Polyadenylation in mycobacteria: evidence for oligo(dT)-primed cDNA synthesis

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The ability of mRNA to direct synthesis of cDNA in the presence of oligo(dT) was analysed using a novel application of fluorescein-11-dUTP incorporation into cDNA by reverse transcriptase. Evidence is provided for the first time that a majority of the mycobacterial mRNA pool is polyadenylated. mRNA transcripts of *hsp65* were also amplified with specific primers from the oligo(dT)-primed cDNA preparation in *Mycobacterium bovis* BCG, *M. smegmatis* and *M. vaccae*. Furthermore, PCR amplification of cDNAs for genes *entD*, *entC* and *trpE2* from *M. bovis* BCG yielded the expected products when reverse transcription was primed with oligo(dT), suggesting that polyadenylation is a general phenomenon in mycobacteria.

Keywords: mycobacteria, polyadenylation, mRNA, oligo d(T), fluorescein-11-dUTP

INTRODUCTION

Post-transcriptional modification of the mRNA precursor is an essential step in mRNA maturation in eukaryotes, and defects in mRNA 3'-end formation can profoundly alter cell viability, growth and development (Zhao *et al.*, 1999). In most cases, poly(A) elongation confers translational activation while de-adenylation promotes translational silencing (Richter, 1999). There is growing evidence for the occurrence of polyadenylated mRNAs in a variety of prokaryotes (Sarkar, 1997). Unlike poly(A) tracts in eukaryotic mRNA, which range between 80 and 200 nucleotides, poly(A) tracts in prokaryotes range between 14 and 60 nucleotides and, moreover, polyadenylation occurs at all unprotected RNA 3'-termini irrespective of their secondary structure. The functions of bacterial RNA polyadenylation are quite diverse, ranging from the control of plasmid replication to the modulation of mRNA stability, as well as a possible role in mRNA translation (Sarkar, 1997). The discovery of the oligo(dT)-dependent synthesis of DNA complementary to bacterial mRNA (cDNA) by reverse transcriptase was an important advance in the study of bacterial poly(A) RNA, which paved the way for subsequent analysis at the molecular level. Reverse transcriptase requires a primer that hybridizes to the mRNA prior to cDNA synthesis. The oligo(dT) hybridizes to the 3'-poly(A) tail of the mRNA and allows the enzyme to initiate cDNA synthesis. We report here evidence for oligo(dT)-

dependent cDNA synthesis in three species of mycobacteria by using RT-PCR. We also describe the incorporation of fluorescein-11-dUTP into cDNA as a novel application for fluorescein. This reaction can be performed as a useful control to check the integrity of a bulk mRNA preparation in construction of a cDNA library, since the ability of mRNA to direct synthesis of long molecules of first-strand cDNA is a sign of mRNA integrity. Fluorescein-11-dUTP is commonly used for 3'-end labelling of oligonucleotides (Cunningham *et al.*, 1996) and for *in situ* hybridization (Wiegant *et al.*, 1991).

The *entC* and *entD* genes that have been used in this work were selected because of their possible involvement in iron utilization (Cole *et al.*, 1998). We presume that these genes are involved in the biosynthesis of salicylic acid, which occurs as a response of mycobacteria to deprivation of iron (Ratledge, 1999; Adilakshmi *et al.*, 2000). Another gene, *mbtI* (*trpE2*) was also included in this study as Quadri *et al.* (1998) suggested that the protein product of this gene from *Mycobacterium tuberculosis* was homologous to salicylate- and anthranilate-forming enzymes from various other bacteria and therefore could be responsible for salicylate formation. In addition, *hsp65*, encoding a well-characterized 65 kDa antigen in mycobacteria (Shinnick, 1987), was used.

METHODS

Mycobacterial strains and growth conditions. *Mycobacterium bovis* BCG (Glaxo strain, batch P691), *M. smegmatis* strain mc²155 and *M. vaccae* R877R were grown in

Abbreviation: DEPC, diethylpyrocarbonate.

Fig. 1. Total RNA from *M. bovis* BCG separated by non-denaturing agarose gel electrophoresis (1.5%, w/v) and stained with 0.5 µg ethidium bromide ml⁻¹. Lanes: M, 100 bp DNA ladder; 1, RNA (2 µg) purified from *M. bovis* BCG.

minimal medium containing (g l⁻¹) KH₂PO₄, 5; glycerol, 10; and asparagine, 5; pH 7.6. Prior to inoculation, the medium was supplemented with (µg ml⁻¹) Zn²⁺, 0.45; Mn²⁺, 0.1; Mg²⁺, 40; and Fe²⁺, 0.05, which gives iron-deficient growth and allows expression of genes involved in iron uptake and metabolism. Cultures were grown at 37 °C on an orbital shaker at 200 r.p.m.

Genomic DNA isolation. Genomic DNA was isolated from mycobacteria according to the method of Gonzalez-y-Merchand *et al.* (1996). Cells were collected from growth medium by centrifugation at 4000 *g* for 10 min and resuspended in 10 µl lysis buffer per mg cells (wet wt). Lysis buffer was 6 M guanidinium chloride, 1 mM 2-mercaptoethanol, 10 mM EDTA and 0.1% Tween 80. The cell suspension was subjected to two cycles of freeze-thawing by placing it in liquid N₂ or ethanol/CO₂ freezing mixture for 1 min and then transferred to a water bath at 65 °C for 10 min. The resulting suspension was chilled on ice for 5 min and extracted twice with 2 vols chloroform/isoamyl alcohol, (24:1, v/v). Two volumes of cold ethanol were added gradually and mixed gently by inverting the tube. This mixture was left at -20 °C for 30 min. The DNA precipitate was recovered by centrifugation and dissolved in TE buffer (10 mM Tris/HCl, 1 mM EDTA; pH 7.5) and stored at -20 °C for further use.

RNA extraction. The method of Rajagopalan *et al.* (1995) was used to extract total RNA. One millilitre of the culture (OD₆₀₀ 0.5) was harvested, and centrifuged for 5 min at 4000 *g* at 4 °C; the supernatant was discarded and 1.5 ml Trizol reagent (phenol and guanidinium thiocyanate in a monophasic solution; Life Technologies) was added to the cell pellet. The suspension was ultrasonicated (Dawe Soniprobe) for 20 s followed by three cycles of freezing in liquid N₂ and thawing at 4 °C. The cell debris was removed by centrifugation for 2 min at 4000 *g*. The supernatant was extracted with 0.75 ml chloroform. RNA was precipitated by adding 0.6 ml propan-2-ol and incubating for 10 min at room temperature. The RNA pellet was recovered by centrifugation at 12000 *g* for

15 min at 4 °C. It was then washed once with 70% (v/v) ethanol prepared in diethyl pyrocarbonate (DEPC)-treated water and resuspended in 40 µl RNase-free, DEPC-treated water using the method of Sambrook *et al.* (1989). RNA was quantified in a GeneQuant II analyser (Pharmacia Biotech) by measuring A₂₆₀. The A₂₆₀/A₂₈₀ ratios for the RNA preparation were in the range 1.8–2.0.

DNase treatment. RNA (1 µg) was treated with 5 units DNase I (Life Technologies) and incubated at 25 °C for 10 min to remove contaminating chromosomal DNA. EDTA (1 µl of 25 mM) was then added and the enzyme inactivated by heating to 65 °C for 15 min. The reaction mix was extracted with chloroform; the aqueous phase was precipitated with chilled propan-2-ol and centrifuged at 12000 *g* for 10 min at 4 °C. The supernatant was discarded and the pellet was once again washed with 70% (v/v) ethanol and reprecipitated at 12000 *g* for 10 min at 4 °C. The pellet was then resuspended in 20 µl DEPC-treated water and requantified using the GeneQuant II analyser. The integrity of the RNA was checked on a 1.5% non-denaturing agarose gel as described by Mahenthiralingam (1998).

cDNA synthesis. Reverse transcription into cDNA was performed in 50 µl containing 1 µg RNA, 2 µg oligo(dT₁₂₋₁₈), 20 units RNasin (both from Pharmacia), 10 mM DTT, 10 µl 5× nucleotide mix (dATP, dGTP, dCTP, dTTP and fluorescein-11-dUTP; Amersham), 10 units Superscript RNase H⁻ reverse transcriptase (Life Technologies) and an appropriate volume of DEPC-treated water. The mixture was incubated for 1 h at 37 °C, chilled rapidly on ice and stored at -20 °C.

Southern blotting. cDNA samples were electrophoresed at 100 V through a 0.8% agarose minigel in 1× TBE (45 mM Tris/borate, 1 mM EDTA). The cDNA could be seen directly under UV as a green fluorescent smear. However, resolution was better when it was stained with 0.5 µg ethidium bromide ml⁻¹ for 30 min. The transfer of DNA from the gel to a nylon membrane was carried out overnight in 10× SSC (3 M NaCl, 0.3 M sodium citrate, pH 7.0) by capillary blotting and the DNA was fixed by UV cross-linking (UV Stratalinker, Stratagene).

Detection of cDNA. The membrane was briefly rinsed with buffer A (100 mM Tris/HCl, 600 mM NaCl; pH 7.5) (2 ml cm⁻²) and incubated in anti-fluorescein-horse radish peroxidase antibody conjugate (ECL; Amersham), diluted 1000-fold in freshly prepared 0.5% (w/v) bovine serum albumin fraction V in buffer A for 30 min. Unbound conjugate was removed by washing the membrane for 3×10 min periods in excess buffer A containing 0.1% (v/v) Tween 20. The washed membrane was treated with detection solution mixture according to the manufacturer's instructions and exposed for 1 h to Hyperfilm (Amersham), which was then developed.

PCR. The technique of message-amplification phenotyping (mRNA phenotyping), an application of the PCR technique, was used to detect the mRNA transcripts of *hsp65*, one of the well-characterized genes of mycobacterial metabolism (Shinnick, 1987), and *entD*, *entC* and *trpE2* (see Introduction). Primers were designed using the Oligo 5.0 software and obtained from MWG-Biotech. All the other reagents used in the PCR reaction were from Life Technologies. The PCR mixture contained 2.5 µl nascent cDNA, 2.5 µl 10× PCR buffer, 1.5 mM MgCl₂, 200 µM each of the dNTPs, 0.5 µg each of oligonucleotide primers and 2 units *Taq* DNA polymerase, and was made up to a final volume of 25 µl with sterile distilled water. Amplification was performed on a Peltier thermocycler (MJ Research) using the following thermocycle: (94 °C, 1 min; 55 °C, 1 min; 72 °C, 2 min)×30 cycles; (72 °C,

10 min) $\times$ 1 cycle. DNA bands were visualized on agarose gel electrophoresis and photographed using the UVIPhoto V.97 software on Windows 95. The identity of the PCR products was confirmed by restriction enzyme analysis. All restriction enzymes were from Life Technologies.

RESULTS

Total RNA was prepared from *M. bovis* BCG, *M. smegmatis* (mc²155) and *M. vaccae* as described in Methods. Low-iron ($0.05 \mu\text{g ml}^{-1}$) growth medium was used to ensure the full expression of *entD*, *entC* and *trpE2*. To assess the quality of the RNA, a small aliquot ($2 \mu\text{g}$) of the preparation was electrophoresed on a 1.5% non-denaturing agarose gel. The appearance of the sharp bands corresponding to the 23S and 16S rRNA indicates the integrity of the RNA (Sambrook *et al.*, 1989) (Fig. 1). To confirm the integrity of the bulk mRNA preparation without the use of radioisotopes, we used fluorescein-11-dUTP as a nucleotide precursor for cDNA synthesis catalysed by reverse transcriptase, since the ability of mRNA to direct the synthesis of long molecules of first-strand cDNA is a sign of its integrity. To study whether polyadenylation is a universal

phenomenon in mycobacteria, cDNA was prepared in the presence and absence of oligo(dT). A cDNA preparation with random nonamer primers was used as a positive control and a cDNA reaction mix without mRNA was used as a negative control. The cDNA was then analysed on 0.8% agarose gel and visualized under UV light after staining with ethidium bromide. A continuous smear of cDNA can be observed starting from 500 bp to $>4 \text{ kb}$ (Fig. 2a, lanes 4 and 5) and no bands were observed in the negative controls (Fig. 2a, lanes 2 and 3).

The gel was blotted on to a nylon membrane, which was treated with anti-fluorescein specific antibody and then exposed to Hyperfilm. The smear was seen at positions corresponding to positive controls (Fig. 2b, lanes 4 and 5) whereas no bands could be detected at the negative control positions (Fig. 2b, lanes 2 and 3). It is evident that the cDNA preparation made in the presence of oligo(dT) (lane 4) reflects the complete integrity of mRNA equivalent to that observed with random priming. Synthesis of cDNA appears to be primed by oligo(dT), suggesting the occurrence of a polyadenylated mRNA population in mycobacteria.

Fig. 2. Synthesis of cDNA in presence and absence of oligo(dT) or random nonamer primer. (a) The cDNA prepared from RNA in the presence of oligo(dT) was separated on a 0.8% agarose gel and stained with $0.5 \mu\text{g ethidium bromide ml}^{-1}$. Lanes: 1, λ -HindIII marker; 2, cDNA reaction mix without mRNA (negative control); 3, cDNA reaction mix without oligo(dT) (negative control); 4, cDNA preparation with random nonamer primers (positive control); 5, cDNA in presence of oligo(dT). (b) Immunoblotting of cDNA with anti-fluorescein-horseradish peroxidase conjugate antibody. Lanes as in (a).

Table 1. PCR primers, position of primers, product sizes and restriction enzyme analysis of the genes used in this study

Gene	Primers (5'–3')	Nucleotide positions in the gene	Expected product size (bp)	Restriction enzyme and product size (bp)
<i>hsp65</i>	ACC AAC GAT GGT GTG TCC AT (Forward) CTT GTC GAA CCG CAT ACC CT (Reverse)	396–415 817–836	441	<i>Hinf</i> I (385 + 56)
<i>entD</i>	GGC GTG CGT AAC CAC CGA TTG CTA CT (Forward) GCA GAT CCT GAT CGA GGC GGT GGG CA (Reverse)	4–29 512–537	534	<i>Hinf</i> I (298 + 236)
<i>entC</i>	TTG CAC CCA GAA CCA CCG TTC GCA CT (Forward) TTG CTC GCA CCT GAT AGA CAG CAC CC (Reverse)	22–47 959–984	963	<i>Not</i> I (599 + 364)
<i>trpE2</i>	TAA GGC GTC AAT GTG GAT AGC TTT T (Forward) ATG GTG GAG CTA GAC AGC GAC GAA C (Reverse)	236–261 1359–1384	1148	<i>Bam</i> HI (301 + 847)

Fig. 3. Electrophoresis of cDNA corresponding to *hsp65* mRNA transcript amplified by RT-PCR with gene-specific primers listed in Table 1 from *M. bovis* BCG (lanes 1–4), *M. smegmatis* (lanes 5–8) and *M. vaccae* (lanes 9–12). Lanes 1, 5 and 9, cDNA preparation without RT; lanes 2, 6 and 10, cDNA preparation without oligo(dT); lanes 3, 7 and 11, cDNA with oligo(dT); lanes 4, 8 and 12: genomic DNA of respective species; M, 100 bp size marker.

Specific mRNA transcripts of *hsp65* were amplified with gene specific primers (Table 1) in three species of mycobacteria, *M. bovis* BCG, *M. smegmatis* and *M. vaccae* (Fig. 3). As expected, a 441 bp sequence corresponding to the *hsp65* product was observed. It is clear that the amplification of *hsp65* was markedly pronounced in the cDNA prepared in presence of oligo(dT) alone (lanes 3, 7 and 11) and was equivalent to that obtained with genomic DNA amplification (lanes 4, 8 and 12).

Further, mRNA transcripts of *entD*, *entC* and *trpE2* were amplified from *M. bovis* BCG by RT-PCR using the gene specific primers shown in Table 1 and, again, transcripts could be detected in cDNA preparations made in presence of oligo(dT) (Fig. 4, lanes 3, 5 and 7) but not in the absence of oligo(dT) (lane 2). Negative controls were obtained for *entC* and *trpE2* transcripts as well (data not shown). Again, the size of the RT-PCR product was equal to that amplified from the genomic

DNA preparation (Fig. 4, lanes 4, 6 and 8). The RT-PCR products for *hsp65*, *entD*, *entC* and *trpE2* were confirmed by restriction enzyme analysis as shown in Fig. 5. The products match those listed in Table 1, establishing the identity of the PCR products.

DISCUSSION

In the construction of a cDNA library, the first step consists in the extraction of mRNA and its reverse transcription into cDNA. During these procedures, it is important to control the integrity of the mRNA preparation before it is used as a template for the synthesis of the first strand of cDNA. We describe here a non-radioactive method to check the integrity of mRNA by analysing its ability to direct the synthesis of first-strand cDNA in the presence of fluorescein-11-dUTP. The method of labelling cDNA with fluorescein-11-dUTP is as effective as the radioactive method and it may be

Fig. 4. Electrophoresis of *entD*, *entC* and *trpE2* mRNA transcripts amplified by RT-PCR from *M. bovis* BCG amplified with gene-specific primers listed in Table 1. Lanes: 1, cDNA reaction mix without RT and amplified with primers for *entD* (negative control); lane 2, cDNA reaction mix without oligo(dT) and amplified with primers for *entD* (negative control); lanes 3 & 4, PCR of *entD* carried out with cDNA preparation made in the presence of oligo(dT) and genomic DNA respectively; lanes 5 & 6, PCR of *entC* carried out with cDNA preparation made in the presence of oligo(dT) and genomic DNA respectively; lanes 7 & 8, PCR of *trpE2* carried out with cDNA preparation made in the presence of oligo(dT) and genomic DNA respectively; M, 100 bp size marker.

Fig. 5. Restriction enzyme analysis of RT-PCR products of *hsp65*, *entD*, *entC* and *trpE2* of *M. bovis* BCG. Lanes: 1, *hsp65*; 2, *HinfI* digest of *hsp65*; 3, *entD*; 4, *HinfI* digest of *entD*; 5, *entC*; 6, *NotI* digest of *entC*; 7, *trpE2*; 8, *BamHI* digest of *trpE2*.

preferred because the hazards related to the use of radioactive compounds are eliminated. Owing to the high G + C content of mycobacterial DNA, one might expect self-priming to occur due to the folding back of mRNA. However, we could not detect any cDNA synthesized in the absence of either primer [random/oligo(dT)], thus ruling out a self-priming mechanism. As expected in the presence of random primers, many smaller fragments were evident in the cDNA preparation resulting from incomplete synthesis (Fig. 2b).

Little is known about gene expression patterns of mycobacteria; the definition of differentially expressed genes is central to understanding pathogenesis and virulence at a molecular level. The application of techniques such as differential display and subtractive hybridization, commonly used for the analysis of eukaryotic gene expression, has been limited in prokaryotes, owing to the generally accepted view that 3'-polyadenylation of mRNA is a eukaryotic feature. Differential display in prokaryotes therefore requires the use of non-specific, random arbitrary primers (RAP) for cDNA synthesis, often involving extensive testing and optimization of primers (Kwaik & Pederson, 1996; Fislage *et al.*, 1997; Rivera-Marrero *et al.*, 1998). In addition, reamplification by PCR and subcloning of the isolated cDNA fragments is often difficult and results in failure. The main problem found with the RAP-PCR method is that, due to the abundance of rRNA and variations in the amount of rRNA in RNA preparations from different strains, amplification with arbitrary primers often results in a high number of false positives. Our finding therefore represents a significant advance in the study of gene expression in mycobacteria, as oligo(dT)-primed cDNA synthesis eliminates the pitfalls encountered with techniques such as RAP-PCR.

Sequential addition of adenylate residues to 3'-termini of RNA molecules is catalysed by the enzyme poly(A) polymerase (ATP:polyribonucleotide adenylyltransferase, EC 2.7.7.19). Multiple isoforms of poly(A) polymerases, probably derived from a single gene by alternate splicing or post-translational modification, occur in eukaryotes (Thuresson *et al.*, 1994). In contrast, there is growing evidence for the occurrence of more than one such enzyme encoded by different genes (*pcnA* and *pcnB*) in *Escherichia coli* (Sarkar, 1997). The deduced product of *pcnB* (PAP I), however, showed no homology to eukaryotic poly(A) polymerases and even moderate overexpression of this gene was lethal to *E.*

coli (Cao & Sarkar, 1992). On the other hand, disruption of this gene decreased the growth rate by two-thirds (Liu & Parkinson, 1989). The protein product of *pcnA* of *E. coli* (PAP II) has no significant sequence homology either to PAP I or to the viral and eukaryotic poly(A) polymerases (Kalapos *et al.*, 1994), suggesting that the bacterial poly(A) polymerases have evolved independently. Sarkar (1997) suggested that PAP I and PAP II possibly polyadenylate complementary classes of mRNA, or one enzyme initiates poly(A) chains and the other functions in their extension, and that the deletion of both the genes would be lethal to the organism. In *Saccharomyces cerevisiae*, there is a single gene for poly(A) polymerase whose inactivation leads to the loss of viability (Lingner *et al.*, 1991). The protein product of the *pcnA* gene (Rv3907c; Cole *et al.*, 1998) of *M. tuberculosis* has 80% similarity (BLASTP search) to the poly(A) polymerase-encoding gene *pcnB* of *M. leprae* (accession no. AAB53125; Fsihi *et al.*, 1996), suggesting that this gene is conserved in mycobacteria. In addition, this protein has a high similarity to poly(A) polymerases from various organisms including *Bacillus subtilis*, *E. coli* and *Helicobacter pylori*, supporting our finding that polyadenylation does occur in mycobacteria. Understanding the importance of *pcnA* and *pcnB* to the survival of *M. tuberculosis* and *M. leprae*, respectively, might therefore open a new area for drug design to combat these pathogens.

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