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Development of DNA Probes for Early Diagnosis and Epidemiological Study of Cryptococcosis in AIDS Patients

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We report the isolation of middle-repetitive DNA sequences from *Cryptococcus neoformans* that are species and variety specific. These probes were used for assessing strain relatedness among cryptococcal isolates from patients with and without AIDS who were from Zaire and the United States. Five distinct hybridization patterns were observed for the 60 isolates examined, regardless of the restriction enzyme used for digestion. The most common pattern among the isolates from the patients without AIDS was also the most common among the isolates from the patients with AIDS who were from the United States and was the only pattern observed for all isolates tested from patients with AIDS who were from Zaire. On the basis of the high specificity and sensitivity of the signals observed by hybridization, we suggest that these sequences provide a means for both biotyping and early diagnosis of *C. neoformans*.

Cryptococcosis occurs frequently in patients with AIDS; in the United States, 9 to 15% of such patients were found to develop this disease (1, 2, 4, 7, 24), while in African patients with AIDS, it is one of the most commonly observed opportunistic infections (10). Primary cryptococcosis starts as an airborne infection confined to the lungs, often with few or no symptoms. The subsequent hematogenously induced secondary stage can involve all organs but is especially damaging to the central nervous system (22). Unfortunately, cryptococcosis is often diagnosed only in the secondary stage of the disease, when treatment is least effective, and there is high failure and relapse rate (1, 2, 4, 22). Early diagnosis of the primary infection may, therefore, be the key to successful therapy (4, 22).

In the primary stage of the disease, *Cryptococcus neoformans* cannot be detected by microscopy or serology, but it may be detected by culture (22). However, since most patients with AIDS also suffer from *Candida* infections in the oropharynx (22), an abundant yeastlike fungus can be expected in secretions of the lungs and respiratory tract, making it difficult to specifically identify *C. neoformans* at this primary stage.

Middle-repetitive DNA sequences from *C. neoformans* that are species and variety specific could be used as hybridization probes to distinguish *C. neoformans* from other yeastlike fungi such as *Candida albicans* which are often abundant in the respiratory secretions of patients with AIDS. Oligonucleotide primers developed from these sequences could make it possible to develop polymerase chain reaction-based tests for the diagnosis of cryptococcosis in an early stage of the infection, when it is limited to the lungs and when it is present in the respiratory tract secretions at a level lower than can be detected by current methods, and before dissemination and colonization of internal organs have taken place.

The repeated DNA sequences such as these reported here can also be used in DNA-based biotyping to identify and trace particular strains in epidemiological studies. Hybridization of these sequences to Southern blots of DNA restriction digests yield "DNA fingerprints" containing multiple bands characteristic of individual strains. This method would provide more detailed information than serotyping alone. It could be very useful in tracing strains for epidemiological studies of cryptococcosis in patients with AIDS and also for comparing isolates from different patients.

Middle-repetitive DNA sequences were reported previously for *C. albicans* (12, 16–21), for which several advantages in both diagnostic and epidemiological studies were described.

This report describes the isolation of such DNA probes for *C. neoformans* and their use in showing strain relatedness among cryptococcal isolates from patients with and without AIDS who were from Zaire and the United States.

MATERIALS AND METHODS

Strains and media. All strains from the patients without AIDS used in this work were provided by K. J. Kwon-Chung from the Laboratory of Clinical Investigation, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Bethesda, Md., and I. F. Salkin from the Wadsworth Center for Laboratory Research, New York State Department of Health, Albany, N.Y. All those from patients with AIDS who were from the United States were obtained from I. F. Salkin, and all the isolates from patients with AIDS who were from Zaire were provided by T. Muyembe from the Clinique University, Kinshasa, Zaire. A detailed list of the isolates used in this study and their locations in the figures is exhibited in Table 1. All the isolates from patients with AIDS were identified by conventional testing procedures (5, 11): they all grew at 37°C, were urea and inositol positive and nitrate negative, and produced a brown pigment when grown on Niger Seed Agar (22). They were all further characterized by using the API 20C AUX (API System, Montalieu Vercieu, France) and showed similarity in the assimilation patterns of at least 19 carbohydrates; some showed variation in the assimilation of L-arabinose. All other isolates were obtained from diverse sources

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TABLE 1. Cryptococcal strains used in this study

Figure and lane no.[a]	Strain	Serotype	Source[b]
Fig. 3 and 4a and b			
1	38-1	A	CSF; ATCC 34869
2	372	A	Pigeon droppings; ATCC 34870
3	162	A	CSF; NYS
4	174A	A	CSF; NIH
5	160	A	Blood; NYS
6	164	B	CSF; NYS
7	365	B	CSF; Thailand
8	444	B	Sputum; ATCC 32609
9	997	B	CSF; Brazil
10	3271	B	CSF; NIH
11	191	C	CSF; ATCC 32608
12	298	C	CSF; California
13	401	C	CSF; Oklahoma
14	403	C	Lung; Oklahoma
15	409	C	CSF; NIH
16	12	D	Human bone lesion; ATCC 28957
17	B-3501	D	Laboratory strain; ATCC 34873
18	161	D	Sputum; NYS
19	430	D	Pigeon droppings; ATCC 28958
20	433	D	Pigeon nest; ATCC 34875
Fig. 6 and 7			
1	104	A	CSF; NIH
2	151	A	Blood; NYS
3	152	A	Blood; NYS
4	159	A	CSF; NYS
5	160	A	Blood; NYS
6	162	A	CSF; NYS
7	169	A	CSF; NYS
8	RV-52351	A	CSF; Zaire
9	RV-52348	A	CSF; Zaire
10	RV-51267	A	CSF; Zaire
11	Z-4	A	CSF; Zaire
12	Z-5	A	CSF; Zaire
13	Z-6	A	CSF; Zaire
14	Z-131/10	A	CSF; Zaire
15	43/86	A	CSF; NYS
16	125/86	A	Blood; NYS
17	313/86	A	Lymph node; NYS
18	621/86	A	CSF; NYS
19	813/86	A	CSF; NYS
20	711/86	A	CSF; NYS
21	1440/85	A	CSF; NYS

[a] Location of restricted DNA isolated from these strains in the gels.
[b] Abbreviations: CSF, cerebrospinal fluid; NYS, New York State Department of Health; NIH, National Institutes of Health.

and have been previously described in detail (9). All isolates were serotyped by using specific antibodies and were also identified to the variety level with canavanine-glycine-bromthymol blue agar (6). All isolates were maintained on Sabouraud dextrose agar until used.

Genomic DNA preparation. Genomic DNA was prepared from protoplasts of each of the four serotypes of *C. neoformans* by our recently published method for chromosomal DNA extraction (9), modified as follows. Instead of 800 ml of YEPD (10 g of yeast extract, 20 g of Bacto Peptone, 20 g of glucose, all per liter) in 2-liter flasks, 20 ml of medium in 100-ml flasks was used in all the experiments. The final concentration of Novozyme for obtaining protoplasts was 2 mg/ml, and the yeast cells were washed once with the buffer solution without the enzymes (20 mM citrate phosphate buffer [pH 5.6], 50 mM EDTA, 0.9 M sorbitol). When *C. neoformans* var. *gattii* isolates (serotypes B and C) were used, the concentration of the Novozyme was 0.1 mg/ml and the mercaptoethanol pretreatment was omitted. After protoplast lysis, DNA was extracted with phenol and treated first with RNase and then with proteinase K. After an additional phenol extraction, the DNA was stored at 4°C in Tris-EDTA (TE) buffer. When the carbohydrate concentration in the DNA preparation was high, mainly because of the high-level production of the capsule polysaccharides, CsCl density gradient was used for separating the DNA. A sealed 1.5-ml tube containing 1 mg of DNA per ml in TE buffer, 1.05 mg of CsCl per ml, and 40 μl of 10-mg/ml ethidium bromide was spun for 3.5 h at 100,000 rpm in a Beckman Vertical Rotor (TLV 100) at 20°C; the DNA band which was visualized by long UV light was removed with an 18-gauge needle, washed once with 60% ethanol, and solubilized in TE buffer.

Construction of genomic library. *Sau*3A-restricted DNA of *C. neoformans* B-3501 (serotype D) (9) was cloned into the *Bam*HI site of the plasmid pBluescript SK (Stratagene, La Jolla, Calif.). The ligation was carried out for 24 h at 4°C in the presence of T4 ligase and reaction buffer provided by Bethesda Research Laboratories, Gaithersburg, Md. An additional amount of fresh enzyme was added after 16 h. The plasmids were then transformed into competent DH5 bacterial cells according to the supplier's (Bethesda Research Laboratories) instructions. The transformed colonies were replicated onto nitrocellulose membranes (3). After alkaline treatment, their DNAs, immobilized on a filter, were screened by hybridization with two types of radioactive probes: *C. albicans* ribosomal DNA probe Ca 4, which is based on the conserved region of the ribosomal DNA (12, 19–21), and *Sau*3A-restricted *C. neoformans* genomic DNA. Colonies labeled with the second probe but not with the first probe were chosen for further study. This eliminated clones containing ribosome DNA repeats, which more likely would have given false-positive results with other yeasts, from further consideration.

Isolation of plasmid DNA, labeling, and Southern transfer. The alkaline lysis method (8) was used for the isolation of plasmid DNA, and the insertions were confirmed by agarose gel electrophoresis. The plasmid DNA was labeled with the multiprime DNA labeling system, according to the manufacturer's instructions (Amersham Corp., Arlington Heights, Ill.). All the probes were named after the accession numbers of the clones from which they were identified, as with CND 1.1, CND 1.2, etc., where CND refers to *C. neoformans* serotype D, representing the source of the element; the first digit refers to the ligation and cloning experiment; and the second digit refers to our laboratory catalog number. Sequencing was carried out by the dideoxy method with reagents from the Sequenase kit manufactured by the U.S. Biochemical Corp. (Cleveland, Ohio) (15).

Genomic DNA of each of the four serotypes was digested with *Eco*RI and *Hin*dIII. DNA fragments that had been separated according to size by electrophoresis through an agarose gel (1%) were denatured and transferred to a Zeta-Probe membrane (Bio-Rad Laboratories, Richmond, Calif.) by alkaline Southern blotting, according to the manufacturer's instructions.

Hybridization and screening. The DNA attached to the membrane was incubated for 5 min at 58 to 65°C in a prehybridization solution containing 7% sodium dodecyl

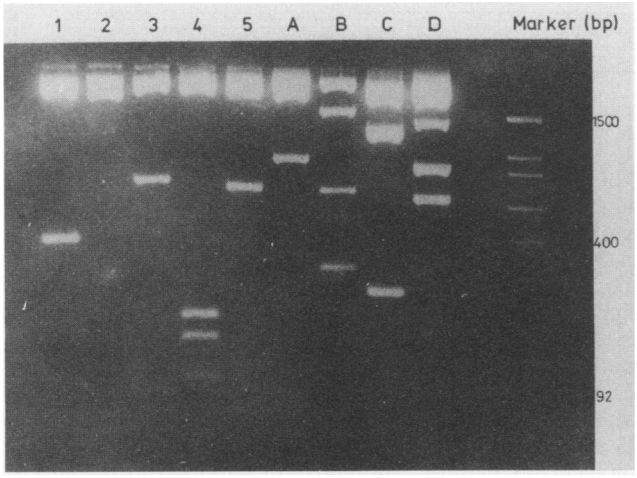

FIG. 1. Cryptococcal inserts in Bluescript plasmid cut with *Eco*RI and *Sst*I. A 1.5-kb ladder was used as a size reference. Lane 4 is CND 1.4, the variety-specific probe.

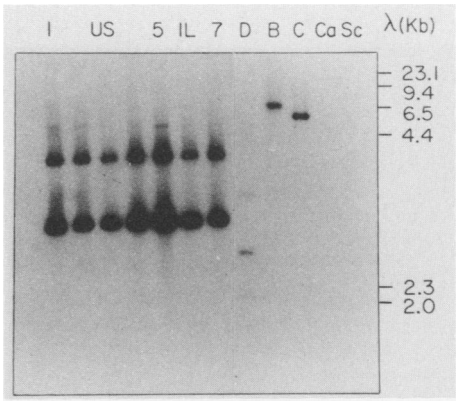

FIG. 2. Hybridization of the ^{32}P-labeled species-specific probe (CND 1.7) to *Eco*RI-restricted DNA isolated from five strains of *C. neoformans* serotype A from patients with AIDS (lanes 1 to 5) (US); two strains of *C. neoformans* serotype A from patients without AIDS (lanes 6 and 7) (IL); one strain each of serotypes D (B-3501) (lane D), B (444) (lane B), and C (191) (lane C); and one strain each of *C. albicans* CBS 562 (Ca) and *S. cerevisiae* 2012 (Sc) (9). The size markers are lambda DNA digested with *Hin*dIII.

sulfate (SDS), 0.5 M Na_2HPO_4 (pH 7.2), and 0.5 mM EDTA and was then hybridized to ^{32}P-labeled DNA probes (overnight at 58 to 65°C). The membranes were washed three times by incubation at 58 to 65°C for 30 min with 50 mM phosphate buffer (pH 7.2) containing 5% SDS and 5 mM EDTA. Autoradiography was used to locate the positions of any bands complementary to the radioactive probe by exposing the filters to Kodak XAR 5 X-ray film in cassettes lined with Lightning-Plus intensifier screen (Dupont, Wilmington, Del.) at −70°C.

RESULTS

Characterization of the DNA probes. Sixty different probes were isolated from individual colonies in two different cloning experiments. All the inserts were in a range of 90 to 1,500 bases. Figure 1 shows a typical picture of the cryptococcal inserts in the Bluescript plasmid, which was cut with *Eco*RI and *Sst*I; all inserts showed between one and four bands. Two types of DNA probes were chosen for further study, one which was species specific (CND 1.7) and one which was variety specific (CND 1.4). Both probes have a high sensitivity; they are found in multiple copies, approximately 10 per genome (approximately 10% of the colonies in the library showed strong hybridization when screened with the probes).

Characterization of the species-specific probe, CND 1.7. The species-specific probe (CND 1.7) showed a high specificity, gave signals only with *C. neoformans*, and did not cross hybridize with DNA from a number of yeastlike fungi, including the following species, which can be found in clinical specimens from patients suffering from fungal infection: *C. albicans*, *Saccharomyces cerevisiae* (Fig. 2), *Candida glabrata*, *Candida guilliermondii*, *Candida krusei*, *Candida lipolytica*, *Candida lusitaniae*, *Candida parapsilosis*, *Candida tropicalis*, *Candida zeylanoides*, *Cryptococcus laurentii*, *Rhodotorula glutinis*, *Rhodotorula rubra*, *Hansenula anomala*, and *Trichosporon beiglii* (data not shown). CND 1.7 gave two bands after the plasmid was cut with *Eco*RI and *Sst*I, with a total size of 1.0 kb (data not shown).

Hybridization analysis with CND 1.7 indicates that with *Eco*RI-restricted DNA, isolates from strains of serotype A gave three bands which were very similar among all isolates tested, whereas three bands of different sizes were detected in isolates of serotype D and a single band was detected in isolates of serotypes B and C (Fig. 2). A similar pattern was obtained with *Hin*dIII-restricted DNA. However, in this case serotype D demonstrated a single band and serotype C demonstrated two bands (data not shown). Electrophoretic karyotype determined by using transversing alternating field electrophoresis (Beckman, Palo Alto, Calif.) demonstrated that CND 1.7 gave two bands with chromosomal elements with each of the four serotypes (data will be published elsewhere).

Characterization of the variety-specific probe, CND 1.4. The variety-specific, probe CND 1.4, contains a single 300-bp *Sau*3A fragment (Fig. 1). Twenty isolates of each serotype were used to ascertain specificity of the probe for the variety. Five representative samples from each serotype were combined for the gels shown in Fig. 3 and 4a and b. A high degree of homogeneity was observed in DNA restriction patterns from the different isolates of the four serotypes of *C. neoformans*, regardless of the endonuclease used. A few bands are highly intense, indicating repetitive DNA (Fig. 3). Figure 4a and b demonstrates that there was no hybridization with isolates of *C. neoformans* var. *gattii* (serotypes B and C), while a typical hybridization pattern was obtained only with isolates of *C. neoformans* var. *neoformans* (serotypes A and D). Hybridization of labeled CND 1.4 DNA to either *Eco*RI- or *Hin*dIII-digested cryptococcal DNA yielded several characteristic fingerprints for each serotype, as shown in Fig. 4a and b. Serotype A exhibits three different patterns (lanes 1, 3, and 5 in these panels are identical), and serotype D exhibits four patterns (lanes 16 and 19 in these panels are identical).

Electrophoretic karyotype determined by using the transversing alternating field electrophoresis system exhibited that CND 1.4 gave two bands only, with chromosomal elements of serotypes A and D (*C. neoformans* var. *neoformans*) but not with those from serotypes B and C (*C. neoformans* var. *gattii*) (data will be published elsewhere). Sequence analysis did not reveal any significant homology to sequences in the GenBank 66 data base (Fig. 5).

Epidemiological study with the species- and variety-specific

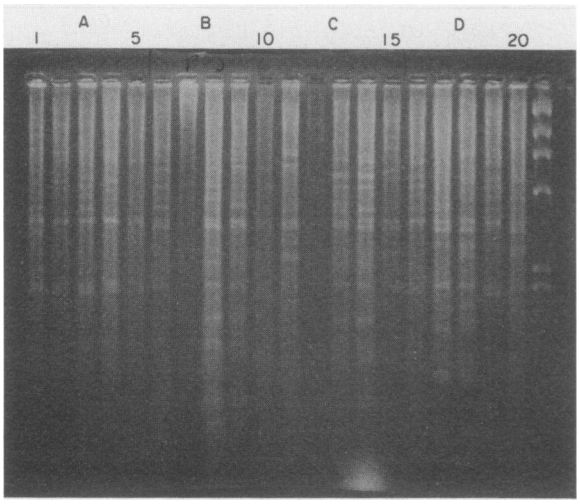

FIG. 3. Gel electrophoresis of DNA from several *C. neoformans* isolates digested with *Eco*RI. Lanes: 1 to 5, serotype A; 6 to 10, serotype B; 11 to 15, serotype C; 16 to 20, serotype D. Rightmost lane, lambda DNA digested with *Hin*dIII. Table 1 shows which isolates are in the various lanes.

probes. Figures 6 and 7 show the relatedness of the Southern blot hybridization patterns among strains isolated from patients with and without AIDS. The species-specific probe, CND 1.7, and the variety-specific probe, CND 1.4, were hybridized to *Eco*RI- and *Hin*dIII-digested DNA of *C. neoformans* isolated from 60 individual patients, including 20 patients without AIDS, 20 patients with AIDS who were from Zaire, and 20 patients with AIDS who were from the United States. Seven representative samples from each group of patients were combined for the gels shown in Fig. 6 and 7. Five distinct hybridization patterns with each probe (CND 1.7 and CND 1.4) were observed for the 60 isolates examined, regardless of the restriction enzyme used for digestion (data with *Hin*dIII-digested DNA are not shown). The most common pattern, exemplified by lanes 4 in Fig. 6 and 7, appeared in 10 of 20 non-AIDS samples, including 4 of

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GATCTCATCC ATGCCTTCTG TATTTGTTTC CAGAGCTCAT CAAGCGACGT GGCGACCCTG     60
GGAAAATGGT GTACCTTTGA CTTTAGGAGA TACATACGTT CTCTATGGGA TTTAGGTCAG    120
GAGAAGAGGG GGGATGGCCA AGGGTTTAGA TTCCCATTTC AGTCTCGCTG GTTTCGCAAC    180
GGCAGTTCGA TGTGAGGGCA TGCCGCCCTC CAACACAAGG ATGTTTTAGA AGCCGGAGGA    240
CTTTAGCCAC CTAAGGATAC CACCCTCGTC CCCCAAGACG CCTTCGATC                289
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FIG. 5. DNA sequence of the variety-specific probe, CND 1.4.

7 of the samples shown in lanes 1 to 7 in these figures. Three additional types appeared in the non-AIDS samples (Fig. 6 and 7, lanes 1 to 3). This common pattern also appeared in 15 of 20 isolates from the patients with AIDS who were from the United States (Fig. 6 and 7, lanes 15 to 21), along with a single additional pattern (Fig. 6 and 7, lanes 18 and 20). The predominance of this common pattern was even more dramatic in isolates from patients with AIDS who were from Zaire. From that group, 20 of 20 samples, including the 7 whose results are shown in Fig. 6 and 7 (lanes 8 to 14), plus 13 additional samples whose results are not presented here showed this pattern. The apparent variation in mobility of the bands in the upper portion of the gel in Fig. 7 was caused by a distortion in the gel and does not appear in other gels in which these samples have been run.

DISCUSSION

The high specificity and sensitivity of the species-specific probe, CND 1.7, suggested that after further development, like coupling with polymerase chain reaction amplification could provide a valuable diagnostic tool to detect *C. neoformans* in the early stage of cryptococcosis in patients with AIDS. At this stage *C. neoformans* is present in respiratory tract secretions or tissue biopsies at levels not detectable by current methods. In addition, middle-repetitive genomic DNA as presented in multiple copies per cell gave a sensitivity comparable to that of ribosomal or mitochondrial DNA but without the potential cross-reactivity expected from probes on the basis of highly conserved sequences of these DNA, such as those described in references 12 and 23.

The variety-specific DNA probe, CND 1.4, in combination with CND 1.7 can be used to distinguish serotypes A and D (*C. neoformans* var. *neoformans*) from serotypes B and C (*C. neoformans* var. *gattii*) and thus facilitate assessment of the prevalence of the specific serotype of *C. neoformans* in

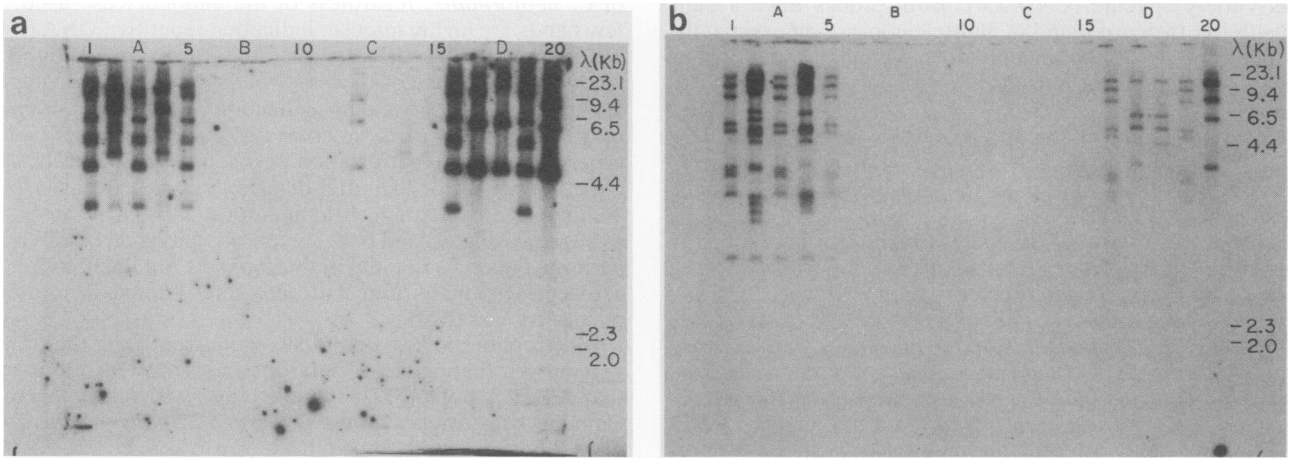

FIG. 4. Hybridization of the ^{32}P-labeled variety-specific probe (CND 1.4) to (a) *Eco*RI- and (b) *Hin*dIII-restricted DNA isolated from five strains of each serotype of *C. neoformans*. Lanes: 1 to 5, serotype A; 6 to 10, serotype B; 11 to 15, serotype C; 16 to 20, serotype D. Hybridization was performed at 60°C with 0.2 M phosphate buffer. The size markers are lambda DNA digested with *Hin*dIII. Table 1 shows which isolates are in the various lanes.

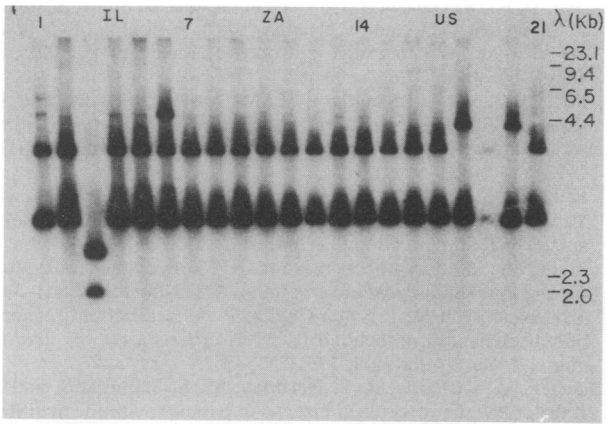

FIG. 6. Southern blot hybridization patterns showing strain relatedness. *Eco*RI-restricted DNA from several *C. neoformans* isolates was hybridized with ^{32}P-labeled species-specific probe (CND 1.7). Cryptococcal DNA was isolated from (left to right) seven patients without AIDS (IL), seven patients with AIDS who were from Zaire (ZA), and seven patients with AIDS who were from the United States (US). Hybridization was carried out at 60°C with 0.2 M phosphate buffer. The size markers are lambda DNA digested with *Hin*dIII. Table 1 shows which isolates are in the various lanes.

patients with AIDS. The large number of bands given by each isolate of *C. neoformans* var. *neoformans* make this method one of the most useful tests for biotyping, for both epidemiological and diagnostic purposes, allowing more detailed analysis of subvarieties than is possible by serotyping alone.

This method could be very useful in tracing strains for epidemiological studies of cryptococcosis in patients with AIDS and also for comparison of isolates from different patients or from the close environment of the patients, especially from suspected vectors.

These probes can also be used to monitor individual *C. neoformans* strains for long periods and through repeated isolations. This permits the clinician to distinguish between treatment failure and reinfection and to monitor changing pathogenic patterns and thus raise the general level of treatment.

The higher frequency of one hybridization pattern among the cryptococcal strains isolated from patients with AIDS than among those isolated from patients without AIDS (Fig. 6 and 7) is an interesting phenomenon that has to be thoroughly studied by using statistical analysis. It may reflect a dominance of a virulent property, but a comparative study with natural isolates will be necessary to elucidate this point. It is beyond the scope of this study to determine whether the difference in frequencies reflects a biological or physiological bias toward a specific strain in patients with AIDS.

Our future strategy will be to create a polymerase chain reaction primer homologous to the most highly repetitive sequence. If such an element has the general structure of retrotransposon, it may be possible to create primers for both terminal repeats and to amplify the core region (13, 14).

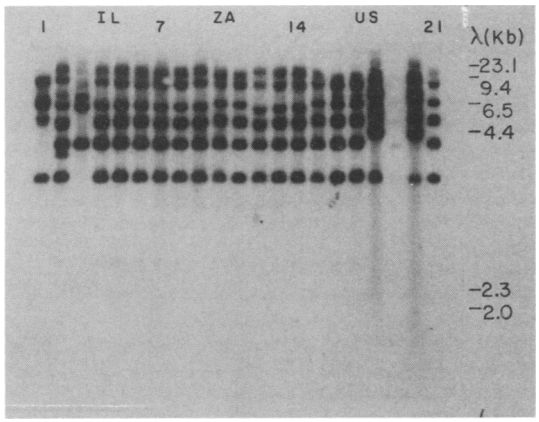

FIG. 7. Southern blot hybridization patterns showing strain relatedness. *Eco*RI-restricted DNA from several *C. neoformans* isolates was hybridized with ^{32}P-labeled variety-specific probe (CND 1.4). Cryptococcal DNA was isolated from (left to right) seven patients without AIDS (IL), seven patients with AIDS who were from Zaire (ZA), and seven patients with AIDS who were from the United States (US). Hybridization conditions were as for Fig. 6. The size markers are lambda DNA digested with *Hin*dIII. Table 1 shows which isolates are in the various lanes.

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