

## Priming reverse transcription with oligo(dT) does not yield representative samples of *Mycobacterium tuberculosis* cDNA

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**Several recent publications have suggested that oligo(dT) can prime reverse transcription of several mycobacterial mRNAs. To determine if this is the case for most *Mycobacterium tuberculosis* mRNA species, reverse transcription reactions of *M. tuberculosis* RNA were primed with oligo(dT) or with other primers that did not target polyadenylated sequences, and the resultant cDNA product was evaluated. Priming with oligo(dT) yielded more cDNA than priming with an arbitrary primer for only 1 of 12 unrelated *M. tuberculosis* genes, as measured by competitive PCR. Priming with oligo(dT) yielded cDNA for only 30% of the genes primed for by 37 *M. tuberculosis* genome-directed oligonucleotides, as assessed by hybridization of cDNA with an *M. tuberculosis* microarray. These data demonstrate that priming of reverse transcription of mycobacterial mRNA with oligo(dT) does not yield representative samples of cDNA.**

Keywords: mycobacteria, gene expression, transcription

### INTRODUCTION

Tuberculosis causes a staggering burden of morbidity and mortality, and is responsible for an estimated 2 million deaths annually worldwide (Dye *et al.*, 1999). The situation is worsened by the rising tide of drug-resistant tuberculosis and the enormous population of susceptible hosts created by the pandemic of human immunodeficiency virus infection. More effective therapeutic and preventive measures for tuberculosis are desperately needed, and these hinge on an improved understanding of the basic biology of *Mycobacterium tuberculosis*.

The evaluation of gene expression by *M. tuberculosis* under different experimental conditions provides important insight into mycobacterial physiology, and these studies have been greatly facilitated by the availability of the genome sequences of *M. tuberculosis* (Cole *et al.*,

1998; <http://www.tigr.org/tigr-scripts/CMR2/CMR-Genomes.spl>). However, analysis of mRNA expression by *M. tuberculosis* is difficult, because prokaryotic mRNA is not extensively polyadenylated, and cannot be separated from total cellular RNA and DNA by binding to an oligonucleotide containing multiple thymidine nucleotides (dT). To overcome this problem, several groups have developed complex methods to study *M. tuberculosis* gene expression, such as selective capture of transcribed sequences and customized amplification libraries (Alland *et al.*, 1998; Graham & Clark-Curtiss, 1999).

In 1986, it was demonstrated that a poly(U) Sepharose column could isolate 0.8% of the total RNA isolated from *Mycobacterium smegmatis*, suggesting that a significant percentage of the mRNA produced by this organism was polyadenylated (Katoch & Cox, 1986). More recently, two publications showed that, after total RNA from *M. tuberculosis*, *Mycobacterium bovis* BCG, *M. smegmatis* and *Mycobacterium vaccae* was reverse transcribed with an oligo(dT) primer, several mycobacterial genes could be amplified by PCR from cDNA,

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**Abbreviation:** gDNA, genomic DNA.

supporting the concept that some mRNAs of *M. tuberculosis* and other mycobacteria are polyadenylated (Adilakshmi *et al.*, 2000; Rindi *et al.*, 1998).

If all mycobacterial mRNAs are polyadenylated, oligo(dT) can be used to isolate and prime reverse transcription of mRNA, permitting accurate quantification of mycobacterial mRNA expression. On the other hand, if only a limited number of mycobacterial mRNAs are polyadenylated or if the extent of polyadenylation of different mRNAs is variable, this strategy will not provide an accurate picture of mycobacterial gene expression. To determine if priming reverse transcription with oligo(dT) yields representative samples of mycobacterial cDNA, we evaluated mRNA expression of multiple mycobacterial genes by competitive RT-PCR and by hybridization of mycobacterial cDNA to an *M. tuberculosis* microarray.

## METHODS

**Bacteria.** *M. tuberculosis* strain H37Ra (no. 25177 from the American Type Culture Collection, Manassas, VA, USA) was grown to exponential phase for 5–7 days in Middlebrook 7H9 medium (Difco) and OADC (Remel) at 37 °C and 5% CO<sub>2</sub>, prior to isolation of total RNA.

**RNA isolation.** Mycobacteria were isolated by centrifugation and resuspended at 5 × 10<sup>6</sup> bacilli ml<sup>-1</sup> in RNazol B (Tel-Test), a chaotropic solution containing guanidine thiocyanate and phenol. The mycobacteria were disrupted by shaking with glass beads in an FP120 cell disrupter (Savant Instruments) at a setting of 6.5 for 23 s, and total RNA was obtained by phenol/chloroform extraction, followed by DNase treatment.

**Quantification of mRNA expression by competitive RT-PCR.** We selected 12 *M. tuberculosis* genes that had the potential to contribute to virulence, based on published studies (Table 1). To quantify mRNA expression for these genes, we used the Omega 1.1 software program (Oxford Molecular) to design primers for each gene, based on the sequences available in the *M. tuberculosis* genome databases maintained by The Institute for Genomic Research and the Sanger Centre. The primer

sequences are shown in Table 1. The forward and reverse primers for 16S rRNA were 5'-GGACTGAGATACGGCCCAGACT-3' and 5'-CGCGACAAACCACCTACGA-3', respectively. Those for 23S rRNA were 5'-GAAACAGCCCAGATCGCC-3' and 5'-CCTACCCACACCCACCACA-3', respectively. In designing primers, the same criteria were used for each gene, thus minimizing differences in amplification efficiencies. Primers were 19–21 bp in length, with a G + C content of 60 mol% and a T<sub>m</sub> of 60–65 °C, yielding amplicons of 150–220 bp. For all genes, competitors were constructed that were amplified by the same set of primers to yield amplicons 100–200 bp larger than those of the target sequences, using the MIMIIC system (Clontech Laboratory).

cDNA was prepared from total mycobacterial RNA by reverse transcription with MMLV reverse transcriptase (Gibco-BRL), and either an arbitrary oligonucleotide primer 5'-TTGAGGT-AGGCGCGAAC-3' or a 15-mer oligo(dT) primer (both from Promega).

Tenfold serial dilutions of known amounts of MIMIIC DNA were added to 1 µl of sample cDNA and amplified by PCR, using oligonucleotide primers specific for each gene. These preliminary experiments allowed a rough estimation of the amount of template cDNA. Serial twofold dilutions of MIMIIC DNA concentrations in the range of the estimated amount of template cDNA were then coamplified with sample cDNA by PCR. In this latter titration, the integrated density of the PCR product bands has a linear relationship to the amount of PCR product, and to the amount of template. PCR amplification was performed by running 28–35 cycles, each cycle consisting of denaturation at 94 °C for 1 min and annealing/extension at 65 °C for 2 min. PCR products were subjected to electrophoresis and visualized by staining with ethidium bromide. To quantify PCR product, gels were photographed with a SPEEDLIGHT gel documentation system (B/T Scientific Technologies) and analysed with Quantity 1 software (Bio-Rad). This imaging and analysis system permits accurate comparison of the integrated density of the PCR product bands for target and MIMIIC DNA. By plotting the log of the ratio of integrated density of sample to MIMIIC PCR product against the log of the number of molecules of MIMIIC substrate DNA, the amount of substrate cDNA was determined, based on the point where the ratio of sample to MIMIIC PCR product was 1:1.

**Table 1.** Primers used for amplification of 12 mycobacterial genes

Gene	Forward primer (5'–3')	Reverse primer (5'–3')	Product size (bp)
Antigen 85C	ATCTGCTGGACGCCTTCAACG	ACTGCTTGCTGCCATCCTGC	159
<i>aroB</i>	AGGTCGCCGTTCGTGCATCAG	CCGATGCGGCCAACACCTC	165
<i>efpA</i>	GCCCTACGGGAAACCAACAA	CCACGATGACAAACGCGA	184
<i>ideR</i>	CGATGTCATCGGGTTGCC	ACGCCAAGTTCACCAGG	159
<i>katG</i>	CCGCCATAGAGAAAGCAGCA	CCACCTACCAGCACCGTCA	241
<i>phoP</i>	CGACGAGGAGACCCACGAA	TGCGGCGCAGATACGACA	197
<i>RNC</i>	GCCGACAAGTCCAGCATTCT	AATCCAATCCCACACCA	151
<i>sigA</i>	GTACAGGCCAGCCTCGAT	CCGATGACGACGAGGAGAT	209
<i>sigB</i>	TTCATCGAGGACGCCGAA	GATCTGACGAACCCGCTCA	207
<i>sirR</i>	TCTGAAGTTCATCTGGACCGCC	TCAACGTACCCGCGCCGTA	164
<i>sodA</i>	CTTACCACAGCAAGCACCA	GTCACCACCGTTAGGCGACA	189
<i>virS</i>	GACCTCATCGAGCGCGAA	ACCGCTGACCCACCATA	180

**Microarray analysis.** Total mycobacterial RNA was isolated as described above. The RNA was reverse transcribed in the presence of Cy3-dCTP (Amersham Pharmacia Biotech), 0.1 M DTT, 10  $\mu$ M dNTP except for 2.5  $\mu$ M dCTP (Gibco-BRL), Superscript II (Gibco-BRL) and its buffer, and either the oligo(dT) or genome-directed primers, which were a mixture of 37 primers specifically designed to amplify all the ORFs in the *M. tuberculosis* genome (Talaat *et al.*, 2000). Reverse transcription was performed at room temperature for 10 min, followed by 42 °C for 2 h. Unincorporated dye was removed by purifying the cDNA with QiaQuick purification kits (Qiagen) according to the manufacturer's protocol. Mycobacterial genomic DNA was nick-translated to generate DNA fragments of approximately 500 bp (gDNA), and 2  $\mu$ g DNA was labelled with Cy5 fluorescent dye, according to the manufacturer's protocol (Promega). The Cy3-labelled cDNA was co-hybridized with Cy-5 labelled *M. tuberculosis* gDNA to an *M. tuberculosis* microarray that contained oligonucleotides (Operon Technologies) representing the 3924 ORFs present in the *M. tuberculosis* genome, as previously described (Kane *et al.*, 2000; Call *et al.*, 2001). Slides were allowed to hybridize overnight at 67 °C before washing in low-stringency buffer ( $1 \times$  SSC/0.1% SDS) for 5 min at room temperature, followed by another 5 min wash in a high-stringency buffer ( $0.1 \times$  SSC) and drying by centrifugation at 1000 r.p.m. for 5 min. Dry slides were scanned at 10 micropixel intensity (GenePix4000, Axon Instruments).

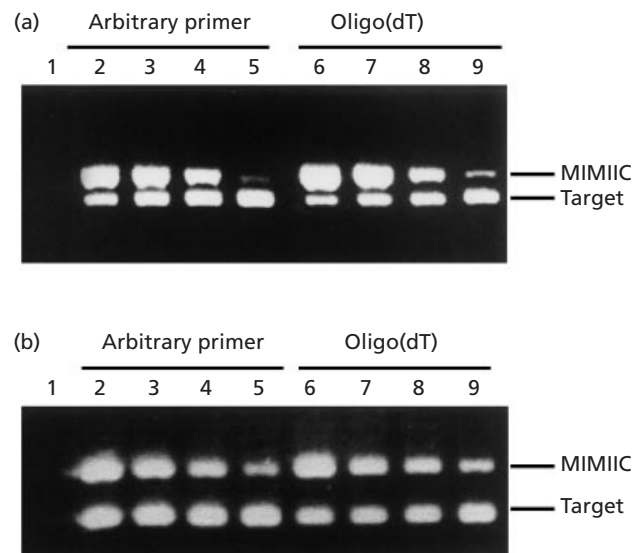
The signal and background Cy3 and Cy5 fluorescence intensities were calculated for each DNA spot, using image analysis software (GenePix 3.0 Pro, Axon Instrument), to average the intensities of every pixel inside the target region. The intensity of each spot was calculated as the difference between mean signal intensity and mean local background intensity (segmentation method). The ratio of intensity for Cy3- to Cy5-labelled probes was determined for each DNA spot, reflecting the abundance of Cy3-labelled cDNA relative to Cy5-labelled gDNA. A ratio of 1 or greater was considered to represent a positive signal where the mRNA of a particular gene was expressed above the hybridization signal generated from its counterpart gDNA. All hybridizations were repeated two to four times before inclusion in the analysis.

## RESULTS

### Oligo(dT) is not more effective than an arbitrary primer in initiating cDNA synthesis of 12 *M. tuberculosis* genes

Previous studies have shown that oligo(dT) can prime cDNA synthesis for several mycobacterial genes (Adilakshmi *et al.*, 2000; Rindi *et al.*, 1998). We compared the efficiency of oligo(dT) and an arbitrary primer in initiating cDNA synthesis. If most mycobacterial mRNAs are polyadenylated, the oligo(dT) primer should bind to most mRNA species much more efficiently than the arbitrary primer, resulting in a far greater yield of cDNA.

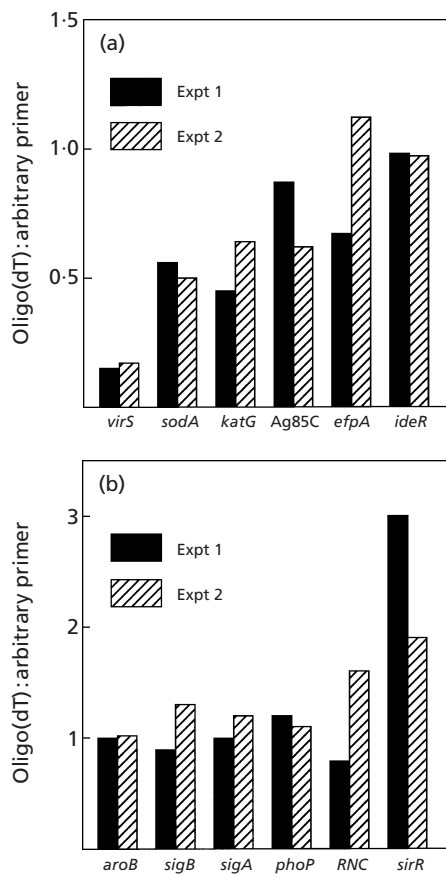
RNA was extracted from two separate cultures of *M. tuberculosis*, and each RNA sample was divided into equal aliquots. Each aliquot was reverse transcribed to cDNA, using either the oligo(dT) primer or the arbitrary primer. The resultant cDNA was amplified by com-



**Fig. 1.** Amplification of mycobacterial cDNA by competitive PCR, after priming reverse transcription of RNA with an arbitrary primer or with oligo(dT). (a) Results for amplification using primers for the *sigA* gene. Equal aliquots of an RNA preparation were reverse transcribed with an arbitrary primer (lanes 2–5) or with oligo(dT) (lanes 6–9), and amplified by competitive PCR. Lane 1, RNA template without reverse transcription; lanes 2–5, RNA template was reverse transcribed with an arbitrary primer, and MIMIIC competitors were added in 10-fold serial dilutions, beginning in lane 2; lanes 6–9, RNA template was reverse transcribed with oligo(dT), and MIMIIC competitors were added in 10-fold serial dilutions, beginning in lane 6. (b) Results for amplification using primers for the gene encoding antigen 85C. Lane 1, RNA template without reverse transcription; lanes 2–5, RNA template was reverse transcribed with an arbitrary primer, and the MIMIIC competitors were added in twofold serial dilutions, beginning in lane 2; lanes 6–9, RNA template was reverse transcribed with oligo(dT), and the MIMIIC competitors were added in twofold serial dilutions, beginning in lane 6.

petitive PCR, using primers for the 12 *M. tuberculosis* genes in Table 1. Serial dilutions of known quantities of MIMIIC DNA were added to template cDNA. Representative results are shown in Fig. 1 for *sigA* and for the gene encoding antigen 85C.

Image analysis software was used to compare the integrated density of the PCR product bands for target and MIMIIC DNA and to calculate the amount of template cDNA for each gene produced by reverse transcription with the oligo(dT) primer or the arbitrary primer. These amounts were expressed as a ratio (Fig. 2). For eight genes, the amounts of cDNA obtained after priming with oligo(dT) were comparable to those obtained after priming with the arbitrary primer. For three genes (*virS*, *sodA* and *katG*), priming with oligo(dT) yielded less cDNA than priming with the arbitrary primer (Fig. 2a). Only in the case of *sirR* did priming with oligo(dT) yield more cDNA (Fig. 2b). Therefore, for most of the genes tested, the amount of

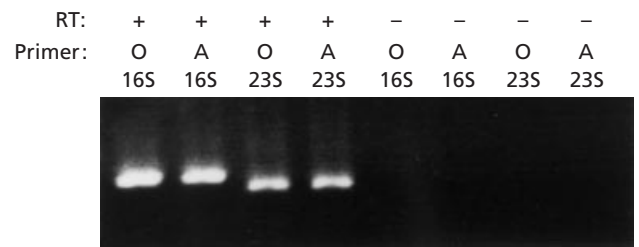


**Fig. 2.** Results of amplification of cDNA for 12 *M. tuberculosis* genes by competitive PCR, after priming reverse transcription of RNA with oligo(dT) or with an arbitrary primer. Equal aliquots of two RNA preparations were reverse transcribed with oligo(dT) or with an arbitrary primer, and amplified by PCR. The ratio of template cDNA after priming with oligo(dT) versus priming with an arbitrary primer is shown on the y-axis. Panels (a) and (b) each show results for six *M. tuberculosis* genes.

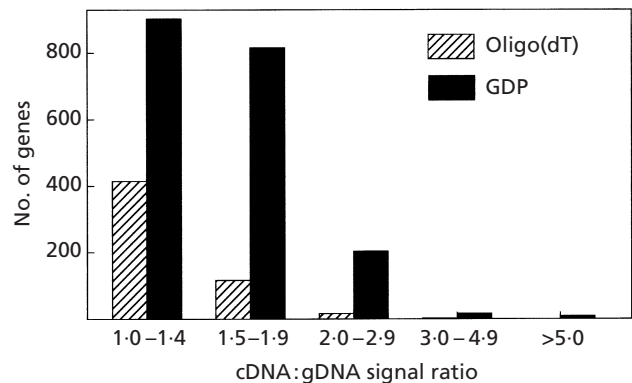
cDNA produced by priming with oligo(dT) was the same or less than that produced by priming with an arbitrary primer.

#### Oligo(dT) primes reverse transcription of rRNA

Recent studies documented polyadenylation in approximately 10% of 23S rRNA of *Escherichia coli* but in only 0.6% of 16S rRNA (Mohanty & Kushner, 2000). If these findings are similar for *M. tuberculosis*, and if reverse transcription with the oligo(dT) primer depends primarily on binding to polyadenylated sequences, 16S or 23S rRNA should be reverse transcribed to a much lesser extent by oligo(dT) than by the arbitrary primer. However, when equal amounts of total *M. tuberculosis* RNA were reverse transcribed by oligo(dT) or the arbitrary primer, the amounts of cDNA for the 16S and 23S rRNA were similar (Fig. 3).



**Fig. 3.** Amplification of cDNA made from mycobacterial 16S and 23S rRNA, after priming reverse transcription with oligo(dT) or with an arbitrary primer. Equal aliquots of an RNA preparation were reverse transcribed with oligo(dT) or with an arbitrary primer, and amplified by PCR with primers for the cDNA for 16S rRNA or 23S rRNA (lanes 1–4). Aliquots of RNA were also amplified in the absence of reverse transcription (lanes 5–8). RT, reverse transcription; O, oligo(dT); A, arbitrary primer.



**Fig. 4.** Microarray analysis of mycobacterial cDNA, after priming reverse transcription of *M. tuberculosis* RNA with oligo(dT) or with genome-directed primers. The cDNA was hybridized to an *M. tuberculosis* microarray. The number of genes with cDNA/gDNA signal ratios of 1 or greater is shown.

#### Microarray analysis demonstrates that a limited fraction of mycobacterial mRNAs is amplified by oligo(dT)-primed cDNA synthesis

As an alternative means to evaluate the extent of polyadenylation of *M. tuberculosis* mRNA, mycobacterial RNA was reverse transcribed with either the oligo(dT) primer or the genome-directed primers. The resultant cDNA was hybridized to an *M. tuberculosis* microarray, and the efficiency of the two methods was compared by the signal ratios of cDNA/gDNA obtained by priming with oligo(dT) or with genome-directed primers (Fig. 4). The same batch of labelled gDNA was used in both hybridizations to normalize for different hybridization efficiencies and different scanning settings. When total RNA was primed with oligo(dT), 550 (14%)

of the 3924 predicted mycobacterial ORFs had signal ratios  $\geq 1$ , the highest value being 3.9. Expression of 114 genes was detected by priming with oligo(dT) but not by priming with genome-directed primers. In contrast, when the same batch of RNA was primed with the genome-directed primers, 1847 (47%) of the genes had signal ratios  $\geq 1$ , the highest ratio being 14.5. This included 1411 genes for which expression was not detected by priming with oligo(dT). Overall, more genes with higher signal intensity were detected by priming with the genome-directed primers than with oligo(dT). If mycobacterial mRNA were extensively polyadenylated, most ORFs would be more efficiently reverse transcribed with oligo(dT). Therefore, these data suggest that the majority of *M. tuberculosis* mRNAs are not extensively polyadenylated.

## DISCUSSION

Our understanding of the biology of pathogenic organisms has been greatly enhanced by evaluation of mRNA expression under different conditions. Previous studies have shown that oligo(dT) can prime cDNA synthesis for mRNA of the mycobacterial genes *hsp65*, *entD*, *entC* and *trpE2* in *M. bovis* BCG, *M. smegmatis* and *M. vaccae* (Adilakshmi *et al.*, 2000), as well as *hsp65* and the gene encoding antigen 85C in *M. tuberculosis* and *M. bovis* BCG (Rindi *et al.*, 1998). In this study, we used competitive PCR to show that the amount of cDNA produced by priming with oligo(dT) was the same as or less than that produced by priming with an arbitrary oligonucleotide for 11 of 12 *M. tuberculosis* genes, including the gene encoding antigen 85C. Microarray analysis showed that genome-directed oligonucleotides primed cDNA synthesis for more *M. tuberculosis* genes than did oligo(dT). These data demonstrate that priming reverse transcription of *M. tuberculosis* RNA with oligo(dT) does not yield a representative sample of mycobacterial cDNA.

Although prokaryotic mRNA has traditionally been regarded as lacking 3' polyadenylated sequences, more recent studies have demonstrated that polyadenylation occurs in a variety of bacteria, including *E. coli* and *Bacillus* species (Sarkar, 1996, 1997). Bacterial polyadenylated tracts are 10–60 nucleotides long, significantly shorter than the 80–200 nucleotides found in eukaryotic cells. In addition, for any single mRNA species, only 2–50% of mRNA molecules are polyadenylated, compared to 100% in the case of eukaryotes (Sarkar, 1997). Polyadenylation is believed to be important for stability, maturation and translation of mRNA in eukaryotes. In contrast, a growing body of evidence suggests that polyadenylation destabilizes certain bacterial mRNA species with stem-loop structures, contributing to their degradation (Sarkar, 1997).

Hybridization with an *M. tuberculosis* microarray revealed that oligo(dT) primed cDNA synthesis for only 550 genes, whereas priming with genome-directed primers yielded hybridization signals for 1847 genes. It is possible that the number of mRNAs primed by oligo(dT)

was underestimated because the large amount of rRNA may depress cDNA synthesis from mRNA templates. In addition, we only considered a significant amount of cDNA to have been synthesized if hybridization of cDNA to the microarray equalled that of an arbitrary amount of gDNA. This method reduces the number of non-specific signals, but it may underestimate the number of mRNAs for which oligo(dT) can prime reverse transcription.

Although the 3' end of mycobacterial mRNA has not been sequenced, it has been hypothesized that polyadenylation is present, because oligo(dT) can prime cDNA synthesis for several mycobacterial genes, which can then be amplified by non-competitive PCR (Adilakshmi *et al.*, 2000; Rindi *et al.*, 1998). Although we confirmed these results, we found that the quantities of cDNA synthesized after priming with oligo(dT) or with an arbitrary primer were very similar, arguing against the presence of extensively polyadenylated mycobacterial mRNA, which would be more efficiently reverse transcribed by oligo(dT).

The mechanism by which oligo(dT) primes reverse transcription of mycobacterial mRNA is uncertain. We speculate that the mRNA is reverse transcribed by non-specific binding of the oligo(dT) primer to the RNA template. When oligo(dT) is used to prime reverse transcription of eukaryotic mRNA, selective binding to the mRNA polyadenylated tail minimizes random binding to other segments of mRNA and rRNA. However, even under these conditions, rRNA is occasionally reverse transcribed. When oligo(dT) is used to prime reverse transcription of mycobacterial mRNA, which is not extensively polyadenylated, genes and rRNA with segments of several contiguous adenosines may function as the favoured binding sites for oligo(dT) at the relatively low temperature of 37 °C used for reverse transcription.

Our results do not exclude the possibility that oligo(dT) primes reverse transcription of mycobacterial mRNA by specific binding to the polyadenylated 3' ends, and that a variable percentage of *M. tuberculosis* mRNA species are polyadenylated. This could explain the variable priming efficiency of oligo(dT) for different mycobacterial mRNAs, compared to a random primer (Fig. 2). In addition, microarray analysis revealed that oligo(dT) primed reverse transcription of 114 genes more efficiently than genome-directed primers, suggesting that these genes may be polyadenylated. However, if variable degrees of limited polyadenylation are present, reverse transcription with oligo(dT) cannot be used to obtain a representative assessment of mRNA expression under different conditions or by different *M. tuberculosis* strains. Such studies are best pursued by using a mixture of primers that are specifically designed to amplify all the ORFs in the *M. tuberculosis* genome (Talaat *et al.*, 2000). Alternatively, because some mRNAs were amplified by oligo(dT) but not by the genome-directed primers, a combination of oligo(dT) and genome-directed primers may yield the most com-

prehensive collection of cDNAs. Others have used a combination of oligo(dT) and multiple arbitrary primers to perform mRNA differential display in two *M. tuberculosis* strains (Rindi *et al.*, 1999).

In summary, using competitive PCR and microarray analysis, we found that priming of reverse transcription of mycobacterial mRNA species with oligo(dT) amplifies a limited fraction of mycobacterial mRNAs, and does not yield representative samples of cDNA.

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