Repetitive DNA Sequence of *Mycobacterium tuberculosis*:
Analysis of Differential Hybridization
Pattern with Other Mycobacteria[1]

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Tuberculosis is a global problem of enormous dimensions. *Mycobacterium tuberculosis,* the etiological agent of tuberculosis, cannot be easily differentiated from other members of the tuberculosis complex and from the atypical mycobacteria of the nontuberculosis complex. Conventional methods used in identifying mycobacteria, such as acid-fast stains and culture, are often time consuming, insensitive, and nonspecific ([20]). Phage typing has been a useful tool in epidemiological investigations but there are limitations ([3, 10, 13, 21]). The nonavailability of a specific antigen and variation in the reproducibility of ELISA results make serodiagnosis of tuberculosis difficult ([16, 17]).

There is, therefore, a pressing need for the rapid and specific diagnosis of tuberculosis. Recombinant DNA methods and deoxyribonucleic acid probe technology can facilitate the diagnosis of tuberculosis. This technique utilizes a specific DNA sequence as a probe to detect only the nucleic acids of the target organisms, even in the presence of excess nucleic acids from other organisms. To be useful as a probe for the diagnosis of tuberculosis, the DNA fragment should be of a repetitive nature and specific to *M. tuberculosis,* with very little or no cross hybridization to other mycobacteria of the tuberculosis as well as the nontuberculosis complex, which could infect humans.

Restriction endonuclease analysis has recently been applied to generate molecular markers that could distinguish between different strains of mycobacterial species ([4, 5, 12, 19]). Eisenach, *et al.,* used randomly picked recombinant clones from a library of *M. tuberculosis* H37Rv to probe restriction digests of mycobacterial DNA to differentiate laboratory strains from wild-type strains of *M. tuberculosis* ([7]).

In our study, we included *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, and *M. microti* from the tuberculosis complex; *M. avium, M. intracellulare,* and *M. scrofulaceum* from the MAIS complex; and some of the cultivable mycobacteria such as *M. smegmatis, M. vaccae, M. cheloni,* and *M. kansasii.* We analyzed mycobacterial DNA with restriction endonucleases *Hind*III, *Bam*HI, *Eco*RI, *Sal*I, *Pst*I, *Alu*I, and *Sau*3A, and looked for a species-specific banding pattern. Among the various enzymes tested, the restriction endonuclease *Alu*I gave specific bands in *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra DNA digests which were totally absent in corresponding *Alu*I DNA digests of the other mycobacteria tested. In the present communication, evidence is presented on the specificity and repetitive nature of one of these fragments. This DNA fragment was also used to isolate genomic sequences from a λgt11 library of *M. tuberculosis* H37Rv ([23]).

MATERIALS AND METHODS

Bacterial strains and growth conditions. *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, *M. microti, M. avium, M. intracellulare, M. scrofulaceum, M. cheloni, M. smegmatis,* and *M. vaccae* were obtained from the standard collection of The Trudeau Institute, Saranac Lake, New York, U.S.A., and grown in Middlebrook 7H9 medium at 37°C.

DNA extraction. Mycobacterial cultures grown to logarithmic phase were treated with glycine to destabilize the cell wall, centri-

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fuged, and the DNA was isolated as described previously ([12, 14]).

Purification of DNA from low-melting agarose gel. An agarose gel slice was melted at 65°C for 20 min in the presence of one half the volume of TE (10 mM Tris, pH 8.0, 1.0 mM EDTA). To this an equal volume of TE-saturated phenol was added, and the extraction was carried out for 3–5 min at room temperature. The upper aqueous phase was reextracted with phenol followed by three times with chloroform : isoamyl alcohol (24:1) and two times with ether. Traces of ether were removed by evaporation at 60°C for 10 min. DNA was precipitated from the aqueous phase by the addition of 2.5 volumes of chilled ethanol in the presence of $\frac{1}{10}$th volume of 3 M sodium acetate, pH 5.2. The procedure was repeated twice to ensure the purity of the fragment.

Radiolabeling of DNA. DNA was radiolabeled with [^{32}P] deoxycytidine triphosphate (Amersham Corp., Arlington Heights, Illinois, U.S.A.) by nick translation ([18]) to a specific activity of 10^6 cpm/μg of DNA.

Restriction endonuclease digestion. Varying amounts of mycobacterial DNA (see figure legends) were digested with 20 units each of the following restriction endonucleases, *Eco*RI, *Bam*HI, *Pst*I, *Hin*dIII, *Sau*3A, and *Alu*I, for a period of 18 hr according to conditions specified by the manufacturer (Bethesda Research Laboratories, Gaithersburg, Maryland, U.S.A.). To ensure complete digestion, 10 units more of each enzyme were added, and the incubation period was continued for a further 4–6 hr.

Hybridization. Denatured DNA was transferred from agarose gels onto nitrocellulose filters (Schleicher & Co., Keene, New Hampshire, U.S.A.) by the methods of Southern ([22]). After transfer, the filter was air dried and baked *in vacuo* at 80°C for 2 hr followed by prehybridization at 42°C for 2 hr with a solution containing 50% formamide, 6X standard saline citrate (SSC), 5X Denhardt's solution ([6]), 100 μg/ml of calf thymus DNA. The [^{32}P]-labeled probe DNA was denatured in a boiling water bath for 5 min, and was added to the filters (2 × 10^6 cpm/filter) in the same mixture, and hybridization was continued for 24 hr. After hybridization, the filters were washed three times with 2X SSC and 0.1% sodium duo-decyl sulfate (SDS) (100 ml each) at 55°C followed by three more washings with 0.1X SSC and 0.05% SDS at 65°C, air dried, and exposed to X-ray film with a duPont intensifying screen at −70°C.

Screening of λgt11 gene libraries of *M. tuberculosis*. From the genomic libraries of *M. tuberculosis* made in λgt11 ([23]), recombinant phages were screened by plaque hybridization ([15]) using the [^{32}P]-labeled 5.6-kb DNA fragment as probe. The recombinant phages picked on the basis of a positive hybridization signal were purified to homogeneity through a second and third screening.

RESULTS

DNA from *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, *M. microti, M. avium, M. intracellulare, M. scrofulaceum, M. kansasii, M. cheloni, M. smegmatis,* and *M. vaccae* was digested to completion with restriction endonucleases recognizing both hexanucleotide and tetranucleotide sequences, and the banding pattern of the restricted fragments was studied by agarose gel electrophoresis. The restriction patterns obtained with *Eco*RI, *Sal*I, *Pst*I, *Hin*dIII, *Bam*HI, and *Sau*3A did not reveal any species-specific differences among members of the tuberculosis complex but showed intense bands over the background smear (data not presented). However, in the case of digestion with *Alu*I, which recognizes the sequence AGCT, the restriction patterns had distinct features. The overall size of the DNA fragments generated with *Alu*I ranged from 0.2 kb to 3 kb, and appeared as a smear on staining with ethidium bromide. In addition, in the high-molecular-weight region, a specific banding pattern was observed (Fig. 1, A and B). Two bands 5.6 kb and 4.8 kb in size were observed which were only seen in *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra and were absent in the *Alu*I digests of *M. bovis* BCG (Fig. 1A). The 4.8-kb and 5.6-kb *Alu*I fragments seen only in *M. tuberculosis* were also found to be absent in *M. microti, M. avium, M. intracellulare, M. scrofulaceum, M. smegmatis, M. vaccae, M. cheloni,* and *M. kansasii* (Fig. 1B). The structural organization of these *Alu* fragments in the *M. tuberculosis* H37Rv genome and their relat-
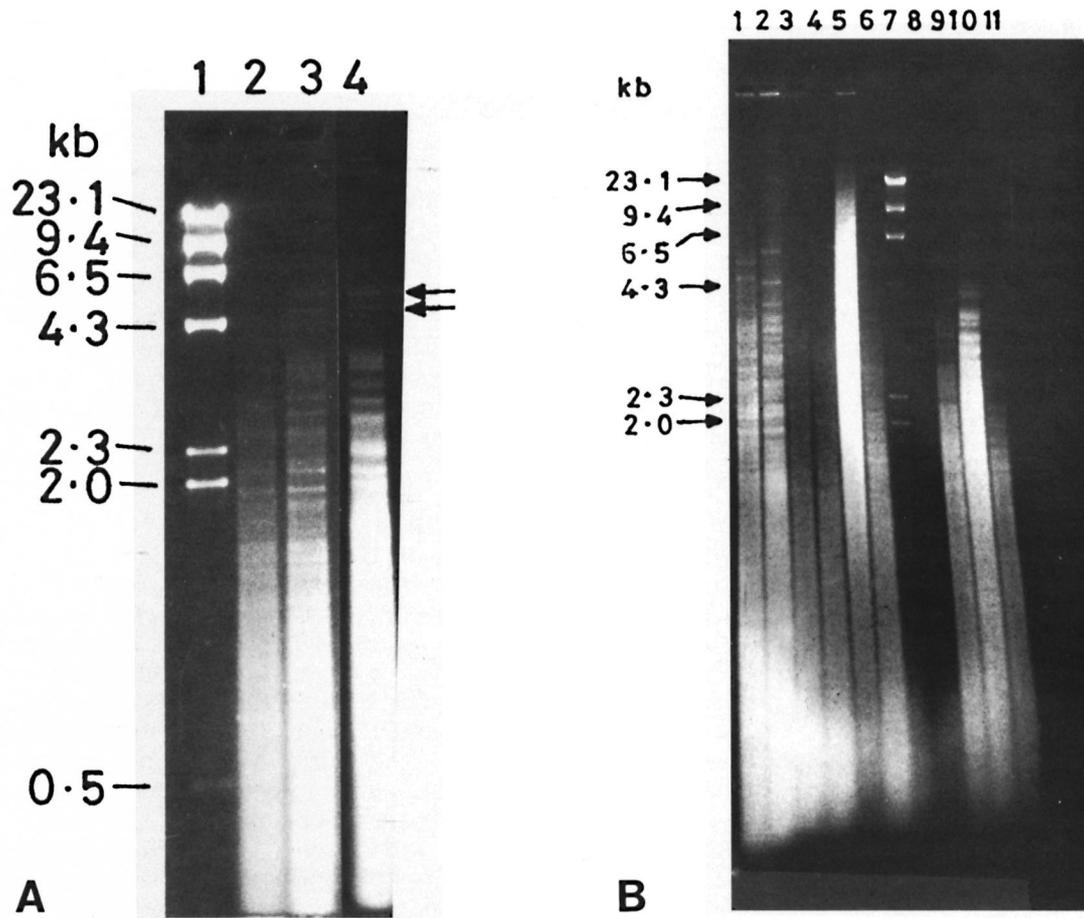
FIG. 1.   For restriction analysis of mycobacterial DNAs with *Alu*I, 2 μg each of DNA from different myco-bacteria were digested to completion with restriction endonuclease *Alu*I. DNA fragments were separated by agarose gel electrophoresis (1.2%) and visualized by ethidium-bromide staining. **A.** Lanes 1–4. *Hin*dIII digest of DNA (lane 1); *Alu*I digests of *M. bovis* BCG (lane 2), *M. tuberculosis* H37Ra (lane 3), *M. tuberculosis* H37Rv (lane 4); arrows indicate 5.6-kb and 4.8-kb *Alu*I fragments of *M. tuberculosis.* **B.** Lanes 1–11. *Alu*I digests of 5 μg each of DNA of *M. tuberculosis* H37Rv, *M. bovis* BCG, *M. microti, M. avium, M. intracellulare, M. scro-fulaceum; Hin*dIII digest of λDNA; *Alu*I digests of *M. cheloni, M. kansasii, M. smegmatis,* and *M. vaccae,* respectively.

edness to other mycobacterial DNAs were studied by Southern blot hybridization ex-periments. The 5.6-kb *Alu*I fragment, purified on low-melting agarose gel electro-phoresis (see Methods), was labeled with [³²P]-dCTP by nick translation ([18]). Hybrid-ization of the *Alu*I digests of *M. tuberculosis* H37Rv with the 5.6-kb fragment gave sev-eral bands which indicated the repetitive nature of this sequence in the *M. tubercu-losis* H37Rv genome. Although ethidium-bromide staining did not reveal the presence of a 5.6-kb fragment in the *Alu*I digest of *M. bovis* BCG DNA (Fig. 1A, lane 1) upon

hybridization, a banding pattern was ob-tained which was comparable to that of *M. tuberculosis* H37Rv (Fig. 2A, lane 1). On the contrary, no hybridization signal was detected even after 8 days of exposure in the case of mycobacterial DNA digests of the nontuberculosis complex (Fig. 2A, lanes 5–7) with the only exception being *M. kan-sasii* where a hybridization signal with an 8–10-kb fragment was obtained (Fig. 2A, lane 4). Similar studies carried out with the 4.8-kb *Alu*I yielded a multiple hybridizable banding pattern with *M. tuberculosis* and *M. bovis* BCG, and also showed cross hybrid-
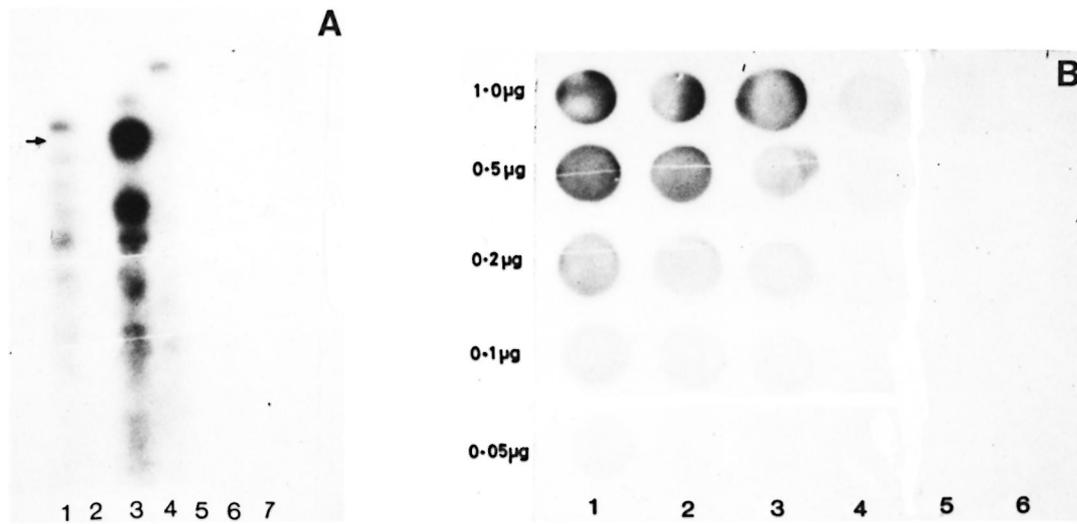
FIG. 2. Hybridization of different mycobacterial DNAs with 5.6-kb *Alu*I fragment of *M. tuberculosis* H37Rv. **A.** Three µg each of mycobacterial DNA were digested to completion with restriction endonuclease *Alu*I, fractionated on 1.2% agarose gel, and hybridized with 2×10^6 cpm of the radiolabeled, purified 5.6-kb *Alu* fragment under high-stringency conditions as described in Methods. Lanes 1 = *M. bovis* BCG, 2 = control DNA, 3 = *M. tuberculosis* H37Rv, 4 = *M. kansasii*, 5 = *M. smegmatis*, 6 = *M. vaccae*, 7 = *M. avium*; arrow indicates position of 5.6-kb *Alu* fragment in *M. tuberculosis Alu*I DNA digest. **B.** DNA was dotted on nitrocellulose filter and hybridized with the [^{32}P]-labeled 5.6-kb *Alu* fragment. Concentration of DNA dotted is marked on the left. Lanes 1–6 are *M. tuberculosis* H37Rv, *M. bovis* BCG, *M. kansasii*, *M. smegmatis*, *M. vaccae*, and *M. avium*, respectively.

ization with the other mycobacteria tested which were the same as for the 5.6-kb *Alu*I fragment (data not presented).

The apparent specificity of the 5.6-kb *Alu* probe, evident in Southern blot, is also reflected in the DNA dot hybridization. Upon hybridization of the mycobacterial DNAs dotted on nitrocellulose membrane with the 5.6-kb *Alu*I fragment, it was possible to differentiate between mycobacterial species of the tuberculosis complex from the other mycobacteria tested. *M. avium, M. smegmatis,* and *M. vaccae* did not give any hybridization signal with the 5.6-kb *Alu* probe at 50 ng concentration of DNA (Fig. 2B). *M. kansasii,* on the other hand, gave a hybridization of similar intensity on dot blot when compared to *M. tuberculosis* H37Rv.

Screening of λgt11 libraries of *M. tuberculosis* H37Rv. The 5.6-kb *Alu*I fragment was used to isolate the genomic counterparts from the λgt11 library of *M. tuberculosis* H37Rv ([23]) by the plaque hybridization technique. A total of 10^5 phages were screened, and 15 recombinant phages, giving hybridization signals in the primary screening, were further purified to homogeneity (Fig. 3), and the DNA isolated was characterized by Southern hybridization to mycobacterial DNAs (data not presented).

DISCUSSION

Deoxyribonucleic acid probe technology offers an alternate approach for the rapid and accurate diagnosis of tuberculosis due to the specificity and sensitivity of molecular hybridization. Furthermore, the sensitivity of DNA probes also depends upon the nature of the sequences, whether repetitive or a single copy ([9]).

With a view to generate a specific DNA probe for *M. tuberculosis,* we analyzed the restriction fragment profiles of the DNA digests of a few mycobacterial species that fall within the tuberculosis complex and compared them with the mycobacteria of the MAIS complex and some atypical mycobacteria. The restriction endonucleases *Eco*RI, *Pst*I, *Bam*HI, *Sal*I, and *Hin*dIII did not show any species-specific differences among the members of the tuberculosis complex, which is in agreement with earlier

FIG. 3. Isolation of recombinants specific to 5.6-kb *Alu* fragment from λgt11 library of *M. tuberculosis* H37Rv. Recombinant phages plated at a density of 10⁴ plaque forming units per plate were transferred to nitrocellulose membrane filter; the DNA was then denatured and fixed at 80°C for 2 hr under vacuum. Prehybridization and hybridization were carried out (see Methods) with 10⁵ cpm per filter of the radiolabeled fragment DNA. Autoradiograph of one of the filters is shown.

reports [4, 11, 20]. However, the restriction endonuclease *Alu*I-digested DNA of *M. tuberculosis* H37Rv and *M. tuberculosis* H37Ra yielded a specific pattern with bands of 5.6 kb and 4.8 kb which appeared to be species specific (Fig. 1, A and B). A species-specific banding pattern was also obtained with other mycobacterial DNA digests as well (Fig. 1B). This would distinguish *M. tuberculosis* from the other members of the tuberculosis complex which were studied, such as *M. bovis* BCG and *M. microti* (Fig. 1B). The reactivity of the 5.6-kb fragment with the DNAs of other mycobacteria, as studied in Southern blot hybridization experiments, revealed restriction-based polymorphism with *M. bovis* BCG. These results also reflect the DNA-relatedness between *M. tuberculosis* and other members of the tuberculosis complex which is reported to be between 90%–100% [1, 2, 11]. It is interesting to note that this 5.6-kb DNA fragment shows a specific hybridization signal with *M. kansasii* DNA (Fig. 2A) in the range of 9–10 kb but not with *M. avium,* although the reported DNA-relatedness of these two

species with *M. tuberculosis* is 32% and 27%, respectively [11]. The fact that no hybridization signal was observed with *M. smegmatis* and *M. vaccae* indicates that these species are evolutionarily distant to *M. tuberculosis* [8].

M. tuberculosis-specific DNA sequences with restriction endonucleases *Bst*EII and *Mbo*I have been reported previously [4, 19]. However, no information is available regarding the nature of these fragments. Similarly, Eisenach, *et al.* [7] reported recombinant clones from a library of *M. tuberculosis* H37Rv which showed strain-specific hybridization signals in the *M. tuberculosis* H37Rv genome as compared to clinical isolates. But the reactivity of these clones with the atypical mycobacteria which could be found in the sputum is now known. Shoemaker, *et al.* [20] used the whole genome of *M. tuberculosis* as a probe to distinguish *M. tuberculosis* from other non-mycobacteria found in sputum. The probe, however, could not distinguish *M. tuberculosis* from other mycobacterial species. This could be due to varying degrees of nucleotide sequence homology between different mycobacterial species.

Our studies show the discriminatory reactivity of the 5.6-kb *Alu* fragment with mycobacteria from the tuberculosis complex as compared to *M. avium, M. smegmatis,* and *M. vaccae* (Fig. 2, A and B). The 5.6-kb probe crossreacts with *M. kansasii,* which could be an advantage because *M. kansasii* is a known pathogen. A clear distinction is observed in DNA dot hybridization. This suggests the potential utility of the 5.6-kb DNA probe in sputum analysis, where the amount of target DNA is the limiting factor. The concentration of mycobacteria found in sputum varies from 10³ to 10⁵ per ml. This concentration may be much lower in samples from treated patients (100 pg of *M. tuberculosis* DNA corresponds to approximately 10⁴ bacteria). The sensitivity of the probe can be increased by an order of one log by increasing the specific activity. Additionally, the target DNA can be amplified, making use of the polymerase chain reaction (PCR) technique. Further, evidence of the species specificities and the repetitive nature of the 5.6-kb *Alu*I fragment has been found with genomic clones obtained from the λgt11 library (results not presented). De-

tailed analysis of the insert DNA of the *M. tuberculosis* genomic clones, presently under investigation, will enable us to understand more regarding the nature of the repetitive element present in the insert. Nevertheless, the present communication is evidence of the potential utility of the 5.6-kb *Alu*I fragment in the development of DNA probes for *M. tuberculosis*. Although the present probe has been tested for cross-reactivity with representative mycobacteria from the tuberculosis complex, the MAIS complex, and atypical mycobacteria, a larger number of species including clinical isolates need to be tested.

SUMMARY

In order to generate specific DNA probes for *Mycobacterium tuberculosis*, restriction fragment length analysis was carried out with some of the mycobacterial species that fall within the tuberculosis complex. The presence of specific bands of 5.6 kb and 4.8 kb was revealed in the *Alu*I DNA digest of *M. tuberculosis*. The hybridization profile of the 5.6-kb *Alu*I DNA sequence, as judged by the Southern blot and dot blot hybridization experiments, revealed the presence of this sequence in *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG as multiple copy and *M. kansasii* as single copy but this sequence was not present in *M. avium, M. smegmatis,* or *M. vaccae* genomes. Genomic clones corresponding to the 5.6-kb *Alu*I fragment from *M. tuberculosis* H37Rv library made in the λgt11 expression vector were isolated.

RESUMEN

Para generar sondas específicas para el DNA del *Mycobacterium tuberculosis* se hizo el análisis de fragmentos de restricción con algunas de las especies micobacterianas incluídas dentro del complejo de tuberculosis y con aquellas fuera de este complejo. Se encontraron bandas específicas de 5.6 kb y de 4.8 kb en el digerido *Alu*I del DNA de *M. tuberculosis*. El perfil de hibridización de la secuencia 5.6 kb *Alu*I, reveló la presencia de esta secuencia en *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra y *M. bovis* BCG, como copia múltiple, y en *M. kansasii* como copia única pero esta secuencia no estuvo presente en los genomas de *M. avium, M. smegmatis,* o *M. vaccae*. De la genoteca de *M. tuberculosis* H37Rv fabricada en el vector de expresión λgt11 se aislaron clones genómicos correspondientes al fragmento 5.6 kb *Alu*I.

RÉSUMÉ

En vue de mettre au point des sondes d'ADN spécifiques pour *Mycobacterium tuberculosis*, une analyse de la longueur des fragments de restriction a été pratiquée chez quelques unes des espèces mycobactériennes qui appartiennent au complexe de la tuberculose, et chez d'autres qui n'appartiennent pas à ce complexe. On a ainsi mis en évidence la présence de bandes spécifiques de 5,6 kb et 4,8 kb dans les produits de digestion de *M. tuberculosis* par *Alu*I ADN. Le profil d'hybridisation de la séquence 5,6 kb *Alu*I ADN, telle qu'elle apparaissait par le Southern-blot et par des expériences d'hybridisation à blot ponctuel, montrait clairement la présence de cette séquence chez *M. tuberculosis* H37Rv, *M. tuberculosis* H37Ra, *M. bovis* BCG, en copie multiple dans tous ces cas, et en simple copie chez *M. kansasii*. Par contre, cette séquence était absente dans les génomes de *M. avium, M. smegmatis* ou *M. vaccae*. On a isolé des clones génomiques qui correspondaient au fragment, 5,6-kb *Alu*I de la bibliothèque de *M. tuberculosis* H37Rv, constitué à partir du vecteur d'expression λgt11.

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