J. Protozool. **36:**21S–22S.
31. **Mazars, E., C. Odberg-Ferragut, E. Dei-Cas, M.-E. Fourmaux, E. M. Aliouat, M. Brun-Pascaud, G. Mougeot, and D. Camus.** 1995. Polymorphism of the thymidylate synthase gene of *Pneumocystis carinii* from different host species. J. Eukaryot. Microbiol. **42:**26–32.
32. **Pascale, R. M., M. R. DeMiglio, M. R. Muroni, M. M. Simile, L. Daino, M. A. Seddaiu, A. Nufris, L. Gaspa, L. Deiana, and F. Feo.** 1996. c-myc amplification in pre-malignant and malignant lesions induced in rat liver by the resistant hepatocyte model. Int. J. Cancer **68:**136–142.
33. **Roetger, A., B. Brandt, and A. Barnekow.** 1997. Competitive-differential polymerase chain reaction for gene dosage estimation of erbB-1 (egfr), erbB-2, and erbB-3 oncogenes. DNA Cell Biol. **16:**443–448.
34. **Sidler, B., L. Alpert, J. E. Henderson, R. Deckelbaum, N. Amizuka, J. E. Silva, D. Goltzman, and A. C. Karaplis.** 1996. Amplification of the parathyroid hormone-related peptide gene in a colonic carcinoma. J. Clin. Endocrinol. Metab. **81:**2841–2847.
35. **Stringer, S. L., J. R. Stringer, M. A. Blase, P. D. Walzer, and M. T. Cushion.** 1989. *Pneumocystis carinii*: sequence from ribosomal RNA implies a close relationship with fungi. Exp. Parasitol. **68:**450–461.
36. **Toth, I., G. Lazar, and H. Goodman.** 1987. Purification and immunochemical characterization of a dihydrofolate reductase-thymidylate synthase enzyme complex from wild-carrot cells. EMBO J. **6:**1853–1858.
37. **Tsolaki, A. G., R. F. Miller, A. P. Underwood, S. Banerji, and A. E. Wakefield.** 1996. Genetic diversity at the internal transcribed spacer regions of the rRNA operon among isolates of *Pneumocystis carinii* from AIDS patients with recurrent pneumonia. J. Infect. Dis. **174:**141–156.
38. **Valerón, P. F., R. Chirino, L. Fernandez, S. Torres, D. Navarro, J. Aguiar, J. J. Cabrera, B. N. Diaz-Chico, and J. C. Diaz-Chico.** 1996. Validation of a differential PCR and an ELISA procedure in studying HER-2/neu status in breast cancer. Int. J. Cancer **65:**129–133.
39. **Vavra, J., and K. Kucera.** 1970. *Pneumocystis carinii* Delanöe, its ultrastructure and ultrastructural affinities. Protozoology **17:**463–483.
40. **Ypma-Wong, M. F., W. A. Fonzi, and P. S. Sypherd.** 1992. Fungus-specific translation elongation factor 3 gene present in *Pneumocystis carinii*. Infect. Immun. **60:**4140–4145.