Effects of Xenopus laevis Mitochondrial Single-stranded DNA-binding Protein on Primer-Template Binding and 3′ → 5′ Exonuclease Activity of DNA Polymerase γ*

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Mitochondrial DNA (mtDNA) is replicated by DNA polymerase γ by a strand displacement mechanism involving mitochondrial single-stranded DNA-binding protein (mtSSB). mtSSB stimulates the overall rate of DNA synthesis on singly primed M13 DNA mainly by stimulating the processivity of DNA synthesis rather than by stimulating primer recognition. We used electrophoretic mobility shift methods to study the effects of mtSSB on primer-template recognition by DNA pol γ. Preliminary experiments showed that single mtSSB tetramers bind tightly to oligo(dT) single strands containing 32 to 48 residues. An oligonucleotide primer-template was designed with an 18-mer primer annealed to the 3′-portion of a 71-mer template containing 40 dT residues at its 5′-end as a binding site for mtSSB. DNA pol γ bound to this primer-template either in the absence or presence of mtSSB in complexes that remained intact and enzymatically active following native gel electrophoresis. Association of mtSSB with the 5′-dT_{40}-tail in the 187:1-mer primer-template reduced the binding of DNA polymerase γ and the efficiency of primer extension. Binding of mtSSB to single-stranded DNA was also observed to block the action of the 3′→5′ exonuclease of DNA polymerase γ. The size of fragments protected from 3′→5′ exonuclease trimming increases with increasing ionic strength in a manner consistent with the known salt dependence of the binding site size of Escherichia coli SSB.

Single-stranded DNA binding proteins (SSB) act as a group of proteins which bind preferentially and with high affinity to single-stranded DNA. SSB proteins are present in prokaryotic and eukaryotic cells and are assumed to be essential for DNA metabolism in all organisms. One of the best characterized prokaryotic SSBs, Escherichia coli SSB, is a stable homotetramer of subunits containing 177 amino acids (18.8 kDa). Both biochemical and genetic data indicate that E. coli SSB is involved in DNA replication, repair, and recombination (1). Depending on the ionic conditions this protein binds to DNA in multiple modes with an apparent binding site size varying from 35 to 56 or 65 nucleotides (2, 3). Eukaryotes contain two different families of nuclear and mitochondrial SSB proteins. Heterotrimeric nuclear SSBs (RPA) (subunits of about 70, 32, and 14 kDa) have been found in all eukaryotic cells examined. These proteins are essential for replication, are involved in recombination and repair, and interact specifically with other proteins involved in DNA metabolism although they have little sequence homology with prokaryotic SSBs (4).

Mitochondrial SSBs have been isolated from Xenopus laevis oocytes (5), rat liver (6, 7), yeast (8), and Drosophila (9). Two Xenopus mtSSBs have been described. One of these, mtSSB-1 has been fully sequenced (10) and is represented in a full-length cDNA clone (11). The second, mtSSB-2, has not been cloned and has been only partially sequenced. The first 80 residues of mtSSB-2 reveal 91% identity to mtSSB-1, which contains 129 amino acids (14.6 kDa). Both Xenopus (12) and human (13) mtSSB form tetramers in solution. mtSSB sequences share similarity to the N-terminal portion of E. coli SSB (11, 14, 15). In particular, Xenopus mtSSB-1 is 30% identical to the first 129 residues of E. coli SSB. However, sequence differences account for a dramatic difference in overall charge of these two proteins. Xl-mtSSB-1 has a pI of 9.92 while E. coli SSB has a pI of 5.23.

The putative biological function of mtSSB is to stabilize single-stranded regions of mtDNA in b-loop structures and other replicative intermediates (7, 16). The binding of various mtSSBs to single-stranded DNA has been studied using spectrophotometric methods. A binding site size of 8 or 9 nucleotides per monomer was estimated using fluorescence quench titration with poly(dT) for mtSSB from rat liver (17). A higher value for the binding site size, 59 nucleotides per tetramer, was obtained by fluorescence titration of human mtSSB at salt concentrations between 0.05 and 2 M NaCl (13). Recently, Thommes et al. (9) have used the fluorescence quench titration method to measure a binding site size on M13 DNA of 17 bases per monomer for Drosophila mtSSB.

The Saccharomyces cerevisiae mtSSB product of the rim1 gene is required for maintenance of mtDNA (8). Similar genetic evidence of an essential role for mtSSB is not available in other organisms. Nevertheless, mtSSB may interact with many proteins and enzymes participating in mtDNA replication, transcription, and repair. However, only a few biological effects of mtSSB have been documented to date. In vitro experiments showed that mtSSB isolated from X. laevis oocytes, Drosophila melanogaster rat liver, and cultured human cells stimulates the activity of homologous DNA polymerase γ (pol γ) on some DNA primer-templates (9, 17–19). E. coli SSB can also stimulate replication by Drosophila pol γ (20).

Despite results suggesting that mtSSB may interact with DNA pol γ, no data have been published on details of the interaction of DNA pol γ with a primer-template complex associated with mtSSB. Since DNA pol γ is not available in the quantities required for physical chemical methods, such exper-
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**Experimental Procedures**

Nucleotides and Nucleic Acids—Poly(dT) was purchased from Sigma. Concentrations of digo(dT) and poly(dT) were determined spectrophotometrically using an extinction coefficient, ε 260o of 8.100 M⁻¹ cm⁻¹ (21), and an ε 260o of 0.350 M⁻¹ cm⁻¹ was used to estimate concentrations of nucleotide residues. [γ-³²P]ATP was obtained from ICN Radiochemicals. A 71-mer template was prepared by ligation of a 31-mer (CAGGCCGTCTTCTACTTCTACCATCTTTCCATTT) to dT₉₀, to provide a binding site for mtSSB adjacent to a unique sequence that hybridizes to specific primers. The 31-mer and 40-mer were annealed to a 19-mer oligo-GGCCGAGTGAAAAAAAAAA to facilitate ligation. Following reaction with T₄ DNA ligase the resulting 71-mer was purified by gel electrophoresis. Either of two primers, an 18-mer (AAATGGAGAAGGAATG) or a 31-mer (AAATGGAGAAGGAAAGTGGAGAGGCGGAT) were annealed to the 71-mer to prepare primer-template templates referred to in the text as 18:71-mer or 31:71-mer. In some experiments the primer strands were labeled at the 5'-end with [γ-³²P]ATP using T₄ polynucleotide kinase. The reaction was carried out at 30°C with 1.04 ml mg⁻¹ of 1.04 ml mg⁻¹ of [γ-³²P]ATP before annealing to the 71-mer template.

A set of digo(dT)ₙ, with n varying from 12 to ~100 nucleotides was prepared by elongation of digo(dT)ₜₕ₋₂₈ using calf thymus terminal transferase (Life Technologies). Oligonucleotides were 5'-end labeled with [γ-³²P]ATP using T₄ polynucleotide kinase and [γ-³²P]ATP before annealing to the 71-mer template.

**DNA Binding Reactions and Electrophoretic Mobility Shift Analysis**

Unless noted otherwise, binding reactions were carried out in buffer A (10 mM Tris-HCl (pH 8.0), 2 mM DTT, 200 μg/ml BSA). Reactions, usually 8 or 10 μl, were assembled at 0°C and then incubated for 15–30 min at 23°C. For each reaction (4 or 5 μl) was loaded onto a single-stranded DNA cellulose column. After washing with buffer C (50 mM imidazole-HCl (pH 6.6), 10 mM MgCl₂, 5 mM DTT, and 15 units of the phage T₄ polynucleotide kinase), the reaction was incubated at 37°C for 30 min, terminated by the addition of EDTA to 15 mM, and then incubated at 65°C for 15 min to inactivate polynucleotide kinase. Oligonucleotides were precipitated with ethanol, and then fractionated on 10% polyacrylamide sequencing gels, or on 5% native polyacrylamide gels by electrophoresis in an 8% polyacrylamide, 8M urea sequencing gel. Oligonucleotides were precipitated with ethanol, and then fractionated on 10% polyacrylamide sequencing gels, or on 5% native polyacrylamide gels by electrophoresis in an 8% polyacrylamide, 8M urea sequencing gel. The radioactive bands were visualized by autoradiography and gel slices containing sets of three adjacent oligonucleotide bands were excised to prepare the digo(dT)ₙ subsets dTₙ₋₉₀, dTₙ₋₈₀, and dTₙ₋₇₀. For simplicity, these size ranges are referred to in the text according to their respective average sizes. The gel pieces were mechanically disrupted and then extracted overnight in 0.3 ml of 0.3 M sodium acetate in TE buffer (10 mM Tris-HCl, pH 7.5, 1 mM EDTA) while stirring. The samples were centrifuged through 0.2-μm filters (Millipore) at 8,000 rpm for 10 min, and the oligonucleotides were precipitated by the addition of 2.5 volumes of ethanol. After washing with 70% ethanol, the plat was dissolved in 10 μl of TE buffer. The yield of oligonucleotides was monitored by Cerenkov counting. Unless otherwise noted, all oligonucleotide quantities or concentrations in the text are expressed in terms of moles of nucleotides.

Other Reagents—E. coli SSB was purchased from Pharmacia, phage T₄ polynucleotide kinase from U. S. Biochemical Corp., and phage T₄ polynucleotide kinase was from New England Biolabs. Leupeptin, aprotinin, and tritium deoxynucleotidy transferase were from Boehringer Mannheim. X. laevis females were obtained from Xenopus I. DE81 ion exchange paper was from Whatman. Prepackaged phenyl-Superox, heparin-Sepharose Hi-Trap, and Superdex 200 columns were from Pharmacia. The PhoPh column was from Preparative BioSystems. Single-stranded DNA cellulose and protease inhibitors phenylmethylsulfonyl fluoride, pepstatin, and benzamidine-HCl were obtained from Sigma. Other chemicals were reagent grade products obtained from Fisher Scientific or Sigma.

Purification of X. laevis DNA Pol γ—Initial stages in purification of DNA Pol γ from a Triton X-100 lysate of X. laevis ovary mitochondria were as described (22). Following chromatography on phenyl-Superox or PhoPh, the polymerase was further purified by preparative gel filtration on a Superdex 200 (1.6 × 60 cm) column at 0.5 M KCl in buffer S (10 mM Tris-HCl, pH 8.0, 1 mM MgCl₂, 2 mM benzamidine-HCl, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml E-64, 1 μg/ml pepstatin). The peak of DNA pol γ activity was diluted with S buffer to 100 mM KCl, loaded on a 1 ml heparin-Sepharose column and eluted with a gradient of 0.1 to 1 M KCl. Fractions containing peak activity were pooled, adjusted to contain 40% glycerol, and stored in aliquots at –80°C. This preparation contained no mtSSB detectable by Western blot analysis with a polyclonal antibody raised against purified mtSSB. For routine assays during purification, polymerase activity was measured using a poly(A):oligo(dT) template as described (22). For the purposes of this paper, 1 unit of DNA polymerase activity was defined as incorporation of 1 nmol of dTMP on poly(dA) at 37°C in 10 min at 30°C.

Purification of X. laevis mtSSB—mtSSB was prepared from the same mitochondrial lysates used to prepare DNA pol γ. mtSSB bound to S-Phage-Sepharose and eluted at approximately 200 mM KCl, before DNA pol γ (23). Fractions containing single-stranded DNA binding activity recovered from the S-Sepharose column were loaded onto a single-stranded DNA cellulose column. Following extensive washing with S buffer containing 800 mM NaCl, mtSSB was step eluted with 3 mM NaCl in buffer S. mtSSB was essentially homogeneous following chromatography on single-stranded DNA cellulose as judged by Coomassie Blue and silver staining of overloaded protein gels and by N-terminal sequence determination (23). mtSSB was dialyzed against a solution containing 50% glycerol, 10 mM Tris-HCI (pH 8.0), 1 mM EDTA, 2 mM DTT, 2 μg/ml leupeptin, 2 μg/ml aprotinin, 2 μg/ml E-64, 0.2 mM phenylmethylsulfon fluoride, 1 μg/ml pepstatin and stored at –20°C.

Protein Quantification—During purification, total protein was quantified using the Bradford assay (Bio-Rad) with bovine serum albumin as a standard (24). The concentration of mtSSB in the final preparation was determined by spectrophotometry using an extinction coefficient, ε 280 of 3.04 ml mg⁻¹ cm⁻¹, as calculated from the amino acid content of Xenopus mtSSB-1 using the method of Gill and von Hippel (25). It should be noted that this is an approximation since the complete sequence of Xenopus mtSSB-2 is not known. Concentrations of X. laevis mtSSB (14.6 kDa) and E. coli SSB (18.8 kDa) are given in units of monomers throughout the text.

DNA Binding Reactions and Electrophoretic Mobility Shift Analysis—Unless noted otherwise, binding reactions were carried out in buffer A (10 mM Tris-HCl (pH 8.0), 2 mM DTT, 200 μg/ml BSA). Reactions, usually 8 or 10 μl, were assembled at 0°C and then incubated for 15–30 min at 23°C. Half of each reaction (4 or 5 μl) was loaded onto a 6.5% polyacrylamide slab gel (6 × 10 × 0.7 cm), prepared in a buffer containing 10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 2 mM thiglycolic acid. Prior to loading of the samples, the gel was prerun at 80 V for 1 h. Electrophoresis was performed at 80 V for 70–90 min at room temperature or at 4°C. The gel was dried onto DE81 paper under vacuum, and then exposed to Kodak XAR-5 film at room temperature without intensifying screens.

In experiments with SSB proteins and Xenopus DNA pol γ, the SSB protein was added first to the reaction mixtures containing [γ-³²P]dATP, at the concentrations indicated. DNA substrates were preincubated with mtSSB for 15 min at 23°C before addition of DNA pol γ to initiate reaction. After reaction the 3–5' exonucleolytic activity of DNA pol γ, MgCl₂, and KC1 were added at final concentrations indicated in the figure legends to the standard reactions containing 10 mM Tris-HCI (pH 8.0), 2 mM DTT, 200 μg/ml BSA. The reactions containing MgCl₂ were terminated by the addition of EDTA to 5 mM, and a portion of each reaction was analyzed by mobility shift electrophoresis as described above.

DNA Polymerase Reactions—Polymerase reaction mixtures contained 0.275 μg/ml 18:71-mer (5'-³²P-labeled primer) or 5 μg/ml M13mp7 DNA annealed to a single 5'-labeled 15-mer universal sequencing primer. Reactions included 10 μM Tris-HCI (pH 8.0), 8 mM MgCl₂, 50 mM KC1, 2 mM DTT, 200 μg/ml BSA, and 50 μM each of dATP, dGTP, and dTTP except for reactions in which dCTP was omitted.

2 T. Whitford and D. F. Bogenhagen, unpublished observation.
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Mobility Shift Analysis of the Interaction of mtSSB with Single-stranded DNA—We characterized the behavior of mtSSB in electrophoretic mobility shift analysis as a prelude to the study of the effects of mtSSB on primer-template recognition by DNA pol γ. Fig. 1 shows a comparison of the binding of Xenopus mtSSB with that of two well-characterized single-stranded DNA-binding proteins, E. coli SSB and T4 gene 32 protein. These proteins were incubated with a set of 5′-end labeled oligonucleotides, oligo(dT)_{12-100}, and the products were subjected to electrophoresis as described under “Experimental Procedures.” A homopolymeric DNA was used in these experiments to avoid complications that would result from sequence-specific interactions that might be observed with DNAs having heterogeneous sequences. Binding of either X. laevis mtSSB or E. coli SSB to the heterogeneous oligo(dT)_{12-100} resulted in the appearance of three discrete shifted species, which were tentatively identified as complexes with one to three SSB tetramers bound per oligonucleotide. In contrast, binding of T4 gene 32 protein produced a single, broad shifted band, as expected for a protein with a small binding site size and a highly cooperative binding pattern.

An additional experiment was performed to study the binding of mtSSB to narrowly fractionated size classes of oligo(dT)$_n$, ranging from 24 to 92 nucleotides in length (Fig. 2). Oligo(dT)$_{24}$ bound poorly to mtSSB, resulting in a smear of radioactive fragments in gel lane 2. Oligos containing 32, 40, or 48 residues bound mtSSB to form discrete complexes for which the electrophoretic mobility increased as the length of the oligonucleotide increased. This seemingly anomalous behavior may be expected for the binding of short oligonucleotides to the very basic mtSSB (calculated pI of 9.92). We conclude that oligonucleotides as short as 24 nucleotides can associate with mtSSB, although more stable complexes are formed with oligonucleotides of ≥32 residues. As the size of oligo(dT)$_n$ was increased to 58 residues or longer, more slowly migrating complexes were formed that were consistent with the binding of two mtSSB tetramers (or three tetramers for oligo(dT)$_{92}$). The dimer complexes formed on oligo(dT)$_{58}$ and oligo(dT)$_{92}$ showed the same unusual gel mobility observed for complexes containing single mtSSB tetramers. Complexes containing oligo(dT)$_{92}$ migrated more rapidly than those containing oligo(dT)$_{58}$.

The data shown in Figs. 1 and 2 suggest that the mode of binding of Xenopus mtSSB to oligo(dT)$_{12-100}$ at low salt concentration is similar to that previously characterized for E. coli SSB. At low salt, E. coli SSB is thought to bind in the SSB$_{35}$ mode, exhibiting “unlimited” cooperativity that suggests extensive protein-protein interactions between SSB tetramers (3). Electron micrographs of E. coli SSB bound to DNA have been...
Interpreted as showing nucleosome-like octamers or paired tetramers of the protein decorating the DNA (Ref. 26; reviewed in Refs. 1 and 3). Therefore, we performed a mobility shift experiment to see whether mtSSB and E. coli SSB would employ species-specific protein-protein interactions in forming pairs of tetramers on single-stranded DNA. Oligo(dT)$_{80}$, which is large enough to bind two tetramers of Xenopus mtSSB or E. coli SSB was used in the experiment. Prior to the binding reaction, mtSSB and E. coli SSB were mixed at different molar ratios with a constant total molar concentration of SSB. Fig. 3 shows that reactions containing either pure mtSSB or pure E. coli SSB formed predominantly complexes containing two tetramers of SSB bound to dT$_{80}$ (marked X2 and E2 in Fig. 3, lanes 2 and 8, respectively). In the reactions with mixtures of Xenopus mtSSB and E. coli SSB, a complex of an intermediate mobility between X2 and E2 appeared (marked XE in Fig. 3, lanes 3-7). This hybrid complex presumably contained one Xenopus mtSSB tetramer and one E. coli SSB tetramer. The ratio of the hybrid double complexes to the homologous double complexes formed by E. coli SSB depended on the molar ratio of Xenopus mtSSB to E. coli SSB in the reaction, and was close to 2 when both proteins were presented in equimolar concentrations (Fig. 3, lane 5). These results are consistent with a random choice of the second tetramer bound to create a double complex. If cooperative protein-protein interactions are required for the formation of double complexes on dT$_{80}$, it appears that Xenopus mtSSB is not precluded from forming such complexes with E. coli SSB.

Effects of mtSSB on the Binding of DNA Pol $\gamma$ to Primer-Template—We tested the effects of addition of mtSSB on DNA synthesis on singly primed M13 DNA as shown in Fig. 4 by monitoring the extension of a 5'-labeled 15-mer primer. Reactions were performed in duplicate with either a full complement of dNTPs or with omission of dCTP. In the absence of mtSSB, the majority of primers were extended by DNA pol $\gamma$ under both conditions. In the presence of all four dNTPs, addition of mtSSB resulted in the production of longer extension products but with a progressive decrease in the utilization of primers. In particular, the fraction of extended chains arrested at a hairpin in M13mp7 was markedly reduced in the presence of mtSSB. These results indicate that the major mechanism by which mtSSB stimulates DNA pol $\gamma$ is to increase the overall processivity of DNA synthesis, not to enhance primer recognition. The decrease in primer utilization in the presence of mtSSB is not simply a consequence of the involvement of DNA polymerase in processive synthesis, since decreased primer utilization was observed in reactions in which extension was limited by omitting dCTP.

We performed experiments similar to those shown in Fig. 4 with oligonucleotide primer-templates that would not be expected to show a significant effect due to the processivity of DNA synthesis. We studied the effect of mtSSB on the ability of DNA pol $\gamma$ to bind and extend an 18-mer primer annealed to a 71-mer template (prepared as described under “Experimental Procedures”). The template strand includes a mixed-sequence 31-mer ligated to the 3' end of dT$_{40}$ to provide a unique sequence capable of specifically binding primers adjacent to a preferred binding site for mtSSB. As shown in Fig. 5, DNA pol $\gamma$ was capable of utilizing this primer-template efficiently, fully extending nearly 100% of the primers in less than 10 min at 30°C in the presence of a full complement of dNTPs. Addition of mtSSB reduced primer utilization on this substrate. Fig. 5B shows the results of a similar set of reactions performed in the absence of dCTP. DNA pol $\gamma$ extended a smaller fraction of primers in the absence of dCTP. We suggest that DNA pol $\gamma$ may remain bound more persistently to the 3' end of the primer when it is in contact with a template strand in the absence of one nucleotide, and may not participate in multiple rounds of primer binding and extension under these conditions. Addition
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![Fig. 6. In situ activity of DNA pol \(\gamma\) on a specific primer-template following mobility shift electrophoresis. 2 ng of 31:71-mer primer-template was preincubated for 15 min at 0°C in the absence of mtSSB (lanes 2, 6, and 9) or in the presence of either 1.8 ng of mtSSB (lanes 1, 3, 5, and 7) or 5.4 ng of mtSSB (lanes 4 and 8). After the addition of 2 \(\mu\)l of pol \(\gamma\) (0.064 units) to the final reaction volume of 8 \(\mu\)l (lanes 2–4 and 6–9), the incubation at 0°C was continued for 15 min, and then the reactions were loaded onto a native 6% polyacrylamide gel. Samples in lanes 1–4 contained 5\(\mu\)prime-\[^32P\]-labeled 31:71-mer (5 \(\times\) 10^5 dpm per sample) while those in lanes 5–8 contained unlabeled 31:71-mers. The sample in lane 9 contained no added primer-template. After electrophoresis, the gel region with lanes 1–4 was dried, while the other half with lanes 5–9 was placed in a reaction mixture with [\(\alpha\]-\[^32P\]]dATP for in situ assay of DNA polymerase activity as described under “Experimental Procedures.”](image)

of mtSSB also resulted in a slight decrease in primer extension in reactions performed in the absence of dCTP. It is interesting to note that the presence of mtSSB resulted in an increase in the fraction of primers shortened by one or two nucleotides by the 3'→5' exonuclease activity of DNA pol \(\gamma\). Similar results were obtained when primers 12 or 31 nucleotides in length were used to vary the size of the single-stranded DNA tail at the 5' end of the template (data not shown).

Effect of mtSSB on the Exonuclease Activity of DNA Pol \(\gamma\)—Xenopus pol \(\gamma\) possesses a potent proofreading 3'→5' exonucleolytic activity (27) which efficiently digests single-stranded oligonucleotides in the presence of Mg^{2+} ions by a distributive mechanism. Fig. 8 shows the results of an experiment to study the effect of mtSSB on exonucleolytic digestion of dT_{12-100} by pol \(\gamma\). As shown in panel A, complexes of oligo(dT) remained stably bound to mtSSB throughout the course of treatment with the exonuclease activity of DNA pol \(\gamma\). The sizes of oligonucleotide fragments remaining following treatment of free oligo(dT) or oligo(dT) complexed with mtSSB were analyzed on a sequencing gel as shown in panel b. In the absence of mtSSB, pol \(\gamma\) digested dT_{12-100} to 10–17 nucleotides in the course of a 10-min reaction at 30°C (lanes 1–5). In the presence of mtSSB, pol \(\gamma\) efficiently digested the shorter oligo(dT), \((n < 40\) nucleotides\) which did not form stable complexes with mtSSB. However, mtSSB protected a fraction of dT_{12-100} from 45 to 65 nucleotides from digestion during incubations as long as 1 h (lanes 6–10). We conclude that mtSSB inhibits exonucleolytic digestion of oligonucleotides stably bound to the protein.
Effects of mtSSB on DNA Pol γ—mtSSB is well adapted to play a role in replication of mtDNA. The strand displacement mechanism employed in mtDNA replication features intermediates with extensive single-stranded regions. Binding of mtSSB to exposed single-stranded DNA protects against nucleases and stimulates the overall rate of DNA synthesis by DNA pol γ. Only a few studies have focussed on the mechanism by which mtSSB stimulates the homologous DNA pol γ. In this paper we present experiments to study the effect of mtSSB on primer-template recognition by Xenopus DNA pol γ. We have used electrophoretic mobility shift assays as a sensitive method to detect complexes containing DNA polymerase γ bound to specific oligonucleotide primer-templates. As a first step in this approach, we characterized the ability of mtSSB to bind single-stranded oligonucleotides using electrophoretic mobility shift analysis. To avoid potential sequence-specific effects, we characterized the ability of mtSSB to bind either heterogeneous mixtures of oligo(dT)12-100 (Fig. 1) or specific narrow size class of oligo(dT) (Fig. 2). Oligonucleotides containing 32 to 48 residues bound stably to single mtSSB tetramers in the gel shift assay while longer oligonucleotides bound two or three tetramers. Most of the binding experiments presented in this paper were performed at low ionic strength. However, other experiments not shown established that binding reactions performed at higher salt (up to 5 mM MgCl₂ or 200 mM KCl) could be analyzed with the same protection pattern for mtSSB could be obtained by treating complexes with the 3′-5′ exonuclease of T4 DNA polymerase. 

**DISCUSSION**

Effects of mtSSB on Primer-Template Binding by DNA Pol γ. The results of two representative experiments are shown in Fig. 9A. Exonuclease treatment of complexes at 3 mM MgCl₂ resulted in a progressive decrease of the size of bound oligomers to approach a fairly stable limit of 32 nucleotides at 20 mM KCl and of 44 nucleotides at 50 mM KCl. A longer incubation time was required at 50 mM KCl to permit complete digestion of unbound oligonucleotides to the limit fragment size of =6 nucleotides. Longer treatments with the DNA pol γ exonuclease resulted in a progressive loss of protected complexes. Fig. 9B shows the results of similar protection experiments performed under several solution conditions. Increasing the KCl concentration resulted in an increased size of protected fragments at either 3 or 15 mM MgCl₂. Increasing the concentration of MgCl₂ from 0.3 to 3 mM did not appreciably affect the size of protected fragments (data not shown), while a further increase to 15 mM MgCl₂ markedly increased the size of protected fragments (compare curves 1 and 2). Control experiments showed that a similar protection pattern for mtSSB could be obtained by treating complexes with the 3′-5′ exonuclease of T4 DNA polymerase (curve 4). An additional control with E. coli SSB using this exonuclease protection assay revealed an increase in fragment size with increasing KCl, consistent with the well known behavior of this protein established using fluorometric techniques (3). Overall, these results indicate that mtSSB responds to increasing ionic strength in a manner similar to that of E. coli SSB.

**FIG. 7. Effect of mtSSB on the binding of DNA pol γ to primer-template.** In each 10-μl reaction, 2 ng of 5′-32P-labeled 18:71-mer (≈5 × 10⁴ dpm) was incubated for 15 min at 23°C in the absence of mtSSB (lanes 1–5) or presence (lanes 6–10) of 2 ng of mtSSB, then pol γ (0.033 units/μl) was added in the amounts of 0.4 μl (lanes 2 and 7), 0.7 μl (lanes 3 and 8), 1.1 μl (lanes 4 and 9), or 1.7 μl (lanes 5 and 10) and incubated was continued for 15 min at 23°C. 5-μl portions of the reactions were analyzed by electrophoresis in a native 6% polyacrylamide gel. Labeled: a, free primer-template complex; b, the primer-template bound to mtSSB; c, complex of pol γ with the primer-template; d, complex of pol γ with the primer-template bound to mtSSB.

**FIG. 8. Binding of mtSSB to dT₁₂₋₁₀₀ inhibits exonucleolytic digestion by DNA pol γ.** Two reactions were assembled containing 16.5 pmol of 5′-32P-labeled dT₁₂₋₁₀₀ (≈5 × 10⁴ dpm) in a final volume of 35.7 μl of 10 mM Tris-HCl (pH 8.0), 3 mM MgCl₂, 50 mM KCl, 2 mM DTT, 200 μg/ml BSA. After incubation for 15 min at 23°C in the absence of mtSSB (reaction I) or in the presence of 3.3 pmol of mtSSB (reaction II), 6.5-μl portions were removed to serve as starting points (shown in lanes 1 and 6, respectively), then the reactions were transferred to 30°C and 13.3 μl (0.36 units) of pol γ was added. At different times during incubation, 9-μl portions from the reactions were transferred on ice and mixed with 1 μl of 50 mM EDTA. Samples were taken from reaction I after incubation for 1, 2, 5, or 10 min (lanes 2–5) and from reaction II after incubation for 5, 10, 20, or 60 min (lanes 7–10). 3-μl portions from each sample were subjected to electrophoresis in a native 6% polyacrylamide gel (a) or in a 15% polyacrylamide, 8 M urea gel (b). Migration of the standards, 5′-32P-labeled dT₁₅ and MspI digest of pUC9 DNA is shown in lanes 0 and 11, respectively, in panel b.
interactions between SSB tetramers in such higher order complexes are not well understood. We tested whether mtSSB and E. coli SSB could form mixed octamers in the experiment shown in Fig. 3. The results show that the heterologous mtSSB tetramers associate randomly with homologous or heterologous partners in octamer complexes. Thus, neither SSB tetramer requires exclusive species-specific protein-protein interactions to form double complexes.

After characterizing the behavior of mtSSB in electrophoretic mobility shift assays, we studied the ability of mtSSB and DNA pol γ to bind to defined oligonucleotide primer-templates. We found that DNA pol γ binds specifically to a 31:71-mer primer-template in the absence of Mg\(^{2+}\) ions to form a complex that can survive native gel electrophoresis and exhibits in situ polymerase activity following addition of MgCl\(_2\) and dNTPs (Fig. 6). We then used mobility shift electrophoresis to test the hypothesis that binding of mtSSB to the template adjacent to a primer might facilitate binding of DNA pol γ. The 18:71-mer primer-template was designed to have a 53-nucleotide single-stranded tail that should be able to accommodate both DNA pol γ and, at the dT\(_{40}\) terminus, mtSSB. Oligonucleotide primer-templates, including the 31:71-mer, are clearly capable of binding both proteins (Figs. 6 and 7). However, this experiment revealed that the presence of mtSSB actually reduces the affinity of DNA pol γ for the 18:71-mer primer-template. The decreased binding by DNA pol γ observed in this experiment could reflect some degree of steric hindrance or perhaps the ability of mtSSB to destabilize the 3' end of the DNA helix. Since we have shown that both DNA pol γ and mtSSB are able to bind to a 31:71-mer primer-template (Fig. 6), it seems unlikely that the presence of mtSSB bound to the longer 53-nucleotide tail of the 18:71-mer primer-template would block binding of pol γ. One reason we began these experiments was to determine whether a protein-protein interaction between mtSSB and DNA pol γ might increase the efficiency of primer binding by DNA pol γ. This was not observed. In addition, we have not observed binding of pol γ to an affinity column of immobilized recombinant mtSSB under conditions that permit active binding of single-stranded DNA to the column.

The observation that mtSSB does not increase primer binding by DNA pol γ is entirely consistent with other kinetic experiments we have performed. Addition of mtSSB stimulates overall DNA synthesis but reduces the efficiency of primer utilization on singly-primed M13 templates and on poly(dT): oligo(dA) (Fig. 4). Other studies using Drosophila DNA pol γ have reported increased primer utilization in the presence of E. coli SSB or Drosophila mtSSB (9, 20) in assays using singly primed M13 DNA in the absence of dCTP. It is not clear at this point whether this results from species-specific differences in binding properties or other technical considerations. Our experiments have consistently shown that stimulation of DNA pol γ activity by mtSSB results from an increase in the polymerization rate and processivity of DNA pol γ, not on primer recognition by the polymerase. These effects are most notable on pyrimidine-rich templates or on templates containing hair-pinned structures, which are unfavorable templates for DNA replication by highly purified DNA pol γ.

Effects of mtSSB on the 3'→5' Exonuclease Activity of DNA Pol γ—Most of the binding experiments in this paper were performed in the absence of Mg\(^{2+}\). In the presence of Mg\(^{2+}\) ions, DNA pol γ efficiently digested oligo(dT)\(_{12-100}\) via its intrinsic 3'→5' exonucleolytic activity (Fig. 8). Binding of mtSSB inhibited this degradation, resulting in trimmed complexes.
containing single mtSSB tetramers complexed with single-stranded DNA. The data are consistent with the possibility that mtSSB may slide toward the 5' end of the kinase-labeled oligo(dT) to permit DNA pol γ to trim the 3' end. This method appears to provide a reasonable estimate of the binding size for mtSSB. The length of single-stranded DNA retained in these complexes was found to increase with increasing ionic strength in a manner reminiscent of the behavior of E. coli SSB (Fig. 9). Both the exonuclease protection and fluorescence quenching methods have practical limitations. For the exonuclease assay, we do not know how closely the exonuclease of pol γ can approach an mtSSB tetramer. This method may either overestimate the true binding site size if the exonuclease is sterically hindered as it approaches mtSSB or may underestimate the binding site size if the exonuclease begins to unwrap single-stranded DNA associated with mtSSB. The fluorescence quenching method is similarly limited by the fact that single-stranded DNA associated with mtSSB. The fluorescence quenching method is similarly limited by the fact that single-stranded DNA “linkers” between adjacent SSB tetramers are included in the binding site size measurement. Therefore, we suggest that the exonuclease method may be a useful adjunct to the classical approach employing fluorescence quenching to measure the binding size.

To our knowledge, this is the first demonstration that mtSSB blocks the 3'→5' exonuclease activity of DNA pol γ. While this is not a surprising result, it does serve to illustrate a potentially important role for this protein that is not frequently considered. One model for the generation of deletions in mtDNA observed in certain human diseases is that the extensive single strands of mtDNA replication intermediates may be cleaved by endonuclease to generate 3'-OH termini that may mispair at other sites, occasionally causing deletions (28). Protecting these single strands from endonucleases and from additional exonucleolytic degradation by DNA pol γ (or other enzymes) is surely a major function of mtSSB. Zeviani (29) has speculated that a deficiency of mtSSB may play a role in the development of deletions in mtDNA. We suggest that the effects of mtSSB on both the polymerase and exonuclease activities of DNA pol γ should be considered as factors potentially involved in mtDNA mutagenesis.

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