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Repetitive DNA Sequences as Probes for *Mycobacterium tuberculosis*

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Three cloned segments of *Mycobacterium tuberculosis* DNA which are promising as clinical probes were identified. An *MboI* digest of DNA from a clinical isolate of *M. tuberculosis* was cloned into bacteriophage M13. To identify recombinants specific for the *M. tuberculosis* complex, plaque lifts were hybridized with *M. bovis* and *M. kansasii* DNA. Recombinants which selectively hybridized with *M. bovis* DNA were characterized by probing slot blots and restriction digests of DNA from various mycobacteria. Three recombinants that did not hybridize to a significant extent with DNA from nontuberculous mycobacteria were identified. These three probes are of special interest because they are each repeated multiple (10 to 16) times in the *M. tuberculosis* chromosome. These probes were also shown to be useful for fingerprinting strains for epidemiological studies.

Tuberculosis is an ancient disease that remains a significant global health problem. In the United States, tuberculosis continues to be a significant problem, especially in underprivileged and minority populations, immigrants from high-prevalence countries, and other high-risk groups, such as persons with human immunodeficiency virus infection. The rapid and specific diagnosis of tuberculosis is one of the most pressing needs in efforts to eradicate the disease.

Although a presumptive diagnosis of tuberculosis can be made on the basis of patient history, clinical and radiological findings, and the presence of acid-fast bacilli in smears, the isolation of *Mycobacterium tuberculosis* is required for a definitive diagnosis. Routine cultures are highly technical, expensive, and time-consuming. Examination of direct smears for acid-fast bacteria is the most rapid method for the detection of mycobacteria. Direct microscopy requires technical expertise in interpreting the smears, and a large number of acid-fast bacteria ($\geq 10^5$/ml) must be present for detection. The radiometric BACTEC system (Johnston Laboratories, Inc., Towson, Md.) for mycobacterial cultures has greatly decreased the time for detection, final identification, and susceptibility testing of *M. tuberculosis*. As with conventional procedures, this system is growth dependent. Noncultural methods, such as radioimmunoassay, latex agglutination, and enzyme immunosorbent assay, for the direct detection of *M. tuberculosis* in clinical material have been described elsewhere (9, 10, 12, 21). Although the immunodiagnostic approach appears to be promising for the rapid diagnosis of tuberculosis, the major limitation is the lack of sensitivity. Recent advances in recombinant DNA and hybridization techniques have provided a new approach to rapid diagnosis of disease, that is, direct detection of specific nucleotide sequences in clinical material by using nucleic acid probes. Nucleic acid probe technology has been successfully applied to the diagnosis of a number of infectious diseases (20).

Recently, Roberts et al. (18) reported the use of whole chromosomal DNA as hybridization probes for rapid identification of clinical isolates of *M. tuberculosis* and the *M. avium* complex. DNA probes for culture confirmation of isolates of the *M. tuberculosis* complex, *M. avium*, and *M. intracellulare* are now available commercially from Gen-Probe (San Diego, Calif.) (4, 6, 11). These DNA probes consist of sequences complementary to mycobacterial rRNA. Also, Gen-Probe has developed DNA probes for detecting strains of the *Mycobacterium* genus and the *M. tuberculosis* complex directly in sputum (K. A. Murphy, E. D. Dean, M. S. Hoppe, D. K. Cabanas, W. J. Daly, M. J. Alden, and D. L. Kacian, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, C311, p. 375; E. D. Dean, D. K. Cabanas, W. J. Daly, A. L. Holden, M. S. Hoppe, K. A. Murphy, and D. L. Kacian, Program Abstr. 27th Intersci. Conf. Antimicrob. Agents Chemother., abstr. no. 1355, 1987).

We previously described cloned fragments of *M. tuberculosis* H37Rv which proved to be useful in highlighting restriction fragment heterogeneity among strains of the *M. tuberculosis* complex (5). Hybridization results demonstrated that considerable differences in DNA sequence exist in some regions of the chromosomes of these strains. The recombinants containing conserved sequences were used to probe slot blots of DNA from nontuberculous mycobacteria (K. D. Eisenach and J. T. Crawford, Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, U32, p. 103). All cross-hybridized to a significant extent with DNA from nontuberculous mycobacteria. In terms of specificity, *M. kansasii* was more of a problem than were the *M. avium* complex strains. In this study, we describe the cloning of smaller fragments of DNA from a clinical isolate of *M. tuberculosis* and the identification of those recombinants which are *M. tuberculosis* specific. We also describe the use of these cloned sequences for differentiating strains of *M. tuberculosis* by chromosomal probe fingerprinting.

MATERIALS AND METHODS

Mycobacterial strains. Reference strains were from the Trudeau Collection and were obtained from the National Jewish Center for Immunology and Respiratory Medicine, Denver, Colo. Clinical isolates of *M. tuberculosis* and *M. kansasii* were obtained from the microbiology laboratories of McClellan Memorial Veterans Hospital and Baptist Medical Center, Little Rock, Ark. Clinical isolates of *M. kansasii* were obtained from the laboratory of the Arkansas Depart-

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ment of Health, Little Rock. Clinical isolates of the *M. avium* complex were obtained from the Centers for Disease Control, Atlanta, Ga.

Preparation of mycobacterial DNA. DNA was prepared as described previously (5). Briefly, strains were incubated on a rotary shaker at 37°C in Middlebrook 7H9 broth containing 0.05% Tween 80. When the cultures were in log phase, D-cycloserine was added to a final concentration of 1 mg/ml (3). Cultures were reincubated until there was a drop in density; then the cells were harvested by centrifugation and lysed with sodium dodecyl sulfate (SDS). DNA was extracted with phenol-chloroform-isoamyl alcohol, purified by dialysis, and concentrated by ethanol precipitation. The DNA concentration was determined by optimal density at 260 nm.

Digestion with restriction endonucleases. Reaction mixtures contained 1 to 2 μg of DNA and 4 to 10 U of enzyme. Digest conditions were as specified by the manufacturer (Bethesda Research Laboratories, Inc., Gaithersburg, Md., or New England BioLabs, Inc., Beverly, Mass.).

Cloning into bacteriophage M13. DNA from a clinical isolate of *M. tuberculosis*, designated T2, was digested with *Mbo*I and ligated with a *Bam*HI digest of M13mp18 replicative form. *Escherichia coli* JM101 cells were transfected and plated. The recombinants, which appear as colorless plaques, were picked to freshly seeded agar plates. Duplicate plaque lifts were made from each plate (13). Recombinant phage were propagated in log-phase cultures of *E. coli* JM101. Single-stranded DNA was isolated from culture supernatants by polyethylene glycol precipitation. Double-stranded (replicative-form) DNA was isolated from cell pellets by the alkaline lysis method (1, 13). To excise the insert DNA, replicative-form DNA was digested with *Eco*RI and *Hin*dIII, which cleave at the opposite ends of the polylinker region. The sizes of the excised fragments were determined by agarose gel electrophoresis.

Labeling DNA probes. Total chromosomal DNA was labeled with [α-^{32}P]dCTP (3,000 mCi/mmol) by nick translation (16). Single-stranded M13 DNA was labeled with ^{32}P by primer extension, using the procedure of Hu and Messing (7). Specific activity of the probes was about 10^8 cpm/μg of DNA, and approximately 10^6 to 10^7 cpm was used in each hybridization.

Slot blot hybridizations. DNA was denatured in 0.4 N NaOH at room temperature for 10 min, neutralized with an equal volume of 2 M ammonium acetate (pH 7), and then loaded on a slot blotter (Minifold II; Schleicher & Schuell, Inc., Keene, N.H.) containing a BA85 nitrocellulose membrane. Membranes were baked in a vacuum oven at 80°C. The prehybridization solution consisted of 6× SSC (0.9 M NaCl plus 0.09 M sodium citrate), 5× Denhardt solution (0.1% polyvinylpyrrolidone, 0.1% Ficoll, 0.1% bovine serum albumin), 0.5% SDS, and 100 μg of denatured salmon sperm DNA per ml. EDTA (0.01 M) and labeled probe DNA were added for the hybridization solution. Membranes were hybridized overnight at 68°C and washed as follows: 2× SSC and 0.5% SDS at room temperature for 5 min, 2× SSC and 0.1% SDS at room temperature for 10 min, and 0.1× SSC and 0.5% SDS at 68°C for 2.5 h.

Southern blot hybridizations. Digests were electrophoresed on 0.8% agarose gels containing ethidium bromide and photographed. DNA fragments were denatured and transferred to GeneScreen Plus membrane (Dupont, NEN Research Products, Boston, Mass.) by using either a modified Southern transfer method as described by Maniatis et al. (13) or the alkaline transfer method of Chomczynski and Qasba (2). GeneScreen Plus was hybridized according to the instructions of the manufacturer, except that the hybridization was performed at 68°C without dextran sulfate. Membranes were washed as follows: twice with 2× SSC at room temperature for 5 min, twice with 2× SSC and 0.1% SDS at 68°C for 30 min, and twice with 0.1× SSC at room temperature for 30 min.

Autoradiography. Membranes were exposed for variable lengths of time at −70°C, using Kodak X-Omat film (Eastman Kodak Co., Rochester, N.Y.) and Cronex Lightning Plus intensifying screens (E. I. du Pont de Nemours & Co., Wilmington, Del.).

RESULTS

Screening of recombinants for specificity. The procedure used to screen the M13 recombinants was based on the premise that those recombinants which hybridized with *M. bovis* DNA and did not hybridize with *M. kansasii* DNA would be specific for the *M. tuberculosis* complex. To ensure that the cloned fragments would hybridize with the whole spectrum of strains of the tuberculosis complex, *M. bovis* was selected as a distant member of the complex (8). A total of 553 colorless plaques were picked to agar plates, and plaque lifts were prepared in duplicate. One set was hybridized with total *M. bovis* DNA, and the other was hybridized with total *M. kansasii* DNA. Recombinants that hybridized only with *M. bovis* DNA and produced a strong ^{32}P signal on the autoradiograph were selected for further study.

Slot blot hybridizations. To assess the potential of these recombinants for use as *M. tuberculosis*-specific probes, slot blot hybridizations were performed. Slot blots containing 128 ng of purified DNA from representative strains of the *M. tuberculosis* complex (*M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis*, and clinical isolates of *M. tuberculosis*) were hybridized with the labeled single-stranded form of the recombinants. Three recombinants, designated M13KE37, M13KE49, and M13KE115, hybridized strongly with all strains and appeared to be good candidates for probes (not shown). To assess specificity, slot blots of DNA from mycobacteria which are commonly found in sputum were hybridized with these three probes. Results with M13KE115 are representative of those observed with all three probes. No significant hybridization occurred with DNA from clinical isolates and reference strains of *M. kansasii* or with reference strains of *M. fortuitum*, *M. chelonei*, *M. gordonae*, and *M. simiae* (Fig. 1). Similarly, no significant hybridization was detected with clinical and reference strains of *M. avium* serotypes 1, 4, and 8, the types most commonly isolated from acquired immunodeficiency syndrome patients, or with other reference strains representative of the *M. avium* complex. When slot blots containing twofold dilutions of *M. tuberculosis* DNA were hybridized with these probes, 2 to 4 ng of DNA (approximately 10^6 bacilli) was detected.

Southern blot hybridizations. Since Southern blot hybridizations yield more specific information, the recombinants were hybridized with membranes containing *Bam*HI digests of DNA from members of the tuberculosis complex. Surprisingly, all three recombinants hybridized strongly with multiple chromosomal fragments. M13KE37 hybridized strongly with 16 *Bam*HI fragments of T2 DNA (Fig. 2). The band patterns of T2 and H37Rv were very similar, although M13KE37 hybridized only with 12 fragments of H37Rv. M13KE37 hybridized with nine fragments in the *M. tuberculosis* T1 (another clinical isolate) digest and with five in the

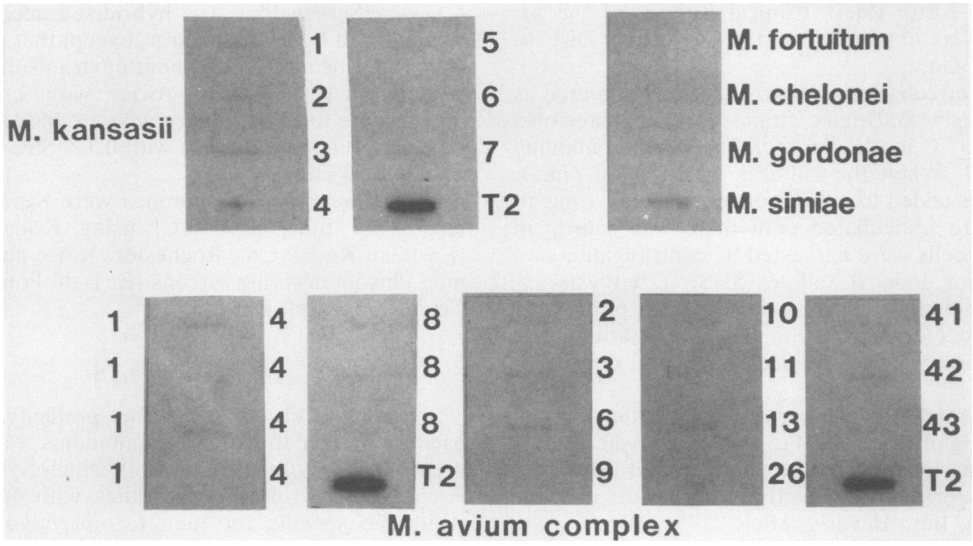

FIG. 1. Slot blot hybridization with M13KE115. Samples containing 128 ng of DNA were slot blotted and probed with M13KE115. Top left, DNA from seven strains of *M. kansasii*; top right, DNA from the indicated strains; bottom, DNA from strains of the *M. avium* complex, with the serotype of each strain indicated. T2 indicates *M. tuberculosis* T2.

M. bovis digest. The fragments ranged from 18 to 1 kilobase pairs. Thus, this sequence is repeated in the chromosomes of strains of the tuberculosis complex, but it does not occur with the same frequency in all strains. A Southern blot containing *Bam*HI digests of representative strains of nontuberculous mycobacteria was probed with M13KE37 (Fig. 3). Inefficient hybridization occurred with several fragments in all five digests of the nontuberculous strains. The insert in M13KE37 was determined to be 790 base pairs based on the size of the fragment excised by *Eco*RI-plus-*Hin*dIII digestion.

M13KE49 hybridized with a total of 10 *Bam*HI fragments in the digests of T2 DNA, strongly with 4 fragments and less efficiently with 6 (Fig. 4). The band pattern of H37Rv was identical to that of T2, and those of T1 and *M. bovis* were very similar. Thus, this segment of DNA is conserved among the *M. tuberculosis* complex strains. Inefficient hybridization occurred with multiple fragments in the digests of two *M. kansasii* strains and one *M. scrofulaceum* strain. No hybridization occurred with the *M. avium* and *M. fortuitum* strains (not shown). The M13KE49 insert was determined to be 570 base pairs.

With M13KE115, hybridization occurred strongly with nine fragments in the T2 digest and less efficiently with seven

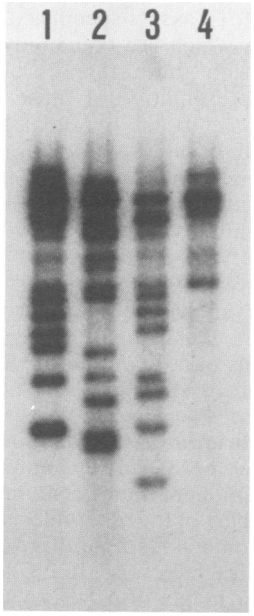

FIG. 2. Southern blot of *Bam*HI digests of DNA from *M. tuberculosis* complex strains hybridized with M13KE37. Lane 1, *M. tuberculosis* T2; lane 2, *M. tuberculosis* T1; lane 3, *M. tuberculosis* H37Rv; lane 4, *M. bovis* TMC 401.

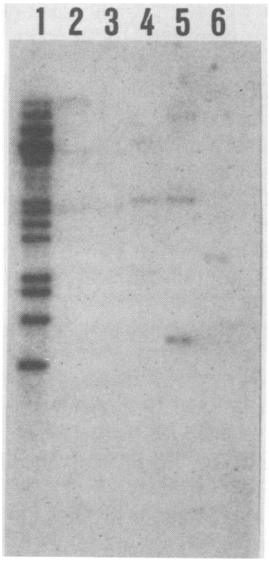

FIG. 3. Southern blot of *Bam*HI digests of mycobacterial DNA hybridized with M13KE37. Lane 1, *M. tuberculosis* T2; lane 2, *M. kansasii* TMC 1201; lane 3, *M. kansasii* TMC 1204; lane 4, *M. avium* LR147; lane 5, *M. scrofulaceum* TMC 1309; lane 6, *M. fortuitum* TMC 1529.

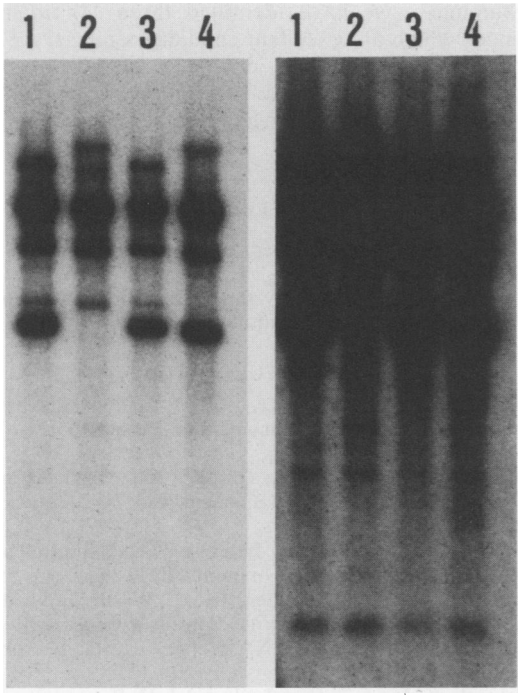

FIG. 4. Southern blot of *Bam*HI digests of DNA from strains of the *M. tuberculosis* complex hybridized with M13KE49. Lane 1, *M. tuberculosis* T2; lane 2, *M. tuberculosis* T1; lane 3, *M. tuberculosis* H37Rv; lane 4, *M. bovis* TMC 401. Membrane was exposed overnight (left) and for 6 days (right).

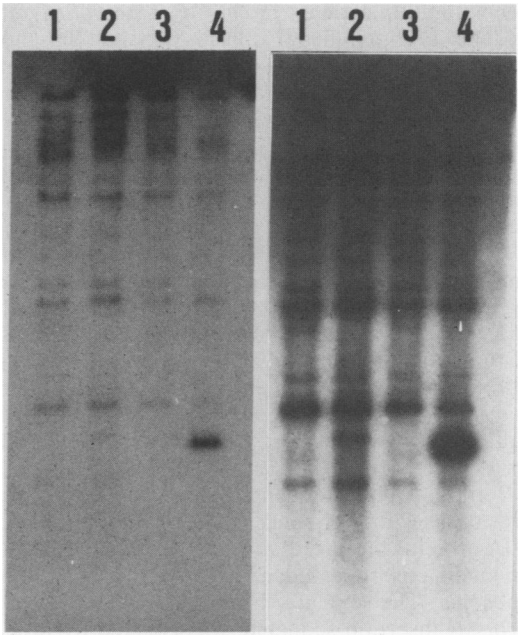

FIG. 5. Southern blot of *Bam*HI digests of DNA from *M. tuberculosis* complex strains hybridized with M13KE115. Lane 1, *M. tuberculosis* T2; lane 2, *M. tuberculosis* T1; lane 3, *M. tuberculosis* H37Rv; lane 4, *M. bovis* TMC 401. Membrane was exposed overnight (left) and for 9 days (right).

others (Fig. 5). The band patterns of all of the *M. tuberculosis* complex strains were almost identical; thus, these sequences are highly conserved. No hybridization occurred with DNA from nontuberculous mycobacteria (not shown). The *Hin*dIII and *Xba*I sites of the M13 polylinker region were deleted during cloning, and thus the M13KE115 insert could not be excised intact. The size was determined from various digests to be approximately 1,600 base pairs. M13KE115 looks especially promising, since it contains a large fragment which occurs in the chromosome at least 16 times and is highly specific.

Chromosomal fingerprinting of clinical isolates. The fact that these cloned fragments contained conserved sequences which hybridized with multiple chromosomal fragments suggested they would be useful for epidemiological studies. To examine this potential, M13KE37 and M13KE115 were used to probe various restriction digests of DNA from several strains of *M. tuberculosis* (Fig. 6). Two isolates, thought to be the same strain, were cultured from patients who had undergone bronchoscopy. The DNAs were digested with *Sma*I, *Pst*I, and *Bam*HI and probed. The probes hybridized with 16 to 20 or more fragments in these digests. The patterns obtained with these two isolates and with strain T2 were quite similar but differed by several distinct fragments; we concluded that these two isolates were not the same strain. Our results demonstrate that these probes can be used to fingerprint strains.

DISCUSSION

We isolated three *M. tuberculosis* DNA fragments which appear to be excellent candidates for probes to detect this organism. These cloned sequences are conserved among strains of the *M. tuberculosis* complex. Although we have not tested a wide variety of mycobacteria, the probes do not hybridize to a significant extent with DNA from mycobacteria which are commonly found in sputum: *M. avium* complex, *M. kansasii*, *M. fortuitum*, and *M. chelonei*.

The most interesting feature of these probes is that they hybridize with multiple chromosomal fragments of *M. tuberculosis*. Most likely, the approach used to screen recombinants for specificity to the *M. tuberculosis* complex enhanced the selection of recombinants containing sequences

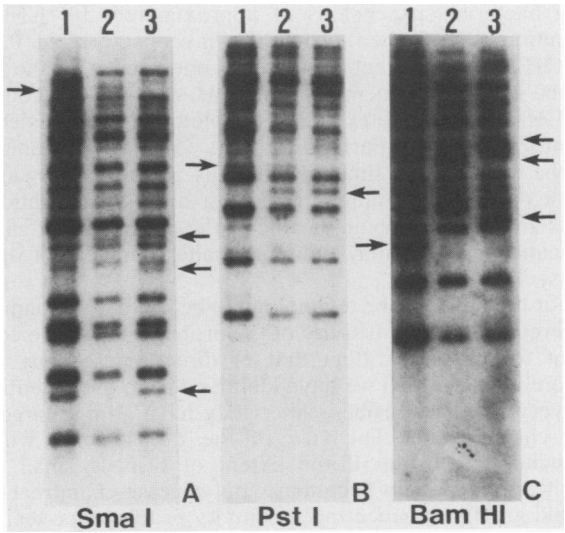

FIG. 6. Southern blots of *M. tuberculosis* DNA digested with the indicated restriction endonucleases. Lane 1, Strain T2; lanes 2 and 3, recent clinical isolates. Panels A and B were probed with M13KE115; panel C was probed with M13KE37. Arrows indicate fragment differences.

that occur multiple times in the *M. tuberculosis* chromosome. This is a significant observation that has not been described previously. Repeated sequences are not commonly found in bacterial genomes, and little is known about their functions and interactions (17). The large repeated sequences (>1 kilobase pair) are present in low copy number, and their regions of homology are extensive. Examples include rearrangement hot-spot (*rhs*) loci, insertion sequences, and rRNA genes. The small repeated sequences (<50 base pairs) are present in high copy number, the most common example being the repetitive extragenic palindromic sequences. Insertion sequences, which are common constituents of bacterial chromosomes and plasmids, have been studied extensively in *E. coli* and *Salmonella* species. Insertion sequences have been recently described for *Corynebacterium diphtheriae* and *Bordetella pertussis* (15; M. A. McLafferty, K. Bromberg, D. R. Harcus, and E. L. Hewlett, Abstr. Annu. Meet. Am. Soc. Microbiol. 1987, C322, p. 377). Both nucleotide sequences show the typical structural features of insertion sequences. The *C. diphtheriae* segment of DNA which is inserted within the *tox* gene of bacteriophage gamma is repeated approximately 30 times in the chromosome. The sequence is about 1.5 kilobase pairs. The *B. pertussis* insertion sequence was isolated by McLafferty et al. while developing DNA probes for diagnosing pertussis. This 1-kilobase-pair segment is highly repeated and conserved within the *B. pertussis* chromosome. Experiments are under way to characterize the repeated sequences which we have identified in the *M. tuberculosis* chromosome. Each of these segments is probably only a portion of a larger repeated sequence. On the basis of their large size, copy number, and extent of conservation among strains of the *M. tuberculosis* complex, we speculate that these sequences are insertion sequences.

For the probes to have practical application, i.e., to be used for detecting *M. tuberculosis* in sputum directly, a procedure for preparing samples for hybridization must be developed. The most challenging aspect will be to develop an efficient and reproducible technique for lysing mycobacteria. Two techniques for lysing mycobacterial cultures have been recently described. Roberts et al. (18) described spotting bacterial suspensions of approximately 10^8 CFU on membranes and then lysing the mycobacteria in 0.5 M NaOH. A second technique is the one used with the Gen-Probe kits for the identification of *M. tuberculosis* complex and *M. avium* complex isolates. Colony suspensions equivalent to a no. 1 McFarland turbidity standard are sonicated in the presence of small glass beads and a lysing reagent. Both of these techniques require a high concentration of cells and may not be applicable to the processing of clinical specimens, which may contain smaller numbers of organisms.

For the DNA probe technology to be useful for diagnosing tuberculosis, the sensitivity of the probe test must be equivalent to or greater than that of direct microscopy. The recombinants which we have identified have the potential to be very sensitive probes, since they have a large target on the chromosome. The size of the target DNA will be especially important if the extent of lysis is small. The application of a new technique, polymerase chain reaction, would greatly enhance the sensitivity of the probe test (14, 19). The polymerase chain reaction allows for specific amplification of discrete fragments, which, in this case, would be a target sequence on the mycobacterial chromosome that is already repeated. Thus, theoretically only one organism needs to be lysed for the DNA to be detected.

In summary, we have identified three *M. tuberculosis* sequences which are excellent candidates for *M. tuberculosis*-specific probes for the detection of *M. tuberculosis* directly in clinical specimens. In addition, the probes will be useful for chromosomal fingerprinting in epidemiological studies.

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