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Molecular Basis of Rifampin Resistance in *Mycobacterium leprae*

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Rifampin is currently the most potent drug used in leprosy control programs. We show that the rifampin resistance which emerged in nine patients with lepromatous leprosy, who had received rifampin monotherapy, stemmed from mutations in the *rpoB* gene, which encodes the beta subunit of RNA polymerase of *Mycobacterium leprae*. In eight cases missense mutations were found to affect a serine residue, Ser-425, while in the remaining mutant a small insertion was found close to this site. These findings will be of use for the development of a rapid screening procedure, involving the polymerase chain reaction, for monitoring the emergence of rifampin-resistant *M. leprae* strains.

Rifampin is a key component in the chemotherapeutic regimens used to combat both leprosy and tuberculosis. Owing to the exquisite rifampin susceptibility of *Mycobacterium leprae*, this drug is the backbone of the multidrug therapy currently recommended by the World Health Organization for the treatment of leprosy (7, 21). It is conceivable, however, that rifampin resistance could compromise control programs, and owing to the very slow growth rate of *M. leprae* (11), there is a real need for a rapid and efficient means of determining whether strains are rifampin susceptible or resistant.

The molecular basis of rifampin resistance has been extensively studied in *Escherichia coli*, in which it has been clearly demonstrated that the drug target is the beta subunit of DNA-dependent RNA polymerase, which is encoded by the *rpoB* gene (14, 15). Comparison of the primary structures of RpoB proteins from several bacteria, deduced from the corresponding gene sequences, led to the identification of six regions of highly conserved sequence (regions I to VI [23]) with obvious homologs in the large subunits of RNA polymerases of eukaryotic origin (1, 5, 17, 22). A detailed molecular genetic analysis of rifampin-resistant mutants of *E. coli* revealed that, in all cases, drug resistance resulted from missense mutations within these conserved regions (9, 12, 14, 15). Furthermore, the majority of these mutations were tightly clustered in a short region, cluster I of region II, near the middle of *rpoB* (9). The nucleotide sequence of the *M. leprae rpoB* gene was determined recently (8), and comparisons revealed extensive homology to the other prokaryotic genes and proteins. In particular, the structures of those sites identified on the basis of their interactions with rifampin were well maintained. The goal of the present study was the characterization of the *rpoB* genes from rifampin-resistant mutants, because this should lead to better understanding of the resistance mechanism and the development of a rapid diagnostic test.

MATERIALS AND METHODS

***M. leprae* isolates.** The properties of the rifampin-resistant and -susceptible strains used in the present study are summarized in Table 1 and elsewhere (6). Rifampin susceptibility was determined in mice as described previously (6), and *M. leprae* cells were obtained either from mouse footpads or human biopsy specimens by standard procedures. All resistant strains were capable of growth in mice that received daily doses of rifampin (>20 mg/kg of body weight), while susceptible strains were unable to grow in mice that received weekly doses of rifampin (10 mg/kg).

PCR procedures and synthesis of biotin end-labeled products. DNA was extracted from 200-μl samples of *M. leprae* suspensions (usually 10^4 to 10^5 bacteria), which were overlaid with 100 μl of mineral oil (Sigma Chemical Co.), by the freezing-boiling technique (19). Aliquots (10 μl) were then used directly in a polymerase chain reaction (PCR) as described previously (19, 20). Two pairs of primers were used to amplify the regions of *rpoB* that were of interest—Brpo24 and rpo25, and Brpo22 and rpo23—and these yielded PCR products of 310 and 710 bp, respectively (Fig. 1). Cycle times for the 310-bp product were 1 min at 92°C, 2 min at 59°C, and 2 min at 72°C for 35 cycles and then 10 min at 72°C for completion. The cycles required for the 710-bp product were 1 min at 92°C, 2 min at 61°C, and then 2 min at 72°C. A PHC-1 thermal cycler (Techne) was used throughout.

The biotinylated primers Brpo24 and Brpo22 were synthesized on an Applied Biosystems apparatus (model 391) by using biotinylated phosphoramidite (RPN 2012 from Amersham International), while all other PCR and sequencing primers were synthesized by standard procedures. The sequences of the various primers are given in Table 2, and their locations are depicted in Fig. 1.

Concentration and sequencing of PCR products. Biotinylated PCR products were concentrated from 80-μl PCR mixtures by using 200 μg of streptavidin-coated Dynabeads (M-280), which were prepared according to the manufacturer's instructions (Dynal, Oslo, Norway). After 15 min at room temperature with occasional mixing, beads were concentrated and were then washed in TE buffer containing 2 M NaCl by using a magnetic particle concentrator (MPC-E). Beads were then suspended in 10 μl of NaOH (0.1 M), to separate the strands of the PCR products, and were left at room temperature for 10 min prior to washing. The biotinylated strand was then recovered by using the particle concentrator and was resuspended in 15 μl of TE buffer. The complementary strand, which was present in the superna-

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TABLE 1. Properties of the *M. leprae* isolates used in the present study

Strain	Allele	Rifampin resistance[a]	Origin
82007	*rpoB1*	R	Martinique
85054	*rpoB2*	R	Paris (Martinique)
81030	*rpoB3*	R	Paris (Guadeloupe)
82061	*rpoB4*	R	New Caledonia
86030	*rpoB5*	R	Guadeloupe
83004	*rpoB6*	R	Martinique
82073	*rpoB7*	R	Paris
83013	*rpoB8*	R	Martinique
87038	*rpoB9*	R	Martinique
88056	*rpoB*$^+$	S	Guadeloupe
90049	*rpoB*$^+$	S	New Caledonia
92002	*rpoB*$^+$	S	Martinique
88063	*rpoB*$^+$	S	Senegal
89033	*rpoB*$^+$	S	Paris

[a] R, resistant; S, susceptible.

TABLE 2. Nucleotide sequences of the primers used in the present study

Primer	Sequence
Brpo22	CAGGACGTCGAGGCGATCAC
rpo23	AACGACGACGTGGCCAGCGT
Brpo24	CAGACGGTGTTTATGGGCGA
rpo25	TCGGAGAAACCGAAACGCTC
rpo32	TCCTCGTCAGCGGTCAAGTA
rpo33	CTTCCCTATGATGACTG
rpo34	GGTGATCTGCTCACTGG
rpo35	GCCGCAGACGCTGATCA
rpo36	TTGACCGCTGACGAGGA
rpo37	GCCAGCGTCGATGGCCG

tant, was neutralized by the addition of 3 μl of HCl (0.33 M). DNA sequences were obtained by the modified dideoxy procedure (2) by using 1.25 μl of template per sequence reaction, appropriate primers (0.6 ng), and the Klenow fragment of DNA polymerase I.

RESULTS

Experimental rationale. In *E. coli*, 19 point mutations conferring rifampin resistance are confined to three areas of the *rpoB* gene (9). One mutation was found in region I and 17 mutations were found in region II, which is subdivided into clusters I and II, harboring 14 and 3 mutations, respectively (9, 12). A single mutation conferring drug resistance has been described in region III (9).

To determine the nucleotide sequence of the corresponding regions of *rpoB* from various *M. leprae* isolates, we devised a PCR strategy (Fig. 1) which exploited our knowledge of the sequence of the gene from a drug-susceptible isolate (8). When primers Brpo24 and rpo25 were used, a 310-bp product, encompassing region I, was obtained. Likewise, primers Brpo22 and rpo23 directed the synthesis of a 710-bp product spanning regions II and III.

Nucleotide sequence analysis of *rpoB* from rifampin-resistant *M. leprae* mutants. A series of rifampin-resistant mutants of *M. leprae* has previously been isolated from patients with lepromatous leprosy who had received rifampin monotherapy, and these mutants have been characterized in depth (6). The relevant details about these and a number of drug-susceptible strains are given in Table 1. When these *M. leprae* isolates (from mouse footpads) were subjected to PCR analysis by using the strategy described above, the expected products were obtained in all cases. To facilitate direct DNA sequencing and limit artifacts caused by polymerase and cloning errors, one of the PCR primers was end labeled with biotin, thus allowing the PCR products to be purified in a rapid and simple manner by means of streptavidin-coated magnetic beads. After strand separation, the nucleotide sequences of the regions of interest were obtained by the dideoxy method. On average, about 1,000 nucleotides of the sequences from both strands were generated and compared with the sequence of the wild-type *rpoB* gene. Typical results obtained with a rifampin-susceptible strain and four resistant mutants are shown in Fig. 2.

In all nine of the rifampin-resistant mutants, sequence differences were found in a short region of the gene, equivalent to cluster I of *E. coli* (9), and these are summarized in Fig. 3A. They ranged from a single C→T transition, affecting codon 425, in six cases (*rpoB* alleles 1, 2, 4, 5, 6, and 7) to a six-base insertion between codons 408 and 409 (*rpoB8*). Two consecutive base changes were found in mutants *rpoB3* and *rpoB9* (Fig. 3), and again these affected the serine codon at position 425. Despite the different geographical origins of the isolates (Table 1), the same codon was mutated on eight separate occasions. For control purposes, PCR was performed on five rifampin-susceptible strains present in human biopsy material. None of these showed any deviation from the wild-type *rpoB* sequence (8), and none of the mutants described above contained additional alterations in the stretches examined.

The findings of the present study are summarized in Fig.

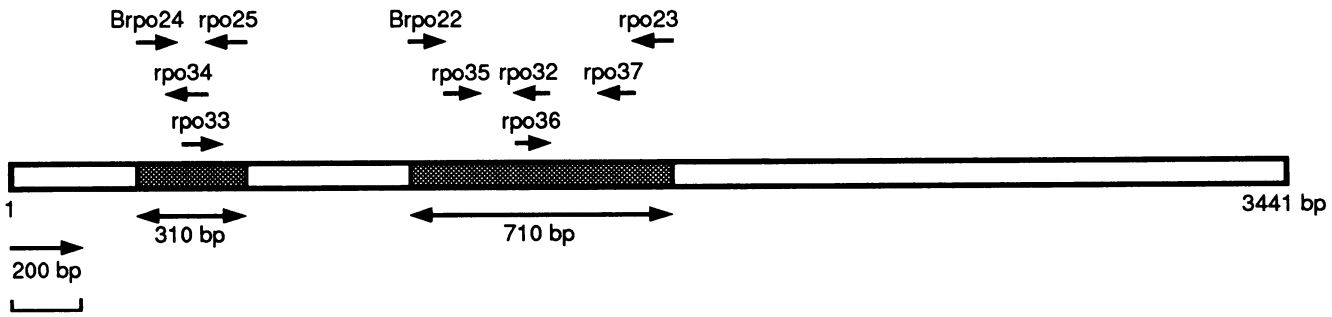

FIG. 1. PCR strategy used to examine various *M. leprae* isolates. The 3,441-nucleotide *rpoB*-coding sequence is represented by the rectangle, and the regions sequenced are depicted as hatched boxes. The primers used to amplify the 310- and 710-bp PCR products are indicated in the top line, while those used as sequencing primers are shown below.

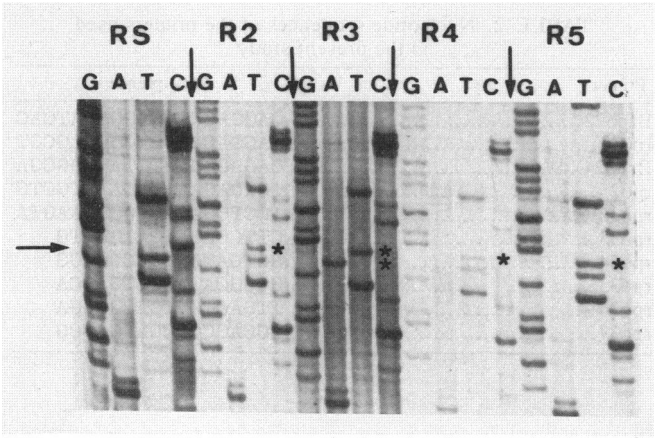

FIG. 2. Nucleotide sequence of a short region of *rpoB* harboring mutations conferring rifampin resistance. The wild-type sequence ladder (RS) is shown along with those of the various mutant alleles *rpoB2* (R2), *rpoB3* (R3), *rpoB4* (R4), and *rpoB5* (R5). Mutated bases are indicated by asterisks. The arrow indicates the cytosine which is most frequently mutated. These sequences were generated from PCR products obtained from mouse footpad biopsy specimens containing *M. leprae*.

3B, in which the amino acid sequence of part of the putative rifampin-binding domain is compared with the corresponding region of the beta subunit of *E. coli* RNA polymerase. The sequence of the conserved region II is shown, and those residues identified by the mutant analysis as being functionally important are indicated. Apart from a few conservative substitutions, the sequences of regions I to III were identical in *E. coli* and *M. leprae* (data not shown) (8).

In eight of the *M. leprae* mutants, Ser-425 in cluster I of region II was changed to a hydrophobic amino acid: leucine (six times), methionine, or phenylalanine. In the ninth mutant, two additional residues, lysine and phenylalanine, were inserted between Phe-408 and Met-409. This mutation probably stemmed from a replication error because there was a perfect duplication of a 6-bp sequence (Fig. 3A). It is surely no coincidence that a similar insertion has been found at almost the same place in the *rpoB* gene of rifampin-resistant *E. coli* (9).

Conservation of *rpoB* in mycobacteria. Rifampin resistance has recently become a major problem in patients with tuberculosis (16). To examine whether the *M. leprae rpoB* gene could be used to identify its counterpart in other mycobacteria, it was used as a probe to detect homologous sequences in chromosomal DNA extracted from *Mycobacterium tuberculosis*, *Mycobacterium avium*, *Mycobacterium gordonae*, and *Mycobacterium szulgai*. Strong signals were obtained in all cases (Fig. 4), thus indicating that the *rpoB* gene sequence has been well conserved during the evolution of the mycobacterial genus.

DISCUSSION

The principal goal of the present study was the elucidation of the molecular basis of rifampin resistance in *M. leprae*. In theory, two possible resistance mechanisms are conceivable: mutation of the putative drug target, RNA polymerase, or alterations in the permeability of the cell wall that lead to reduced drug uptake. Although the results of the present study do not exclude the latter possibility, it seems unlikely because conclusive evidence was obtained for missense mutations in *rpoB*, the gene encoding the beta subunit of RNA polymerase.

Furthermore, the mutations were all located in a short, evolutionarily well conserved region, cluster I of region II, which is known to be the site of mutations that confer rifampin resistance in *E. coli* (9). A comparison of the amino acid residues mutated in cluster I of *M. leprae* and *E. coli* is presented in Fig. 3B, in which it can be seen that the mutation Ser-425 → Phe was found in both organisms, although it was encountered more frequently in *E. coli*.

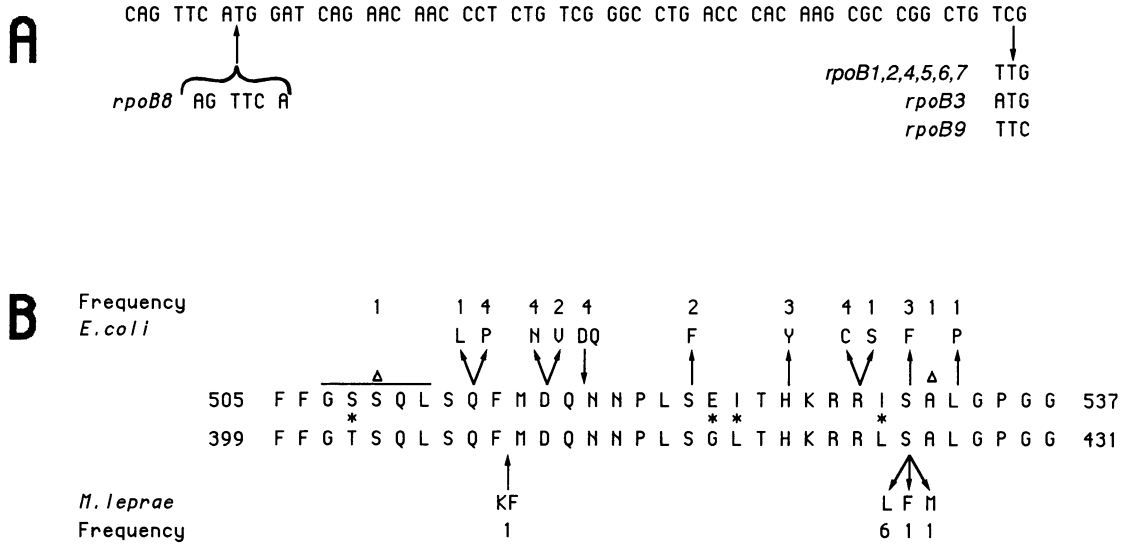

FIG. 3. (A) Nucleotide sequence of a short region of *rpoB* harboring mutations conferring rifampin resistance. The base changes and the corresponding alleles are indicated. (B) Amino acid sequence comparison of cluster I, from region II, of the beta subunits of RNA polymerases from *E. coli* and *M. leprae*. Residue numbers are indicated together with amino acid differences (*). The mutated amino acid residues associated with rifampin resistance are shown along with the frequency with which a given mutation was isolated. Data for *E. coli* were taken from the summary of Jin and Gross (9).

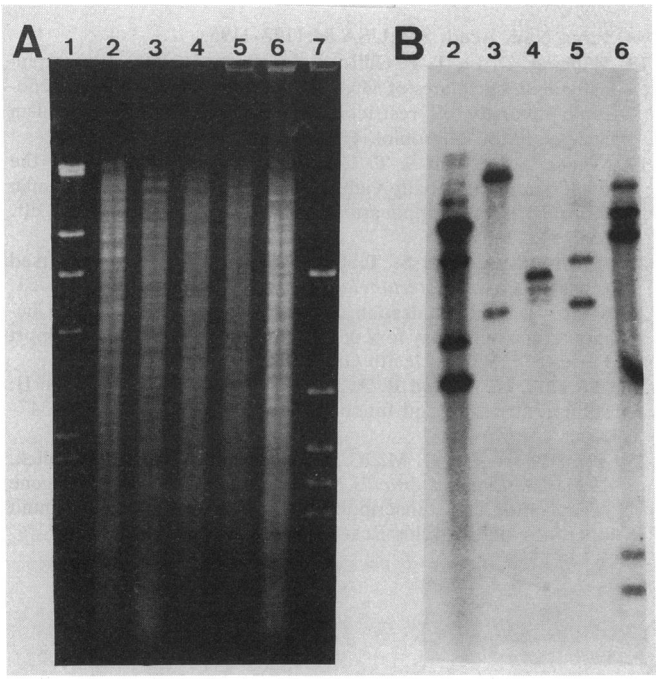

FIG. 4. Southern blot analysis of *rpoB* in mycobacteria. (A) Samples of different bacterial genomic DNAs (1.5 μg) were digested with *Rsr*II, separated on an agarose gel (0.6%), and stained with ethidium bromide. Lanes 1 and 7, size markers; lane 2, *M. leprae*; lane 3, *M. tuberculosis* H37Rv; lane 4, *M. gordonae*; lane 5, *M. szulgai*; lane 6, *M. avium*. (B) The gel shown in panel A was blotted and hybridized with a ^{32}P-labeled probe spanning the *rpoB* region (8) as described previously (4).

Curiously, the replacement of this Ser residue by Leu has not been described in *E. coli*, although it is predominant in drug-resistant *M. leprae* and has been detected in organisms from four different geographical locations. The majority of the mutations found in rifampin-resistant *E. coli* have not yet been detected in *M. leprae*, and there may be a sound, physiological reason for this apparent difference. Many of the mutant *rpoB* alleles of *E. coli* encode beta subunits which display pleiotropic conditional defects in addition to rifampin resistance (9, 10). These include slow growth, cold sensitivity, and temperature sensitivity, and the corresponding strains can be maintained only under laboratory conditions (9, 10). It is quite conceivable that such in vitro mutations would not be isolated in the case of *M. leprae*, because they would result in the death of this obligately intracellular parasite.

Full understanding of the mechanism of rifampin resistance will require detailed knowledge of the three-dimensional structure of RNA polymerase, although this will be an extremely difficult undertaking. This would allow us to situate the mutations with respect to the active site of the enzyme and its substrate and binding domains. At present, one can only speculate with respect to the mechanistic effects of the mutations. It is possible that the substitution of the small, polar side chain of Ser-425 by the bulkier, hydrophobic groups of Met, Leu, and Phe would result in a physical obstruction for rifampin as it diffuses into a functionally important crevice within the enzyme.

Another important conclusion which can be drawn from the present study concerns the remarkable stability of the genome of *M. leprae*, because even though strains from four different continents were examined, no polymorphic nucleotides were found in the segments examined. This observation confirms and extends the results of restriction fragment length polymorphism studies (3, 18) and is indicative of a slow rate of evolutionary divergence.

Finally, it was very encouraging that the number of sites involved in rifampin resistance was so limited that a simple PCR-based approach for the rapid screening of strains for the presence of resistant alleles can be envisaged. This could be done by means of oligotyping, single-stranded conformational polymorphism analysis (13), or even direct sequencing. Furthermore, the hybridization results presented in Fig. 4 suggest that it should be a relatively simple matter to extend this approach to multidrug-resistant *M. tuberculosis* isolates.

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