Functional Interactions of Mitochondrial DNA Polymerase and Single-stranded DNA-binding Protein

TEMPLATE-PRIMER DNA BINDING AND INITIATION AND ELONGATION OF DNA STRAND SYNTHESIS*  

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Single-stranded DNA-binding proteins (SSBs)1 serve critical roles in DNA replication, repair and recombination (1). Whereas high affinity DNA binding by SSBs can occur independently of other proteins, both functional and physical interactions between SSBs and a variety of enzymes involved in the above processes have been documented. In particular, interactions between SSBs and replicative DNA polymerases have been demonstrated in bacterial, nuclear, and viral systems (1).

The near-homogeneous mitochondrial polymerase from Drosophila embryos catalyzes relatively efficient DNA synthesis on both predominantly double- and single-stranded DNA templates (2, 3), yet its activity and processivity are greatly affected by reaction conditions (4). Mitochondrial SSBs share similar physical and biochemical properties with Escherichia coli SSB (5–10), with which they exhibit a high degree of amino acid sequence conservation (10–12). Considering the roles

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The abbreviations used are: SSB, single-stranded DNA-binding protein; pol, DNA polymerase; mtSSB, mitochondrial single-stranded DNA-binding protein; nt, nucleotide(s); DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; ssDNA, single-stranded DNA.

served by E. coli SSB in bacterial replication in helix destabilization (13) and in enhancing DNA polymerase processivity (14, 15) and fidelity (16, 17), we purified Drosophila mtSSB and studied its effects in in vitro DNA synthesis by pol γ, in an assay that mimics lagging DNA strand synthesis in mitochondrial replication (9). These studies allowed the first demonstration of stimulation by a mtSSB of DNA synthesis by a near-homogeneous pol γ. Our biochemical data are consistent with an important role for mtSSB in mitochondrial DNA replication that has been documented genetically by the fact that a null mutation in the gene for the yeast homolog (RIM1) results in complete loss of mitochondrial DNA in vivo (7). Furthermore, we found that Drosophila mtSSB stimulates pol γ by a mechanism highly similar to that which we found for E. coli SSB (9, 18). Here we demonstrate a dual role for mtSSB in initiation and elongation of DNA strand synthesis catalyzed by pol γ, and evaluate for the first time the effects of mtSSB on the mispair-specific 3′→5′ exonuclease in pol γ.

EXPERIMENTAL PROCEDURES

Materials

Nucleotides and Nucleic Acids—Unlabeled deoxy- and ribonucleotides were purchased from Amersham Pharmacia Biotech. [3H]dTTP, [α-32P]dATP, and [γ-32P]dATP were purchased from ICN Biochemicals. Recombinant M13 DNAs (M13trx22, 10,650 nucleotides (nt); M13mp19, 7249 nt; and M13mp7, 7238 nt) were prepared by standard laboratory methods. Oligodeoxynucleotides complementary to the M13 viral DNAs were synthesized in an Applied Biosystems oligonucleotide synthesizer. Primers for primer extension and 3′→5′ exonuclease assays were 17 nt in length to produce a 3′-terminal base pair (dGMP-dCMP) on M13trx22 DNA. The sequences of the 17-mer and 15-mer are complementary to positions 993–1014 and 987–993 in M13mp19 DNA; this primer was the gift of Dr. Charles McHenry (University of Colorado Health Science Center). The sequence of the 38-nt primer used in the template-primer binding and idling experiments is complementary to positions 6291–6329 in M13mp7 DNA.

Enzymes and Proteins—Drosophila DNA polymerase γ (Fraction VI, >90% homogeneous) was prepared as described by Wernette et al. (3). Drosophila mtSSB (~90% homogeneous) was prepared from embryonic mitochondria essentially as described by Thommes et al. (9). Bovine pancreatic DNAase I (Type IV) and T4 polynucleotide kinase were purchased from Sigma and Roche Molecular Biochemicals, respectively. Sequenase, version 2.0, was purchased from United States Biochemical Corp.

Methods

Bacterial Subcloning, Overexpression, and Purification of Recombinant mtSSB—The 972-base pair coding sequence of Drosophila mtSSB was engineered by polymerase chain reaction amplification of a full-length cDNA clone (12) to contain NdeI restriction endonuclease sites at its ends; on the amino-terminal end, an NdeI site was created to contain an ATG at amino acid position 16, corresponding to the residue prior to the first amino acid in the mature Drosophila mtSSB (9), and on the
carboxyl-terminal end, the Ndel site was positioned at a site 11 base pairs distal to the termination codon. The resulting DNA fragment was purified by gel electrophoresis, cleaved with NdeI, and cloned into the bacteriophage T7 promoter-based expression vector pET-11a (Novagen) at its unique Ndel site. The E. coli strain BL21 (DE3) (Novagen) was used for transformation, and ampicillin-resistant plasmid-containing cells were screened for ampicillin resistance.

For overexpression, pET-11a recombinant plasmid-containing BL21 (DE3) cells (400 ml) were grown at 37 °C with aeration, in Luria broth containing 100 μg/ml ampicillin. When the bacterial cells reached an optical density of 0.6 at 595 nm, isolated Fraction VI enzyme (0.2 unit) was added to 0.3 ml, and the culture was incubated further for 2 h. Cells were harvested by centrifugation, washed in 50 ml Tris-HCl, pH 7.5, 10% sucrose, recrystallized, centrifuged in liquid nitrogen, and stored at −80 °C.

For preparation of cell extracts and purification of recombinant mtSSB, frozen cells were thawed on ice, and all further steps were performed at 0 to 4 °C. Cells were suspended in 1/5 volume of original cell culture in 50 ml Tris-HCl, pH 7.5, 10% sucrose, 2 ml EDTA, 5 mM dithiothreitol (DTT), 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mM sodium metabisulfite, 2 μg/ml leupeptin. Cells were lysed by incubation for 30 min in the presence of 0.3 mg/ml final concentration of hen egg white lysozyme (Roche Molecular Biochemicals) and 20 μM spermidine followed by freezing in liquid nitrogen and thawing on ice. The suspension was then centrifuged at 17,500 × g for 30 min. The supernatant fluid was recovered for use as the soluble pol fraction for Cibacron blue agaroze chromatography.

The soluble extract (~70 mg of total protein, ~4 mg of mtSSB) was diluted to an ionic equivalent of 100–120 mM NaCl and loaded onto a Cibacron blue agaroze column (1.9 × 35 cm) equilibrated with buffer containing 30 mM Tris-HCl, pH 7.5, 10% glycerol, 100 mM NaCl, 2 mM EDTA, 2 mM DTT, 1 mM PMSF, 10 mM sodium metabisulfite, 2 μg/ml leupeptin. The column was washed with 40 ml of the same buffer containing 800 mM NaCl, and the bound protein was eluted with 40 ml each of buffer lacking NaCl and containing 0.5, 1.0, and 1.5 mM NaSCN. Recombinant mtSSB eluted at 1.5 mM NaSCN; fractions were pooled; dialyzed against buffer containing 50 mM KPO4, pH 7.6, 200 mM KCl, 0.1 units of Fraction VI enzyme. mtSSB (0.4 unit of Fraction VI enzyme) was added as indicated in the figure legends. Incubation was at 30 °C for the indicated times. Samples were then made 1% in SDS and 10 mM in EDTA, heated for 10 min at 65 °C, and precipitated with ethanol in the presence of 1 μg of sonicated salmon sperm DNA. Samples were electrophoresed, the gel was processed, and the data were quantitated as described above.

DNA Nase I Footprinting—Pol γ interactions at the template-primer terminus were examined by DNAase I footprinting on singly primed M13 DNA in an experimental scheme modified from Reems and McHenry (19). 70-nt primer was annealed to M13mp19 DNA (at a position corresponding to 993–1041 in the latter) and radiolabeled at its 3′-end essentially as per Reems and McHenry (19), except that the 3′-end was extended by 2 nt with Sequenase, version 2.0, in the presence of [α-32P]dATP (3000 C/mm, 0.13 μM) and dDTTP (0.8 μM). The radiolabeled template-primer DNA was separated from excess unannealed primer and unincorporated nucleotide by gel filtration on a Sephadex G-50 column equilibrated with 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.

The DNase I digestion reactions were performed in a reaction mixture (4 μl) containing 50 mM Tris-HCl, pH 8.5, 4 mM MgCl2, 5 mM DTT, 30 mM KCl, 400 μg/ml bovine serum albumin, 400 μg/ml tRNA, 500 μM of each of dGTP, dATP, and dTTP, and [32P]dCTP, and [3H]dTTP (1000 cpm/pmol), 10 μM each of dGTP, dATP, and dTTP, and 0.04 ml) was added as indicated in the figure legends. Incubation was at 30 °C for the indicated times. Samples were then made 1% in SDS and 10 mM in EDTA, heated for 10 min at 65 °C, precipitated with ethanol in the presence of 1 μg of sonicated salmon sperm DNA. Samples were electrophoresed, the gel was processed, and the data were quantitated as described above.

DNA Synthesis Assay for the Stability of Pol γ DNA Complexes—The stability of pol γ-DNA complexes in template-primer binding and elongation was determined by an experimental scheme modified from Hacker and Alberts (20). Reaction mixtures contained 50 mM Tris-HCl, pH 8.5, 4 mM MgCl2, 10 mM DTT, 400 μg/ml bovine serum albumin, 30 or 120 mM KCl, and 4 μM singly primed M13mp7 DNA (as nt). For experiments to examine the effect of mtSSB on the stability of primer binding, mtSSB (0.4 μg/200 μmol of DNA as nt) was added to the reaction mixture and incubated for 1 min at 30 °C. Pol γ (0.2–0.7 unit) was then added, and the incubation was continued for 1 min. After incubation with pol γ, a 50-μl aliquot was removed and terminated as described below. DNase I-activated calf thymus DNA as the substrate.

Preparation of 5′-32P-Labeled M13 DNA Substrates for Product Analysis—Synthetic oligodeoxynucleotides (15, 17, or 35 nt) as described under “Experimental Procedures” were 5′-end-labeled. The kinase reaction (0.04 ml) contained 50 mM Tris-HCl, pH 8.3, 10 mM MgCl2, 0.1 mM EDTA, 5 mM dithiothreitol, 0.1 mM spermidine, γ-32PATP (0.2 μM, 4500 Ci/mmol), 28 or 54 pmol (as nt) of oligonucleotide (15-mer and 17-mer or 38-mer, respectively), and 10 units T4 polynucleotide kinase. Incubation was for 30 min at 37 °C. Recombinant M13 DNA (M13srz22 DNA for 15- and 17-nt primers and M13mp7 DNA for 38-nt primer) was added to a concentration of ~70 μM (as nt, in 4-fold molar excess over homologous oligonucleotide), and the DNA mixture was precipitated with ethanol. The pellet was resuspended in a buffer (0.1 ml) containing 10 mM Tris-HCl, pH 8.0, 0.3 M NaCl, and 0.05 M sodium citrate and was incubated at 65 °C for 1 h, followed by incubation at 37 °C for an additional 1 h in order to anneal the primer to the template.

The reaction mixture and incubated for 1 min at 30 °C. Pol γ (0.2–0.7 unit) was then added, and the incubation was continued for 1 min. After incubation with pol γ, a 50-μl aliquot was removed and terminated as described below. DNase I-activated calf thymus DNA (180–600 μM as nt) was added to the remainder of the reaction mixture to serve as a DNA trap. After DNA trap addition, 50-μl aliquots were removed at varying times to tubes containing dGTP, dATP, and dTTP at a final concentration of 0.3 mM each. Reactions were incubated for an additional 2 min at 30 °C to allow primer extension to the GGG trinucleotide position. Reactions were terminated and samples were processed and analyzed as described for the 3′ → 5′ exonuclease assay.

Experiments to examine pol γ idling were conducted essentially as above. Pol γ, in the presence or absence of mtSSB was preincubated with the M13 DNA substrate for 1 min at 30 °C dGTP, dATP, and dTTP for 5 min with a final concentration of 0.3 mM each. Reactions were then added to a final concentration of 0.3 mM each. Reactions were incubated for an additional 2 min at 30 °C to allow primer extension to the hairpin by pol γ molecules still associated with
Mitochondrial DNA polymerase activity of Drosophila DNA polymerase γ—We showed previously that SSB stimulates the DNA polymerase activity of Drosophila DNA polymerase γ on singly-

RESULTS

Bacterial Overexpression and Purification of Recombinant Drosophila mtSSB—Drosophila mtSSB was purified previ-

Mitochondrial SSB Stimulates Both the DNA Polymerase and 3′ → 5′ Exonuclease Activities of Drosophila DNA Poly-

FIG. 3. mtSSB increases the rate of initiation of DNA synthesis by Drosophila pol γ. Primer extension was performed at 30 mM KCl in the absence of dCTP as described under “Experimental Procedures.” Reactions were performed in the absence (A, closed circles, and B) or presence (A, open circles, and C) of recombinant mtSSB. B and C, DNA product strands were isolated, denatured, and electrophoresed in 18% polyacrylamide gels; quantitation of the data obtained in three experi-

ments is shown in A. Lane 1 (B and C) represents controls lacking both pol γ and mtSSB. Lanes 2–5 and 6–8 represent time points of 5, 10, 20, and 40 s and 1, 2, and 4 min, respectively.

FIG. 2. mtSSB stimulates DNA synthesis and mispair hydroly-

FIG. 1. Bacterial overexpression and purification of Drosoph-

"Mitochondrial SSB Stimulates Both the DNA Polymerase and 3′ → 5′ Exonuclease Activities of Drosophila DNA Poly-

mously to homogeneity from both whole embryos and embryonic mitochondria with a high yield of 0.5–1.0 μg/ml of embryos (9).

To pursue further biochemical and physical studies of the in-

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ments is shown in A. Lane 1 (B and C) represents controls lacking both pol γ and mtSSB. Lanes 2–5 and 6–8 represent time points of 5, 10, 20, and 40 s and 1, 2, and 4 min, respectively.
primed M13 DNA, in an assay that mimics lagging DNA strand synthesis in mitochondrial replication (9, 18). To extend these results and provide a comparative analysis for the native and recombinant proteins, we assayed both forms in the DNA synthesis reaction, and also evaluated their effects in mispair hydrolysis by pol γ on the same DNA substrate containing 3’-terminal mispaired primers. Native and recombinant Drosophila mtSSBs stimulate similarly the DNA polymerase activity of pol γ over a broad range of KCl concentrations (Fig. 2). DNA polymerase activity is stimulated 18- and 21-fold, respectively, at 15 mM KCl, thereby lowering the KCl concentration required to achieve optimal DNA synthetic rate 8-fold relative to the 120 mM KCl concentration that is optimal in the absence of SSB.

Likewise, the native and recombinant Drosophila mtSSBs stimulate the 3’ → 5’ exonuclease activity of pol γ. Both the maximal stimulation, 16- and 13-fold, respectively, and the KCl titration curves are highly similar to those for the DNA synthetic reaction. This suggests the likelihood of effective coordination of the two activities in native pol γ under the fluctuating ionic conditions present in the mitochondrial matrix (21, 22).

Notably, the mispair specificity of the 3’ → 5’ exonuclease is unchanged in the presence of mtSSB; over the entire KCl range, less than 10% of the paired termini generated by 3’-terminal mispair hydrolysis were hydrolyzed (data not shown). Thus, although we have shown previously that mtSSB enhances the processivity of pol γ in nucleotide polymerization, contributing severalfold to the overall stimulation of DNA synthetic rate (9), the mechanism of stimulation of the 3’ → 5’ exonuclease is clearly unrelated to enhanced processivity. At the same time, it may be likely that mtSSB would enhance the processivity of pol γ in hydrolysis of multiple 3’-terminal mispairs.

Our working hypothesis for the mechanism of pol γ stimulation is that mtSSB increases the rate of initiation on single-stranded substrates for both DNA synthesis and exonuclease-catalyzed hydrolysis. We examined the former experimentally using a time course analysis of DNA synthesis that involves limited primer extension on M13 DNA in the absence of dCTP, such that DNA strand termination occurs after polymerization of 8 or 11 deoxynucleotides. We find that mtSSB stimulates the production of short nascent strands 10–30-fold from 5–120 s of incubation (Fig. 3). This stimulation results in 40 versus 1.3% of the substrate being utilized at 2 min of incubation in the presence versus the absence of mtSSB, respectively.

Assuming a binding site size of 68 nt per tetramer (9), mtSSB was used in this analysis at a level 2.5-fold in excess of that required to saturate the ssDNA and was preincubated with the DNA substrate in the presence of reaction mix containing dNTPs for 5 min prior to pol γ addition. Thus, the lag before maximal stimulation of DNA strand synthesis likely relates to the time required for formation of productive pol γ complexes.

mtSSB Enhances Primer Binding by Pol γ—mtSSB may stimulate the rate of initiation of DNA synthesis by enhancing primer recognition and binding, by enhancing formation of stable and/or productive pol γ-template-primer DNA complexes, and/or by stimulating nucleotide polymerization per se. To evaluate these possibilities, we began with a DNase I footprinting analysis of template-primer DNA binding. Because pol γ has a high affinity for ssDNA, we examined primer-terminus interactions directly using an M13 DNA substrate to which was annealed an oligonucleotide primer (49 nt) labeled at its 3’-terminus. We find that in the absence of accessory proteins, the pol γ heterodimer forms a stable complex with template-primer DNA that results in DNase I protection of 20 nt of the substrate being utilized at 2 min of incubation in the presence versus the absence of mtSSB, respectively.

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**Fig. 4.** DNase I footprinting of pol γ on template-primer DNA. DNase I footprinting was performed at 30 mM KCl on singly primed M13 DNA as described under “Experimental Procedures.” A, reactions were performed in the absence of mtSSB and in the presence of 0, 7, 14, 27, 55, 83, or 0 fmol of Drosophila pol γ (lanes 1–8, respectively). B, reactions were performed in the presence of saturating recombinant mtSSB and 0, 7, 14, 27, 55, 83, or 0 fmol Drosophila pol γ (lanes 2–8, respectively). Lane 1 represents a control reaction lacking pol γ and mtSSB. The sizes of the DNA fragments were determined relative to a DNA sequencing ladder electrophoresed in a parallel lane (not shown). 7 fmol of Drosophila pol γ corresponds to 1.1 ng and 0.09 units.
DNA (Fig. 4B). Thus, mtSSB enhances primer recognition and binding, yet the 3–4-fold increase can account only partially for the 30-fold stimulation of the rate of initiation observed under similar low salt reaction conditions (Fig. 3). In this regard, the DNase I footprinting analysis does not relate directly substrate DNA binding to catalysis and so does not measure the potential contribution of mtSSB to productive complex formation, which might exhibit a shorter half-life than that required to observe a stable footprint. The footprinting analysis of primer binding may therefore represent an underestimate of the effect of increased binding on catalysis.

**Stability of Pol γDNA Complexes in Template-Primer Binding and Enzyme Idling—**mtSSB may stimulate primer recognition and template-primer binding indirectly, by eliminating nonproductive binding of pol γ to ssDNA regions. Alternatively or in addition, mtSSB may stabilize enzyme-DNA interactions, increasing the lifetime rather than the formation of productive complexes. We examined the latter by measuring dissociation rates using a strategy developed by Hacker and Alberts (20) to study the bacteriophage T4 holoenzyme. The experimental scheme is shown in Fig. 5. M13 DNA primed with a 5′-end-labeled oligonucleotide is used as the substrate for DNA binding and catalysis. By staging the addition of a DNA trap either before or after dNTP addition, we measured by DNA product strand analysis the rate of dissociation of pol γ in the presence or absence of mtSSB, either from the primer terminus or after the incorporation of eight nucleotides, where the enzyme pauses in the absence of dCTP at the position of the three consecutive template dGMP residues.

We find that mtSSB has a modest effect on the dissociation rate of pol γ bound at the primer terminus and measured in terms of productive complexes, that is, enzyme bound with the capacity for nucleotide polymerization (Fig. 6). At 30 mM KCl, where stimulation by mtSSB is 30-fold at 2 min in the initiation assay, the half-life of pol γ primer binding is 13.2 ± 2.3 and 4.6 ± 0.82 min in the absence and presence of mtSSB, respectively. Because the half-life for dissociation in either case is longer than the time required to observe the effect on initiation, the negative contribution of mtSSB on complex stability is minimized. The relatively long half-life of the pol γDNA complexes suggests that formation rather than the stability of productive complexes is rate-limiting in the overall replication scheme. The apparently negative effect of mtSSB on complex stability at low salt is not present at 120 mM KCl, where stimulation is less than 3-fold, and pol γ activity alone is highest. Notably, the dissociation rate at 120 mM KCl is 15-fold higher than at 30 mM KCl, with a half-life of 0.87 ± 0.15 min. This likely reflects a salt-stimulated rapid recycling of pol γ upon nonproductive DNA binding to ssDNA regions.

As with template-primer DNA binding, we find that mtSSB has only a modest effect on the dissociation rate of pol γ upon enzyme idling after polymerization of 8 nt (Fig. 7). The dissociation rate of pol γ upon idling is 16–40-fold slower at 30 mM than at 120 mM KCl, with half-life values of 17.9 ± 3.2 and 1.08 ± 0.18 min, respectively, in the absence of mtSSB, and 33.8 ± 6.0 (30 mM KCl) and 0.84 ± 0.15 min (120 mM KCl) in its presence. The stabilizing effect of mtSSB on the idling complex likely reflects the structural difference between an elongation complex versus an initiation complex. The functional or physical interactions between the enzyme and mtSSB may also change once pol γ engages in processive DNA synthesis. Notably, whereas DNA synthesis is stalled at the position of the 22-base pair hairpin helix in the DNA substrate (see Fig. 5) in the absence of mtSSB, pol γ polymerizes 3 nt into the helix at 120 mM KCl (Fig. 7D) and completely through it at 30 mM KCl (Fig. 7B) in the presence of mtSSB. This and the longer half-life of the stalled complex at 30 mM KCl are likely a reflection of both the substantially higher processivity of pol γ at low salt (18), and the further enhancement of enzyme processivity by the helix destabilizing function of mtSSB (9).

Taken together, the dissociation experiments indicate that whether bound at the primer terminus in the absence of nucleotides, or stalled in the polymerization mode by a single nucleotide omission, pol γDNA complexes are remarkably stable. Thus, the major contribution of mtSSB in stimulating the rate of initiation of DNA synthesis is most likely in complex formation.

**DISCUSSION**

We have evaluated mechanistically the effects of mtSSB on the catalytic activities of pol γ under in vitro conditions that mimic lagging DNA strand synthesis in mitochondrial replication. Our finding that mtSSB stimulates similarly both the DNA polymerase and 3′ → 5′ exonuclease activities of pol γ over a broad range of KCl concentrations suggests functional coordination of the two activities at the replication fork. In evaluating 3′ → 5′ exonuclease activity, we measured specific mispair hydrolysis on oligonucleotide-primed ssDNA. At the replication fork, the growing 3′-terminus is paired to the template strand that is coated with mtSSB (23). Thus, coordination of DNA polymerase and 3′ → 5′ exonuclease in pol γ is anticipated in the catalysis of proofreading DNA synthesis that is required to ensure replication fidelity, and our results support this. In fact, that mispair hydrolysis is specifically stimulated by mtSSB over base pair hydrolysis may reflect a lower cost of editing in mitochondrial DNA replication as compared with bacterial and bacteriophage systems with replicative DNA polymerases that exhibit high nucleotide turnover (1).
We have shown previously that SSB increases severalfold the processivity of nucleotide polymerization by pol γ (18). Nevertheless, neither the major contribution of mtSSB to increasing the rate of nucleotide polymerization nor its stimulation of 3'→5' exonuclease are apparently a consequence of it.

We have shown here that in stimulating the rate of initiation of DNA strand synthesis by pol γ, mtSSB increases the fraction of substrate molecules utilized 30-fold and thus likely recruits pol γ to the primer terminus. Whether this occurs by an active or passive mechanism remains to be elucidated, but our DNase I
footprinting analysis supports the recruiting model. Remarkably, the footprinting data show that unlike most replicative DNA polymerases, the native pol γ heterodimer forms stable complexes with template-primer DNA, and template-primer binding is enhanced in the presence of mtSSB. mtSSB alone does not protect the primer from digestion, nor does it alter the footprinting pattern of pol γ, which protects two helical turns of the DNA template strand at the primer terminus.

Mikhailov and Bogenhagen (24) found mtSSB to inhibit binding of Xenopus pol γ to template-primer DNA, in a gel mobility shift analysis on short oligonucleotides. On such substrates, we would anticipate efficient binding of pol γ alone because the ssDNA present is less than 50 nt per substrate molecule. Indeed, we find that oligonucleotide template-primer binding by Drosophila pol γ is not stimulated by mtSSB nor is the initiation of DNA synthesis on such substrates.2 We suggest that initiation of lagging DNA strand synthesis in mitochondrial replication should require mtSSB-facilitated primer recognition and binding by pol γ where, as in our model M13 assay in vitro, the displaced lagging DNA strand template is thousands of nucleotides in length (25). Our data show clearly that the rate of initiation of DNA strand synthesis to produce an 8–11-mer on a long ssDNA is stimulated up to 30-fold by mtSSB.

What factors mediate the functional interactions of pol γ with mtSSB? Functional interactions may occur upon DNA binding and/or by specific physical interactions. E. coli SSB is known to bind DNA in several modes depending on ionic conditions (26). Studies of mtSSBs also show salt-dependent effects on DNA binding and, in particular, on binding site size and in cooperativity of DNA binding (5, 8, 9). Ionic conditions likely affect both DNA conformation and SSB structure, and these may vary on different template primers. Likewise, pol γ binding to the template DNA at the primer terminus may differ from that in long ssDNA regions. Our data show that mtSSB stimulates DNA synthesis by pol γ on M13 DNA over a broad range of salt concentrations, under which the effects on mtSSB on template-primer binding and dissociation by pol γ vary substantially. This suggests flexibility in presumptive protein-protein interactions and perhaps in the mode of mitochondrial DNA replication under the fluctuating ionic conditions that occur in vivo (21, 22).

Specific physical interactions have been demonstrated between replicative DNA polymerases and SSB proteins in several systems. Both bacteriophage T4 and T7 DNA polymerases interact physically with their cognate SSBs, the gene 32 and 2.5 proteins, respectively (27–30). That T7 DNA polymerase is known to interact physically with their SSBs, the gene 32 and 2.5 proteins, respectively (27–30). That T7 DNA polymerase is known to interact physically with their cognate SSBs, the gene 32 and 2.5 proteins, respectively (27–30). That T7 DNA polymerase is known to interact physically with their cognate SSBs, the gene 32 and 2.5 proteins, respectively (27–30). That T7 DNA polymerase is known to interact physically with their cognate SSBs, the gene 32 and 2.5 proteins, respectively (27–30). That T7 DNA polymerase is known to interact physically with their cognate SSBs, the gene 32 and 2.5 proteins, respectively (27–30). That T7 DNA polymerase is known to interact physically with their cognate SSBs, the gene 32 and 2.5 proteins, respectively (27–30). That T7 DNA polymerase is known to interact physically with their cognate SSBs, the gene 32 and 2.5 proteins, respectively (27–30).

The acidic carboxyl-terminal tail of the latter and has a dual effect on in vitro DNA synthesis under elevated salt conditions in DNA strand initiation and in chain elongation. Our functional data resemble those studies: mtSSB stimulates both primer recognition by pol γ and enhances the processivity of nucleotide polymerization. Interestingly, although mtSSB is a homolog of bacterial SSB, sharing significant sequence (7, 10–12) and structural similarity (36), it lacks the acidic carboxyl terminus required for physical interaction in both E. coli SSB and in T7 gene 2.5 protein (30). In fact, the carboxyl terminus appears as a disorganized loop in the crystal structure of the human mtSSB (36). Thus, future studies to link functional with physical interactions between pol γ and mtSSB have the potential to reveal novel interaction domains.

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