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Use of a Dispersed Repetitive DNA Element To Distinguish Clinical Isolates of *Cryptococcus neoformans*

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We isolated a recombinant phage from a *Cryptococcus neoformans* genomic library that contains a member of a dispersed family of repetitive DNA elements. This clone, CNRE-1, hybridized to at least seven chromosomes in *C. neoformans* on the basis of pulsed-field gel analysis. Hybridization of CNRE-1 to restriction digests of genomic DNA confirmed that there are multiple copies of this element and that restriction fragment length polymorphisms are present in strains from different serotypes of *C. neoformans*. The utility of this probe as an epidemiologic marker was determined by testing cryptococcal isolates from a single hospital. Five isolates from four patients were closely related to a serotype A reference strain, whereas five other isolates from four additional patients exhibited distinct patterns. In two patients, the isolates obtained during recurrent cryptococcal infections were identical to the original isolates.

Cryptococcus neoformans is an encapsulated yeastlike fungus that is present in the environment worldwide. Inhalation of the organism usually results in a self-limited asymptomatic pulmonary infection; however, *C. neoformans* can also cause disseminated disease, particularly in patients with depressed cell-mediated immunity. The most common site of extrapulmonary infection is the meninges. Infections caused by *C. neoformans* have assumed new importance as a result of the AIDS epidemic (4, 8). Cryptococcal meningitis occurs in 5 to 15% of patients with AIDS and is the leading cause of life-threatening fungal infections in that population. Because of the high incidence of recurrent infections following the cessation of antifungal therapy, long-term suppressive therapy with amphotericin B or fluconazole has been recommended.

C. neoformans is divided into two varieties, *C. neoformans* var. *neoformans* (serotypes A and D) and *C. neoformans* var. *gatti* (serotypes B and C). Most clinical isolates from the United States (outside of Southern California) belong to *C. neoformans* var. *neoformans* (5). Genetic differences among strains of *C. neoformans* have been demonstrated by the presence of restriction fragment length polymorphisms (RFLPs) in mitochondrial DNA (18) and chromosome length polymorphisms have been demonstrated by pulsed field gel electrophoresis (9, 10), yet little is known about the extent of genotypic variation among clinical isolates from a restricted geographic region or whether patients with AIDS are infected with a distinct set of strains. Recent studies with *Candida albicans*, another major cause of fungal opportunistic infections, have shown the utility of middle-repetitive DNA sequences as polymorphic markers for distinguishing clinical isolates (12, 13). In this report we describe the isolation of a polymorphic repetitive DNA element from *C. neoformans* and its application as an epidemiologic marker in patients with cryptococcal infections.

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MATERIALS AND METHODS

Strains. Clinical isolates of *C. neoformans* were obtained from the Clinical Microbiology Laboratory, University Hospital at Stony Brook. These strains are identified by the prefix SB and a patient number (A and B designations indicate serial isolates from the same patient). All of these isolates were identified as *C. neoformans* var. *neoformans* on the basis of their reactions on CGB agar (6). Reference strains of *C. neoformans* were obtained from the American Type Culture Collection (ATCC; Rockville, Md.). *Saccharomyces cerevisiae* EG123 was obtained from S. Fields.

Purification of *C. neoformans* DNA. Purification of cryptococcal DNA involved preparation of protoplasts (11) and then extraction with cetyltrimethylammonium bromide (CTAB) (7) to remove capsular polysaccharides. *C. neoformans* was grown to a density of 5×10^7 cells per ml in 1% yeast extract–2% peptone–2% glucose at 30°C. Three milliliters of culture was centrifuged in a microcentrifuge, resuspended in 0.5 ml of 10 mM Tris (pH 7.5)–50 mM EDTA–10 mM dithiothreitol, and incubated for 20 min with gentle agitation at room temperature. Cells were then pelleted in a microcentrifuge for 3 min and resuspended in 0.5 ml of 40 mM citric acid–120 mM Na_2HPO_4–50 mM EDTA (pH 6.0)–1.2 M sorbitol–5 mM dithiothreitol containing 1.2 mg of Novozym 234 (Novo Biolabs, Danbury, Conn.) per ml. The suspension was incubated for 30 to 45 min at 30°C until >70% of the cells were converted to spheroplasts. Cells were pelleted and resuspended in 300 μl of 0.45 M EDTA–10 mM Tris (pH 7.5)–1% sodium dodecyl sulfate (SDS) containing 0.50 mg of proteinase K per ml and incubated for 60 min at 37°C and then for 15 min at 65°C. The lysate was diluted with an equal volume of TE (10 mM Tris [pH 7.6], 1 mM EDTA), adjusted to 0.7 M NaCl–1% CTAB, incubated at 65°C for 10 min, and extracted with phenol-chloroform and chloroform. DNA was precipitated by the addition of 0.6 volume of isopropanol, and the DNA was then washed in 70% ethanol and resuspended in TE buffer. RNA was removed by treatment with RNase. For some strains, the procedure was scaled for 50 to 100 ml of culture.

Construction of a genomic library and isolation of CNRE-1. Total cellular DNA from *C. neoformans* ATCC 6352 was partially digested with *Sau*3A to produce fragments of 10 to

20 kb, partially filled in, and ligated to *Xho*I-digested, partially filled in lambda GEM-11 arms (Promega Corp., Madison, Wis.). To screen for repetitive sequences, individual clones were tested for the ability to hybridize to more than one *C. neoformans* chromosome. DNA was purified from randomly selected recombinant phage with Lambda-Sorb (Promega), labeled with ^{32}P (Random Primers DNA Labelling System; GIBCO Bethesda Research Laboratories, Gaithersburg, Md.), and hybridized to filters containing *C. neoformans* chromosomes that were separated by pulsed-field gel electrophoresis (see below). Clone CNRE-1, which hybridized to several chromosomes, was selected for further study.

RFLP analysis. DNA from strains of *C. neoformans* was digested with restriction enzymes, separated on 0.7% agarose gels in TAE buffer (40 mM Tris-acetate, 2 mM EDTA), and transferred to Nytran membranes (Schleicher & Schuell, Keene, N.H.) by using a positive pressure apparatus (Posiblot; Stratagene, La Jolla, Calif.). Southern hybridizations were performed in 5× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–5× Denhardt's solution–50 μg of salmon sperm DNA per ml–25 mM potassium phosphate (pH 7.4)–50% formamide at 42°C. Filters were sequentially washed with 2× SSC–0.1% SDS and 0.5× SSC–0.1% SDS at room temperature and with 0.1× SSC–0.1% SDS at 42°C.

Pulsed-field gel electrophoresis. Electrophoresis of intact chromosomes was based on previously described methods (9, 10). *C. neoformans* protoplasts were prepared with Novozym 234 as described above and mixed with an equal volume of 1.0% low-melting-point agarose (SeaPlaque; FMC, Inc., Rockland, Maine) in 0.125 M EDTA (pH 8). Cell plugs were then treated with 0.5 mg of proteinase K per ml in 10 mM Tris (pH 8)–0.45 M EDTA–1% Sarkosyl overnight at 50°C, washed with 0.5 M EDTA (pH 8), and stored at 4°C. Chromosomes were separated by contour-clamped homogeneous electric field electrophoresis (CHEF) (3). Cell plugs were loaded in 1% agarose gels and electrophoresed in 0.5× TBE buffer (1× TBE is 89 mM Tris, 89 mM boric acid, 2 mM EDTA) in a CHEF-DRII apparatus (Bio-Rad Laboratories, Richmond, Calif.) at 14°C. For chromosome hybridizations, CHEF gels were treated with 0.25 N HCl for 30 min to depurinate DNA, denatured in 0.5 N NaOH–1.5 M NaCl for 30 min, and then neutralized in 1 M ammonium acetate for 30 min. DNA was transferred to nylon membranes and hybridized as described above.

RESULTS

Isolation of a repetitive element from *C. neoformans*. In order to isolate clones containing members of families of dispersed repetitive DNA, individual random clones from a *C. neoformans* genomic library were purified and screened for the ability to hybridize to multiple *C. neoformans* chromosomes that had been separated by CHEF. One clone, CNRE-1, hybridized to several chromosome-sized bands and was selected for further study. CNRE-1 contained a 16-kb insert composed of seven *Sst*I fragments ranging in size from 0.6 to 3.8 kb. The ability of this clone to hybridize to multiple chromosomes was not the result of artifactual cloning of noncontiguous single-copy DNA sequences, since rescreening of the genomic library with an internal *Sst*I fragment from CNRE-1 led to the isolation of three nonidentical clones with overlapping restriction maps (16).

Figure 1A shows ethidium bromide-stained chromosomes from *S. cerevisiae* and four strains of *C. neoformans* separated by CHEF. Eight to 10 bands were seen in the chromosomes from the different strains of *C. neoformans*, the smallest being approximately 850 kb. These data are similar to those obtained in previous studies (9, 10). Since *C. neoformans* is haploid (19), each band represents at least one chromosome (heavily staining bands indicate comigration of two or more chromosomes). Although some of the high-molecular-mass bands (>2,200 kb) were faint, they were consistently seen in repeat gels. Strains ATCC 6352 (serotype A) and clinical isolate SB4A had very similar banding patterns, whereas strains ATCC 28958 (serotype D) and SB6A each differed from ATCC 6352 at three or more bands. Figure 1B shows the same gel hybridized to CNRE-1. This clone hybridized to at least seven bands in ATCC 6352 and SB4A and at least four bands in ATCC 28958 and SB6A. Hybridization was not due to homology with lambda DNA, since the same hybridization pattern was seen when the filter was probed with internal *Sst*I fragments of 3.4, 5.0, and 1.7 kb isolated from the recombinant phages (16). These data also suggest that the repetitive element is at least 5 to 10 kb. Under the conditions tested, CNRE-1 did not hybridize to *S. cerevisiae*, indicating the lack of any highly conserved CNRE-1-like sequences in this species. In all of the *C. neoformans* strains tested, two of the faster-migrating bands (at approximately 850 and 1,200 kb) hybridized much more intensely to CNRE-1 than did the more slowly migrating bands (>2,200 kb). This pattern is probably due to the presence of comigrating chromosomes and/or multiple copies of the repetitive element on individual chromosomes. CNRE-1 hybridized less intensely to ATCC 28958, even though the sample was overloaded (Fig. 1B, lane 3).

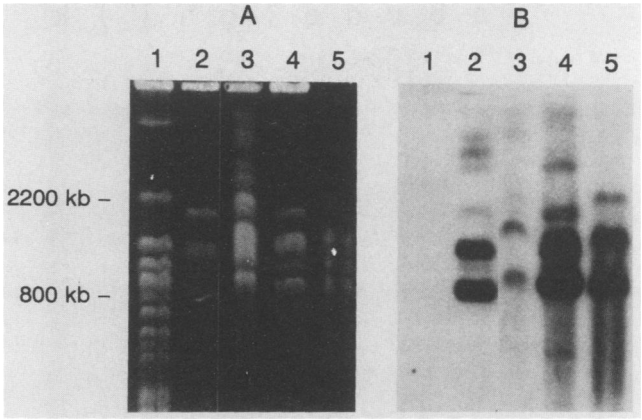

FIG. 1. Hybridization of CNRE-1 to intact chromosomes of *C. neoformans*. Chromosomes were separated by CHEF in 1% agarose at 150 V with pulse intervals of 90 s for 9 h, which was followed by a linear ramp from 120 to 360 s for 39 h. (A) Ethidium bromide-stained CHEF gel. (B) The gel shown in panel A hybridized to CNRE-1. Lanes 1, *S. cerevisiae*; lanes 2, *C. neoformans* ATCC 6352; lanes 3, *C. neoformans* ATCC 28958; lanes 4 and 5, *C. neoformans* clinical isolates SB4A and SB6A, respectively.

Identification of CNRE-1 RFLPs. To determine whether the structure of the repetitive element is conserved in different strains, Southern hybridization was performed on different serotypes of *C. neoformans*. As seen in Fig. 2, hybridization of CNRE-1 to an *Sst*I digest of ATCC 6352 produced a complex pattern of 12 bands with various intensities. Strongly hybridizing bands at 1.7, 2.5, and 3.4/3.5 kb comigrated with fragments that were present in the phage clone. The other bands probably represent junction fragments spanning repetitive and single-copy DNAs or diver

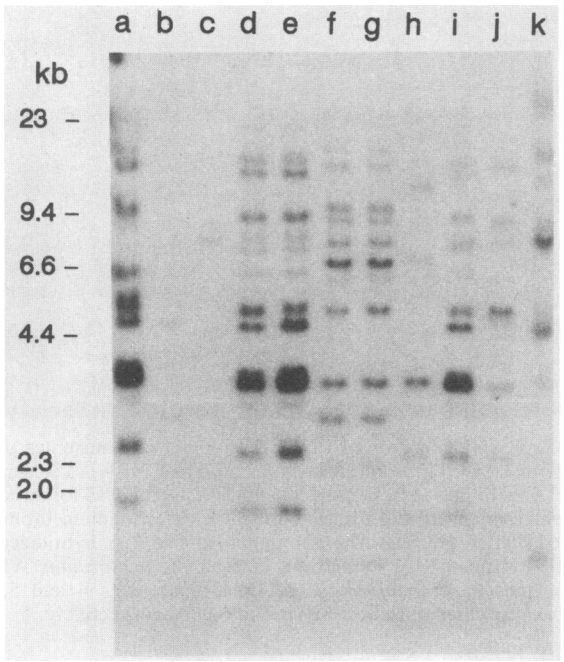

FIG. 2. Hybridization of CNRE-1 to restriction digests of *C. neoformans* isolates. Whole-cell DNA was digested with *Sst*I, fractionated on a 0.7% agarose gel, and hybridized to ^{32}P-labeled CNRE-1. Lane a, ATCC 6352; lane b, ATCC 32608; lane c, ATCC 28958; lanes d and e, SB4A and SB4B, respectively; lanes f and g, SB6A and SB6B, respectively; lane h, SB7; lane i, SB8; lane j, SB9; lane k, 10-fold more ATCC 28958.

gent sequences. The complexity of this pattern was not due to incomplete restriction digests, since the same hybridization pattern was seen with different DNA preparations from this strain digested under a variety of reaction conditions. CNRE-1 hybridized less intensely and to fewer bands in ATCC 28958 (Fig. 2, lane c), which is consistent with the weak hybridization seen on the CHEF gel. By using 10 times as much DNA from this strain, it was shown that CNRE-1 hybridized to a unique set of bands (Fig. 2, lane k). Although serotypes A and D both belong to *C. neoformans* var. *neoformans*, others have also noted genetic differences between these serotypes (9, 10, 18). ATCC 32608, a serotype C (*C. neoformans* var. *gatti*) strain, exhibited weak hybridization and a pattern that differed from those of the *C. neoformans* var. *neoformans* strains (Fig. 2, lane b). The 3.4-kb *Sst*I fragments from CNRE-1 hybridized to only three *Sst*I bands in ATCC 6352, suggesting that at least a portion of the elements is relatively conserved (16).

RFLP analysis of clinical isolates. RFLP analysis was performed on recent clinical isolates to determine whether CNRE-1 was useful as an epidemiologic marker. These studies were made practical by the development of a method for purification of cryptococcal DNA from small-scale cultures. The method eliminates interference from capsular polysaccharides. *C. neoformans* isolates from patients with cryptococcal meningitis in the New York City area exhibited a variety of restriction digest patterns. Isolates from a patient with AIDS (isolate SB4A) and a renal transplant recipient (isolate SB8) were very similar to ATCC 6352, whereas isolates from three patients with AIDS (isolates SB6A, SB7, and SB9) differed from each other and all of the ATCC strains tested. Isolates from three other patients with AIDS at our hospital have been tested; isolates SB1 and SB2 resembled ATCC 6352, whereas isolate SB3 was distinct. Serial isolates were available from two patients with AIDS and cryptococcal meningitis. Isolates SB4B and SB6B were obtained from cerebrospinal fluid cultures in two patients who developed symptomatic cryptococcal infections approximately 4 months after their initial episodes of cryptococcal meningitis. In both patients, the later isolates (SB4B and SB6B) were indistinguishable from the initial isolates (SB4A and SB6A) (Fig. 2, lanes d and e and lanes f and g). Since isolates SB6A and SB6B had patterns that were very different from those of other strains, the second isolate most likely represents reactivation of a latent infection rather than infection with a new strain. Isolate SB4B was obtained from a patient who was initially infected with a strain that had a common RFLP pattern; thus, it was more difficult to distinguish reactivation versus reinfection. Despite the overall similarity of SB4A and SB4B to ATCC 6352, these two clinical isolates exhibited two bands at 7.5 to 8.0 kb that are not present in ATCC 6352; therefore, these results are also consistent with reactivation of the original infecting strain.

DISCUSSION

In order to isolate repetitive DNA sequences from *C. neoformans*, random genomic clones were screened for the ability to hybridize to more than one chromosome. This procedure has the advantage that it selects for dispersed nuclear sequences rather than mitochondrial or ribosomal DNA, which constitute a substantial fraction of repetitive DNA in yeasts (12). CNRE-1 hybridizes to a polymorphic family of repetitive sequences containing at least 7 to 10 members which are a minimum of 5 to 10 kb in length. Individual elements are being sequenced to determine whether they possess features typical of transposable elements such as the retrotransposons of *S. cerevisiae* and other eukaryotes (2). Families composed of relatively large repetitive elements have been described in other opportunistic pathogens. *C. albicans* contains a family of elements, represented by the sequences Ca3, Ca24 (12), and 27A (13), that are at least 10 kb in length and present at 20 copies per genome. The Rp3-1 elements of *Pneumocystis carinii* also have a minimum length of 10 kb and are present at approximately 70 copies per genome (17).

CNRE-1 recognizes multiple RFLPs among clinical isolates of *C. neoformans*. Fifty percent of clinical isolates from our hospital in the New York City area were closely related, suggesting the presence of a predominant clone in this geographic region. The resemblance of this group of strains to a serotype A strain is consistent with surveys indicating that 80% of strains from the United States are serotype A (5). Although DNA hybridization analysis has shown approximately 90% relatedness among strains within each of the two varieties of *C. neoformans* (1), the ability of CNRE-1 to detect multiple polymorphisms among clinical isolates and reference strains belonging to *C. neoformans* var. *neoformans* suggests that there may be many genotypic variants circulating in the environment. This is consistent with a previous study in which two *C. neoformans* cDNA probes hybridized to identical restriction fragments in isolates ATCC 6352, SB1, and SB2 but hybridized to distinct fragments in isolate SB3 (15). Our data also indicate that even within a restricted geographic area, patients with AIDS are infected with more than one genotypic variant of *C. neoformans*. The clinical significance of this variability is not known. It will be useful to examine the CNRE-1 restriction

digest patterns of *C. neoformans* strains from other geographic areas to determine whether there are regional differences in disease-causing strains. Recent studies of *Histoplasma capsulatum*, another environmentally acquired systemic fungal pathogen, have demonstrated geographic differences among strains that infect patients with AIDS (14).

Recurrent cryptococcal infection is a major problem in the management of patients with AIDS. The demonstration of identical CNRE-1 RFLP patterns in serial isolates provides evidence at the DNA level that recurrent cryptococcal infection in patients with AIDS results from reactivation of the original infecting strain rather than reinfection with a second strain. The ability of this probe to distinguish among clinical isolates should prove useful in characterizing the pathogenesis of recurrent infection in immunosuppressed hosts and in monitoring the development and spread of strains resistant to antifungal agents. By using the polymerase chain reaction to amplify conserved CNRE-1 sequences, it may also be possible to detect latent foci of infection, particularly in patients who are receiving suppressive antifungal therapy.

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