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# Insertion Element IS*987* from *Mycobacterium bovis* BCG Is Located in a Hot-Spot Integration Region for Insertion Elements in *Mycobacterium tuberculosis* Complex Strains

PETER W. M. HERMANS,[1]* DICK VAN SOOLINGEN,[1] ELISABETH M. BIK,[1] PETRA E. W. DE HAAS,[1] JEREMY W. DALE,[2] AND JAN D. A. VAN EMBDEN[1]

*National Institute of Public Health and Environmental Protection, P.O. Box 1, 3720 BA Bilthoven, The Netherlands,[1] and Department of Microbiology, University of Surrey, Guildford, Surrey GU2 5XH, United Kingdom[2]*

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**Most strains of the *Mycobacterium tuberculosis* complex carry multiple copies of an IS*3*-like element, and these strains are highly polymorphic with regard to the site of integration in the chromosome. In contrast, *Mycobacterium bovis* BCG contains a single copy of the insertion element, and in all strains this copy is integrated at the same site in the chromosome. In this study, we determined the sequence of the single-copy insertion element from *M. bovis* BCG, IS*987*, and its flanking regions. The analysis of IS*987* revealed that this element was virtually identical to the sequence of IS*986* from *M. tuberculosis*. IS*987* is located in a region containing direct repeats (DRs). The cloned flanking regions contained 20 virtually identical DRs of 36 bp, each separated by 35 to 41 bp of spacer DNA. Analysis of chromosomal DNA by the polymerase chain reaction revealed the presence of a cluster of 49 DRs, and IS*987* is inserted in the 30th DR. Furthermore, the DR sequences were found to occur only in species of the *M. tuberculosis* complex and not in nine other mycobacterial species tested. Analysis of 14 *M. tuberculosis* strains revealed the presence of one insertion sequence element in the DR-containing region of eight strains, two insertion sequence elements were located in the DR region of five strains, and one strain did not contain an insertion sequence element in this region. Additionally, the DR-containing regions of these 14 *M. tuberculosis* strains were polymorphic in length and composition. We conclude that the DR cluster is a specific, hot-spot region for integration of insertion elements in the chromosome of *M. tuberculosis* complex strains.**

Insertion sequences (ISs) and transposons are mobile genetic elements encoding genes which are essential for transposition. Insertion of these elements into structural genes leads to gene inactivation (2, 9). Insertion elements were first discovered as a result of their strong polar effects due to insertions near the *lac* (13) and *gal* (11) operons of *Escherichia coli*. Mobile elements are known to occur in gram-positive bacteria such as *Bacillus* (12) and *Staphylococcus* (1) spp., gram-negative bacteria such as *Agrobacterium* (20), *Pseudomonas* (19), *Rhizobium* (22), *Salmonella* (10), and *Shigella* (15) spp., and archaebacteria (3).

Mycobacteria have recently been added to the list of insertion element-containing bacteria. Martin et al. (14) have isolated the transposable element Tn*610* from *Mycobacterium fortuitum* FC1, encoding sulfonamide resistance. Tn*610* is 4,070 bp in length and is flanked at both sites by an insertion element, IS*6100*, which shares similarity with the enterobacterial IS*6* family. Four copies of IS*6100* are present in the chromosome of *M. fortuitum* FC1, whereas no copy of IS*6100* has been found in *Mycobacterium bovis* BCG, *Mycobacterium tuberculosis*, or other *M. fortuitum* strains. Transposition of Tn*610* has been shown to occur in *Mycobacterium smegmatis* (14).

The analysis of repetitive DNA in mycobacteria has led to the identification of putative insertion elements. McFadden et al. have isolated a repetitive DNA element, IS*900*, from *Mycobacterium paratuberculosis* (17). IS*900* is present in 10 to 15 copies in the chromosome of *M. paratuberculosis* and some *Mycobacterium avium* strains (6, 18). IS*900* consists of 1,451 bp and lacks terminal inverted repeats. The 399-amino-acid open reading frame (ORF) of IS*900* shows significant homology with a putative gene product of IS*110* in *Streptomyces coelicolor* A3 (5).

Analysis of repetitive DNA from *M. tuberculosis* has revealed the identification of putative IS elements of the enterobacterial IS*3* family. Two such elements, IS*986* and IS*6110*, have been characterized, and sequence analysis has revealed them to be virtually identical (16, 27). The host range of these IS*3*-like elements is limited to mycobacterial species belonging to the *M. tuberculosis* complex, and therefore these IS elements are suitable as target DNA for the direct detection of *M. tuberculosis* in clinical specimens by the polymerase chain reaction (PCR) (8, 26). Furthermore, these IS elements are an extremely useful tool in the epidemiology of tuberculosis because of the highly variable copy number and the great variability of insertion sites in the chromosome of the different mycobacterial strains. This variability allows easy typing of strains of the *M. tuberculosis* complex by restriction fragment length polymorphism analysis (8, 30). In contrast, no such polymorphism was observed among vaccine strains and clinical isolates of *M. bovis* BCG. All seven *M. bovis* BCG strains tested contained a single copy of the IS element, and the chromosomal site of integration was identical in these strains (8). The absence of any polymorphism of IS-containing DNA among these *M. bovis* BCG strains indicates that no transposition of the IS element has taken place during culture of these strains. This suggests that the IS element of *M. bovis* BCG has lost its capacity to transpose or that the transposition frequency is extremely low. One may speculate that the IS element is involved in the attenuation of virulence of *M. bovis* BCG

* Corresponding author.

TABLE 1. Bacterial strains used in this study

| Bacterial strain | Species | Property or origin | Source or reference |
|---|---|---|---|
| 110, 111, 134–148 | *M. tuberculosis* | Clinical isolates | This laboratory |
| 38, 153 | *M. africanum* | Clinical isolates | This laboratory |
| 41, 154 | *M. bovis* | Clinical isolates | This laboratory |
| 43, 105, 149–152 | *M. bovis* BCG | Clinical isolates | This laboratory |
| 102 | *M. bovis* BCG | Vaccine strain | Organon Teknika[a] |
| 103 | *M. bovis* BCG | Vaccine strain | Armand Frappier[b] |
| 44, 104 | *M. bovis* BCG | Vaccine strains | This laboratory |
| 46 | *M. microti* | Clinical isolate | F. Portaels[c] |
| 158 | *M. asiaticum* | | This laboratory |
| 49 | *M. avium* | | This laboratory |
| 156 | *M. flavescens* | | This laboratory |
| 159 | *M. gordonae* | | This laboratory |
| 160 | *M. kansasii* | | This laboratory |
| 157 | *M. malmoense* | | This laboratory |
| 163 | *M. terrae* | | This laboratory |
| 162 | *M. chitae* | ATCC 25805 | ATCC[d] |
| 161 | *M. intracellulare* | ATCC 19422 | ATCC[d] |
| | *E. coli* K-12 | | This laboratory |
| | *B. pertussis* | Wellcome 28 | 23 |

[a] Organon Teknika N.V., Veedijk 58-2300 Turnhout, Belgium.
[b] Institut Armand Frappier, Laval, Quebec, Canada.
[c] Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium.
[d] American Type Culture Collection.

because of insertional inactivation of a chromosomal gene(s) essential for virulence.

This study was undertaken to characterize the insertion element of *M. bovis* BCG and the chromosomal DNA flanking this IS element. The insertion element of *M. bovis* BCG was analyzed to investigate the possible differences of this element with IS*986* and IS*6110* of *M. tuberculosis*, which might explain the apparent transposition incapacity of the insertion element. Furthermore, both flanking regions of the IS element of *M. bovis* BCG were characterized to investigate the possible involvement of the chromosomal insertion of the IS element in the attenuation of virulence of *M. bovis* BCG. Finally, the homologous IS*987* integration region of *M. bovis* BCG was characterized in various *M. tuberculosis* strains.

## MATERIALS AND METHODS

**Bacterial strains, genomic DNA, and plasmids.** The bacterial strains and plasmids used in this study are listed in Tables 1 and 2. Media, reagents, and enzymes were used as described by Thole et al. (28). The culture of mycobacterial strains and the isolation of genomic DNA were performed as reported previously (7).

**Synthetic oligonucleotides.** Oligonucleotides were synthesized by using a DNA synthesizer (Applied Biosystems, Inc., Foster City, Calif.). The oligonucleotides used for Southern blot analysis and PCR are listed in Table 3 and depicted schematically in Fig. 4. On the basis of the direct repeat (DR) sequence, the 36-bp oligonucleotides DR-r and DR-l were developed. The previously described synthetic oligonucleotides INS-1 and INS-2 were used for the amplification of the 245-bp fragment of IS*986* in plasmid pRP5000 (8). This DNA fragment was used as a probe in colony blot and Southern blot analyses.

**Colony blot and Southern blot hybridization.** Genomic

TABLE 2. Plasmids used in this study

| Plasmid | Derivation | Source or reference |
|---|---|---|
| pBluescript II KS+ | | Stratagene[a] |
| pPH1001 | pBluescript recombinant containing a 1.75-kb *Pst*I-*Xho*I fragment of *M. bovis* BCG | |
| pPH1002 | pBluescript recombinant containing a 1.9-kb *Pvu*II fragment of *M. bovis* BCG | |
| pRP5000 | pAT153 derivative containing insertion element IS*986* from *M. tuberculosis* | 16 |

[a] Stratagene Cloning Systems, La Jolla, Calif.

DNA from *M. bovis* BCG strain 44 was digested with *Pvu*II and *Xho*I-*Pst*I and cloned into the vector pBluescript II KS+ cleaved with *Sma*I and *Xho*I-*Pst*I, respectively. From each gene library, 5,000 *E. coli* recombinants were transferred onto GeneScreen Plus membranes and screened as recommended by the manufacturer (Dupont, NEN Research Products, Boston, Mass.) for the presence of IS-homologous DNA. The Southern blot hybridization procedure was performed as described previously (8).

**Labeling of DNA probes.** For colony blot hybridization, the amplified 245-bp fragment of IS*986* was labeled with [$\alpha$-$^{32}$P]dCTP by using the multiprime DNA labeling kit (Amersham International plc). For Southern blot hybridization experiments, the amplified 245-bp fragment of IS*986* (8) and the synthetic oligonucleotide DR-r were labeled with horseradish peroxidase by using the enhanced chemiluminescence gene detection system (Amersham International plc).

**DNA sequencing.** The mycobacterial DNA inserts of pPH1001 and pPH1002 were sequenced by the chain termination method (25). The DNA sequencing was initiated by the pBluescript vector primers M13-forward (5′ TGTAAAA CGACGGCCAGT) and M13-reversed (5′ CAGGAAACAG CTATGACC).

**Sequence analysis.** The search for homology of the DR DNA sequence with published sequences was performed by using the GenBank sequence library v. 65 (21).

**PCR.** The PCR was performed as described by Saiki et al. (24). The PCR buffer was supplemented with 100 ng of chromosomal DNA, 1 μM each deoxynucleoside triphosphate, 0.85 μM amplimers IS-l, IS-r1, IS-r2, and US-r and/or 2.13 μM amplimers DR-p and DR-q, plus 0.63 U of Taq polymerase (Perkin-Elmer Cetus) in a final volume of 25 μl. After 20 cycles of 1 min at 94°C, 1 min at 65°C, and 2.5 min at 72°C in a thermo-cycler (Perkin-Elmer Cetus), 10 μl was analyzed by agarose gel electrophoresis in 1.5% agarose. The amplification of the 245-bp fragment of IS*986* was performed as described by Hermans et al. (8).

## RESULTS

**Cloning and sequence analysis of IS*987* from *M. bovis* BCG.** The Dutch vaccine strain *M. bovis* BCG 44 was used as a source of DNA to clone the single-copy insertion element, which hybridizes with IS*986* from *M. tuberculosis*. Gene libraries of *Pvu*II- and *Xho*I-*Pst*I–cleaved chromosomal DNA from *M. bovis* BCG were constructed in the cloning vector pBluescript II KS+. Both gene libraries were

TABLE 3. Oligonucleotides used in the PCR and Southern blot analysis[a]

| Oligonucleotide | Sequence | Sequence position (bp) |
|---|---|---|
| IS-l | 5'C A C C T G A C A T G A C C C C A T | 547–530 |
| IS-r1 | 5'G A G A C C A G C C G C C G G C T G | 1,781–1,798 |
| SP-1 | 5'T C T C C T G G C G A G G T C A A G | 158–141 |
| US-r | 5'C T G C A G A T G G T C C G G G A G | 1–18 |
| IS-r2 | 5'T T C A A C C A T C G C C G C C T C | 1,701–1,718 |
| SP-r | 5'C C C A C A C C G T C G A A G C G C | 2,680–2,697 |
| DR-r | 5'G T C G T C A G A C C C A A A A C C C C G A G A G G G G A C G G A A A C | |
| DR-l | 5'G T T T C C G T C C C C T C T C G G G G T T T T G G G T C T G A C G A C | |
| INS-1 | 5'C G T G A G G G C A T C G A G G T G G C | —[b] |
| INS-2 | 5'G C G T A G G C G T C G G T G A C A A A | —[b] |

[a] Abbreviations: I(N)S, insertion element; SP, spacer DNA; US, unique spacer DNA; DR, direct repeat; l, left; r, right.
[b] —, previously described by Hermans et al. (8).

screened by using the 245-bp DNA fragment of IS*986* as a probe. The recombinant clones pPH1001 and pPH1002 were selected and used for further study. Comparison of the physical maps of the mycobacterial inserts showed that both plasmids contained overlapping fragments. The restriction maps were partially identical to that of IS*986* (Fig. 1) (8). By comparing the physical maps of pPH1001 and pPH1002 with that of IS*986*, we deduced that the entire insertion element of *M. bovis* BCG was cloned. Sequence analysis of mycobacterial DNA inserts of pPH1001 and pPH1002 revealed that *M. bovis* BCG contained a sequence of 1,355 bp which is virtually identical to that of IS*986* and IS*6110* from *M. tuberculosis* (Fig. 2) (16, 27). The *M. bovis* BCG insertion element is designated IS*987*. This element carries two inverted repeats of 28 bp each, which are completely identical to the *M. tuberculosis* IS elements IS*986* and IS*6110*. Similar to the latter IS elements, the left and right inverted repeats in IS*987* differ in three residues in the central part of the repeat. The IS*987* sequence contains three ORFs, ORFa, ORFb, and ORFc. The derived amino acid sequence of ORFc is identical to the homologs in IS*986* and IS*6110*. ORFb differs from the IS*986* homolog in the first two amino acids and is identical to the IS*6110* homolog. However, ORFa in IS*987* is one single ORF, whereas the homologous segment in IS*986* and IS*6110* is composed of two different ORFs, ORFa1 and ORFa2, as described by McAdam et al. (Fig. 3) (16).

**Flanking sequences of IS*987*.** Analysis of the complete mycobacterial DNA inserts of pPH1001 and pPH1002 disclosed the sequence of a segment 486 bp left of, and 999 bp right of, IS*987*. A striking feature of these flanking sequences is the presence of multiple DRs of 36 bp, each separated by spacer DNA, 35 to 41 bp in length. These spacers are nonrepetitive, except for two, each of which was found twice in the sequence (Fig. 2). Seventeen of 20 DRs were completely identical, whereas one nucleotide substitution was observed in two DRs. One DR was split by the insertion of IS*987*. This DR contained a 3-bp duplication (5'CCC), supposedly due to the integration of IS*987*. Such a 3-bp duplication has also been found in the target DNA of IS*6110* (27). No significant homology of the DR with DNA sequences present in the GenBank data library was found.

**Determination of the number of DRs flanking IS*987* in *M. bovis* BCG.** To investigate the number of DRs flanking IS*987*, we synthesized oligonucleotides that allowed the amplification of DR-containing sequences to the left and right of the insertion element. The primers used for this purpose are given in Table 3 and depicted schematically in Fig. 4. The PCR that used the primer pairs IS-l/DR-r and SP-l/DR-r resulted in multiple fragments with maximum sizes of 2.2 and 1.8 kb, respectively (Fig. 5a). This result indicates that a 2.1-kb DR-containing segment is located to the left of IS*987*. In a similar way, when the primer pairs IS-r1/DR-l and SP-r/DR-l were used, the DR-containing region flanking the right border of IS*987* was determined to be 1.5 kb in size. To

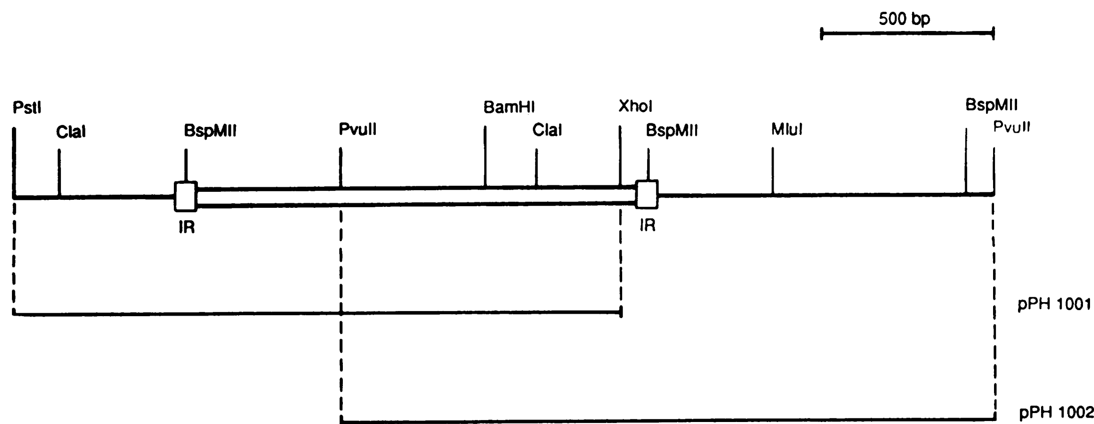


FIG. 1. Physical map of the 2.85-kb *Pst*I-*Pvu*II DNA fragment of *M. bovis* BCG strain 44 containing IS*987* flanked by inverted repeats (IR). The DNA fragments present in the recombinant clones pPH1001 and pPH1002 are also depicted.

```
CTGCAGATGGTCCGGGAGGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACTGGATT   60 (DR24)
 PstI
GCGCTAACTGGCTTGGCGCTGATCCTGGTGGTCGTCAGACCCAAAACCCCGAGAGGGGAC  120 (DR25)

GGAAACTCCACATCGATTTCCTTGACCTCGCCAGGAGAGAAGATCACGTCGTCAGACCCA  180 (DR26)

AAACCCCGAGAGAGGACGGAAACTCGTCGACGATCGCGTCGATGTCGATGTCCCAATCGT  240

CGAGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACTTGGAGCGTGTCACCGCAGAC  300 (DR27)

GGCACGATTGAGACAAGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACCCTCAGCT  360 (DR28)

CAGCATCGCTGATGCGGTCCAGCTCGTCCGTGTCGTCAGACCCAAAACCCCGAGAGGGGA  420 (DR29)

CGGAAACCCAACCTCACCGCCTGCTGGGTGAGACGTGCTCGCCGCGAGTCGTCAGACCCA  480 (DR30)
      ┌═════════════IR-1═════════════┐
AAACCCTGAACCGCCCCGGCATGTCCGGAGACTCCAGTTCTTGGAAAGGATGGGGTCATG  540
  ORFa → T  A  P  A  C  P  E  T  P  V  L  G  K  D  G  V  M

TCAGGTGGTTCATCGAGGAGGTACCCGCCGGAGCTGCGTGAGCGGGCGGTGCGGATGGTC  600
 S  G  G  S  S  R  R  Y  P  P  E  L  R  E  R  A  V  R  M  V

GCAGAGATCCGCGGTCAGCACGATTCGGAGTGGGCAGCGATCAGTGAGGTCGCCCGTCTA  660
 A  E  I  R  G  Q  H  D  S  E  W  A  A  I  S  E  V  A  R  L

CTTGGTGTTGGCTGCGCGGAGACGGTGCGTAAGTGGGTGCGCCAGGCGCAGGTCGATGCC  720
 L  G  V  G  C  A  E  T  V  R  K  W  V  R  Q  A  Q  V  D  A

GGCGCACGGCCCGGGACCACGACCGAAGAATCCGCTGAGCTGAAGCGCTTGCGGCGGGAC  780
 G  A  R  P  G  T  T  T  E  E  S  A  E  L  K  R  L  R  R  D
                             ORFb → A  E  A  L  A  A  G

AACGCCGAATTGCGAAGGGCGAACGCGATTTTAAAGACCGCGTCGGCTTTCTTCGCGGCC  840
 N  A  E  L  R  R  A  N  A  I  L  K  T  A  S  A  F  F  A  A
Q  R  R  I  A  K  G  E  R  D  F  K  D  R  V  G  F  L  R  G

GAGCTCGACCGGCCAGCACGCTAATTACCCGGTTCATCGCCGATCATCAGGGCCACCGCG  900
 E  L  D  R  P  A  R
R  A  R  P  A  S  T  L  I  T  R  F  I  A  D  H  Q  G  H  R

AGGGCCCCGATGGTTTGCGGTGGGGTGTCGAGTCGATCTGCACACAGCTGACCGAGCTGG  960

E  G  P  D  G  L  R  W  G  V  E  S  I  C  T  Q  L  T  E  L

GTGTGCCGATCGCCCCATCGACCTACTACGACCACATCAACCGGGAGCCCAGCCGCCGCG 1020

G  V  P  I  A  P  S  T  Y  Y  D  H  I  N  R  E  P  S  R  R

AGCTGCGCGATGGCGAACTCAAGGAGCACATCAGCCGCGTCCACGCCGCCAACTACGGTG 1080

E  L  R  D  G  E  L  K  E  H  I  S  R  V  H  A  A  N  Y  G

TTTACGGTGCCCGCAAAGTGTGGCTAACCCTGAACCGTGAGGGCATCGAGGTGGCCAGAT 1140
                           G  Q  V  T  L  A  D  L  H  G  S
V  Y  G  A  R  K  V  W  L  T  L  N  R  E  G  I  E  V  A  R

GCACCGTCGAACGGCTGATGACCAAACTCGGCCTGTCCGGGACCACCCGCGGCAAAGCCC 1200
A  G  D  F  P  Q  H  G  F  E  A  Q  G  P  G  G  A  A  F  G
C  T  V  E  R  L  M  T  K  L  G  L  S  G  T  T  R  G  K  A

GCAGGACCACGATCGCTGATCCGGCCACAGCCCGTCCCGCCGATCTCGTCCAGCGCCGCT 1260
A  P  G  R  D  S  I  R  G  C  G  T  G  G  I  E  D  L  A  A
R  R  T  T  I  A  D  P  A  T  A  R  P  A  D  L  V  Q  R  R
```

FIG. 2. Sequence of the 2,840-bp *Pst*I-*Pvu*II fragment from *M. bovis* BCG strain 44 containing IS*987* and its flanking regions. The inverted repeats flanking IS*987*, IR-l and IR-r, and the derived amino acid sequences of the ORFs of IS*987*, ORFa, ORFb, and ORFc, including their orientation, are depicted. DR25 to DR44 present in the flanking regions of IS*987* are marked in bold, whereas the other repeated areas are single and double underlined. IS*987* differs from IS*986* at the positions 648 to 650 GGT (GAT), 670 to 672 GGC (G-C), and 759 to 763 GCTGA (GATAA), and IS*987* differs from IS*6110* at the positions 770 to 771 T-G (TAG), 867 to 871 ACCCG (AACC-G), and 933 to 934 T-C (TTC). The IS*986* and IS*6110* sequences are depicted in parentheses. These sequence data will appear in the EMBL, GenBank, and DDBJ Nucleotide Sequence Databases under the accession number X57835.

confirm that the multiple bands corresponded to the DRs, the DNA was transferred onto a nylon filter and hybridized with the oligonucleotide DR-r. Since the multiple-banding pattern was clearly visible in this Southern blot (Fig. 5b), we concluded that these bands indeed contain DR sequences which extend 2.1 kb to the left and 1.5 kb to the right of IS*987*. The individual bands were clearly visible on the original X-ray film and could be counted. The total number of DR copies flanking IS*987* is 49. IS*987* is inserted into the 30th DR (DR30) from the left. We investigated the DR cluster of three other *M. bovis* BCG strains, the vaccine strains 102 and 104 and the clinical isolate 43. In all three strains we found the same number of DR copies and DR30 as the IS-containing copy (data not shown).

```
TCGGACCACCAGCACCTAACCGGCTGTGGGTAGCAGACCTCACCTATGTGTCGACCTGGG 1320
E  S  W  W  C  R  V  P  Q  P  Y  C  V  E  G  I  H  R  G  P
F  G  P  P  A  P  N  R  L  W  V  A  D  L  T  Y  V  S  T  W

CAGGGTTCGCCTACGTGGCCTTTGTCACCGACGCCTACGCTCGCAGGATCCTGGGCTGGC 1380
C  P  E  G  V  H  G  K  D  G  V  G  V  S  A  P  D  Q  A  P
A  G  F  A  Y  V  A  F  V  T  D  A  Y  A  R  R  I  L  G  W

GGGTCGCTTCCACGATGGCCACCTCCATGGTCCTCGACGCGATCGAGCAAGCCATCTGGA 1440
P  D  S  G  R  H  G  G  G  H  D  E  V  R  D  L  L  G  D  P
R  V  A  S  T  M  A  T  S  M  V  L  D  A  I  E  Q  A  I  W

CCCGCCAACAAGAAGGCGTACTCGACCTGAAAGACGTTATCCACCATACGGATAGGGGAT 1500
G  A  L  L  F  A  Y  E  V  Q  F  V  N  D  V  M  R  I  P  S
T  R  Q  Q  E  G  V  L  D  L  K  D  V  I  H  H  T  D  R  G

CTCAGTACACATCGATCCGGTTCAGCGAGCGGCTCGCCGAGGCAGGCATCCAACCGTCGG 1560
R  L  V  C  R  D  P  E  A  L  P  E  G  L  C  A  D  L  R  R
S  Q  Y  T  S  I  R  F  S  E  R  L  A  E  A  G  I  Q  P  S

TCGGAGCGGTCGGAAGCTCCTATGACAATGCACTAGCCGAGACGATCAACGGCCTATACA 1620
D  S  R  D  S  A  G  I  V  I  C ← ORFc
V  G  A  V  G  S  S  Y  D  N  A  L  A  E  T  I  N  G  L  Y

AGACCGAGCTGATCAAACCCGGCAAGCCCTGGCGGTCCATCGAGGATGTCGAGTTGGCCA 1680

K  T  E  L  I  K  P  G  K  P  W  R  S  I  E  D  V  E  L  A

CCGCGCGCTGGGTCGACTGGTTCAACCATCGCCGCCTCTACCAGTACTGCGGCGACGTCC 1740

T  A  R  W  V  D  W  F  N  H  R  R  L  Y  Q  Y  C  G  D  V

CGCCGGTCGAACTCGAGGCTGCCTACTACGCTCAACGCCAGAGACCAGCCGCCGGCTGAG 1800

P  P  V  E  L  E  A  A  Y  Y  A  Q  R  Q  R  P  A  A  G

GTCTCAGATCAGAGAGTCTCCGGACTCACCGGGGCGGTTCACCCCGAGAGGGGACGGAAA 1860 (DR30)
             └═══════════════IR-r════════════════┘
CTCGGGGAGCCGATCAGCGACCACCGCACCCTGTCAGTCGTCAGACCCAAAACCCCGAGA 1920 (DR31)
GGGGACGGAAACCTTCAGCACCACCATCATCCGGCGCCTCAGCTCAGCATGTCGTCAGAC 1980 (DR32)
CCAAAACCCCGAGAGGGGACGGAAACCCTTCGACGCCGGATTCGTGATCTCTTCCCGCGG 2040
ATAGGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACTGCCCCGGCGTTTAGCGATC 2100 (DR33)
ACAACACCAACTAATGGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACCAGCGAAA 2160 (DR34)
TACAGGCTCCACGACACGACCACAACGCGTCGTCAGACCCAAAACCCCGAGAGGGGACGG 2220 (DR35)
AAACTCTTGACGATGCGGTTGCCCCGCGCCCTTTTCCAGCCGTCGTCAGACCCAAAACCC 2280 (DR36)
CGAGAGGGGACGGAAACAGGTTCGCGTCAGACAGGTTCGCGTCGATCAAGTCCGGTCGTC 2340 (DR37)
AGACCCAAAACCCCGAGAGGGGACGGAAACTCGGGGAGCCGATCAGCGACCACCGCACCC 2400
TGTCAGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACTTTATCACTCCCGACCAAA 2460 (DR38)
TAGGTATCGGCGTGTTCAAGTCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACTCGAC 2520 (DR39)
ACCGACATGACGGCGGTGCCGCACTTGACGCAGTCGTCAGACCCAAAACCCCGAGAGGGG 2580 (DR40)
ACGGAAACTCGACACCGACATGACGGCGGTGCCGCACTTGACGCAGTCGTCAGACCCAAA 2640 (DR41)
ACCCCGAGAGGGGACGGAAACCTTTGCGAAGTCACCTCGCCCACACCGTCGAAGCGCCTG 2700 (DR42)
TCGTCAGACCCAAAACCCCGAGAGGGGACGGAAACCTGCATCCGGAAAGTCCGTACGCTC 2760
GAAACGCTTCCAACGTGTCGTCAGACCCAAAACCCCGAGAGGGGACGTAAACTCGAAATC 2820 (DR43)

CAGCACCACATCCGCAGCTG 2840
              PvuII
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FIG. 2—*Continued.*

**Occurrence of the DRs among the various mycobacterial species.** To determine whether other mycobacterial species contained the DRs identified in *M. bovis* BCG, the oligonucleotide DR-r was used as a DNA probe in Southern blot experiments. We investigated *Nru*I-digested genomic DNA from nine strains of the *M. tuberculosis* complex, the atypical mycobacterial species *M. asiaticum*, *M. avium*, *M. chitae*, *M. flavescens*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. malmoense*, and *M. terrae*, and, furthermore, the bacterial species *E. coli* K-12 and *Bordetella pertussis* Wellcome 28. All strains of the *M. tuberculosis* complex contained a single *Nru*I fragment of about 10 kb which hybridized with the DR probe, indicating that all DR copies are clustered within this fragment. None of the other species contained DR-hybridizing DNA (Fig. 6a).

**Linkage of the DR-containing region and the insertion element in *M. tuberculosis* strains.** To investigate whether an insertion element is present within or near the DR-containing region of other *M. tuberculosis* complex strains, we rehybridized the blot used in the previous section with the 245-bp fragment of IS*986*. Only strains of the *M. tuberculosis* complex hybridized with IS*986* DNA and, consistent with

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IS6110 ORFa1: TAPACPETPVLGKDGVMSGGSSRRYPPELRERAVRMVAEIRGQHDSEWAA

IS986  ORFa1: TAPACPETPVLGKDGVMSGGSSRRYPPELRERAVRMVAEIRGQHDSEWAA

IS987  ORFa : TAPACPETPVLGKDGVMSGGSSRRYPPELRERAVRMVAEIRGQHDSEWAA
              **************************************************

IS6110 ORFa1: ISEVARLLGVGCAETVRKWVRQAQVDAGARPGTTTEESAELKRL
IS6110 ORFa2:                                               RRDNAE
IS986  ORFa1: ISEIARLLGV-
IS986  ORFa2:            CAETVRKWVRQAQVDAGARPGTTTEESAEIKRLRRDNAE
IS987  ORFa : ISEVARLLGVGCAETVRKWVRQAQVDAGARPGTTTEESAELKRLRRDNAE
              *** ****** ***************************** *********


IS6110 ORFa2: LRRANAILKTASAFFAAELDRPAR

IS986  ORFa2: LRRANAILKTASAFFAAELDRPAR
IS986  ORFa : LRRANAILKTASAFFAAELDRPAR
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FIG. 3. Alignment of potentially translated sequence of ORFa in IS*6110* and IS*986*, both from *M. tuberculosis*, and IS*987* from *M. bovis* BCG. Asterisks denote identical residues in all three sequences.

previous findings of Hermans et al. (8), all strains of the *M. tuberculosis* complex, except *M. bovis* BCG, showed a multiple-banding pattern unique for each strain. Interestingly, in all strains of the *M. tuberculosis* complex tested, at least one copy of the IS element was located on the *Nru*I fragment containing the DR cluster (Fig. 6b). This finding could suggest that, in these *M. tuberculosis* complex strains, an IS element is located within the DR cluster, as in *M. bovis* BCG. To investigate this possibility, genomic DNA from *M. bovis* BCG and *M. tuberculosis* was digested with *Pvu*II, which cleaves the IS element at a single site (Fig. 1). Blots carrying the separated chromosomal *Pvu*II fragments were hybridized with the oligonucleotide probe DR-r and, subsequently, with the 245-bp fragment of IS*986*. In all *M. bovis* BCG strains tested, a 1.9-kb fragment hybridized with both DNA probes, as expected from the DNA sequence of IS*987* and its flanking regions. In contrast to the IS probe, the DR probe also hybridized with a 4.0-kb *Pvu*II fragment, representing the DR-containing region left of IS*987*, and with a 2.9-kb *Pvu*II fragment, which is located rightwards from the 1.9-kb *Pvu*II DR-containing fragment (Fig. 7a and b). We conclude that all eight *M. bovis* BCG strains do not differ with regard to the chromosomal position of the IS element and copy number of the DRs. Fourteen *M. tuberculosis* strains were analyzed in a manner similar to that used for the *M. bovis* BCG strains. In all strains except one, both the IS probe and the DR probe cohybridized with either one or two *Pvu*II fragments (Fig. 7c and d), suggesting the presence of one or two IS copies, respectively, in the chromosomal DR cluster. In four *M. tuberculosis* strains, a 1.9-kb cohybridizing *Pvu*II fragment was found similar to that in the *M. bovis* BCG strains. This implies integration of an IS element at the same location in the DR cluster as occurs in *M. bovis* BCG.

**Detailed analysis of the IS integration site in the DR-containing region of *M. tuberculosis* strains.** The polymorphic hybridization pattern of the 14 *M. tuberculosis* strains in Southern blot analysis with DR DNA (Fig. 7c) indicated variation in the integration site of the insertion element in the DR region and/or polymorphism in the DR region. We examined the exact location of the insertion element within the DR region in the set of 15 *M. tuberculosis* strains, including the 14 strains which were analyzed in the previous section. This was performed by PCR by using the primer pair US-r/IS-l, which corresponds to a nonrepeated, unique spacer sequence and a sequence in the left part of IS*987* (Fig. 4). By using this primer pair, a 547-bp sequence from *M. bovis* BCG strain 44 was amplified (Fig. 8). An identically sized fragment was amplified from six *M. tuberculosis* strains, indicating that the IS element was located exactly at the same position in the DR region. A limited number of differently sized fragments were amplified from DNA of seven other *M. tuberculosis* strains. In these strains, fragments of either 300 or 400 bp were generated. From one strain, two fragments of 300 and 600 bp were amplified. Three strains did not allow the amplification of DNA. These data again indicate that polymorphism in the DR region occurs among the *M. tuberculosis* strains.

**Polymorphism in the size of the DR-containing region among various *M. tuberculosis* strains.** We further analyzed the DR-containing region of the 8 *M. bovis* BCG strains described in Fig. 7 and the 15 *M. tuberculosis* strains which

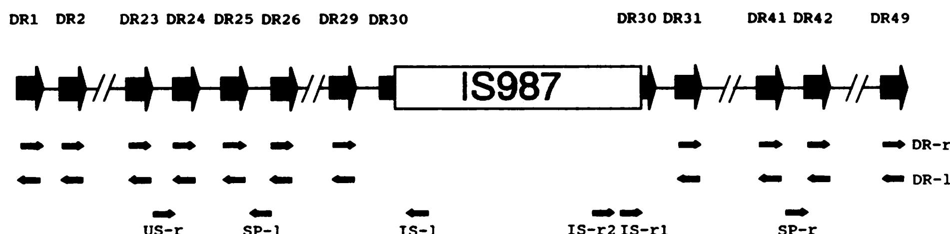


FIG. 4. Schematic drawing of IS*987* and flanking sequences and of the oligonucleotide amplimers used in PCR and Southern blotting. Abbreviations: IS, insertion element; SP, spacer DNA; US, unique spacer DNA; DR, direct repeat; l, left; r, right.

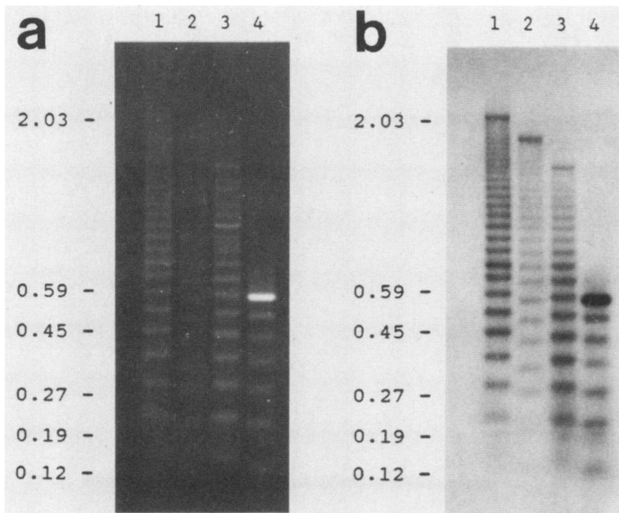


FIG. 5. Determination of the number of DRs flanking IS*987*. Agarose gel electrophoresis (a) and Southern blot analysis (b) of amplified DNA from *M. bovis* BCG strain 44. The oligonucleotide primer pairs IS-l/DR-r (lane 1), SP-l/DR-r (lane 2), IS-r1/DR-l (lane 3), and SP-r/DR-l (lane 4) were used in the PCR. Oligonucleotide DR-r was used as a DNA probe in Southern blot hybridization. Numbers at left indicate sizes of standard DNA fragments in kilobase pairs.

were analyzed in the previous section. To determine the size of the DR-containing DNA, the amplimer pairs IS-l/DR-r and IS-r/DR-l were used in the PCR (Fig. 4). The amplified fragments were analyzed by Southern blotting by using oligonucleotide probe DR-r. Consistent with previous findings (Fig. 7a and b), no polymorphism was observed among the eight *M. bovis* BCG strains (data not shown). However, different multiple-banding patterns were obtained among the *M. tuberculosis* strains (Fig. 9), indicating again polymorphism of the DR-containing region on the chromosome of these *M. tuberculosis* strains. As expected, the *M. tuberculosis* strain which did not contain an IS element in the DR region (Fig. 7c and d; lane 3) lacked the multiple-banding pattern. We established the presence of two IS elements in the DR region of five *M. tuberculosis* strains described in Fig. 7 by using chromosomal DNA and the amplimer pairs IS-l/IS-l, IS-l/IS-r2, and IS-r2/IS-r2 in the PCR (Fig. 4). None of the *M. tuberculosis* DNAs showed an amplified fragment with the amplimer pairs IS-l/IS-l and IS-r2/IS-r2. However, when the amplimers IS-l and IS-r2 were used in the PCR, a fragment of 700 bp was amplified in four *M. tuberculosis* strains, whereas one *M. tuberculosis* strain contained a 350-bp amplifiable fragment (Fig. 10). We conclude that in these two groups of *M. tuberculosis* strains, the two IS elements in the DR region are located in a direct orientation and the distances between the two IS elements are 500 and 150 bp, respectively.

## DISCUSSION

In this study we showed that the *M. bovis* BCG insertion element IS*987* is virtually identical to the previously described IS elements IS*986* and IS*6110* from *M. tuberculosis*. The only biologically significant difference is the presence, in IS*987*, of ORFa in one single ORF, whereas IS*986* and IS*6110* contain ORFa composed of two different ORFs (16). As discussed by McAdam et al., regarding several other members of the IS*3* family, translation of the putative transposase (ORFb) is thought to occur by readthrough from ORFa. Functional expression of ORFb from the latter two insertion elements is presumed to take place by two trans-

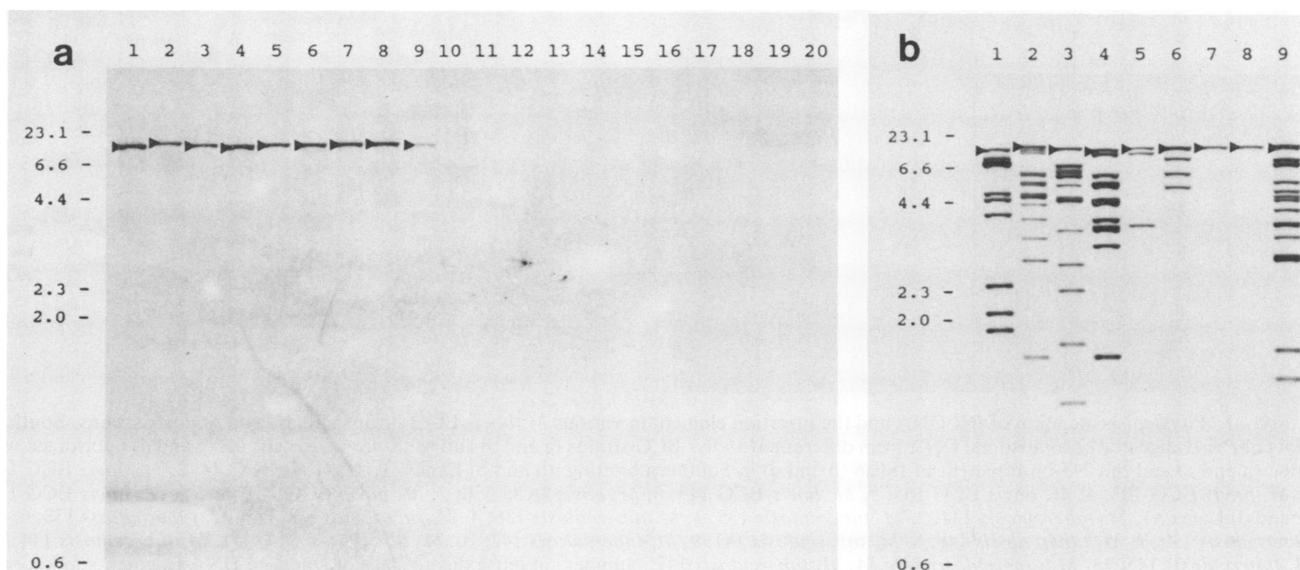


FIG. 6. Presence of DR DNA in various mycobacterial species and physical association of the DR DNA and the insertion element. Southern blot analysis of *Nru*I-digested chromosomal DNA from different bacterial species, by using DR-r (a) and the 245-bp fragment of IS*986* (b) as a DNA probe. Lanes: 1, *M. tuberculosis* 111; 2, *M. tuberculosis* 112; 3, *M. africanum* 38; 4, *M. africanum* 153; 5, *M. bovis* 154; 6, *M. bovis* 41; 7, *M. bovis* BCG 102; 8, *M. bovis* BCG 103; 9, *M. microti* 46; 10, *M. flavescens* 156; 11, *M. malmoense* 157; 12, *M. asiaticum* 158; 13, *M. avium* 49; 14, *M. gordonae* 159; 15, *M. kansasii* 160; 16, *M. intracellulare* 161; 17, *M. chitae* 162; 18, *M. terrae* 163; 19, *B. pertussis* Wellcome 28; 20, *E. coli* K-12. Numbers at left indicate sizes of standard DNA fragments in kilobase pairs. The arrowheads mark the bands hybridizing with both DNA probes.

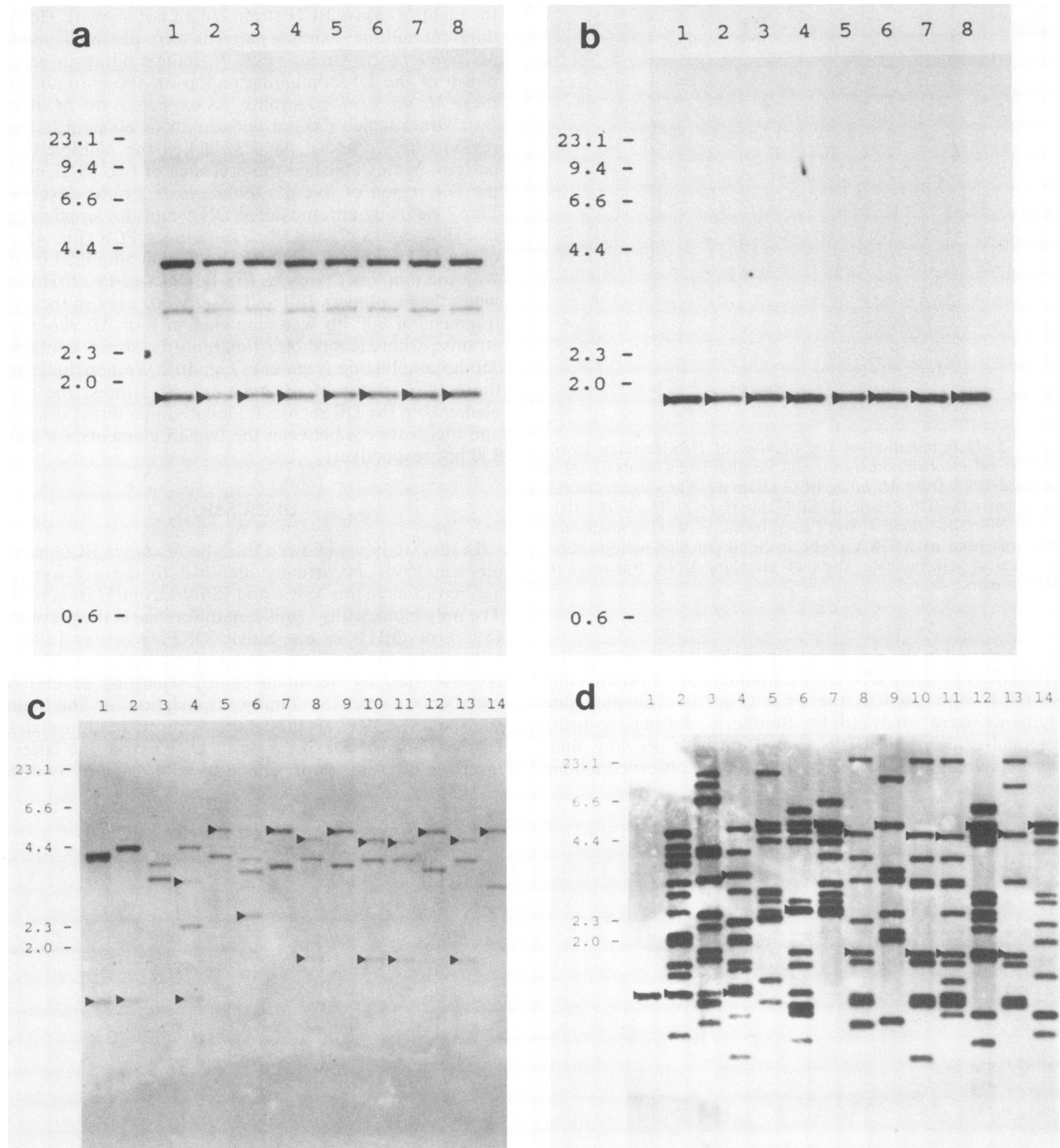


FIG. 7. Physical association of DR DNA and the insertion element in various *M. bovis* BCG strains and *M. tuberculosis* strains. Southern blots of *Pvu*II-digested chromosomal DNA from different *M. bovis* BCG strains (a and b) and *M. tuberculosis* strains (c and d) hybridized with DR-r (a and c) and the 245-bp fragment of IS*986* (b and d) in Southern blotting. (a and b) Lanes: 1, *M. bovis* BCG 44; 2, *M. bovis* BCG 102; 3, *M. bovis* BCG 103; 4, *M. bovis* BCG 105; 5, *M. bovis* BCG 149; 6, *M. bovis* BCG 150; 7, *M. bovis* BCG 151; and 8, *M. bovis* BCG 152. (c and d) Lanes: 1, *M. tuberculosis* 134; 2, *M. tuberculosis* 135; 3, *M. tuberculosis* 136; 4, *M. tuberculosis* 137; 5, *M. tuberculosis* 138; 6, *M. tuberculosis* 139; 7, *M. tuberculosis* 140; 8, *M. tuberculosis* 141; 9, *M. tuberculosis* 142; 10, *M. tuberculosis* 143; 11, *M. tuberculosis* 144; 12, *M. tuberculosis* 145; 13, *M. tuberculosis* 146; 14, *M. tuberculosis* 147. Numbers at left indicate sizes of standard DNA fragments in kilobase pairs. The arrowheads mark the bands hybridizing with both DNA probes.

lational frameshifts in ORFa1 and ORFa2, respectively (16). It seems very unlikely that the capacity of IS*987* to transpose in *M. bovis* BCG would be impaired by the absence of the requirement for two translational frameshifts. On the contrary, one might expect that the putative transposase in *M. bovis* BCG is better expressed during a single translational frameshift in comparison with the necessity for a double translational frameshift expected in IS*986* and IS*6110* of *M.*

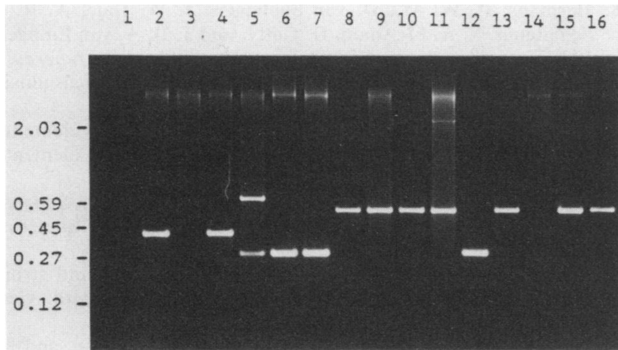


FIG. 8. Polymorphism in the DR-containing region flanking the IS element in various *M. tuberculosis* strains. Agarose gel electrophoresis of amplified DNA from 16 *M. tuberculosis* complex strains. The amplimers IS-l and US-r were used in PCR. The *M. tuberculosis* strains analyzed in lanes 1 to 14 correspond to the *M. tuberculosis* strains described in Fig. 7c and d, lanes 1 to 14, respectively; lane 15, *M. tuberculosis* 148; lane 16, *M. bovis* BCG 44. Numbers at left indicate sizes of standard DNA fragments in kilobase pairs.

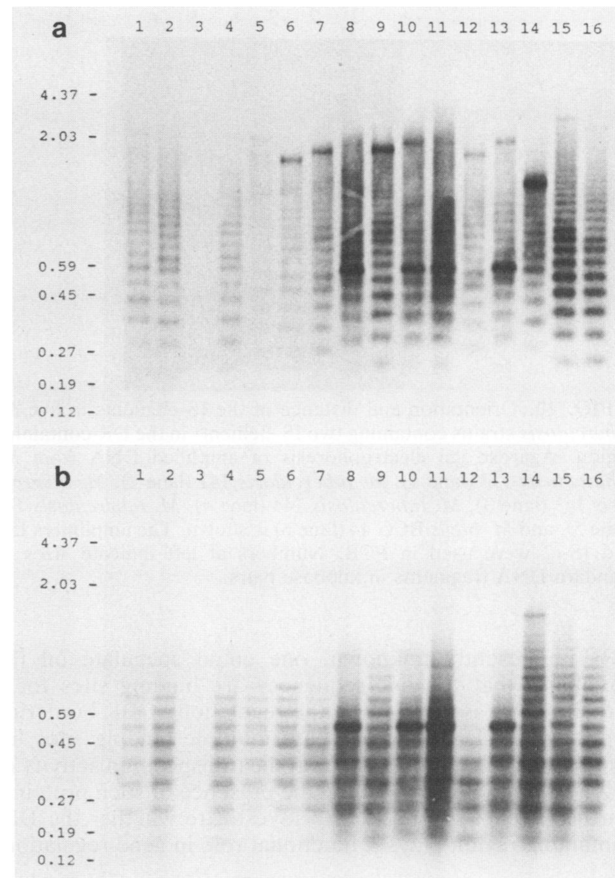


FIG. 9. Polymorphism in the size of the DR-containing region of various *M. tuberculosis* strains. Southern blot analysis of amplified DNA from 16 strains of the *M. tuberculosis* complex using DR-r as a DNA probe is shown. The amplimer pairs IS-l/DR-r (a) and IS-rl/DR-l (b) were used in the PCR. The *M. tuberculosis* strains analyzed in lanes 1 to 14 correspond to the *M. tuberculosis* strains described in Fig. 7c and d, lanes 1 to 14, respectively; lane 15, *M. tuberculosis* 148; lane 16, *M. bovis* BCG 44. Numbers at left indicate sizes of standard DNA fragments in kilobase pairs.

*tuberculosis*. Since the inverted repeats of IS*987* are identical to the homologs of IS*986* and IS*6110* and virtually no differences are present between ORFb and ORFc of IS*987* and the *M. tuberculosis* IS elements, we conclude that there is no reason to believe that IS*987* is not functional with regard to its capacity to transpose. The different *M. bovis* BCG vaccine strains used in this study were recultured frequently for many years at different laboratories. Therefore, the absence of any restriction fragment length polymorphism in IS-containing chromosomal DNA of these *M. bovis* BCG strains suggests that transposition of these IS elements is an extremely rare event. This is consistent with our previous study (8) and more recent observations of van Soolingen et al. (29) that the insertion elements in *M. tuberculosis* are extremely stable, even after many months of reculture in vitro or growth in vivo.

Sequence analysis of the flanking DNA of IS*987* revealed that the insertion element was integrated in a region containing multiple DRs of 36 bp. In the cloned 2.85-kb fragment, 20 virtually identical DRs were present which were separated by spacer DNA, 35 to 41 bp in length. Determination of the total number of DRs in the *M. bovis* BCG chromosome disclosed the presence of 49 repeats. IS*987* was found to be integrated in the 30th DR. No significant homology of the DR with DNA sequences present in the GenBank was observed. The DR hybridized only with chromosomal DNA from species of the *M. tuberculosis* complex and not with DNA from nine other mycobacterial species. Similar to the case with *M. bovis* BCG, the DRs were clustered at a unique position on the chromosome of the other *M. tuberculosis* complex strains tested. The presence and conservation of the DRs in all of the species of the *M. tuberculosis* complex suggest a biological role for these sequences; however, their function remains unknown at present.

Interestingly, the DR region of most *M. tuberculosis* strains tested also contained IS elements. Among 14 *M. tuberculosis* isolates, eight strains contained one IS element and five strains contained two IS elements in the DR region. Only one *M. tuberculosis* strain did not contain an IS element in this region. Analysis of the integration sites of these IS elements and the sizes of the DR regions revealed that the DR-containing region of these *M. tuberculosis* strains is polymorphic in size and composition. Because IS elements are inserted in the DR region on the chromosome of both *M. tuberculosis* strains and *M. bovis* BCG strains, it is unlikely that the lack of virulence of *M. bovis* BCG is due to IS*987* insertional inactivation of a gene which is involved in virulence.

The finding that the size and composition of the DR-containing region are polymorphic among strains of the *M. tuberculosis* complex suggests that this genetic diversity could be due to homologous recombination between DR sequences or to slippage during replication in this region. Since IS elements are known to induce many types of genetic rearrangements like deletion, duplication, and inversion (4), IS*987* might be responsible for the observed polymorphism in the DR-containing chromosomal DNA.

The observation that the DR-containing region of strains of the *M. tuberculosis* complex generally contains one or two insertion elements indicates that this region is a hot spot of IS integration. It is unclear whether this is caused by a preference of insertion elements to integrate into the DR cluster or by a decreased excision frequency of IS elements once integrated in this region. Although the function of the

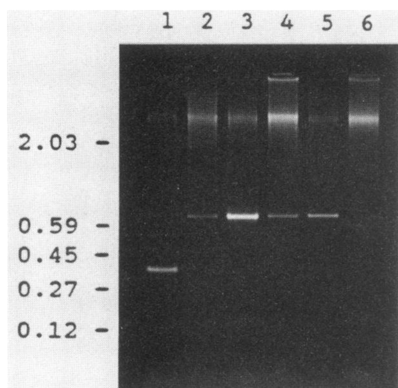


FIG. 10. Orientation and distance of the IS elements in five *M. tuberculosis* strains containing two IS elements in the DR-containing region. Agarose gel electrophoresis of amplified DNA from *M. tuberculosis* 137 (lane 1), *M. tuberculosis* 141 (lane 2), *M. tuberculosis* 143 (lane 3), *M. tuberculosis* 144 (lane 4), *M. tuberculosis* 146 (lane 5), and *M. bovis* BCG 44 (lane 6) is shown. The amplimers IS-l and IS-r2 were used in PCR. Numbers at left indicate sizes of standard DNA fragments in kilobase pairs.

DRs is presently unknown, one could speculate on the possibility that the DR sequences are binding sites for a protein which is involved in the regulation of DR-bordering genes. If the DR sequences are multiple binding sites for proteins, one might envision that the transposition activity of IS*987* is greatly influenced by the presence of such proteins. Further studies are needed to investigate whether the DR-containing region plays a functional role in gene regulation.

## ACKNOWLEDGMENTS

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