Mitochondrial Single-stranded DNA-binding Protein from Drosophila Embryos

PHYSICAL AND BIOCHEMICAL CHARACTERIZATION*

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Using a stringent purification procedure on single-stranded DNA cellulose, we have isolated the mitochondrial single-stranded DNA-binding protein from Drosophila melanogaster embryos. Its identity is demonstrated by amino-terminal sequencing of the homogeneous protein and by its localization to a mitochondrial protein fraction. The mitochondrial protein is immunologically and biochemically distinct from the previously characterized nuclear replication protein A from Drosophila (Mitsis, P. G., Kowalczykowski, S. C., and Lehman, I. R. (1993) Biochemistry 32, 5257-5266; Marton, R. F., Thömmes, P., and Cotterill, S. (1994) FEBS Lett. 342, 139-144). It consists of a single polypeptide of 18 kDa, which is responsible for the DNA binding activity. Sedimentation analysis suggests that D. melanogaster mitochondrial single-stranded DNA-binding protein exists as a homo-oligomer, possibly a tetramer, in solution. The protein binds to DNA in its single-stranded form with a strong preference over double-stranded DNA or RNA, and binds to polyuridylates preferentially over polypurines. Drosophila mitochondrial single-stranded DNA-binding protein exhibits a greater affinity for long oligonucleotides as compared to short ones, yet does not show high cooperativity. Its binding size, determined by competition studies and by fluorescence quenching, is approximately 17 nucleotides under low salt conditions, and increases in the presence of greater than 150 mM NaCl. The homogeneous protein stimulates the activity of mitochondrial DNA polymerase from D. melanogaster embryos, increasing dramatically the rate of initiation of DNA synthesis on a singly primed DNA template.

Many of the processes involved in DNA metabolism including DNA replication, recombination, and repair, generate intermediates containing single-stranded regions of DNA. These regions are stabilized and kept accessible for the various catalytic processes by the binding of single-stranded DNA-binding proteins (SSBs).¹ Prokaryotic SSBs (e.g., Escherichia coli (Eco) SSB and bacteriophage T4 gene 32 protein) are generally small proteins which bind to single-stranded DNA (ssDNA) with high affinity. They show high specificity for ssDNA over double-stranded DNA (dsDNA) and RNA, but display little sequence specificity (reviewed in Refs. 1-3). Although they do not exhibit direct catalytic function, they stimulate DNA replication in vitro.

Mitochondrial DNA replication is independent from chromosomal DNA replication and is carried out largely with specific mitochondrial replication proteins including the mitochondrial DNA polymerase (pol γ) and an SSB (mtSSB) distinct from the nuclear SSB, replication protein A (RP-A). mtSSB appears to have at least two important functions during mtDNA replication, by stabilizing the displaced ssDNA that is the template for lagging DNA strand synthesis (4). mtSSBs have been isolated from several species including rat (4, 5), Xenopus laevis (6), and yeast (7). These proteins consist of a single small (13-16 kDa) polypeptide, which shows a high degree of similarity to Eco SSB in its primary structure (7, 8). Although all the functions of mtSSB in mtDNA metabolism have not been defined, it is critical for replication, because depletion of the yeast protein (RIM1) causes loss of mitochondrial DNA (7). Consistent with a role in mtDNA replication, interactions between mtSSB and other mitochondrial replication proteins have been observed. In vitro studies indicate that under some conditions, the rat and X. laevis mtSSBs stimulate partially purified forms of mitochondrial DNA polymerase (9, 10), and a putative human mtSSB stimulates human pol γ (11). In addition, genetic evidence from yeast suggests an interaction between RIM1 and the mtdNA helicase, Rif1 (7).

We have purified a single-stranded DNA-binding protein from Drosophila embryos (hereafter called Dm mtSSB) to near homogeneity. Its physical and biochemical properties demonstrate that it is distinct from the nuclear SSB, dRP-A, but has a high degree of similarity to Eco SSB and to eukaryotic mtSSBs. Further, its functional interaction with the near-homogeneous mitochondrial DNA polymerase from Drosophila melanogaster embryos (12) suggests that it serves an important role in Drosophila mtDNA replication.

EXPERIMENTAL PROCEDURES

Materials

Chemicals—Ultrapure deoxy- and ribonucleoside 5'-triphosphates, and Mono Q, Mono S, and nick columns were from Pharmacia Biotech Inc. DNase I (RNase-free) was from Boehringer Mannheim. [γ-32P]ATP and [α-32P]CTP were from Amersham Corp.; [3H]dTTP was from ICN Biochemicals. Pepstatin, leupeptin, and ssDNA cellulose were from Pharmacia LKB. Restriction endonucleases were from Northumbria Biologicals Ltd.

Nucleic Acids—Recombinant and wild-type M13 DNAs were prepared by standard laboratory methods (13). Oligonucleotides comple-

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¹ The abbreviations used are: SSB, single-stranded DNA-binding protein; Eco SSB, Escherichia coli SSB; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; pol γ, DNA polymerase γ; mtSSB, mitochondrial SSB; Dm mtSSB, Drosophila melanogaster mtSSB; nt, nucleotide(s); MS, mass spectrometry; RP-A, replication protein A.
mentary to the M13 viral DNAs and those indicated below were synthesized in an Applied Biosystems oligonucleotide synthesizer, according to the manufacturer’s protocol. Homo-oligomeric sequences were \((dA)_{32}, (dC)_{32}, (dG)_{32}, \text{and} (dT)_{32}\). Hetero-oligomeric sequences were \(5'\) GTGCTTCCGGTTCTACG (25 nt), \(5'\) GGGCTCTCAATTGAGCCACGATTTCG (32 nt), \(5'\) TATGCGATCTGTTACGATATGGTTCCG (35 nt), \(5'\) GCAATGGTGCTGACGTTGCG (37 nt), \(5'\) AACAGCTGATGAAATTGTATGCTAAGATGACTGATC (32 nt), and \(5'\) AAGCTTGGCAGCAGTGTGATGATGCCTGTTTAT (58 nt), and \(5'\) AACGTGTCGTCGTTGCGATCTGTTATGCTAAGATGACTGATC (32 nt). Hetero-oligomeric sequences were \(5'\) TGGTCATAG CTGTTTCCTGTGTGAAATTGTTA (75 nt).

**Methods**

Labeling of Nucleic Acids—A 230-base pair fragment of the common region of the tomato golden mosaic virus was labeled as described elsewhere (14). The reaction product of the polymerase chain reaction was radioactively labeled and made amenable to ultraviolet cross-linking to protein by inclusion of \([\alpha-32P]dCTP\) and bromo-dUTP, respectively, in the reaction. To produce a ssDNA substrate, the DNA was heated to 95°C for 5 min and then cooled rapidly in an ice bath. Oligonucleotides were labeled at their 5’-ends as previously described (13), using 5 pmol of DNA and 10–25 \(\mu\)Ci of \([\alpha-32P]dATP\).

**Gel Mobility Shift Assay—** Enzyme fractions containing Dm mSSB, Dm-PrA, or Eco SSb were incubated at 20°C for 30 min in reactions (25 \(\mu\)l) containing 50 fmol of 5’-labeled oligonucleotides in 20 \(\mu\)l Tris-HCl (pH 7.5), 4% sucrose, 8 \(\mu\)l dithiothreitol, and 80 \(\mu\)g bovine serum albumin. Reactions were brought to a final concentration of 5% glycerol (v/v), 0.033 (w/v) bromophenol blue, and 0.03% (w/v) xylene cyanol FF, loaded onto a 10% polyacrylamide gel in 1 \(\times\) TBE (89 mM Tris borate, 89 mM boric acid, and 2 mM EDTA) and electrophoresed at 100 V. The gels were dried onto Whatman No. 3MM paper prior to autoradiography.

**Fluorometry—** Dm mSSB was used at concentrations of 1–5 \(\times\) 10\(^{-7}\) M. Samples were diluted in buffer B containing the indicated amount of NaCl. Fluorescence spectra were recorded from excitation at 284 nm were recorded from 300–400 nm with a scan speed of 1 nm/s on a Baird-Atomic spectrophotometer. For the determination of fluorescence quenching, excitation was at 284 nm with 2-nm slit width, and emission was monitored at 348 nm with 20-nm slit width. Binding site size was calculated as described elsewhere (17, 18).

**Miscellaneous Methods—** SDS-gel electrophoresis was performed according to Laemmli (19); protein determination was performed using the Bio-Rad protein assay kit and bovine serum albumin as standard (20). Silver staining of SDS-gels was performed using the Bio-Rad silver stain plus kit. Amino-terminal sequencing was performed by automated sequential Edman degradation in an Applied Biosystems model 494 pulse liquid peptide sequenator. MALDI-MS analysis was performed using a PerSeptive Biosystems Voyager mass spectrometer (PerSeptive Biosystems).

**Purification of Dm mSSB—** The initial steps of the purification of Dm mSSB followed those used in the isolation of Dm-PrA (14). Briefly, 40 g of 0–6-h D. melanogaster embryos (wild type, Oregon R) were dechorionated and homogenized at 0°C in 2.5 volumes of homogenization buffer (50 mM Hapes (pH 7.5), 10 mM EDTA, 0.5 mM NaCl, 2.5 mM 2-mercaptoethanol, 2 \(\mu\)g/ml leupeptin, 1 \(\mu\)g/ml pepstatin A, 1 \(\mu\)g/ml phenylmethylsulfonyl fluoride, 10 mM sodium metabisulfite, and 10 mM benzamidine) in a dounce homogenizer using a Teflon pestle. The lysate was cleared by threefold centrifugation at 42,000 rpm in a Sorvall T55 rotor for 30 min at 3°C. After filtration through a double layer of Miracloth (Calbiochem), it was loaded onto a 25-cm column (2.5 x 2.5 cm) equilibrated with buffer A (25 mM Hapes (pH 7.5), 10% (v/v) glycerol, 1 mM EDTA, 0.02% (w/v) Brij 58, 2.5 mM 2-mercaptoethanol, 1 \(\mu\)g/ml leupeptin, 0.5 \(\mu\)g/ml pepstatin A, 0.25 \(\mu\)g/ml phenylmethylsulfonyl fluoride, and 5 mM sodium metabisulfite) containing 0.5 M NaCl. The column was washed sequentially with 4 volumes of buffer B containing 0.5 M NaCl and 5 volumes of buffer A containing 0.75 M NaCl. Protein was eluted with buffer A containing 1.5 M NaCl and 50% (v/v) ethylene glycol. Fractions that contained protein were pooled and dialyzed against buffer B (25 mM imidazole hydrochloride (pH 7.5), 10% (v/v) glycerol, 0.1 mM EDTA, 0.02% Brij 58, 2.5 mM 2-mercaptoethanol, 1 \(\mu\)g/ml leupeptin, 0.5 \(\mu\)g/ml pepstatin A, 0.25 \(\mu\)g/ml phenylmethylsulfonyl fluoride, and 5 mM sodium metabisulfite) containing 50 mM NaCl. Precipitated material was removed by centrifugation of the dialysate in a bench-top centrifuge. The supernatant was loaded onto a 1-ml Mono Q column which had been equilibrated with buffer B containing 50 mM NaCl. Dm mSSB did not bind to the column under these conditions. The flow-through of the Mono Q column was loaded onto a 1-ml Mono S column equilibrated in buffer B containing 50 mM NaCl. Again the bulk of Dm mSSB did not bind to the column. At this stage the protein was >95% pure as visualized by silver staining of an SDS-polyacrylamide gel.

**Glyceraldehyde 3-phosphate Dehydrogenase—** A 15–30% glyceraldehyde 4 (4 ml) was formed in buffer B containing 10% sucrose and 100 mM NaCl. The Mono 5 fraction of Dm mSSB (1 \(\mu\)l) was loaded on the gradient and sedimented at 3°C for 2 h and 30 min at 50,000 rpm in a Sorvall T55 rotor. Fractions (120 \(\mu\)l) were collected from the bottom and analyzed for DNA-binding activity. Marker proteins (Sigma) were bovine liver catalase (11.3 S), rabbit muscle aldolase (7.35 S), bovine serum albumin (4.7 S), ovalbumin (3.66 S) and bovine pancreas ribonuclease A (2.2 S).

For DNAse I treatment, samples were adjusted to a final concentration of 10 mM Tris-HCl, 1 \(\mu\)g/ml bovine serum albumin and incubated with DNase I at 37°C for 30 min (100 units) prior to loading onto the gradient. Control samples were treated in the same way with omission of DNase I.

**RESULTS**

**Purification of Dm mSSB**

We followed single-stranded DNA-binding activities during the fractionation of a Drosophila embryonic extract. Two bind-
ing activities were recovered from a single-stranded DNA cellulose column that was run under stringent binding conditions (750 mM NaCl) and eluted with 50% ethylene glycol, 1.5 M NaCl; the activities differed in the extent a 55-mer oligonucleotide was shifted in a gel mobility shift assay. The protein causing the larger shift in migration was identified as the Drosophila homolog of the nuclear SSB, RP-A (14). The two SSBs could be separated by fractionation on a Mono Q column, where dRP-A was retained by the resin but the second SSB was not (Fig. 1). At this stage, the second DNA-binding activity still contained several polypeptides, and also contained a DNA helicase activity. The impurities were removed by Mono S chromatography, where the SSB again flowed through the column while the contaminating proteins were bound. The final preparation was nearly homogeneous as judged by silver staining of an SDS-polyacrylamide gel, that indicated a single band with an apparent molecular mass of \(18\) kDa (Fig. 1, lane 5). The typical yield from a preparation of 60 g embryos was 30–60 mg of purified SSB. Alternatively, the corresponding protein derived from partially purified mitochondria (12) is nearly homogeneous after fractionation on ssDNA cellulose followed by glycerol-gradient centrifugation, yielding \(0.4\) mg per embryo (data not shown).

That the 18-kDa polypeptide is responsible for DNA binding activity was demonstrated by sedimentation analysis on a glycerol gradient (Fig. 2). When the fractions displaying ssDNA binding were analyzed by silver staining, a single band was observed upon autoradiography (Fig. 2, lower panel). The band at \(50\) kDa that appears in all of the lanes is an artifact of the silver-staining method. Marker proteins were run in a parallel gradient, and their positions of migration indicated above the upper panel.

The amino-terminal sequence of the purified SSB was determined by automated sequential Edman degradation. Using the resulting 29-mer ATTTAAAPAKVEKTNTVTILGRVXADP as query sequence in a data base search, we found 96% identity to a sequence that had been assigned as the mitochondrial single-stranded DNA-binding protein from D. melanogaster (22): the amino-terminal sequence of the 18-kDa polypeptide is identical to that deduced from a recently reported cDNA sequence, beginning at residue 17 in the latter, indicating a leader peptide of 16 amino acids. Further, analysis of the 18-kDa SSB by MALDI-MS yields a mass of 13,845 \(\pm\) 14 Da, in agreement with the predicted size of 124 amino acids for the mature protein, with a mass of 13,832 Da. Also consistent with...
with 50% activity loss upon heating to 40°C. Particularly, dRP-A is heat labile and exhibits decreased binding activity after incubation at temperatures above 20°C, while mtSSB was found to be very heat stable, showing no change in DNA binding activity upon heating to 90°C. Under the same conditions, E. coli SSB retains ~75% of its DNA binding activity. In contrast, dRP-A is heat labile and exhibits decreased binding activity after incubation at temperatures above 20°C, with 50% activity loss upon heating to 40°C.

**Physical and Biochemical Properties of Dm mtSSB**

**Heat Stability**

SSBs were incubated at increasing temperatures for 10 min, cooled to room temperature and analyzed for their DNA binding ability in a gel mobility shift assay (data not shown). Dm mtSSB was found to be very heat stable, showing no change in DNA binding activity upon heating to 90°C. Under the same conditions, E. coli SSB retains ~75% of its DNA binding activity. In contrast, dRP-A is heat labile and exhibits decreased binding activity after incubation at temperatures above 20°C, with 50% activity loss upon heating to 40°C.

**DNA Binding Properties**

**Binding Site Size**—Observations made during the course of purification showed that Dm mtSSB could bind to a 32-mer but not to a 17-mer oligonucleotide substrate (data not shown), suggesting that the binding site size is larger than 17 nucleotides. To define further the effects of length on DNA binding, a 32-mer oligonucleotide was used as a labeled substrate in gel mobility shift analyses, and unlabeled oligonucleotides of increasing length were added as competitors (Fig. 3). We found that the 17-mer was unable to compete with the 32-mer oligonucleotide, even in 200-fold molar excess. In fact, only oligomers larger than 32 nt could compete efficiently for mtSSB binding, although a 25-mer oligonucleotide competed partially in 100- and 200-fold molar excess. The efficiency of binding increased with the length of the oligonucleotide. By comparison, in similar experiments with dRP-A, binding is less dependent on the length of the oligonucleotide and a 25-mer can compete efficiently, although the 17-mer still is a weak competitor (data not shown) (14).

Further evaluation of the binding site size was performed by fluorescence quenching. Analysis of emission spectra showed that excitation of Dm mtSSB at 284 nm caused maximum emission at 348 nm (data not shown). Fluorescence is quenched up to 60% upon addition of ssDNA, indicating interaction of the protein with DNA. The binding site size was determined from fluorescence titration curves in which increasing amounts of oligo- or polynucleotides were added sequentially to fixed amounts of protein. Using Dm mtSSB at concentrations of 1 × 10^{-7} M, ssM13 DNA or 75 mer oligonucleotide were added stepwise to the solution. Under low salt conditions complex formation was rapid, and equilibrium was attained within the mixing time. Because under high salt conditions the rate of binding was slower, readings were taken when the fluorescence had stabilized after addition of the DNA. Linear approximations were fitted to the initial and final slopes of the curves and the intersection of the two lines taken as the apparent binding site size. Under low salt conditions (50 mM) the calculated binding site size was 17 ± 3 nt (Fig. 4A). This size did not change significantly at lower protein concentrations or with different DNAs. However, under higher salt conditions a significant increase in the binding site size was observed. Binding site sizes of 28 ± 2 nt and 34 ± 2 nt were determined at 320 mM and 480 mM NaCl, respectively (Fig. 4A).

The salt stability of DNA binding under these conditions was monitored by increasing the NaCl concentration once maximum quenching had been achieved (Fig. 4B). There was no reduction in the quenching observed up to 500 mM NaCl. At 800 mM NaCl, 50% of the initial complex was disrupted, and only 20% of the initial quenching was retained above 1 mM NaCl. The residual quenching most likely is due to some tight form of DNA-protein complex, which is not disrupted at this salt concentration.

**Cooperativity**—The cooperativity of DNA binding by Dm mtSSB was evaluated in gel mobility shift assays using heat-denatured radiolabeled 230 mer DNA as substrate (data not shown). At low protein concentrations, a single diffuse band was observed at both 50 mM and 300 mM KCl that migrates with lower mobility than the free DNA, suggesting low cooperativity of binding (18). At 300 mM KCl, the cooperativity appears to be higher, because at higher protein concentrations a distinct band of unbound DNA is observed in addition to that corresponding to a mtSSB-DNA complex.

**DNA Composition**—To assess whether or not Dm mtSSB shows nucleotide preferences in DNA binding, homopolymeric oligonucleotides were examined in a competitive gel mobility shift assay (Table I). Adding unlabeled oligonucleotides to reactions containing radiolabeled 32-mer oligonucleotide, we found that both poly(dT) and poly(dC) were efficient competitors, while poly(dA) and poly(dG) were not. This suggests preferential binding of Dm mtSSB to pyrimidine nucleotide-containing DNAs. A similar result was obtained with Eco SSB. In contrast, dRP-A showed less pronounced base preferences, discriminating only slightly against (dA)_{10} (data not shown).

The same assay was used to examine the ability of Dm...
mtSSB to bind to dsDNA and RNA (Table I). While heat-denatured plasmid DNA competed efficiently for binding of the oligonucleotide, the same DNA in its double-stranded form did not. Similarly, tRNA did not compete, suggesting that Dm mtSSB does not bind RNA.

Stimulation of Dm Mitochondrial DNA Polymerase

We examined the effects of Dm mtSSB on DNA polymerase \( \gamma \) from \( D. \) melanogaster embryos. We found that Dm mtSSB stimulates pol \( \gamma \) activity on singly primed M13 DNA up to 18-fold, depending on the KCl concentration (Fig. 5). To evaluate the mechanism by which mtSSB stimulates Drosophila pol \( \gamma \), we examined its effects on pol \( \gamma \) processivity and on the rate of initiation of DNA synthesis by pol \( \gamma \). The processivity of Dm Pol \( \gamma \) decreases with increasing salt concentration (25).

Upon addition of Dm mtSSB, processivity is increased at each of three salt concentrations tested (30, 65, and 120 mM KCl; Fig. 6), but in a disproportional manner relative to stimulation of DNA polymerase activity at the same salt concentrations. Pol \( \gamma \) processivity is increased 2-fold from ~2000 to 2300 nt at 30 mM KCl, where stimulation is 18-fold, and ~5-fold from 145 to 780 nt at 120 mM KCl, where stimulation is 5-fold. Thus, Dm mtSSB increases pol \( \gamma \) processivity, yet this increase cannot account for the substantially greater stimulation observed over a broad range of KCl concentrations. In contrast, we found in a primer extension analysis that the stimulation of pol \( \gamma \) activity by Dm mtSSB corresponds closely with an increase in the rate of initiation of DNA synthesis by Drosophila pol \( \gamma \). We performed limited DNA synthesis on singly primed M13 DNA in the absence of dCTP, to cause DNA strand termination after the incorporation of 8 or 11 nucleotides, the first and second positions where dCTP is the required substrate (Fig. 7). Upon quantitation of the DNA product strands produced in the presence and absence of SSB, we found that Dm mtSSB stimulates primer extension 14-fold at 30 mM KCl, a value that corresponds closely with the 18-fold stimulation of pol \( \gamma \) activity observed under the same conditions in the presence of all four dNTPs.

DISCUSSION

We have purified the mitochondrial single-stranded DNA-binding protein from \( D. \) melanogaster embryos. The identity of Dm mtSSB was demonstrated by amino-terminal sequencing and MALDI-MS analysis of the single 19-kDa polypeptide, and by its localization to a mitochondrial protein fraction. Recently, a cDNA clone of the Dm mtSSB was isolated by screening of an expression library for DNA-binding proteins (22). The deduced amino acid sequence exhibits a high degree of similarity with those of mtSSBs from \( X. \) laevis (8, 24), yeast (7), rat (23), and human tissues (23) and with that of Eco SSB (26). The cDNA encodes a protein of 15.6 kDa. The amino-terminal sequence of Dm mtSSB and the deduced amino acid sequence of cDNA are identical apart from a leader sequence of 16 aa present in the latter, and MALDI-MS analysis indicates a mass of 13.8 kDa for the mature protein. An overproduced fusion protein generated from the cDNA clone migrates on SDS-polyacrylamide gels with a similar size as Dm mtSSB, and binds preferentially to ssDNA as compared to dsDNA (22).

We have found that Dm mtSSB binds to ssDNA very tightly, and that binding is resistant to 750 mM NaCl (Fig. 4B), 4 M urea and 0.25% SDS (data not shown). Dm mtSSB discriminates strongly in favor of ssDNA over dsDNA and RNA. Fluorescence quenching titrations indicate a coverage size of 17 nucleotides of DNA per monomer of protein. This is also consistent with the observed mobility shift of Dm mtSSB when it is photocross-linked to DNA. Given a binding site size of 17 nt, it is perhaps surprising that Dm mtSSB does not appear to bind to a 17-mer oligonucleotide. This cannot be explained by cooperativity of the binding reaction, since under low salt conditions Dm mtSSB shows low cooperativity. Instead, a possible explana-
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Fig. 5. Dm mtSSB stimulates the rate of DNA synthesis by Drosophila pol γ over a broad KCl range. A, DNA synthesis was measured on singly primed M13 DNA as described under "Methods," in the presence of the indicated amounts of KCl and in the absence (closed circles) or presence (open circles) of Dm mtSSB (0.8 μg). B, the data from A were replotted to show the ratio of nucleotide incorporation by Dm pol γ in the presence versus absence of Dm mtSSB at each KCl concentration.

Fig. 6. Dm mtSSB increases the processivity of Drosophila pol γ. DNA synthesis was performed on singly primed M13 DNA (6407 nucleotides) as described under "Methods," and the DNA product strands were isolated, denatured, and electrophoresed on a 1.5% denaturing agarose gel (16). Reactions were performed in the absence (lanes 1–3) or presence (lanes 4–6) of Dm mtSSB (2 μg) and incubated at 30 °C for 8 min. Lanes 1 and 4, 30 mM KCl; lanes 2 and 5, 65 mM KCl; lanes 3 and 6, 120 mM KCl. Numbers at left indicate the positions and sizes (in nt) of HindIII restriction fragments of λ-DNA and HpaII fragments of M13Gori1 DNA (28) that were electrophoresed in an adjacent lane. The reaction products were also electrophoresed in a 6% denaturing polyacrylamide gel as described by Williams et al. (25), in order to quantitate product DNA strands of ≥150 nt (data not shown).

Fig. 7. Dm 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 "Methods." DNA product strands were isolated, denatured, and electrophoresed on an 18% denaturing polyacrylamide gel (16). Reactions were performed in the absence (lane 2) or presence of Dm mtSSB (2 μg, lane 3) or Eco SSB (2 μg, lane 4). Lane 1 represents a control reaction lacking both pol γ and SSB.

Possibility is suggested by the observation that on glycerol gradients Dm mtSSB exhibits a sedimentation coefficient of 4.04 S. Thus, the native protein apparently exists as an oligomer, most likely a tetramer, leading to an effective binding site size of 68 nucleotides per tetramer. Under these circumstances, while cooperativity appears to be low between tetramers, the formation of protein-DNA complexes by the individual protomers may be highly cooperative, such that stable binding may not be observed until more than one subunit is bound to the DNA. Consistent with this explanation is the increase in efficiency of binding with an increasing oligonucleotide size of up to at least 59 nt, and a lack of protein:DNA intermediates in gel mobility shift assays that were performed to evaluate binding to the 59 mer (data not shown). We cannot however, rule out entirely the possibility that the methods used for determining the concentration of Dm mtSSB have led to an overestimate of the amount of protein used in the DNA-binding reactions, and therefore an underestimate of binding site size.

The DNA-binding parameters of Dm mtSSB show some variation at higher salt concentrations. An increase in the binding site size from 17 to 28–34 is observed, and there is also an apparent increase in the cooperativity of binding. It therefore seems likely that Dm mtSSB has more than one mode of DNA binding and that the DNA-binding mode is modulated by ionic strength. Several DNA-binding modes resulting in distinct binding site sizes have been demonstrated for Eco SSB (17). For Eco SSB, changes in cooperativity with varying salt concentrations have also been observed, although in this case binding is with high cooperativity at low salt, while it is low at salt concentrations above 200 mM (18).

Overall, the physical and biochemical characteristics of Dm mtSSB are similar to those of Eco SSB (2) and to various features of mtSSBs from rat liver (5, 9), X. laevis (6, 8), yeast (7), and a recombinant form of the human protein (27). To date, however, the effects of mtSSB on the function of mitochondrial DNA polymerase are unclear. The rat (9) and frog (10) mtSSBs have been shown to stimulate partially purified forms of pol γ on homopolymeric DNA substrates, yet the frog protein was found to be completely inhibitory to mitochondrial DNA polymerase activity on singly primed single-stranded viral DNA (10). More recently, an SSB isolated from human mitochondria was shown to enable highly purified human pol γ to utilize singly primed M13 DNA (11). Here, it is curious that the human enzyme alone cannot replicate a single-stranded DNA template; as a result, the mechanism by which the SSB exerts its
function cannot be readily assessed. At the same time, it is not known if the human SSB used in that study represents the Eco-like mtSSB reported by others.

We have shown here that the homogeneous Dm mtSSB stimulates greatly the activity of its cognate DNA polymerase, the well-characterized pol γ from D. melanogaster embryos (12, 16, 25). Our primer extension data show that the stimulation of pol γ by Dm mtSSB is due primarily to an increased rate of primer recognition and binding. Further, Dm mtSSB increases the processivity of Drosophila pol γ, reducing or eliminating pausing of DNA polymerase at specific sites on the template DNA strand where stable secondary structure is predicted. In fact, the effects of Dm mtSSB on Dm pol γ function are strikingly similar to those found previously for Eco SSB (16). Taken together with the high amino acid conservation between mtSSBs and Eco SSB and their similar physical and biochemical properties, the parallel effects of Dm mtSSB and Eco SSB on mtDNA polymerase suggest that the important role ascribed to Eco SSB in bacterial DNA replication also reflects that of mtSSB in the replication of mitochondrial DNA.

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