

Preparation of a Specific RNA Probe for Detection of *Mycobacterium paratuberculosis* and Diagnosis of Johne's Disease

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A species-specific recombinant clone (F57) was obtained from a genomic library of *Mycobacterium paratuberculosis* in the transcription vector pGem 3Z. This clone proved to be specific for all mycobacteria tested, including *M. avium*, and was able to recognize all of the tested *M. paratuberculosis* strains isolated from animals and humans (patients with Crohn's disease). The F57 insert was sequenced, and a segment of 620 bp with a G+C content of 58.9% was identified. Comparison of the sequence with sequences in the EMBL and UGEN data banks revealed the uniqueness of the F57 sequence, which had no resemblance to other known genes.

Mycobacterium paratuberculosis, the etiological agent of paratuberculosis (Johne's disease), a chronic infectious disease of wild and domestic ruminants, multiplies within the macrophages of the gastrointestinal tract and the associated lymphoid tissues (7). Infected animals excrete increasing quantities of bacilli, thus propagating infection (4). Microbiological analysis of fecal material is rendered difficult by the use of decontamination procedures, the slow growth rate of *M. paratuberculosis* (27), and its kinship with other mycobacterial species, such as those of the *M. avium-M. intracellulare-M. scrofulaceum* group (6, 20, 24, 36, 45).

Nucleic acid hybridization offers an alternative approach to the diagnosis of paratuberculosis (42). DNA and RNA probes have been successfully used to detect different microorganisms (9, 29, 40, 44), including mycobacteria (15, 32). Although several probes have been proposed for use in the detection of *M. paratuberculosis* (1, 16, 19, 31, 41), heterologous hybridization was invariably observed with the highly homologous DNA of mycobacteria of the *M. avium-M. intracellulare-M. scrofulaceum* complex. A probe specific to *M. paratuberculosis* has recently been described; it represented a part of an insertion sequence (IS900) found at 15 to 20 copies per chromosome (10, 16, 30).

Mycobacteria, some identified as *M. paratuberculosis* (5, 7, 23), are frequently isolated from human patients with Crohn's disease (a chronic enteritis of humans resembling Johne's disease). Epidemiological investigation with a specific probe would provide investigators with a means of studying the etiology of this disease.

In this report, we describe the cloning of the *M. paratuberculosis* genome in a transcription vector and the isolation of a specific DNA fragment that was used as a diagnostic probe.

Construction and screening of an *M. paratuberculosis* genomic library. The DNA of *M. paratuberculosis* 2E was extracted and purified as described previously (12, 17) and was then partially digested with the restriction endonuclease *Sau*3A to produce DNA fragments with an average size of

500 bp. The degree of DNA digestion was monitored by agarose gel electrophoresis. DNA fragments were dephosphorylated with alkaline phosphatase (Boehringer, Mannheim, Germany) and ligated to the *Bam*HI-digested plasmid pGem 3Z (Promega Corp., Madison, Wis.). The ligated DNA was used to transform *Escherichia coli* JM 109 cells; 60% of the clones were recombinants. To measure the sizes of the inserts, plasmids were extracted from 10 randomly chosen recombinant clones by the alkaline lysis procedure (3). Restriction endonuclease analysis showed mycobacterial DNA inserts of 0.2 to 0.8 kb. Some 28,000 recombinant clones were screened by colony hybridization. After UV-mediated fixation, nylon filters (Hybond N; Amersham, Slough, United Kingdom) were incubated for 60 min at 37°C in TNE buffer (30 mM Tris-HCl [pH 7.5], 0.3 M NaCl, 2 mM EDTA) containing 100 µg of proteinase K per ml. Probes were prepared from the chromosomal DNAs of *M. paratuberculosis* 2E and *M. avium* serotype 4 by nick translation with [α -³²P]dCTP (21). Unlabeled DNA samples were preincubated for 60 min at 55°C in fivefold-concentrated SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate [pH 7]) containing 0.1% bovine serum albumin, 0.1% Ficoll, 0.1% polyvinylpyrrolidone, 0.1% sodium dodecyl sulfate (SDS), 100 µg of sheared herring sperm DNA per ml, and 50% deionized formamide. After the addition of denatured labeled DNA, hybridization was performed for 16 h at 55°C in the same solution. Filters were washed twice (15 min at 60°C in 2× SSC-0.1% SDS and 30 min in 0.2× SSC-0.1% SDS), dried, and autoradiographed. Some 78 clones hybridized with the *M. paratuberculosis* probe at a high level of efficiency. Their specificities were checked by extraction of plasmid DNA (rapid alkaline method) and hybridization (dot blot technique) with three probes that were nick translated from the genomic DNAs of *M. paratuberculosis* 2E, *M. avium* serotype 4, and *M. intracellulare*. Fourteen clones hybridized with *M. paratuberculosis* but not with *M. avium* or *M. intracellulare*.

Analysis of clone specificity. The 14 cloned species-specific fragments of mycobacterial DNA were used as templates for radiolabeled RNA probes. Recombinant plasmids were cleaved with *Ava*I downstream of the cloned fragment,

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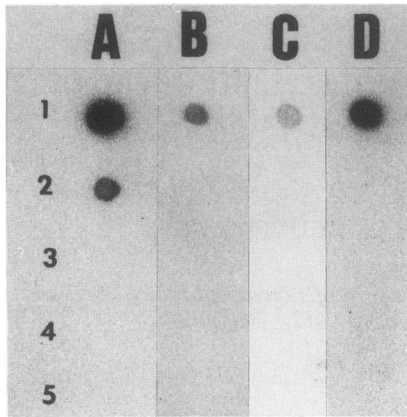


FIG. 1. Specificities of four *M. paratuberculosis* radiolabeled RNA probes toward several mycobacterial species. Chromosomal DNA samples (40 ng) from *M. paratuberculosis* 2E (row 1), *M. avium* serotype 2 (row 2), *M. bovis* BCG (row 3), *M. phlei* (row 4), and *M. intracellulare* (row 5) were denatured and spotted onto nylon filters. After irradiation with UV light, the DNA samples were hybridized with four radiolabeled RNA probes prepared by in vitro transcription from clones 7 (A), 2 (B), 26 (C), and 57 (D) and were then autoradiographed.

extracted with phenol and chloroform, precipitated with ethanol, and transcribed (11, 26). The reaction mixture contained 4 μ l of fivefold-concentrated RNA transcription buffer (200 mM Tris-HCl [pH 7.5], 30 mM MgCl₂, 10 mM spermidine, 50 mM NaCl), 2 μ l of 100 mM dithiothreitol, 20 U of RNase inhibitor; 4 μ l of 2.5 mM ATP, GTP, and UTP, 2.4 μ l of 100 μ M CTP (final concentration, 12 μ M), 1 μ g of linearized DNA template, 5 μ l of 10 mCi of [α -³²P]CTP per ml, and 10 U of SP6 RNA polymerase. After 60 min of incubation at 37°C, digestion with 1 U of RNase-free RQ1 DNase (15 min at 37°C), and phenol extraction, transcripts were purified by chromatography on a Sephadex G50 spun column (Pharmacia, Uppsala, Sweden) (21).

The 14 radiolabeled probes (5 \times 10⁸ cpm/ μ g) were hybridized by dot blotting with unlabeled DNAs from *M. bovis*, *M. phlei*, *M. avium*, *M. intracellulare*, and *M. paratuberculosis* 2E. After a prehybridization step (1 h at 60°C) in 5 \times SSC containing 50 mM Na₂HPO₄-NaH₂PO₄ (pH 6.5), 1% SDS, 250 μ g of sheared salmon sperm DNA per ml, 500 μ g of yeast RNA per ml, 0.16% bovine serum albumin, 0.16% Ficoll, and 0.16% polyvinylpyrrolidone, samples were incubated for 16 h at 60°C. The nylon filters were washed, dried, and autoradiographed. All probes hybridized with the DNA of *M. paratuberculosis*, but none hybridized with *M. bovis* or *M. phlei* DNA. Eleven probes hybridized with either *M. avium* or *M. intracellulare*, or both.

The nonspecific probe produced by in vitro transcription of clone 7 (Fig. 1A) hybridized to *M. paratuberculosis* with a greater intensity than did clones 2, 26, and 57 (Fig. 1B, C, and D, respectively). Those high-intensity signals could be due to the presence of high-copy-number fragments in the *M. paratuberculosis* genome. Such a possibility was supported by the finding that in Southern blots with *Pst*I- or *Pvu*II-digested *M. paratuberculosis* DNA, several fragments were autoradiographically identified by the clone 7 probe (data not shown). This putative repeated fragment could be an insertion element similar to those described for *M. paratuberculosis* (16) and other mycobacteria (14). Three probes that hybridized with *M. paratuberculosis* DNA alone (clones 2, 26, and 57 in Fig. 1) were analyzed further.

TABLE 1. Characteristics of the microorganisms analyzed in the present study

Bacterial species	Strain	Need for mycobactin	Source ^a	
<i>Mycobacterium</i> spp.				
<i>M. paratuberculosis</i>	2E	No	NVIN	
	316F	No	NVIN	
	19698	Yes	ATCC	
	2887 (Crohn's disease patient)	Yes	ITMA	
	2888 (Crohn's disease patient)	Yes	ITMA	
	2890 (bovine)	Yes	ITMA	
	2895 (goat)	Yes	ITMA	
	2893 (monkey)	Yes	ITMA	
	2891 (bovine)	Yes	FVM	
	<i>M. tuberculosis</i>	7787	No	ITMA
<i>M. bovis</i>	BCG	No	PIB	
<i>M. avium</i>	D4	No	NVIN	
	serotype 4	No	ITMA	
	serotype 2	No	ITMA	
	serotype 8	No	ITMA	
	A3	No	PIB	
	A84	No	PIB	
	8715	No	PIB	
	87537	No	PIB	
	<i>Corynebacterium</i> spp.			
	<i>C. renale</i>		No	ITMA
<i>C. xerosis</i>		No	ITMA	
<i>Nocardia caviae</i>				
		No	ITMA	

^a Sources of microorganisms were as follows: INRV, National Institute for Veterinary Research, Brussels, Belgium; NVIN, National Veterinary Institute, Oslo, Norway; ATCC, American Type Culture Collection, Bethesda, Md.; ITMA, Institute of Tropical Medicine, Antwerp, Belgium; PIB, Pasteur Institute of Brabant, Brussels, Belgium; FVM, Faculty of Veterinary Medicine, University of Liège, Liège, Belgium.

The specificities of the three selected probes were tested with bacterial strains of the related genera *Corynebacterium*, *Mycobacterium*, and *Nocardia* and *E. coli* (host of the bovine gut). All the probes hybridized with *M. paratuberculosis* DNA, while probe 2 hybridized with *M. avium* D4, and that from clone 26 hybridized with the *M. avium*-*M. intracellulare*-*M. scrofulaceum* strain A84 (Table 1). The remaining part of the present work was restricted to clone 57, which proved to hybridize selectively with *M. paratuberculosis*.

Hybridization of the F57 transcript to different *M. paratuberculosis* isolates. Additional controls were made by hybridization of the F57 RNA probe with the DNAs from different isolates of *M. paratuberculosis* from humans (patients with Crohn's disease), bovines (with paratuberculosis), goats, and a monkey. All the tested strains hybridized with the F57 probe (Fig. 2).

The occurrence of a single highly conserved sequence corresponding to the fragment from clone 57 was explored as follows. The chromosomal DNAs from three *M. paratuberculosis* strains were digested with *Sau*3A and *Bam*HI. After agarose gel electrophoresis, denaturation, and transfer to nylon membranes, the resulting DNA fragments were hybridized (38) with the F57 RNA probe. Identical Southern blotting patterns were obtained in all cases. When the F57 probe was hybridized with *Sau*3A-digested DNA, two fragments of 253 and 128 bp were labeled (Fig. 3, lanes 1 to 3). On the other hand, when the probe was hybridized with the

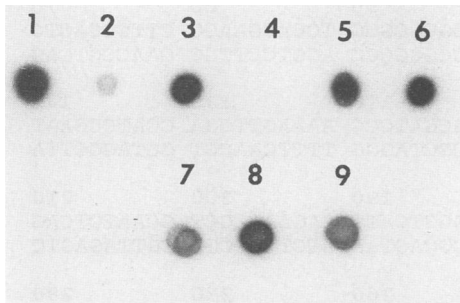


FIG. 2. Hybridization of the probe F57 with different strains of *M. paratuberculosis*. Chromosomal DNAs (40 ng) from different strains of *M. paratuberculosis* were denatured, spotted onto nylon filters, UV irradiated, hybridized with the radiolabeled F57 probe, and submitted to autoradiography. The following strains of *M. paratuberculosis* were tested: reference strain 2E (dot 1), isolates from patients with Crohn's disease (dots 2 and 3), bovines (dots 5, 6, and 7), a goat (dot 8), and a monkey (dot 9). Chromosomal DNA of *M. avium* serotype 2 was also analyzed as a negative control (dot 4).

*Bam*HI fragments, only one band with a high molecular mass (>20 kb) was revealed (Fig. 3, lanes 4 to 6), suggesting the occurrence of a single copy of the F57 sequence in the *M. paratuberculosis* genome.

Sequence determination of clone F57. Sequence analysis of the DNA insert of the recombinant clone F57 was done by the primer extension and dideoxy termination T7 DNA polymerase methods (35). For sequencing, the SP6 and T7 primers were used in a first step and two internal primers were used in a second step. The insert sequence in Fig. 4

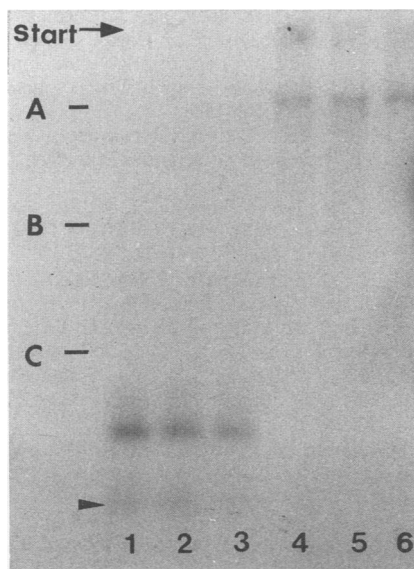


FIG. 3. Hybridization of the F57 probe with restriction fragments of chromosomal DNAs from three strains of *M. paratuberculosis*. *Sau*3A-digested (lanes 1 to 3) and *Bam*HI-digested (lanes 4 to 6) DNAs from *M. paratuberculosis* 2E (lanes 1 and 4), 316F (lanes 2 and 5), and ATCC 19698 (lanes 3 and 6) were electrophoresed on a 1% agarose gel, Southern blotted onto a nylon filter, UV irradiated, and hybridized with the radiolabeled F57 RNA probe. The arrow indicates the hybridization of a *Sau*3A restriction fragment of 128 bp. Molecular weight markers were as follows: 21,226 bp (A), 1,584 bp (B), and 564 bp (C).

shows a 620-bp DNA segment flanked by two *Sau*3A extremities. The G+C content of this segment, 58.9%, agrees with the 66% G+C content of the *M. paratuberculosis* genome. Computer-aided analysis of the F57 sequence by the COD-FICK program (PC-GENE, Intelligenetics, Inc./Betagen, Mountain View, Calif.) shows an open reading frame through the whole fragment. Searches of the EMBL and UGEN data banks yielded no known homologous sequence.

Discussion. Radioactive and nonisotopic DNA probes have repeatedly been applied to the identification of different types of pathogens, including protozoa (2, 44), viruses (9, 33, 39), bacteria (29, 37, 40), and mycobacteria (13, 15, 32, 34), in clinical samples (22). Our work described the isolation from *M. paratuberculosis* of a DNA fragment which served as a template for the production of a radiolabeled RNA probe by the SP6 in vitro transcription system (26). RNA probes are devoid of self-hybridization, which decreases the sensitivity of annealing reactions (42). Indeed, growth of mycobacteria in the stool specimen is a dispensable preliminary step, in view of the highly sensitive identification procedure, the F57 probe hybridization procedure, as preliminary work (data not shown) seems to indicate. RNA probes detected enterotoxigenic *E. coli* in clinical isolates (8).

M. paratuberculosis DNA fragments used as probes for restriction fragment length polymorphism analysis (43) showed heterologous hybridization with the DNAs of the highly homologous strains of the *M. avium-M. intracellulare-M. scrofulaceum* group (18, 45). The DNA fragment F57 described here did not hybridize with the DNA of the tested microorganisms of the *Corynebacterium*, *Mycobacterium*, and *Nocardia* group, including *M. bovis* and *M. phlei* strains isolated from bovines. This was also the case for DNA fragments cloned by others laboratories (19, 31), as expected because of the reduced level of homology of the genome for these mycobacterial species to *M. paratuberculosis* DNA (45). The unique feature of the F57 probe, however, is its lack of annealing with the closely related genomes of mycobacteria of the *M. avium-M. intracellulare-M. scrofulaceum* group (Fig. 1), which have very high levels of homology with the *M. paratuberculosis* genome (18).

A basic requirement for a diagnostic nucleic acid probe is its ability to recognize all clinical isolates within the targeted mycobacterial species. Although derived from a mycobactin-independent strain of *M. paratuberculosis* (strain 2E), the F57 DNA fragment hybridized with several mycobactin-dependent strains of *M. paratuberculosis* (Fig. 2 and 3), including the type strain *M. paratuberculosis* ATCC 19698. Moreover, F57 recognized all the *M. paratuberculosis* strains tested, irrespective of their human or animal origin (Fig. 2). On the other hand, this specific fragment, unlike the reported fragment IS900 (16), seems to be present as a single copy in the *M. paratuberculosis* genome, since only one band was recognized when the corresponding probe was hybridized to *Bam*HI-digested chromosomal DNAs from three different *M. paratuberculosis* strains (Fig. 3). The F57 sequence (Fig. 4) has no homology with known sequences, including the insertion sequence IS900 (16).

In conclusion, the present work described the cloning of an *M. paratuberculosis* DNA segment (F57) endowed with species specificity. F57 in vitro transcription with labeled precursor yielded a RNA probe with high specific activity that may be used as a diagnostic reagent for Johne's disease. This probe recognized all *M. paratuberculosis* isolates tested

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      10          20          30          40          50          60          70
GGATCTCGGC CCCGATAGCT TTCCTCTCCT TCGTCACCAA CTGGCGCGGG TCCAGGAACG CTTGGCAGCTC
CCTAGAGCCG GGGCTATCGA AAGGAGAGGA AGCAGTGGTT GACCGCGCCC AGGTCCTTGC GAACCGTGAG

      80          90          100         110         120         130         140
GTCAATCACC ACGAACACCA GCGGAACCTG CGGCGTCCGG CCGCGATCCC AAAAGTTGGA CGATCCGAAT
CAGTTAGTGG TGCTTGTGGT GCCTTGGAC GCCGCAGCCC GGGCTAGGG TTTTCAACCT GCTAGGCTTA

      150         160         170         180         190         200         210
ATGTTGTTGC CCTGTCTAAT TCGATCACGG ACTAGACCGG TCGCGTCATT CAGAATCGCT GCAATCTCAG
TACAACAACG GGACAGATTA AGCTAGTGCC TGATCTGGCC AGCGCAGTAA GTCTTAGCGA CGTTAGAGTC

      220         230         240         250         260         270         280
GCAGCTCCAG ATCGTCATTG ATGAATCGGC AGCTACGAGC ACGCAGGCAT TCCAAGTCCT GACCACCCCTT
CGTCGAGGTC TAGCAGTAAG TACTTAGCCC TCGATGCTCG TGCGTCCGTA AGGTTTCAGGA CTGGTGGGAA

      290         300         310         320         330         340         350
CCCGTCGATG ACAGCGAACT GGACCGCCGC TGACGCACCG AACGACCCCA GAGCACTCGT CAGCCACGCG
GGGCAGCTAC TGTCGCTTGA CCTGGCGGCG ACTGCGTGCC TTGCTGGGGT CTCGTGAGCA GTCGGTGGCG

      360         370         380         390         400         410         420
GTTTTCCCCG ACCCTGGTAC GCCTCCCCTT ACAACGCCCG ATACGTTCCG CAACGGGAGC GACTGGGTAGA
CAAAGGGGGC TGGGACCATG CCGAGGGTGA TGTTCGGGGC TATGCAAGCG GTTGCCCTCG CTGACCATCT

      430         440         450         460         470         480         490
CGCCCATTTT ATCGATACCC AAACCTCAGAG ACCACGAGTG GGCGGCTGGC ACAGACGACC ATTCAAGCGG
GCGGGTAAAG TAGCTATGGG TTTGAGTCTC TGGTGCTCAC CCGCCGACCG TGTCTGCTGG TAAGTTCCGC

      500         510         520         530         540         550         560
CGAACTAAGC GGATCGACAA TTCTCAGCTG CAACTCGAAC ACACCTGGGA CCGGCTCGGT GACAACAACA
GCTTGATTCC CCTAGCTGTT AAGAGTCGAC GTTGAGCTTG TGTGGACCCT GGCCGAGCCA CTGTTGTTGT

      570         580         590         600         610         620
TTCGGTACAC CAATAGCTGA CGCCAGCTTC GGAGCCGCCA CCTGCCACTG TCTGAGATCC
AAGCCATGTG GTTATCGACT GCGGTCGAAG CCTCGGCGGT GGACGGTGAC AGACTCTAGG

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FIG. 4. Base sequence of the F57 cloned fragment of *M. paratuberculosis* DNA.

but none of the other tested mycobacteria, including those of the *M. avium-M. intracellulare-M. scrofulaceum* group.

Nucleotide sequence accession number. The nucleotide sequence of the F57 DNA fragment of *M. paratuberculosis* has been submitted to the EMBL data library under the accession number X70277.

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