Eur. J. Biochem. **264**, 591–598 (1999) © FEBS 1999

Cloning, over-expression and biochemical characterization of the single-stranded DNA binding protein from *Mycobacterium tuberculosis*

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The single-stranded DNA binding protein (SSB) plays an important role in DNA replication, repair and recombination. To study the biochemical properties of SSB from *Mycobacterium tuberculosis* (*Mtu*SSB), we have used the recently published genome sequence to clone the *ssb* open reading frame by PCR and have developed an overexpression system. Sequence comparison reveals that the *Mtu*SSB lacks many of the highly conserved amino acids crucial for the *Escherichia coli* SSB (*Eco*SSB) structure–function relationship. A highly conserved His55, important for homotetramerization of *Eco*SSB is represented by a leucine in *Mtu*SSB. Similarly, Trp40, Trp54 and Trp88 of *Eco*SSB required for stabilizing SSB–DNA complexes are represented by Ile40, Phe54 and Phe88 in *Mtu*SSB. In addition, a group of positively charged amino acids oriented towards the DNA binding cleft in *Eco*SSB contains several nonconserved changes in *Mtu*SSB. We show that in spite of these changes in the primary sequence *Mtu*SSB is similar to *Eco*SSB in its biochemical properties. It exists as a tetramer, it has the same minimal size requirement for its efficient binding to DNA and its binding affinity towards DNA oligonucleotides is indistinguishable from that of *Eco*SSB. Furthermore, *Mtu*SSB interacts with DNA in at least two distinct modes corresponding to the SSB_{35} and $SSB_{56/65}$ modes of *Eco*SSB interaction with DNA. However, *Mtu*SSB does not form heterotetramers with *Eco*SSB. *Mtu*SSB therefore presents us with an interesting system with which to investigate further the role of the conserved amino acids in the biological properties of SSBs.

Keywords: DNA–protein interaction; EMSA; functional genomics; *M. tuberculosis*; single-stranded DNA binding protein.

Single-stranded DNA binding proteins (SSBs) bind to single-stranded DNA (ssDNA) in a sequence-independent manner [1]. SSBs are found in different oligomeric states in various organisms: SSB is monomeric in T4 phage (gene 32 protein), dimeric in filamentous bacteriophages, heterotrimeric in eukaryotes and homotetrameric in eubacteria and mitochondria [2–6]. Of the tetrameric SSBs, *Escherichia coli* SSB (*Eco*SSB) has been studied extensively [7–9]. Studies with the *Eco*SSB N-terminal fragments obtained by cleavage at Arg115 by trypsin (SSB_T) or at Trp135 by chymotrypsin (SSB_C) have established that the tetramerization and DNA binding sites are contained within the first 115 amino acids [10]. His55 has a central role in tetramerization as its substitution with residues containing more bulky side chains leads to destabilization of the tetramer [11,12]. The crystal structure of *Eco*SSB shows that His55 of one monomer contacts the main chain carbonyl oxygen of Leu83 and the side chain of Asn6 of the other monomer at the dimeric interface [5]. The 12 C-terminal amino acids contain a high number of negatively charged residues which are required for interaction of SSB with various proteins in *E. coli* and other eubacteria but are not essential for DNA binding [8,13]. In fact, their deletion results in a protein of higher affinity for ssDNA [10,13]. Homotetrameric forms of SSBs bind to DNA in three distinct modes with site sizes of approximately 35, 56 and 65 nucleotides respectively (SSB_{35}, SSB_{56} and SSB_{65}) [8,14,15]. In the SSB_{35} mode, DNA binds with two of the subunits whereas in the $SSB_{56/65}$ mode the DNA wraps over the tetramer [16]. These binding modes are influenced by the salt and SSB concentrations [8]. It has also been suggested that switching among these binding modes might be important in various biological processes [17]. In *Eco*SSB, of the four tryptophan residues at positions 40, 54, 88 and 135, the first three appear to play a role in DNA binding [18]. The Trp40 and Trp54 stack with the nucleotide bases for high affinity binding to DNA whereas mutations at Trp88 have been shown to influence the stability of different DNA binding modes [19,20]. These tryptophan residues and several other residues such as Phe60, Lys49, Lys62, and Arg21, Arg41, Arg56, Arg72, Arg86, and Arg96 are oriented towards the DNA binding cleft, suggesting a possible role for these positively charged side chains in DNA interaction [5].

Recently the complete genome sequence of *Mycobacterium tuberculosis* was published [21]. *M. tuberculosis* continues to be the pathogen that causes the most casualties worldwide; this intracellular pathogen remains dormant inside the host cells and is resistant to several antibiotics making it difficult to cure. Thus it is important to understand the biology of this organism. To understand the basic DNA functions in *M. tuberculosis*, we have cloned, overexpressed and studied the biochemical properties of its SSB (*Mtu*SSB).

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Abbreviations: SSB, single-stranded DNA binding protein; *Mtu*SSB, *Mycobacterium tuberculosis* SSB; *Eco*SSB, *Escherichia coli* SSB; UDG, uracil DNA glycosylase; UgI, uracil DNA glycosylase inhibitor protein; PEI, polyethylenimine; EMSA, electrophoretic mobility shift assay.

(Received 25 January 1998, revised 22 June 1999, accepted 29 June 1999)

MATERIALS AND METHODS

Oligodeoxyribonucleotides

Oligodeoxyribonucleotides were obtained from DNA synthesis facilities at the Indian Institute of Science, Bangalore, India, the University of Calgary, Calgary, Canada and Ransom Hill Bioscience, Ramona, CA, USA. The oligonucleotides were purified from polyacrylamide (15%)/8 M urea gels and desalted by gel filtration on Sephadex G-50 columns. The 37-mer and 69-mer oligonucleotides were from New England Biolabs Inc., as HPLC pure syntheses (a kind gift from D. N. Rao). Working stocks of 10 pmol·μL^{-1} were prepared by diluting the oligonucleotides to a concentration (A_{260}) of $0.1 \times n \cdot ml^{-1}$, where n is the size of oligonucleotide in bases [22,23].

[^{32}P]-labelling and purification of oligonucleotides

Oligonucleotides (10 pmol) were labelled at the 5′ end with [^{32}P] [24] and purified by the spin column [23] method using Sephadex G-50 minicolumns. For use in various experiments the radiolabelled oligonucleotides were diluted with known amounts of their cold oligonucleotides such that contribution from the labelled counterpart was less than 1%.

Cloning of *Mtu*SSB

The sequence of the predicted *M. tuberculosis* SSB (EMBL locus MTCY21D4, accession number Z80775) was obtained from the Sanger Center UK, and amplified from *M. tuberculosis* H37 Ra genomic DNA using Vent DNA polymerase (New England Biolabs). Two synthetic DNA sequences, 5′-GAAAACCATGGCTGGTGACACCAC-3′ and 5′-CTTGT-GATCAGAATGGCGGTTC-3′ were used as the forward and the reverse primers in PCR, according to the supplier's instructions. The PCR product was digested with *Nco*I and *Bcl*I, and fractionated on 1.2% agarose gel. DNA corresponding to ≈ 500 bp (the expected size of the product was 494 bp) was cut out of the gel and spin-eluted through polyester wool. The DNA was purified further by phenol/chloroform extractions and ethanol precipitation and cloned into pET11d (Novagen Inc.) digested with *Nco*I and *Bam*HI. The resulting plasmid was referred to as pET*Mtu*SSB. The authenticity of the clone was ascertained by sequencing of the complete DNA fragment [25].

Overexpression and purification of *Mtu*SSB and *Eco*SSB

*Mtu*SSB was overexpressed using pET*Mtu*SSB in *E. coli* BL21 (DE3). Cells from 1.4 L culture grown for 8 h in L-broth [23] were harvested by centrifugation and suspended in 10 mL buffer consisting of 50 mM Tris/HCl, pH 8.0, 1 mM Na_2EDTA, and 0.2 M NaCl, and lysed by sonication. The crude cell lysate was centrifuged at 20 000 ***g*** for 15 min. Proteins from the resulting S-20 supernatant (Fraction I) were subjected to precipitation with polyethylenimine (PEI) to a final concentration of 0.4% and centrifuged at 10 000 ***g*** for 10 min. The proteins from the pellet were extracted with 10 mL 50 mM Tris/HCl pH 8.0, 0.4 M NaCl (Fraction II), subjected to precipitation with ammonium sulfate to 25% saturation, recovered by centrifugation at 17 500 ***g*** for 30 min and dissolved in 2 mL of 20 mM Tris/HCl pH 8.0, 0.2 M NaCl (Fraction III). Fraction III was loaded on to a 5-mL heparin sepharose FPLC column (Amersham Pharmacia Biotech) equilibrated with 20 mM Tris/HCl (pH 8.0), washed with the same buffer and then eluted with a gradient of 0–1 M NaCl in the wash buffer. SSB eluted in the initial fractions (≈ 100 mM NaCl), which were pooled and dialysed against a buffer containing 50 mM Tris/HCl pH 8.0, 0.5 M NaCl, 1 mM Na_2EDTA, 50% glycerol (v/v) and stored at −20 °C (Fraction IV).

The *Eco*SSB was overexpressed from a construct pTL119 (from T. Lohman [26], provided by K. Muniyappa) and purified [24].

Determination of ε_m coefficient of *Mtu*SSB

Absorbance of *Mtu*SSB (Fraction IV) was determined at 280 nm. Guanidine HCl powder was added to a final concentration of 6 M. The protein was incubated at 37 °C and its absorbance determined again after 1 h and 2 h and the ε_m coefficient was calculated by taking into consideration the numbers of tyrosine, tryptophan and cysteine residues in *Mtu*SSB [27]. Extinction coefficient (ε_{max}) of *Mtu*SSB tetramer was found to be 1.5×10^5 $M^{-1} \cdot cm^{-1}$ and used to determine SSB concentrations.

Subunit exchange

*Eco*SSB and *Mtu*SSB (≈ 7.5 μg each) were mixed in 51 μL volume in a buffer containing 50 mM Tris/HCl, 1 mM Na_2EDTA, pH 8.0, 50% glycerol (v/v), 0.5 M NaCl and kept at room temperature. Aliquots (17 μL) were taken out at 0, 36 and 60 h, frozen and analysed by 12% native PAGE for heterotetramer formation [28].

Analytical gel filtration chromatography

Proteins (up to ≈ 1 mg total) were subjected to gel filtration chromatography on Superdex 200 HR 10/30 (Amersham

Table 1. DNA oligomers.

Size (nt)	Sequence
15	5′ d(ggggcgattatagat) 3′
17	5′ d(caggaaacagctatgac) 3′
19	5′ d(ccttatcccacaccgggac) 3′
22	5′ d(cgtgaagcttgacggtacgggc) 3′
25	5′ d(agcucatagtttacctgaagaatat) 3′
27	5′ d(agcucgccgacgaatatgatacaggag) 3′
33	5′ d(gctaaataggacaaaaaaaccactggatatacc) 3′
37	5′ d(gactggtacagtatcaggcgctgacccacaacatccg) 3′
69	5′ d(tcaagattactcttgatgaaggtcagccagcctatgcgcctggtctgtacaccgttcatctgtcctctt) 3′

Pharmacia Biotech) column equilibrated with 20 mM Tris/HCl pH 7.4, 0.2 M NaCl, 1 mM Na_2EDTA, and eluted with the same buffer at a flow rate of 0.5 mL·min^{-1} and collected in 0.5 mL fractions. Absorbance at 280 nm was recorded to monitor elution profile.

Electrophoretic mobility shift assays (EMSA)

DNA samples were incubated with SSB in the binding buffer containing 20 mM Tris/HCl pH 8.0, 50 mM NaCl, 5% glycerol (v/v), 50 μg·ml^{-1} BSA for 10 min and separated by PAGE at 4 °C on an 8% native gel (30 : 0.5, acrylamide/bisacrylamide) using 0.5 × Tris/borate/EDTA buffer, for 1–2 h at 150 V (≈ 8 V·cm^{-1}) [24]. The complex and the free DNA bands were visualized by autoradiography. The various DNA oligonucleotides used in this study are listed in Table 1.

Fluorescence measurements

Titrations of SSB with synthetic DNA were performed by monitoring the quenching of intrinsic (tryptophan) fluorescence of SSB (λ_{ex} = 295, λ_{em} = 345–349) using a Shimadzu RF-5301 spectrofluorimeter. The protein amount was kept constant and fluorescence changes were recorded upon adding defined aliquots of concentrated 25-mer DNA until saturation. The mixture was allowed to equilibrate for 60–120 s at 25 °C before recording the fluorescence. The measurements were corrected for dilution and the relative

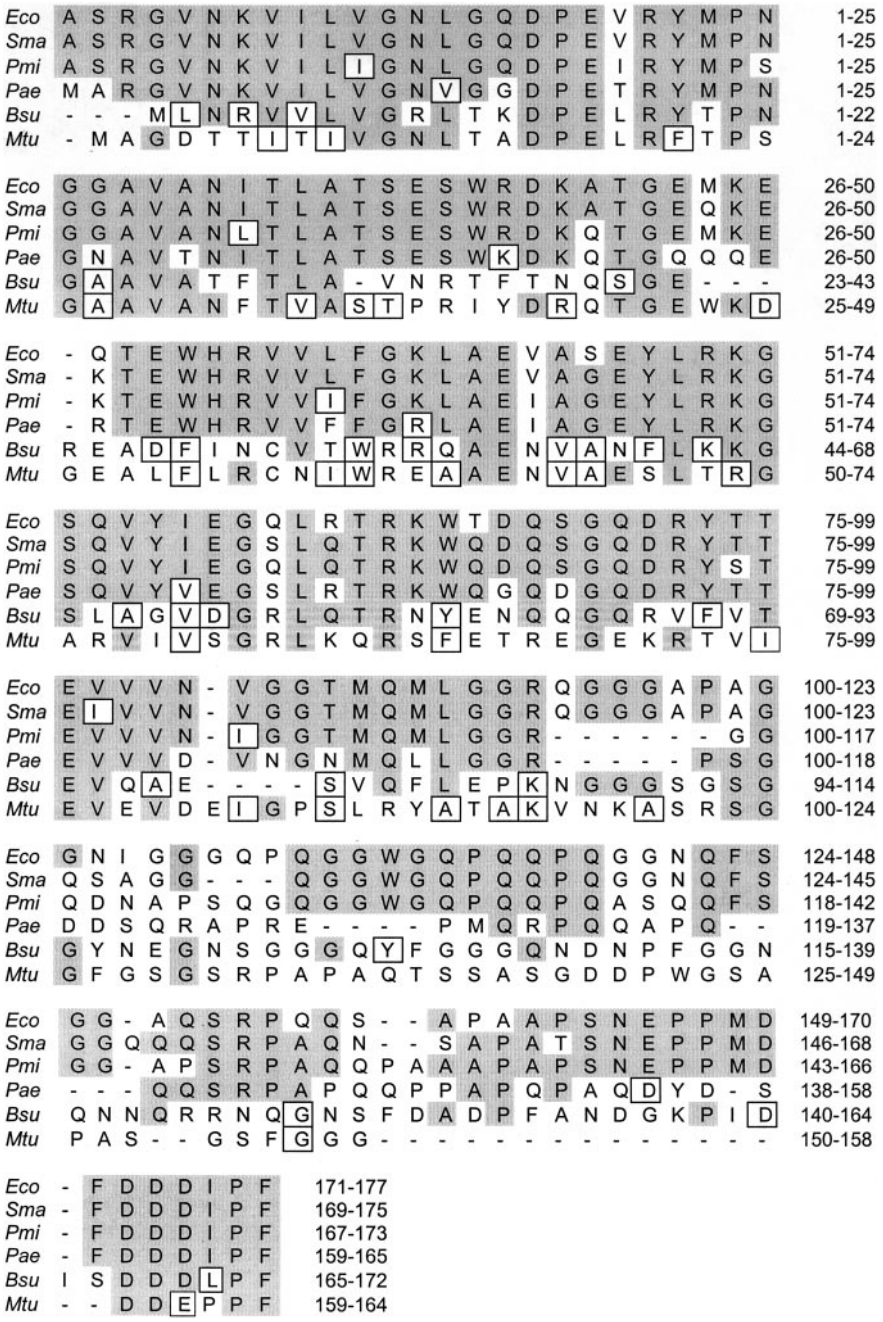

Fig. 1. Sequence comparison of *Eco*SSB with other eubacterial SSBs. The alignment was obtained by using the PILEUP program. Residues that are highly conserved and identical amongst various SSBs are shaded, and the positions that show a conservative change have been boxed. The amino acid position 1 in the *Eco*-, *Sma*- and *Pmi*SSBs corresponds to the second position of the open reading frame in the respective genes. *Eco*, *E. coli*; *Sma*, *S. marcescens*; *Pmi*, *P. mirabilis*; *Pae*, *Ps. aeruginosa*; *Bsu*, *B. subtilis*; *Mtu*, *M. tuberculosis*.

fluorescence [$F_{observed}/F_{initial}$] was plotted as a function of DNA/protein molar ratio [29].

Modes of interaction of SSBs with synthetic DNA

Approximately 2.5 pmol 5′-end [^{32}P]-labelled (≈ 12000 c.p.m.) 37-mer or 69-mer oligonucleotides were mixed with either 1 or 10 pmol *Eco*SSB or *Mtu*SSB and analysed by EMSA.

RESULTS

Comparison of eubacterial SSBs

Figure 1 shows an alignment of *Serratia marcescens*, *Proteus mirabilis*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, *M. tuberculosis* and *E. coli* SSBs as obtained by 'pile up' analysis. Of these, the *Eco*SSB has been characterized extensively both biochemically and biophysically and it serves as a prototype for eubacterial SSBs [5,8]. SSBs from *P. mirabilis* and *S. marcescens* have also been characterized [30]. The analysis shows that *E. coli*, *S. marcescens*, *P. mirabilis* and *Ps. aeruginosa* SSBs are highly conserved. In fact, the sequences of these SSBs towards the N-terminal half are virtually identical. On the other hand, the *B. subtilis* and *M. tuberculosis* SSBs do not share such a high degree of homology with *Eco*SSB or even among themselves. Although some of the changes in amino acids of the *Mtu*SSB which contribute to the low level of identity (≈ 30%) are conservative changes, many others are strikingly different (see Discussion). In addition, the *Mtu*SSB lacks a region corresponding to the

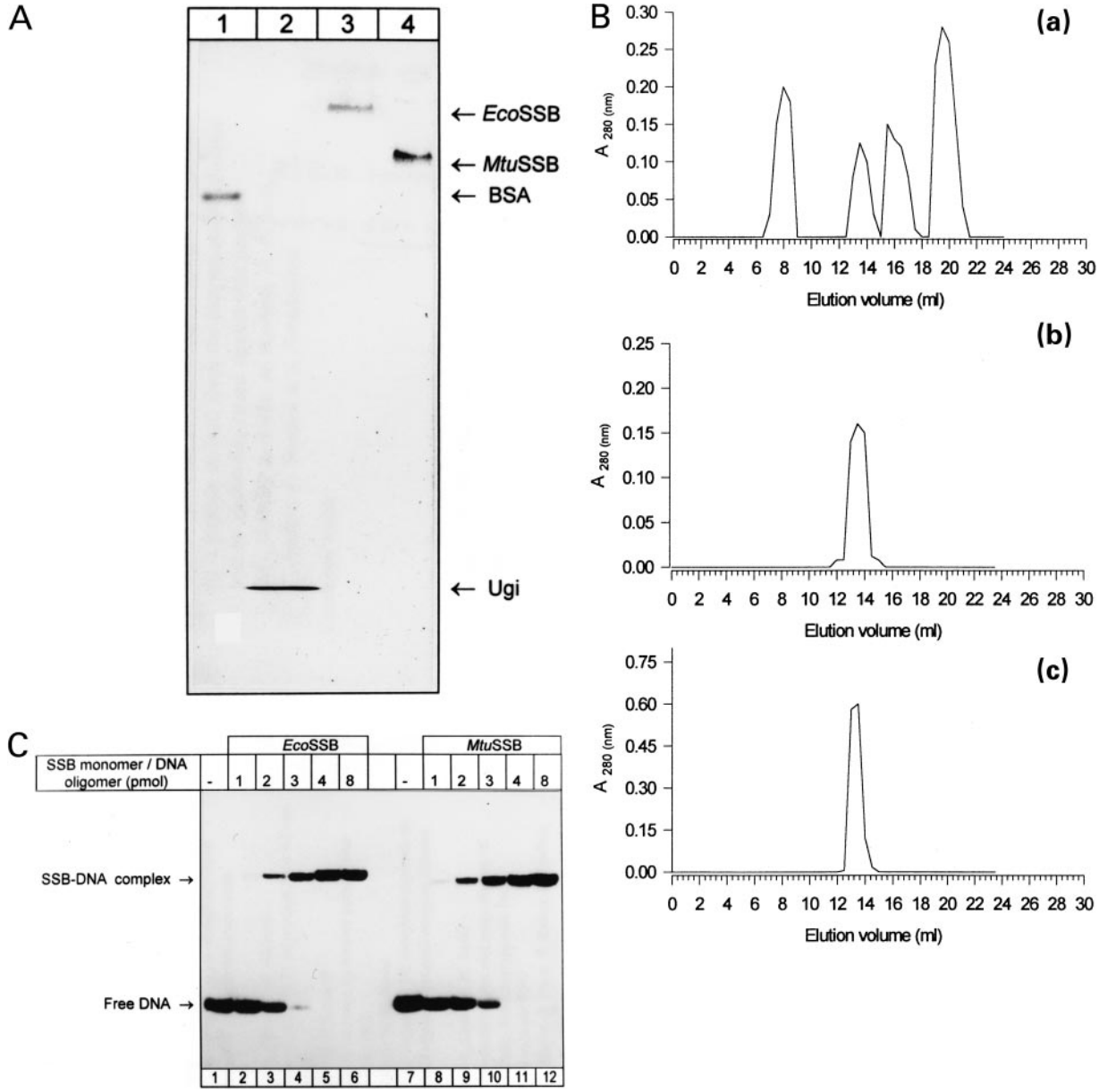

Fig. 2. Oligomerization status of *Mtu*SSB. (A) Analysis of proteins on 12% native PAGE. Lanes: 1, BSA (5 μg); 2, Ugi (5 μg); 3, *Eco*SSB (2.5 μg); 4, *Mtu*SSB (4 μg). (B) Analytical gel filtration chromatography of various proteins on Superdex 200 (HR 10/30, Amersham Pharmacia Biotech). Panels: (a) Molecular mass standards, glyceraldehyde-3-phosphate dehydrogenase from rabbit (140 kDa), BSA (66 kDa), UDG–Ugi complex (36 kDa), and lysozyme (14 kDa); (b) *Mtu*SSB; (c) *Eco*SSB. (C) Determination of the stoichiometry of SSB interaction with a 27-mer DNA by EMSA. Lanes: 2–6, complex formation with *Eco*SSB; 8–12, complex formation with *Mtu*SSB. Molar ratios of SSB (monomer) to DNA are as indicated on top of each lane.

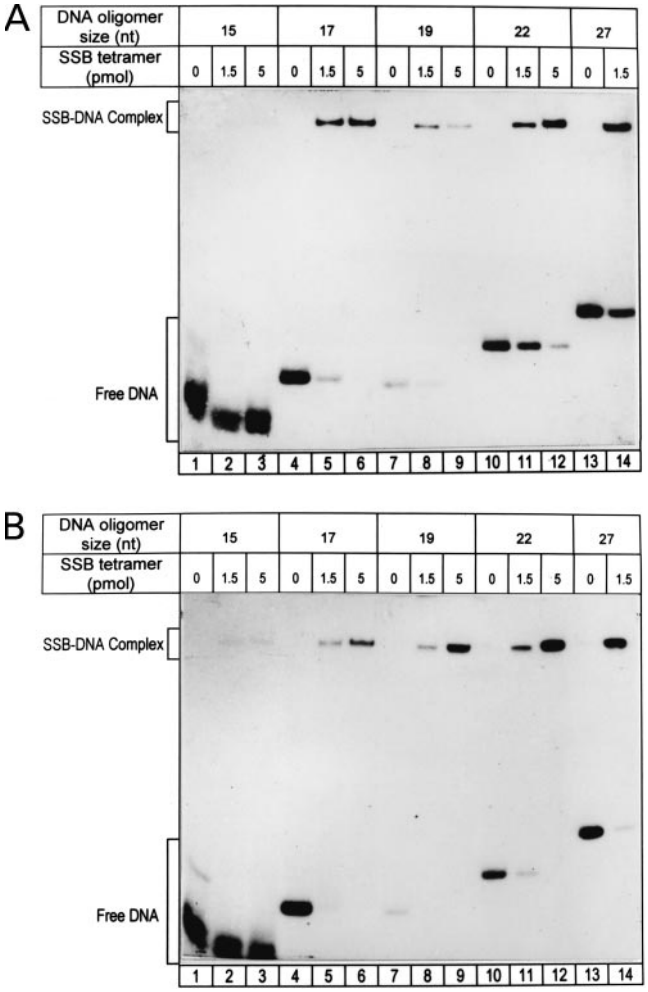

Fig. 3. Minimum DNA binding size determination. Approximately 2 pmol of ^{32}P 5′-end-labelled oligonucleotides, 15-mer (lane 1–3), 17-mer (lane 4–6), 19-mer (lane7–9), 22-mer (lane 10–12) and 27-mer (lane 13, 14) were incubated either with *Eco*SSB (A) or *Mtu*SSB (B) and the SSB-DNA complexes analysed by EMSA. Lanes 1, 4, 7, 10 and 13 contain no SSB. Lanes 2, 5, 8, 11 and 14 contain ≈ 1.5 pmol SSB and lanes 3, 6, 9 and 12 contain 5 pmol SSB.

amino acids 159–171 of *Eco*SSB; notably, the missing amino acids in this region of *Mtu*SSB overlap with a part of the C-terminal 12 amino acid stretch (166–177) of *Eco*SSB, which has been implicated in the interaction of *Eco*SSB with various other proteins in DNA functions [8,13].

Overexpression and purification of *Mtu*SSB

The 494 bp DNA fragment containing the full open reading frame was amplified by PCR and cloned in to a T7 RNA polymerase-based expression vector, pET11d and referred to as pET*Mtu*SSB. This construct resulted in abundant production of *Mtu*SSB in *E. coli* BL21(DE3) even in the absence of induction with isopropyl thio-β-D-galactoside. Therefore, for large-scale purification of *Mtu*SSB, we used the T7 RNA polymerase-based expression system. The extent of *Mtu*SSB overproduction was such that when S-20 extracts were subjected to fractionation with PEI and ammonium sulfate, it resulted in substantial purification of *Mtu*SSB. Further purification on a heparin–sepharose column resulted in an apparently homogeneous preparation (data not shown).

Oligomerization status of *Mtu*SSB

SSBs exist in different oligomeric states. To determine the oligomeric state of *Mtu*SSB, it was electrophoresed under native conditions alongside *Eco*SSB (which exists as a tetramer), BSA and uracil DNA glycosylase inhibitor protein (Ugi) [28] (Fig. 2A). The pI and molecular masses of *Mtu*SSB, 5.12 and ≈ 17.35 kDa, respectively, are less than the corresponding values of 5.44 and ≈ 18 kDa, respectively, of *Eco*SSB. Therefore, although the *Mtu*SSB migrates slightly faster than *Eco*SSB, the close proximity of the two proteins on this gel may suggest that the *Mtu*SSB also exists as a homotetramer (lanes 3 and 4). In addition, the *Mtu*SSB (m = 69 kDa for a tetramer, pI 5.12) migrates somewhat slower than BSA (m = 66 kDa, and pI 4.7–4.9) but far too slow when compared with Ugi (m = 9.8 kDa, pI 4.2). To confirm the tetrameric nature of *Mtu*SSB, we have also analysed its elution profile on a gel filtration column (Superdex 200), and compared it with that of *Eco*SSB and various other protein size markers. As shown in Fig. 2B, both *Eco*SSB and *Mtu*SSB elute at approximately the same volume (13.5 mL) which lies between the elution volume of rabbit glyceraldehyde 3-phosphate dehydrogenase (8 mL, m = 140 kDa) and BSA (14 mL, m = 66 kDa). Taken together, these results confirm that in solution, like *Eco*SSB, the *Mtu*SSB exists as a tetramer.

To investigate if *Mtu*SSB binds to DNA as a tetramer, a 27-mer DNA was mixed with either *Eco*SSB or *Mtu*SSB in the protein (monomeric) to DNA molar stoichiometries of 0–8, and the complexes formed were analysed by EMSA. As shown in

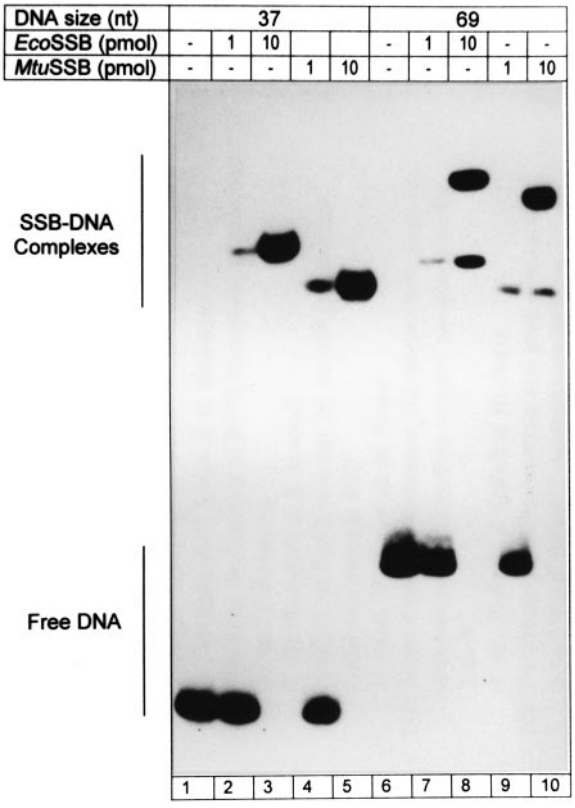

Fig. 4. Analysis of different modes of SSB binding to DNA. Approximately 2.5 pmol of [^{32}P] 5′-end-labeled (≈ 12 000 c.p.m.) oligomers (37-mer, lanes 1–5; 69-mer, lanes 6–10) were mixed with 1 pmol *Eco*SSB (lanes 2 and 7), 1 pmol *Mtu*SSB (lanes 4 and 9), 10 pmol *Eco*SSB (lanes 3 and 8) or 10 pmol *Mtu*SSB (lanes 5 and 10) and analysed by EMSA.

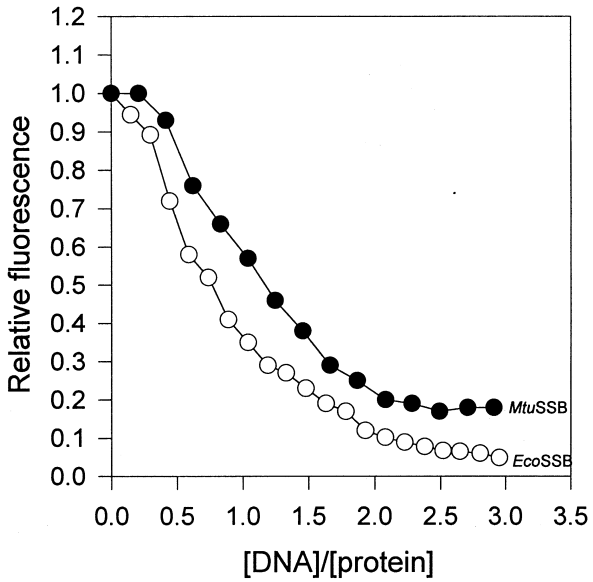

Fig. 5. Interaction of synthetic DNA with SSBs. Intrinsic tryptophan fluorescence quenching of *Mtu*SSB or *Eco*SSB with a 25-mer DNA. The titrations were carried out in 300 μL volumes containing *Eco*SSB (0.2 μM), or *Mtu*SSB (0.14 μM). Aliquots (2 μL) of a concentrated solution of the 25-mer oligo (0.73 μM stock) were gradually added to the above solution, mixed and equilibrated for 2 min each time before recording emission intensity. The titrations were continued until saturation was reached.

Fig. 2C, with the increasing molar excess of SSBs (lanes 1–6, *Eco*SSB; lanes 7–12, *Mtu*SSB), an increasing proportion of SSB–DNA complex was seen. At a 4 : 1 molar excess of SSB (lanes 4 and 11), no free DNA remained. This experiment shows that like *Eco*SSB, *Mtu*SSB also binds to DNA as a tetramer.

Determination of minimum DNA size for efficient binding to SSB

Oligonucleotides (≈ 2 pmol of 15-mer, 17-mer, 19-mer, 22-mer or 27-mer DNA) were 5′-end labelled with ^{32}P and mixed with 1.5 or 5 pmol *Eco*SSB (Fig. 3A) or *Mtu*SSB (Fig. 3B) and the complexes were analysed by EMSA. The complex formation with the 15-mer is weak and barely detectable (lanes 1–3). However, 17-mer or longer sized oligomers show efficient complex formation (compare lane 4 with lanes 5 and 6; lane 7 with lanes 8 and 9; lane 10 with lanes 11 and 12; and lane 13 with 14). These results suggest that 15–17 bases is the minimum length of DNA that is required for efficient binding. Importantly, the data show that both *Eco*SSB and *Mtu*SSB require similar minimum DNA lengths to form a stable complex.

Different modes of SSB binding to DNA

*Eco*SSB binds to DNA in either SSB_{35} or $SSB_{56/65}$ mode. In the SSB_{35} mode, which occurs under high SSB to DNA ratio or low salt conditions, DNA binds to SSB with ≈ 35 nucleotides per SSB tetramer and interacts with two of its subunits. On the other hand, binding in the $SSB_{56/65}$ mode, which occurs under low SSB to DNA ratio or high salt conditions, approximately 56/65 nucleotides of DNA wrap over the SSB tetramer [14–17]. As many of the amino acids shown to be important for DNA binding in *Eco*SSB are not conserved in *Mtu*SSB, it was of interest to analyse the mode of DNA interaction with *Mtu*SSB. We used 37-mer and 69-mer oligonucleotides (Table 1) for complex formation (Fig. 4). As expected, the 37-mer yielded a single complex with both SSBs (*Eco*SSB, lanes 2 and 3; *Mtu*SSB, lanes 4 and 5) at either low (lanes 2 and 4) or high (lanes 3 and 5) SSB to DNA ratios. On the other hand, the 69-mer showed two distinct complexes which differed in their mobility. At low SSB to DNA ratio (*Eco*SSB, lane 7; *Mtu*SSB, lane 9) a single complex was seen, whereas upon increasing the SSB concentration (*Eco*SSB, lane 8; *Mtu*SSB, lane 10) an additional complex with the slower mobility was also seen. Thus it appears likely that similar to *Eco*SSB, *Mtu*SSB also binds to DNA in two distinct complexes. The slow and fast moving complexes with 69-mer could correspond to the SSB_{35} and $SSB_{56/65}$ binding modes, respectively.

Relative affinities of *Mtu*SSB and *Eco*SSB to DNA

To characterize further *Mtu*SSB binding to DNA, we performed fluorescence titration experiments in which the quenching of intrinsic fluorescence of SSBs was monitored upon increasing the concentration of a 25-mer DNA (Table 1). A plot of relative fluorescence vs μmol DNA per μmol protein (Fig. 5) shows that both the *Eco*SSB and *Mtu*SSB bind to DNA with relatively similar affinities.

To substantiate further that both *Eco*SSB and *Mtu*SSB bound to DNA with similar affinities, we performed an EMSA in which *Eco*SSB and *Mtu*SSB were mixed in different molar ratios and used to form a complex with a 33-mer DNA (Table 1). Because of the differences in their molecular masses, the complexes formed with *Eco*SSB and *Mtu*SSB can be resolved by EMSA and quantified to estimate relative levels of each complex. In the experiment (Fig. 6), 5 pmol of the DNA oligomer was either not mixed with any SSB (lane 1) or mixed with 10 pmol of *Mtu*SSB tetramer (lane 2), 10 pmol *Eco*SSB tetramer (lane 12) or with *Eco*SSB and *Mtu*SSB together (lanes 3–11). As shown in Fig. 6, the extent of either the *Eco*SSB–DNA or the *Mtu*SSB–DNA complex was proportional to the *Eco*SSB to *Mtu*SSB ratio, suggesting that the two SSBs bound DNA with equal affinity.

Subunit exchange studies

As deduced from the comparative analysis of the biochemical properties, the *Mtu*SSB mimics *Eco*SSB. To analyse whether

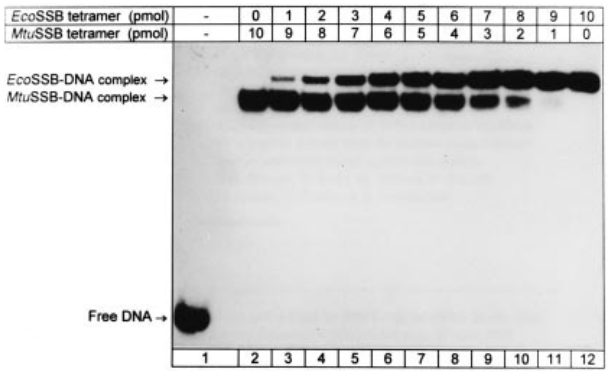

Fig. 6. Interaction of synthetic DNA with SSBs. Binding of DNA to SSB in a mix of *Eco*SSB and *Mtu*SSB. To 5 pmol of the 33-mer DNA (25 000 c.p.m.) either 10 pmol of pure SSB (lanes 2 and 12) or a mixture of *Eco*SSB and *Mtu*SSB in different molar ratios, were added and the complex formation was analysed by EMSA. The complexes of DNA with *Eco*SSB or *Mtu*SSB are indicated by arrows.

they also share overall structural similarity, we analysed their ability to form heterotetramers. In the native gels, *Eco*SSB migrates slower than the *Mtu*SSB and the two SSB tetramers are well resolved (Fig. 2A). In the experiment in which heterotetramer formation was analysed, we did not detect any bands with intermediate mobility, implying that the two SSBs do not form stable heterotetramers (data not shown). This interpretation is supported further by the fact that no SSB–DNA complex with mobility intermediate of *Eco*SSB–DNA and *Mtu*SSB–DNA complexs was detected (Fig. 6).

DISCUSSION

Organisms of the genus *Mycobacterium* constitute one of the most important group of pathogens as they infect and multiply in host macrophages–the cells of the immune system which provide the first-line of host defense. Approximately one-third of the world population is infected with *M. tuberculosis* and yet we know little about the biology of this organism. A large body of research efforts has been focused on the study of host–pathogen interaction and on the mechanisms that lead to suppression of the host immune response against this bacterium. However, little is known about the unique aspects of DNA replication, repair and recombination in these organisms and how these processes differ from the prototypic mechanisms known in *E. coli*. Understanding the distinct mechanisms of DNA–protein interactions in the *Mycobacterium* will facilitate the development of new drugs to intervene in the life cycle of this important pathogen.

The complete genome sequence of *M. tuberculosis* has provided us with the much awaited opportunity to understand the biology of this organism. In this study, we have cloned, overexpressed and studied the biochemical properties of SSB, a key protein the characterization of which is crucial for the study of various aspects of DNA function.

Sequence comparison of *Mtu*SSB with the prototypic *E. coli* SSB shows some striking differences. The equivalent of His55 of *Eco*SSB present at the tetramerization interface is substituted by leucine in *Mtu*SSB. His55 is highly conserved among various SSBs and mutations at this position (e.g. in *Eco*SSB *ssb1* allele, His55Tyr) result in a protein which does not tetramerize efficiently [11]. However, intra-genic second-site suppressors of another temperature-sensitive allele of *ssb*, in which His55 was mutated to Lys55, have been isolated which show either Gln76Leu or Gln110Leu [31]. That the His55 is not absolutely required for tetramerization was also shown by another mutant of *Eco*SSB in which His55Ile did not result in any detectable change in its ability to tetramerize even in the absence of the compensatory mutations at position 76 or 110 [12]. In fact, SSBs from *Klebsiella aerogenes* RK2 plasmid (tetrameric SSB), and *B. subtilis* also lack the equivalent of His55. However, these two SSBs possess the Gln76Leu change (Fig. 1, *Eco* numbering). On the other hand, *M. tuberculosis* SSB which possesses leucine as the His55 equivalent, contains arginine at the equivalents of positions 76 and 110. As our studies show that *Mtu*SSB exists as a tetramer (Fig. 2), could it be that the arginine residues at positions 76 and 110 also facilitate tetramerization of *Mtu*SSB? This SSB is, therefore, an attractive model system with which to study the significance of positions 55, 76 and 110 (*Eco* equivalents). Although at present it is not clear if the alternate combination of amino acids at these positions provides any physiological advantage to the gram-positive bacteria, in this study, we have shown that the biochemical properties of *Mtu*SSB are, mostly, indistinguishable from those of *Eco*SSB.

Both *Eco*SSB and *Mtu*SSB require a size of more than 15 nucleotides for efficient binding to DNA (Fig. 3); as shown in Fig. 4, depending on the molar stoichiometry of SSB to DNA, *Mtu*SSB also binds in at least two distinct modes corresponding to the SSB_{35} and $SSB_{56/65}$ modes of *Eco*SSB binding to DNA. Furthermore, the affinity of both the SSBs towards DNA is similar (Figs 5 and 6). On the other hand, although the *Mtu*SSB possesses tryptophans at positions 48, 60 and 145 (*Eco* numbering), these are located differently from those of *Eco*SSB (positions 40, 54, 88 and 135) and the first three of these (40, 54 and 88) have been shown to be important in DNA binding. DNA concentration dependence of tryptophan fluorescence quenching (Fig. 5), suggests that the tryptophan environment changes upon binding of *Mtu*SSB to DNA. A simple interpretation of this observation would be that DNA interacts with these tryptophans [8,18]. Furthermore, in *Eco*SSB, the positively charged residues such as lysines at position 49 and 62, and arginines at positions 21, 41, 56, 72, 86 and 96 are oriented towards the DNA binding cleft and, therefore, probably define the path of DNA on SSB [5]. In *Mtu*SSB at least three of these positions (62, 41, 72, *Eco* numbering) are substituted by nonconserved amino acids, glutamic acid, tyrosine and threonine, respectively. Taken together, these observations suggest that in spite of their apparent biochemical similarity, the molecular details of DNA interaction with the two SSBs are probably different. The answers to some of these and other questions will require mutational analysis of the *Mtu*SSB and study of its interaction with the proteins involved in various DNA functions [24,32].

ACKNOWLEDGEMENTS

This work was supported by a research grant from the Department of Biotechnology, New Delhi, India. We thank V. Nagaraja, D. N. Rao and P. Handa for their suggestions and critical reading of the manuscript, and S. Thanedar and M. Thomas for useful discussions relating to this work. K. P. is a Dr K. S. Krishnan fellow. Use of the Department of Biotechnology (New Delhi)-supported phosphorimager facility is acknowledged.

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