A Single-Stranded DNA Binding Protein from *Drosophila melanogaster*: Characterization of the Heterotrimeric Protein and Its Interaction with Single-Stranded DNA[†]

Paul G. Mitsis,[*,‡] Stephen C. Kowalczykowski,[§] and I. R. Lehman[‡]

Department of Biochemistry, Beckman Center, Stanford University School of Medicine, Stanford, California 94305, and Sections of Microbiology and of Molecular Biology, Cell Biology and Biochemistry, University of California, Davis, California 95616

ABSTRACT: We describe the purification to near homogeneity of a single-stranded DNA binding protein from 0–18-h embryos of *Drosophila melanogaster*. *Drosophila* SSB (D-SSB) is a heterotrimer with subunits of molecular weight of 70 000, 30 000, and 8000. It has a Stokes radius of 48.6 ± 2 Å and $s_{20,w} = 5.0 \pm 0.2$ S. The interaction of D-SSB with ssDNA was examined by the quenching of intrinsic protein fluorescence. The binding site size was determined to be $n = 22 \pm 4$ nucleotides with a maximum quenching $Q_m = 35 \pm 3\%$. Equilibrium titrations indicate that D-SSB binds with low cooperativity, $\omega = 10$–300, and high apparent affinity, $K\omega = (0.7$–5$) \times 10^7$ M^{-1}, at 225 mM NaCl. Sedimentation of D-SSB bound to small oligonucleotides demonstrates that D-SSB does not require protein association for binding. D-SSB stimulates the extent and processivity of DNA synthesis of its cognate DNA polymerase α. On the basis of these properties, we conclude that D-SSB is the *Drosophila* cognate of the human and yeast SSB/RP-A proteins.

The stable maintenance and propagation of genetic information requires the functional integration of replication, repair, and recombination. A class of proteins required for these three processes is the single-stranded DNA binding proteins (SSBs).[1] SSBs bind tightly and cooperatively to single-stranded DNA (Chase & Williams, 1986; Meyer & Lane, 1990). Examination of the biochemical and physical properties of SSBs from *Escherichia coli* (SSB) and bacteriophage T4 (gene 32 protein) and their interaction with DNA have begun to elucidate the nature of both the protein–DNA and the cooperative protein–protein interactions in these systems (Kowalczykowski et al., 1981; Lohman & Bujalowski, 1990; Meyer & Lane, 1990; Formosa et al., 1983) and provided a theoretical framework for the examination of protein–nucleic acid interactions in general (McGhee & von Hippel, 1974).

Proteins analogous to SSBs have been purified and characterized from several eukaryotes (Wobbe et al., 1987; Wold & Kelly, 1988; Fairman & Stillman, 1988; Brill & Stillman, 1989; Kenny et al., 1989; Thommes et al., 1991; Brown et al., 1992) and are required, both in vitro and in vivo, in many aspects of DNA metabolism. These eukaryotic single-stranded DNA binding proteins (referred to as SSB, RP-A, or RF-A) are heterotrimers of approximately 70, 30, and 10 kDa and stimulate the synthesis and processivity of their cognate DNA polymerase α (Tsurimoto & Stillman, 1989; Kenny et al., 1989). Examination of the activity of the individual subunits

has demonstrated that the large subunit has DNA binding activity (Kenny et al., 1990; Erdile et al., 1991) and the middle subunit undergoes regulated phosphorylation (Din et al., 1990). The function of the small subunit remains unclear.

In vitro the human RP-A is required for SV-40 DNA replication (Wobbe et al., 1987; Wold & Kelly, 1988), where it interacts directly with both the DNA polymerase α-primase and the origin recognition protein, large T-Antigen (Dornreiter et al., 1992). Human RP-A is also required in vitro for excision repair (Coverely et al., 1991). The *Saccharomyces cerevisiae* and human RP-As may also function in recombination, stimulating in vitro their cognate DNA strand exchange proteins (Moore et al., 1991; Heyer et al., 1990; Alani et al., 1992). The yeast RP-A has been shown to be essential in vivo; deletion of the genes of any of the three subunits is lethal, with cells arresting with single or multiple buds (Heyer et al., 1990; Brill & Stillman, 1991).

RP-As are regulated in a cell-cycle-dependent manner. The steady-state levels of mRNA of the three RP-A subunits are regulated coordinately, rising to maximal levels at the G1–S boundary (Din et al., 1990). The ~30-kDa subunit of the human or yeast RPA is multiply phosphorylated at the G1–S boundary and dephosphorylated at mitosis causing a characteristic 2–3-kDa shift in mobility of the subunit upon SDS–polyacrylamide gel electrophoresis. The cyclin-*cdc2* (p34) protein kinase, which is known to have a role in cell cycle progression, can phosphorylate RP-A on the same residues in vitro as are phosphorylated in vivo (Din et al., 1890; Dutta & Stillman, 1992; Fotedar & Roberts, 1992). Although there have been no reports of direct biochemical effects of the phosphorylation of SSB/RP-A on interaction with the purified replication components, a phosphorylation event has been shown in the crude SV-40 system to occur specifically within the replication initiation complex by a non-cell-cycle-dependent kinase (Fotedar & Roberts, 1992). Although this suggests a correlation between the phosphorylation state of SSB/RP-A and its function in DNA replication, the role of the different states of phosphorylation in SSB/RP-A in DNA metabolism is not understood.

---

[*] To whom correspondence should be addressed.
[‡] Stanford University School of Medicine.
[§] University of California, Davis.
[1] Abbreviations: ssDNA, single-stranded DNA; ssM13mp18, single-stranded M13mp18 phage DNA; SSB, single-stranded DNA binding protein; D-SSB, *Drosophila* SSB; Tris, tris(hydroxymethyl)aminomethane; HEPES, *N*-(2-hydroxyethyl)piperizine-*N'*-(2-ethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; EDTA, (ethylenedinitrilo)-tetraacetic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid; PEG$_{6000}$, poly(ethyleneglycol) 6000; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; BSA, bovine serum albumin.

In view of the crucial role of SSBs in DNA metabolism, we have begun an investigation of properties of SSB from *Drosophila melanogaster* and its interaction with nucleic acids and other proteins involved in DNA replication in this system. Aside from being a rich source of replication proteins, *Drosophila* provides a system to examine developmental and tissue-specific control of replication in metazoans and is amenable to genetic analysis to assess in vivo function.

We report here the isolation and initial characterization of the *Drosophila* analogue of RP-A. *Drosophila* SSB (D-SSB) was purified from 0–18-h embryos to 90–95% homogeneity and consists of three subunits with molecular weights of 70 000, 30 000, and 8000. Measurements of the interaction of D-SSB with ssDNA by quenching of intrinsic protein fluorescence showed that the protein binds ssM13mp18 with a site size of 22 ± 4 nucleotides with low cooperativity. D-SSB does not require oligomerization for its interaction with nucleic acids and can bind small DNA oligomers as a 1:1 protein to DNA complex. We also demonstrate that D-SSB increases the synthesis and processivity of *Drosophila* DNA polymerase α on multiprimed homopolymer templates.

MATERIALS AND METHODS

Materials

[γ-^{32}P]ATP (6000 Ci/mmol) was purchased from Amersham. Glycerol gradient, gel filtration, and ultra low SDS–polyacrylamide gel electrophoresis standards, Fast-Q Sepharose, Superdex 200 prep grade, and Mono Q resins were from Pharmacia/LKB. Salmon sperm DNA and SigmaCell Type 50 cellulose were from Sigma. Hydroxylapatite Bio-Gel HT, Bio-Gel P-60, and low molecular weight SDS–polyacrylamide gel electrophoresis standards were from Bio-Rad. Miracloth was from Calbiochem. PMSF, leupeptin, and pepstatin A were from US Biochemical. Bovine serum albumin was Pentax grade from Miles Laboratories. Polynucleotide kinase was from New England Biolabs. All chemicals used were of reagent grade and solutions were made with double-distilled water. For protein purification all glassware and plasticware were pre-chilled to 4 °C, and all operations were done at 4 °C unless otherwise indicated.

Nucleic Acids

Poly(dT) and poly(dA) were purchased from Midland Biochemicals. Single-stranded M13mp18 was purchased from U.S. Biochemical. (dT)$_{20}$ was synthesized by the PAN Facility, Beckman Center, Stanford University School of Medicine. Quantitation of nucleic acids was by spectrophotometry using the following extinction coefficients (per mole of phosphate): poly(dT), ϵ_{260} = 8.1 × 10^3, and poly(dA), ϵ_{257} = 10.0 × 10^3 (Kowalczykowski et al., 1981); ssM13mp18, ϵ_{259} = 7.37 × 10^3 (Berkowitz & Day, 1974).

Protein Determinations and Gel Electrophoresis

Protein concentration was determined by the method of Bradford (1976) as modified by Read and Northcote (1981) (reagent 1). Bovine serum albumin was used as a concentration standard. SDS–polyacrylamide gel electrophoresis was performed by the method of Laemmli (1970) with an acrylamide to bisacrylamide ratio of 233:1.

Buffers

Buffer A: 20 mM HEPES-NaOH, pH 7.5, 5 mM magnesium acetate, 50 mM potassium acetate, 1.0 mM EGTA, 0.5 mM EDTA, 0.1% (v/v) Triton X-100, 10% (v/v) glycerol, 10 mM Na$_2$S$_2$O$_5$, 1 mM DTT, 5 μg/mL leupeptin, and 5 μg/mL pepstatin A. *Buffer B*: 20 mM HEPES-NaOH, pH 7.5, 1.0 mM EGTA, 0.5 mM EDTA, 0.01% (v/v) Triton X-100, 10% (v/v) glycerol, 10 mM Na$_2$S$_2$O$_5$, 1 mM DTT, 1 μg/mL leupeptin, 1 μg/mL pepstatin A, and 0.5 mM PMSF. *Buffer C*: 20 mM Tris-HCl, pH 7.5, 200 mM NaCl, 0.1 mM EGTA, 0.1 mM EDTA, 0.001% (v/v) Triton X-100, 10% (v/v) glycerol, 0.5 mM DTT, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, and 0.5 mM PMSF. *Buffer D*: 20 mM Tris-acetate, pH 7.5, 0.1 mM EDTA, 0.001% (v/v) Triton X-100, 0.5 mM DTT, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin A, and 0.5 mM PMSF. *Buffer E*: 20 mM Tris-acetate, pH 7.5, 200 mM sodium acetate, 0.001% (v/v) Triton X-100, 5% (v/v) glycerol, 0.5 mM DTT, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin A, and 0.1 mM PMSF. *Buffer F*: 20 mM HEPES-NaOH, pH 7.5, 200 mM sodium acetate, 0.1 mM EDTA, 0.001% (v/v) Triton X-100, 50% (v/v) glycerol, 0.5 mM DTT, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin A, and 0.5 mM PMSF. Buffers were adjusted to pH at room temperature and stored at 4 °C. DTT and protease inhibitors were added just before use. All chromatography buffers were filtered through Millipore type GS filters (0.22 μm).

Purification of Drosophila DNA Polymerase α

Drosophila DNA polymerase α was prepared by the method of Kaguni et al. (1983) through the glycerol gradient step. It was purified further as follows. A Mono Q column was equilibrated with 20 mM Tris-HCl, pH 8.0, 100 mM KCl, 0.5 mM EDTA, 10% (v/v) glycerol, 0.5 mM DTT, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin, and 0.5 mM PMSF. A 0.35-mg aliquot of the glycerol gradient fraction of DNA polymerase was loaded on the column and eluted with a linear gradient of 0.1–0.4 M KCl. The peak of undegraded polymerase activity which eluted at approximately 300 mM KCl was pooled, dialyzed vs buffer F (substituting 100 mM sodium acetate for 200 mM sodium acetate), and stored at –80 °C. This preparation is 80% homogeneous as judged by quantitation by densitometry of SDS–polyacrylamide gels after staining with Coomassie Blue G and is substantially free of proteolytic breakdown products of the large (182-kDa) subunit.

Purification of D-SSB

Embryos and Extraction. *D. melanogaster* strain Canton S was cultured in population cages under standard conditions. Zero- to 18-h embryos were collected and bleach dechorionated (Schlief & Wensink, 1981). The dechorionated embryos were filtered through Nitex screen, blotted dry from below, resuspended in 25–50 mL of buffer A, and incubated on ice for 10 min. The embryo slurry was refiltered, blotted dry, and weighed. Embryos were then homogenized with 1 mL of buffer A and 0.04 mL of 100 mM PMSF per gram of embryos in a 40-mL stainless steel homogenizer with a Teflon pestle [or equivalent 40-mL dounce homogenizer with a tight fitting (A) pestle] with 10 up and down strokes. The volume of the homogenate was measured and one-ninth volume of 5 M NaCl was added to produce a final concentration of 0.5 M NaCl. This mixture was then homogenized with a Teflon pestle or B pestle with five up and down strokes and extracted 30 min on ice with occasional stirring with a sterile plastic pipette. The homogenate was then centrifuged at 100000g for 1 h at 4 °C (Beckman SW28 rotor, 28K rpm). The supernatant was collected with a syringe through the side of the tubes carefully avoiding the loose chromatin pellet. The

supernatant was recentrifuged at 100000g for 1 h at 4 °C, and the supernatant was collected. The crude extract was frozen in liquid N_2 and stored at -80 °C.

ssDNA–Cellulose Chromatography. ssDNA–cellulose containing 3–4 mg of ssDNA per gram of dry cellulose was prepared using SigmaCell Type 50 cellulose (Alberts & Herrick, 1971). A 40-mL ssDNA–cellulose column was equilibrated with 30–40 column volumes of buffer B containing 500 mM NaCl. The extract was quickly thawed in a 30 °C water bath and centrifuged at 100000g for 1 h at 4 °C to remove any precipitated material and then was filtered through sterile Miracloth. The clarified extract was loaded on the column at 0.3–0.5 g of protein per mL of ssDNA–cellulose at 0.5 column volume per h. If there is any clogging of the column by the crude extract, the ssDNA–cellulose can safely be resuspended and allowed to repack. The column was washed with 20 column volumes of buffer B containing 500 mM NaCl and eluted with buffer B containing 2.5 M NaCl at 0.5–1.0 column volumes per h, and 7-mL fractions were collected. The protein peak was pooled and dialyzed twice against 1 L of buffer C for 4 h and then against 2 L for 6–12 h. The dialysate was recovered, and precipitated material was removed by centrifugation at 25000g for 30 min at 4 °C.

Fast Q Sepharose Chromatography. A 2.0-mL column of Fast Q Sepharose was packed and washed with 5 mL of buffer D containing 10% (v/v) glycerol (buffer D10) and 1.0 M sodium acetate and then equilibrated with 10 mL of buffer D10 containing 150 mM sodium acetate. The DNA–cellulose fraction was brought to 150 mM NaCl by dilution with buffer D10, loaded by gravity, and washed with 8–10 mL of buffer D10 containing 150 mM sodium acetate. The column was eluted with a 10-mL linear gradient of 0.15–0.5 M sodium acetate in buffer D10 at 0.2 mL/min, and 0.4-mL fractions were collected. D-SSB eluted in a broad peak between 0.4 and 0.5 M sodium acetate. The protein peak was pooled and if necessary the volume was reduced to 2–3 mL by concentration against solid PEG_{6000}.

Superdex 200 Chromatography. The Fast Q Sepharose fraction was cleared by centrifugation at 150000g (Beckman TLA 100.2 rotor at 70K rpm for 20 min at 4 °C). The supernatant was loaded directly onto a Superdex 200 prep grade 16/70 column that had been equilibrated with buffer D containing 5% glycerol and 200 mM sodium acetate and was developed at a flow rate of 0.5 mL/min, and 1.5-mL fractions were collected. The peak of D-SSB protein (approximately fraction 45) was pooled and used directly in the next step.

Hydroxylapatite Chromatography. A 0.5-mL hydroxylapatite column (Bio-Gel HT) was packed and washed with 2.5 mL of buffer E containing 200 mM sodium phosphate and then equilibrated with 10 mL of buffer E. The Superdex fraction was loaded directly by gravity and washed with 5 mL of buffer E at 6.0 mL/h. D-SSB was eluted with a 5-mL linear gradient of 0–200 mM sodium phosphate in buffer E, and 140-μL fractions were collected. The fractions containing the D-SSB (35 mM sodium phosphate) were pooled, and if necessary the volume was reduced to 1 mL by concentration against solid PEG_{6000}. The D-SSB fraction was dialyzed against 500 mL of buffer F for 4–6 h and then against another 500 mL for 6–12 h. The final preparation was frozen in liquid N_2 and stored at -80 °C.

Glycerol Gradient Sedimentation

Triplicate 4.8-mL 10–25% (v/v) linear glycerol gradients were poured at 4 °C with 20 mM HEPES-NaOH, pH 7.5,

0.1 mM EDTA, 0.5 mM DTT, 0.5 μg/mL leupeptin, 0.5 μg/mL pepstatin A, 0.5 mM PMSF, and the indicated salt and were allowed to equilibrate 8 h at 4 °C. The gradients were overlaid with 0.1 mL of sample in the above buffer (no glycerol) (addition of D-SSB and/or standards brought sample to 7% glycerol). Samples contained either standards alone [20 μg of bovine liver catalase (11.3 S), 10 μg of rabbit muscle aldolase (7.35 S), 2.5 μg of bovine serum albumin (4.3 S), 10 μg of hen egg ovalbumin (3.66 S) (Smith, 1968; Gel Filtration Theory and Practice, 1987)], standards plus 10 μg of homogeneous D-SSB, or D-SSB alone. The gradients were sedimented at 60 000 rpm in a Beckman SW65 rotor (350000g) for an $\omega^2 t = 2.0 \times 10^{12}$ rad^2/s. The gradient was fractionated from top to bottom using a Haake Buchler Auto Densi-flow IIC gradient fractionator, and two drop (approximately 95–98 μL) fractions were collected. A 25-μL aliquot was assayed for protein by SDS–polyacrylamide gel electrophoresis followed by Coomassie blue or silver staining (Merril et al., 1981). The relative amounts of each protein were quantitated by densitometry of the stained gels and expressed as percent of maximum peak area.

Analytical Gel Filtration

A 45 × 1.5 cm column of Sephacryl S-300 was equilibrated at 4 °C with the same buffer used for the density gradient sedimentation to which 10% glycerol was added. The column was calibrated by chromatography of the following standards at a flow rate of 0.075 mL/min: bovine liver catalase (Cat), 52.2 Å; rabbit muscle aldolase (Ald), 48.1 Å; BSA, 35.5 Å, bovine pancreas chymotrypsinogen A (Chyg), 20.9 Å (Smith, 1968; Gel Filtration Theory and Practice, 1987). Protein chromatography was continuously monitored at A_{280}, and 1-mL fractions were collected. A 100-μg sample of homogeneous D-SSB was loaded in a sample volume of 1 mL and chromatographed under the same conditions used for the standards. A 20-μL aliquot of each fraction was analyzed as described for the glycerol gradient sedimentation fractions.

Calculation of Molecular Weight and Shape Factor

Molecular weight and shape factors were calculated (Siegel & Monty, 1966) assuming a partial specific volume for the protein of 0.73 mL/g. The axial ratio for prolate ellipsoid of revolution was determined from published shape factors (Cantor & Schimmel, 1980).

DNA Polymerase Processivity Assay

$(dT)_{20}$ was labeled with $[\gamma\text{-}^{32}P]$ATP and polynucleotide kinase by standard methods (Maniatis et al., 1982) and hybridized at a 5-fold molar (molecules) excess to $(dA)_{2000}$ in 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, and 100 mM NaCl by heating to 100 °C for 5 min followed by slow cooling to room temperature. Unincorporated ATP was separated from template on a small Bio-Gel P-60 column.

Processivity was measured in a reaction mixture containing 20 mM HEPES-NaOH, pH 7.5, 5 mM magnesium acetate, 0.1 mg/mL BSA, 0.5 mM DTT, 10% glycerol, and 40 μM dTTP. After addition of template, SSB was added and incubated for 10 min at 20 °C. DNA polymerase α was then added, and the reaction was continued an additional 15 min. The reaction were placed on ice, stopped with 1 μL of 0.5 M EDTA and 2 μL of 0.1% SDS, extracted with phenol/chloroform/isoamyl alcohol (24:24:1) and chloroform/isoamyl alcohol (24:1), ethanol precipitated, and resuspended in

electrophoresis sample buffer. The reaction products were separated by electrophoresis on 5% acrylamide gels containing 8 M urea and visualized by autoradiography.

Exonuclease Assays

The substrate for the exonuclease assay was *Hin*fI cleaved pIC20R (Marsh et al., 1984). It was either labeled at the 3' end with $[\alpha\text{-}^{32}P]dATP$ and the *E. coli* DNA polymerase large fragment or, after dephosphorylation with calf intestinal phosphatase, labeled at the 5' end with $[\gamma\text{-}^{32}P]ATP$ and polynucleotide kinase. For exonuclease assays, 48 fmol (ends) was incubated for 30 min at 20 °C with 1.5 μg of protein in polymerase assay buffer in a final volume of 20 μL. Activity was assayed by measurement of acid-soluble ^{32}P.

Spectrophotometry and Fluorimetry

Protein absorbance spectra were determined with a Cary 118 spectrophotometer in 20 mM Tris, pH 7.5, 0.1 mM EDTA, 10% (v/v) glycerol, 200 mM NaCl, 0.5 mM DTT, 0.5 $\mu g/mL$ leupeptin, and 0.5 $\mu g/mL$ pepstatin A. Initial protein fluorescence spectra were determined with a Perkin-Elmer LS5B spectrofluorimeter using 5 nm for excitation and emission slit widths and a scan speed of 60 nm/min. Stoichiometric and equilibrium titrations were performed on an SLM 8000 spectrofluorimeter. Excitation was at 290 nm with a 0.5-nm slit width, and emission was monitored at 340 nm with a 8-nm slit width. Data were collected in the slow kinetic mode, taking 2-s readings and averaging these over 20 s to obtain a data point.

To obtain stable baselines the following method was used. We observed that our preparations of D-SSB bound reversibly to the walls of quartz cuvettes (even when siliconized). Titrations were therefore performed using methylacrylate cuvettes (Perfector Scientific). A sample of D-SSB was placed in a cuvette in sample buffer to adsorb to the walls for 15–30 min, resulting in irreversible binding of the protein to the walls of the cuvette. The cuvette was washed with 3–4 volumes of water, dried by centrifugation, and subsequently used for titrations. This procedure resulted in up to 2–4% of the added protein adsorbing to cuvette walls and stable baselines over 2–3-h periods. In addition, the fluorescence signal was linear with respect to protein concentration and independent of added salt up to 1.4 M NaCl and up to 375 mM $MgCl_2$.

Titrations were performed at 20 °C in 20 mM HEPES-NaOH, pH 7.5, 0.1 mM EDTA, and 10% glycerol, plus the indicated amount of salt. D-SSB was added to the treated cuvettes with constant stirring with a siliconized stir bar, and the fluorescence signal was allowed to stabilize for 10–15 min. Aliquots of DNA titrant were added and allowed to mix for 2 min, and then readings were averaged over 1.3 min to obtain a final value of the fluorescence. Fluorescence was corrected for dilution. Under the conditions of our experiments, inner filter corrections and photobleaching were not significant.

Data Analysis and Curve Fitting

Equilibrium titration data were fit as described (Kowalczykowski et al., 1986). The value of n was fixed at 22, and Q_m was fixed at 35%. Variation of n by ±4 residues and/or Q_m by ±3% did not change the uncertainty of K and ω beyond the observed scatter in the data.

RESULTS

Purification and Physical Properties of D-SSB. The *Drosophila* SSB was purified from 0–18-h embryos. Ex-

Table I: Purification of D-SSB[a]

fraction	volume (mL)	protein (mg/mL)	total protein (mg)	% homogeneity
extract	515	32	16,400	
ssDNA–cellulose	45	0.08	3.6	35
Fast-Q	4.2	0.47	1.97	81
Superdex-200	10.5	0.13	1.37	96
HAP-concentrate	0.5	1.53	0.77	96

[a] A standard purification for D-SSB is presented. Fast-Q is Fast-Q Sepharose. HAP-concentrate is concentrated hydroxylapatite eluant. Homogeneity was determined by densitometry of the Coomassie Blue G stained gel in Figure 1.
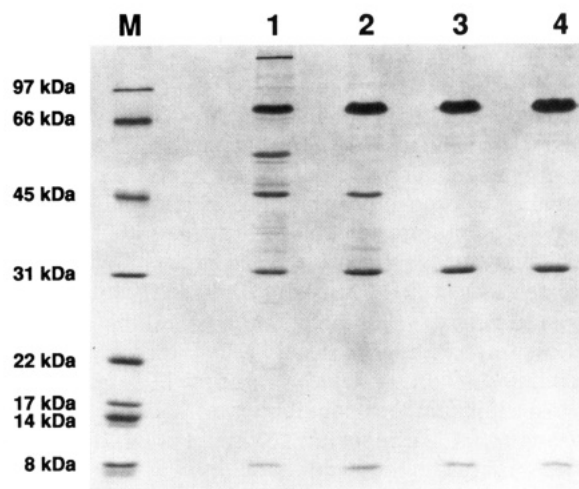

FIGURE 1: SDS–polyacrylamide gel electrophoresis of fractions obtained during the purification of D-SSB. Two micrograms of each of the protein purification fractions listed in Table I were fractionated on a 17% SDS–polyacrylamide gel and then visualized by staining with Coomassie Blue G: (M) molecular weight standards in kDa; (lane 1) ssDNA–cellulose; (lane 2) Fast-Q Sepharose; (lane 3) Superdex 200; (lane 4) hydroxylapatite concentrate.

traction of 375 g of embryos yielded 16.4 g of crude protein (Table I). The high affinity of SSBs for ssDNA made it possible to purify D-SSB to 30–40% homogeneity from the crude extract in a single step (ssDNA–cellulose chromatography, Figure 1, lane 1). This fraction stimulated *Drosophila* DNA polymerase α activity (data not shown) and contained major polypeptides whose molecular weights by SDS–polyacrylamide gel electrophoresis were consistent with those of other eukaryotic SSBs. Although initially fractionation was based on stimulation of *Drosophila* DNA polymerase α activity, the high degree of homogeneity of D-SSB and the simplicity of the peptide composition in the ssDNA–cellulose fraction allowed us routinely to follow the purification directly by SDS–polyacrylamide gel electrophoresis.

Subsequent purification by anion exchange (Fast-Q, Figure 1, lane 2) and gel filtration (Superdex 200, Figure 1, lane 2) chromatography yielded a near homogeneous preparation of D-SSB (Figure 1, Table I). Chromatography on hydroxylapatite, which did not increase the apparent homogeneity (Figure 1, lane 4) and resulted in some loss of protein, is required to remove a low level of exonuclease contamination. The final preparation contained <4 fmol/(min·mg) of 3'–5' exonuclease activity and 4 fmol/(min·mg) of 5'–3' exonuclease activity.

SDS–polyacrylmide gel electrophoresis was used to assess the purity and peptide composition of the fractions obtained during purification (Figure 1). Staining and densitometry of the most purified fraction indicated that the preparation consists of three major polypeptides with apparent molecular
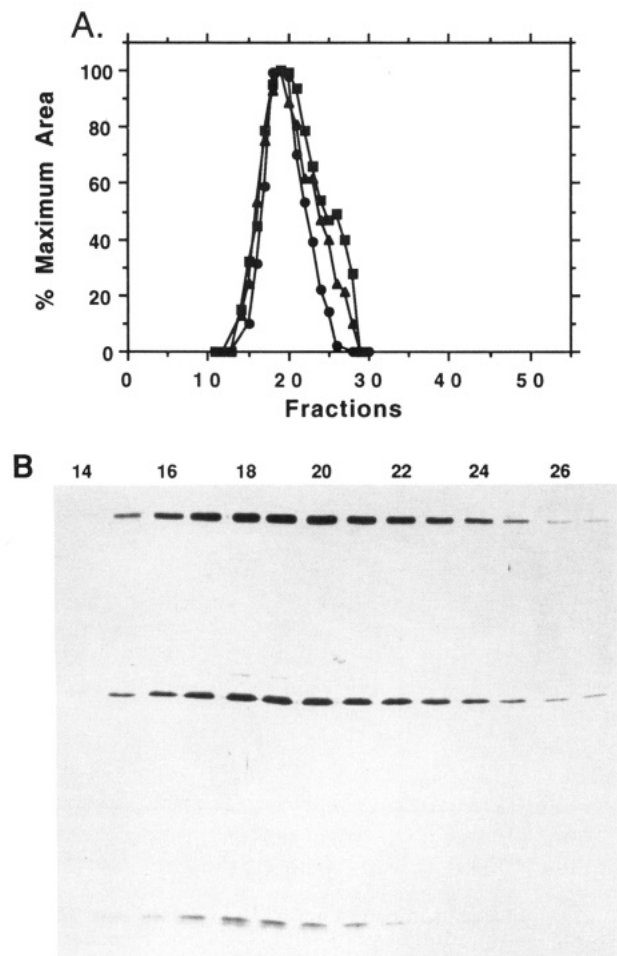
A.

B.

FIGURE 2: Cosedimentation of D-SSB subunits. Purified D-SSB (21 μg) was sedimented through a 10–25% glycerol gradient at 100 mM sodium acetate. The protein was quantitated (A) by densitometry of the silver-stained SDS–polyacrylamide gel shown in panel B. (●) 70 000 molecular weight subunit; (▲) 30 000 molecular weight subunit; (■) 8000 molecular weight subunit. (B) Silver-stained SDS–polyacrylamide gel of sedimentation fractions.
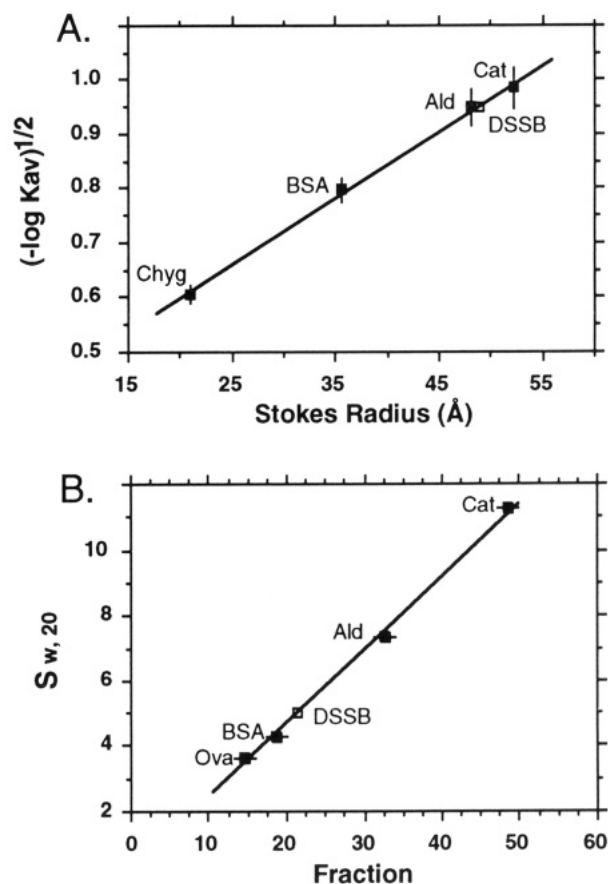
A.

B.

FIGURE 3: Determination of the Stokes radius and sedimentation coefficient of D-SSB. Analytical gel filtration (A) and sedimentation (B) were performed at 200 mM NaCl. Standards (■). D-SSB (□). Abbreviations: Cat, catalase; Ald, aldolase; BSA, bovine serum albumin; Ova, ovalbumin; Chyg, α-chymotrypsinogen. Error bars indicate plus and minus one fraction.

weights of 70 000, 30 000, and 8000 (Figure 1, lane 4). This fraction is 96% homogeneous with respect to the three peptides, which occur at a molar ratio of 1:1:1.3. The same molar ratio was seen in the cruder fractions, indicating that the extent of homogeneity was not overestimated because of coelectrophoresing contaminants. Occasionally the final preparation contained small amounts of polypeptides in the 35 000–70 000 molecular weight range. These are most likely degradation products of the 70 000 molecular weight polypeptide because of their appearance on storage at 4 °C and their cross-reactivity with polyclonal antibodies directed against this polypeptide (data not shown). The 70 000, 30 000, and 8000 molecular weight peptides cofractionated during glycerol gradient sedimentation (Figure 2), and gel filtration, anion exchange, and hydroxylapatite chromatography (data not shown). We therefore conclude that *Drosophila* SSB is a complex of three tightly associated subunits.

By comparing the subunit molecular weights determined by SDS–polyacrylamide gel electrophoresis with the native molecular weight of the protein determined by hydrodynamic measurements, the stoichiometry can be estimated (Siegel & Monty, 1968). Analytical gel filtration of the purified protein yielded a Stokes radius of 48.6 ± 2 Å (Figure 3A), and glycerol gradient sedimentation analysis of D-SSB gave an $s_{20,w}$ = 5.0 ± 0.2 S (Figure 3B), a value lower than expected from the gel filtration. The same sedimentation coefficient was obtained

at 25 and 100 mM NaCl, demonstrating that D-SSB does not aggregate at lower salt in this concentration range. The native molecular weight calculated from these data was 102 000 ± 6000. From SDS–polyacrylamide gel electrophoresis of the subunits, the molecular weight of a heterotrimer would be expected to be 108 000. Therefore, the native protein is an heterotrimer in solution. Using this estimate of the native molecular weight, we calculated a shape factor of f/f_o = 1.57, indicating an asymmetric molecule. Modeling the protein as a prolate ellipsoid of revolution, we obtained a highly asymmetric axial ratio of 11:1. This degree of asymmetry is qualitatively consistent with the anomalous molecular weight prediction from gel filtration chromatography (Figure 3A). We conclude that the native protein is an heterotrimer with highly asymmetric shape.

Interaction with Nucleic Acids. Fluorescence spectroscopy has been used to examine both the site size of SSB–protein interactions and the affinity and cooperativity of binding (Molineaux et al., 1975; Kelly et al., 1976; Lohman & Overman, 1985; Lindberg et al., 1989; Kim et al., 1992; Alani et al., 1992). To investigate the properties of the interaction of D-SSB with nucleic acids by fluorescence spectroscopy, we first characterized the optical properties of D-SSB.

The near-UV absorbance spectrum of D-SSB (Figure 4A) showed a broad maximum at 278 nm consistent with the presence of tyrosine and tryptophan. Excitation at wavelengths from 274 nm (the absorbance maximum of tyrosine) through 290 nm yielded fluorescence spectra of decreasing intensity with increasing wavelength, and maxima at 342 nm, slightly
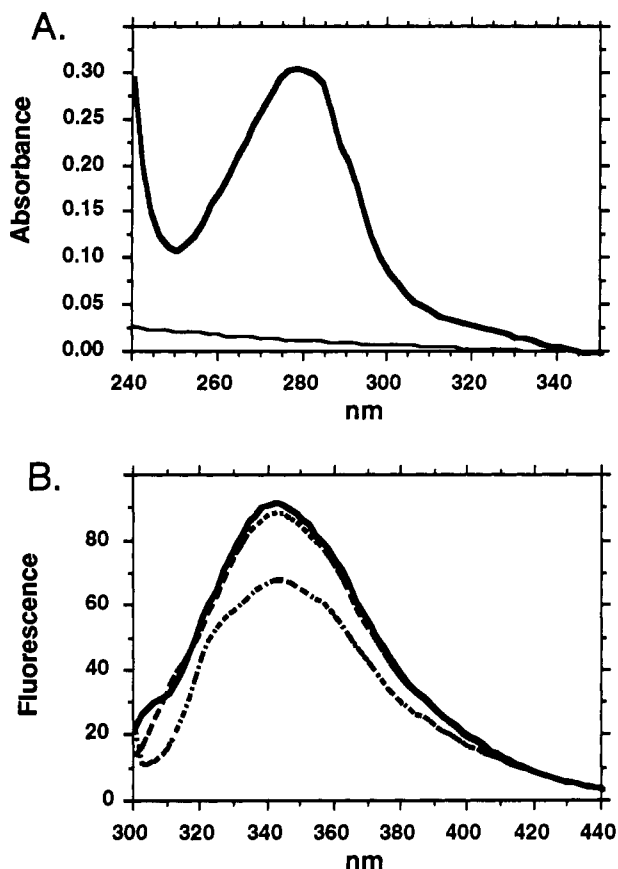
A.

B.

FIGURE 4: Absorbance and fluorescence spectra of D-SSB. D-SSB at 1.7 μM (0.18 mg/mL) was analyzed by absorbance (A) and fluorescence (B) spectroscopy. (A) Absorbance spectra: (thin line) buffer alone; (thick line) buffer plus D-SSB. (B) Fluorescence spectra at different excitation wavelengths: (—) 274 nm; (- - -) 280 nm; (– - –) 290 nM.

less than the emission maximum of tryptophan (Figure 4B). This is most likely due to energy absorption by tyrosine followed energy transfer and emission by tryptophan (Cantor & Schimmel, 1980).

To determine the size of the ssDNA binding site of D-SSB, increasing amounts of ssM13mp18 were added to a constant amount of D-SSB under conditions where the affinity constant was sufficiently great that the binding was stoichiometric. The point at which the initial slope of the fluorescence quenching intersects the fully quenched fluorescence value corresponds to the stoichiometric binding point. The site size is determined by the protein to DNA ratio at this point. Addition of ssDNA to D-SSB resulted in a maximal quenching (Q_m) of the intrinsic fluorescence equal to 38% and yielded a site size of 22 nucleotides per D-SSB heterotrimer (Figure 5A). The average site size (eight titrations) was $n = 22 \pm 4$ nucleotides with an average $Q_m = 35 \pm 3\%$. This value, similar in several preparations of D-SSB, was independent of the addition of 5 mM Mg^{2+} and did not vary in the range of 30–100 nM D-SSB or in the presence of either 25–100 mM NaCl or NaCH$_3$COO (data not shown), confirming that the conditions were stoichiometric.

To control for complicating effects of secondary structure in the ssM13mp18 template, the titrations were repeated with a homopolymer, (dT)$_{3000}$. Four titrations with this polymer under the conditions used for ssM13mp18 (Figure 5A) gave identical site sizes of 18 nucleotides with $Q_m = 33 \pm 2\%$ (data not shown), which is not significantly different from that obtained with ssM13mp18.
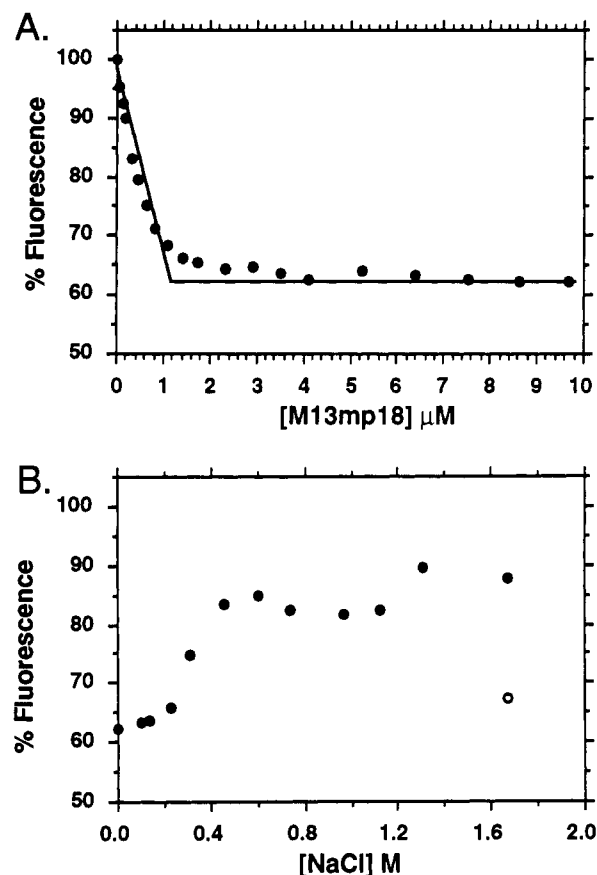
A.

B.

FIGURE 5: Stoichoimetric titration of D-SSB with ssM13mp18. D-SSB (50 nM) was titrated at 50 mM NaCl (A). Fluorescence is given as percent of initial fluorescence. Solid lines were used to determine site size. Salt-induced recovery of fluorescence (B). The same sample as in panel A was subsequently titrated with NaCl. (●) Percent initial fluorescence. (O) Final titration point after the sample was centrifuged for 5 min at room temperature in a microfuge ($\sim$13000g)

To ensure that all the observed quenching is due to the protein–DNA interaction, it is necessary to demonstrate recovery of protein fluorescence when the protein–DNA complex is disrupted. High concentrations of salt can be used to disrupt the SSB–DNA interactions because of their salt sensitivity (Kowalczykowski et al., 1986) and the minimal effect of increased salt concentrations on D-SSB intrinsic protein fluorescence (see Materials and Methods). Increasing the salt concentration disrupted the D-SSB–ssDNA complex (Figure 5B); however, only 60–70% of the initial D-SSB fluorescence was recovered upon addition of 1.7 M NaCl (or 300 mM $MgCl_2$ in other titrations, data not shown) (Figure 5B). We observed that at the elevated salt concentrations the D-SSB–ssDNA solution became a turbid suspension. Centrifugation of this suspension at the end of the titration (1.7 M NaCl) resulted in a loss of fluorescence (Figure 5B). Protein alone, protein–DNA complex below 850 mM NaCl, or protein and DNA added directly to high salt solutions did not show this phenomenon (data not shown). Therefore, the lack of the full recovery of fluorescence may be caused by increasing aggregation of the D-SSB–ssDNA complexes that are formed at low concentrations of salt upon increasing the salt concentration. An alternative possibility is that there may exist a form of the D-SSB–ssDNA complex that does not dissociate at salt concentrations below 1.7 M NaCl (or 300 mM $MgCl_2$) leading to the incomplete recovery of fluorescence.

To measure the cooperativity and affinity of the D-SSB–ssDNA binding interaction, either protein can be titrated
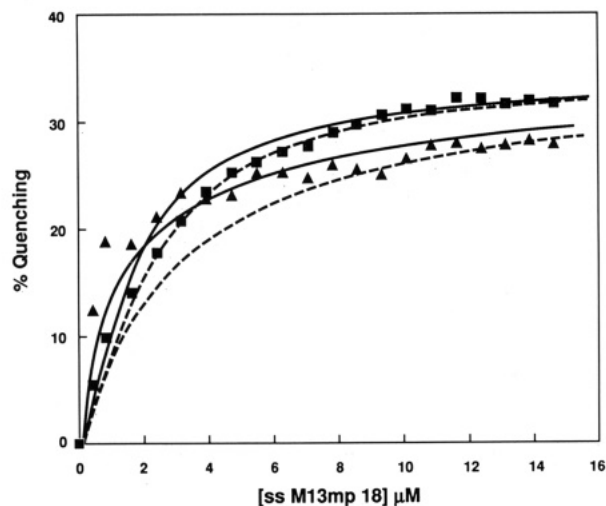
FIGURE 6: Equilibrium reverse titrations of D-SSB with ssM13mp18. Percent quenching is calculated as [(initial fluorescence – fluorescence)/initial fluorescence] × 100. Curves were fit as described under Materials and Methods. The titrations were performed at 80 nM D-SSB (■) and fitted to (—) $\omega = 25$, $K = 7 \times 10^5$, or (- - -) $\omega = 10$, $K = 7.5 \times 10^5$, or at 15 nM D-SSB (▲), and fitted to (—) $\omega = 50$, $K = 2.7 \times 10^5$, or (- - -) $\omega = 200$, $K = 2.2 \times 10^5$.

against a fixed concentration of ssDNA (forward titration) or ssDNA can be titrated against a fixed concentration of protein (reverse titrations) (Kowalczykowski et al., 1986). Forward titrations require observation of either the signal from the ssDNA or the change in incremental increase of protein fluorescence upon addition of protein (Alma et al., 1983). Forward titrations can be followed by the enhancement of fluorescence of the nucleotide analogue poly(etheno-rA) caused by protein binding (Kowalczykowski et al., 1981, 1986); however, D-SSB does not enhance the fluorescence of poly-(etheno-rA) (data not shown). The alternative is to monitor the quenching of D-SSB fluorescence upon addition to ssDNA, but the low amount of quenching seen under stoichiometric conditions makes it difficult to monitor this change accurately (data not shown). We have therefore employed reverse titrations to examine the interaction of D-SSB with ssDNA.

Reverse titrations of protein with ssDNA under nonstoichiometric conditions result in diagnostic titration curves, particularly if the binding is cooperative (Kowalczykowski et al., 1986). If the cooperativity parameter ω exceeds $\sim 10^2$, then the titration curves appear to saturate before maximum quenching is obtained with the value of the apparent saturation dependent on the initial protein concentration. The data in the initial portion of the titration, at high ratios of protein to ssDNA, are subject to error because of potential end effects and ssDNA secondary structure (Kowalczykowski, 1986). Therefore, the data at the higher concentrations of ssDNA are weighted more heavily in fitting the values of K and ω.

Reverse titrations of D-SSB were performed at the elevated salt concentrations defined in Figure 5B that result in a reduction of the apparent affinity for ssDNA. The binding of 80 nM D-SSB to ssM13mp18 at 225 mM NaCl (Figure 6, squares) results in apparent saturation of fluorescence at higher concentrations of ssDNA and at a lower Q_m than the stoichiometric titration (compare Figure 5A and 6) as expected for equilibrium reverse titrations. Decreasing the protein concentration to 15 nM D-SSB resulted in a slightly lower level of quenching (Figure 6, triangles), suggesting some cooperativity in the binding. Consistent with this conclusion, the data from a titration at 30 nM D-SSB (omitted from Figure 6 for clarity) fall between the data from titrations at 15 and 80 nM D-SSB.
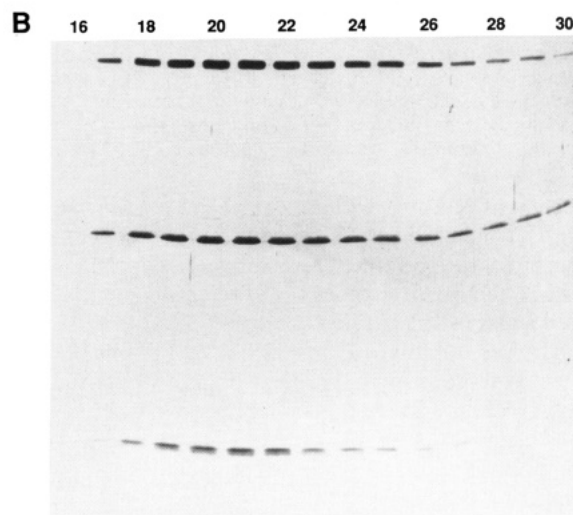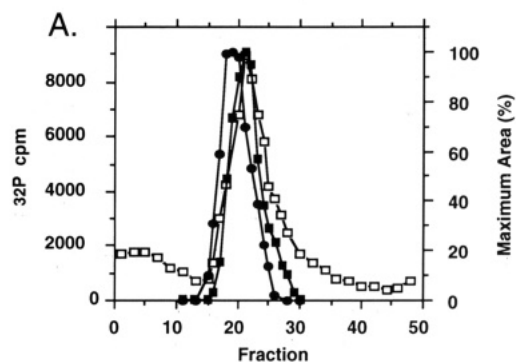

FIGURE 7: Comparison of the sedimentation of D-SSB and D-SSB–(dT)$_{20}$ complex. Conditions are the same as given in the legend to Figure 2. For complex formation, 2 μM D-SSB and 70 μM (nucleotide) (dT)$_{20}$ (end labeled to 140 000 cpm/nmol) in 100 μL were preincubated for 10 min at 20 °C and then loaded on the gradient. Protein was quantitated by densitometry of 70 000 molecular weight subunit (A). Comparison of the sedimentation of free protein (from Figure 2) (●) and of D-SSB–(dT)$_{20}$ complex; cpm DNA (□), protein in complex (■). SDS–polyacrylamide gel electrophoresis of fractions of complex stained with silver (B).

The resulting titration data were fitted to the McGhee–von Hippel equation using the average values for site size and Q_m for ssM13mp18 obtained from the stoichiometric titration. We present two fits of the titration data at each concentration of D-SSB (Figure 6). The titration data can also be fit equally well with an apparent affinity, $K\omega$, ranging from 0.7×10^{-7} to 5×10^7 M^{-1} and a cooperativity parameter, ω, of 10–300.

An implicit assumption in our analysis of the interaction of D-SSB with ssDNA is a protein to DNA stoichiometry of one D-SSB heterotrimer bound per binding site. We tested this assumption directly by examining the sedimentation of a small oligonucleotide of approximately one site size (20 nucleotides) complexed to D-SSB under stoichiometric binding conditions. The complex consisting of DNA and the three subunits of D-SSB (Figure 7, squares) sedimented more rapidly than free protein (Figure 7, circles) with an $s_{20,w} = 5.7 \pm 0.2$ S and an approximate molecular weight of 115 000 ± 6000. This molecular weight is consistent with stoichiometry of D-SSB to (dT)$_{20}$ of 1:1–2. The precision in our measurements does not allow us to distinguish between one or two oligonucleotides bound per monomer; however, the presence of free DNA in the gradient suggests a ratio of D-SSB to (dT)$_{20}$ of 1:1.

To ensure that we did not underestimate the size of the complex due to dissociation during sedimentation, the ex-
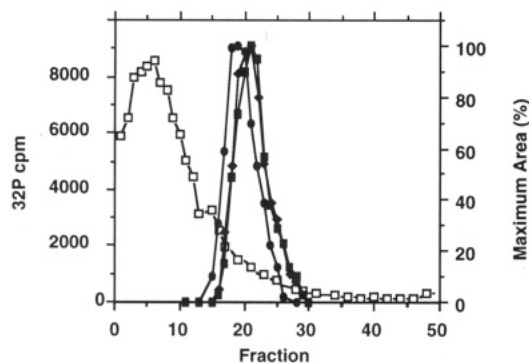
FIGURE 8: Sedimentation of D-SSB in the presence of 70 μM unlabeled $(dT)_{20}$ throughout the gradient. Comparison of the sedimentation of free protein ($\bullet$) (Figure 2), of D-SSB–$(dT)_{20}$ complex in absence of DNA throughout the gradient, protein ($\blacksquare$) (Figure 7), and of D-SSB–$(dT)_{20}$ complex in presence of DNA throughout the gradient, protein ($\blacktriangle$) and cpm DNA ($\square$). Protein was quantitated as described in the legend to Figure 7.

periment was repeated with an equivalent amount of DNA throughout the gradient. The sedimentation of the protein component of the preformed complex was independent of the presence of DNA throughout the gradient (compare Figure 8, filled squares versus filled triangles). The labeled DNA was, however, rapidly exchanged out of the complex and sedimented at the top of the gradient (Figure 8, open squares). This observation suggests that either the exchange time of the free and bound DNA is rapid with respect to the sedimentation rate or there is an active dissociation promoted by the presence of competing DNA.

Stimulation of DNA Polymerase α. Eukaryotic SSBs exhibit specific stimulation of their cognate DNA polymerase α. We have examined D-SSB for its effect on *Drosophila* DNA polymerase α compared to a noncognate SSB from *E. coli*. The homopolymer template $(dA)_{2000}$ was employed to minimize regions of strong secondary structure. Synthesis was performed under a 40-fold excess of primer to polymerase for a brief period to minimize reinitiation and to permit measurement of processivity. D-SSB greatly increased the amount and size of the products synthesized (Figure 9, lane 3) while *E. coli* SSB had only a slight effect (Figure 9, lane 4). We conclude that D-SSB can stimulate DNA synthesis by *Drosophila* polymerase α and its processivity of deoxynucleotide polymerization.

DISCUSSION

We have purified an SSB to greater than 95% homogeneity from 0–18-h embryos of *D. melanogaster*. The SSB can be purified from *Drosophila* embryos in large amounts and good yield, facilitating its functional analysis. We do not discuss measurements of the specific activity of the protein during the purification. Of the two assays that we can use, measurement of DNA binding is nonspecific, and DNA polymerase α stimulation cannot be measured in the crude extracts because of contaminating DNA polymerases. Since the protein is 30–40% homogeneous when it can be first assayed, there would be, at best, a 2–3-fold increase in specific activity. This increase in specific activity also assumes that there are no inhibitory or stimulatory factors in our fractions. In fact, we observe a decrease in specific activity after the Fast-Q Sepharose chromatography (data not shown) suggesting that there may be proteins in the DNA cellulose fraction that can potentiate the stimulation by D-SSB of *Drosophila* DNA polymerase α.
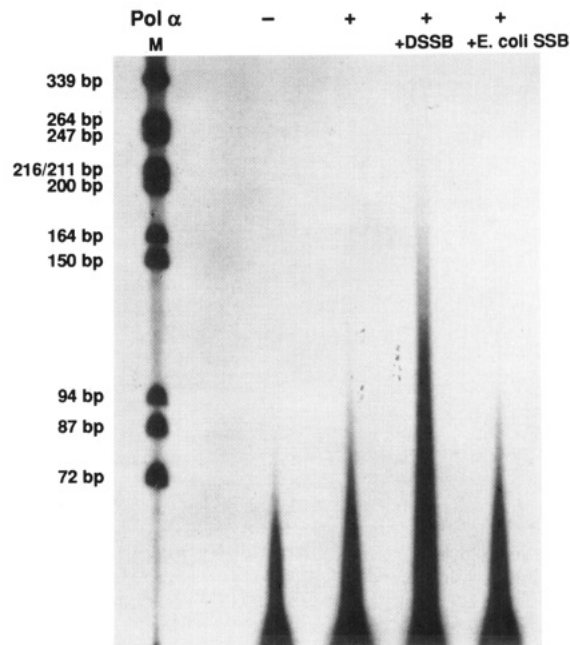

FIGURE 9: Stimulation of processivity of DNA polymerase α by D-SSB. $(dT)_{20}/(dA)_{2000}$ (5:1 molecules) at 20 nM (primers) was preincubated in the absence or presence of 0.66 μM D-SSB (D-SSB to nucleotide ratio of 1:25) or 1.67 μM (monomer) *E. coli* SSB [SSB-(monomer) to nucleotide ratio of 1:15]. DNA polymerase α was added to 0.5 nM and assayed (Materials and Methods). (M) Molecular weight markers in base pairs. Some contaminants are present in the oligomers which cause smearing in gel electrophoresis as is evident in the no addition lane.

The purified protein is a heterotrimer with subunits of molecular weight of 70 000, 30 000, and 8000. This composition is strikingly similar to the human and yeast SSB/RP-A, which are heterotrimers of 70 000, 32 000, and 14 000 and 69 000, 36 000, and 13 000, respectively. Although this structural conservation is maintained at the amino acid level for the yeast and human SSBs (Erdile et al., 1989, 1991; Brill & Stillman, 1991), they cannot substitute for each other either in the stimulation of SV-40 DNA replication (Brill & Stillman, 1989) or in rescue of gene disruptions (Brill & Stillman, 1991), suggesting functional divergence.

The middle subunit of the yeast and human SSBs has been demonstrated to be multiply phosphorylated. Our preparation of D-SSB and similar preparations of D-SSB from rapidly replicating 0–2-h cleavage stage *Drosophila* embryos do not exhibit the characteristic 2–3-kDa shift in mobility during SDS–polyacrylamide gel electrophoresis upon treatment with phosphatase which would be consistent with this modification (data not shown). The abundance of protein phosphatases found in *Drosophila* embryos (Mayer-Jaekal et al., 1992) may make it difficult to isolate the phosphorylated form of D-SSB. It is also possible that the phosphorylation event does not cause a change in mobility, as has been reported with the in vitro phosphorylation of human RP-A by cyclin A-p34[cdc2] kinase (Fodetar & Roberts, 1992).

The interaction of D-SSB with nucleic acids was examined by fluorescence spectroscopy. We determined the site size for the interaction of D-SSB with ssM13mp18 of 22 $\pm$ 4 nucleotides. The accuracy of site size determination is affected by the quantitation of the D-SSB by a colorimetric protein assay. Until we have accurate protein composition information and an extinction coefficient determined for D-SSB, it is not possible to know the error that may be involved. The lack of full recovery of fluorescence may be caused by aggregation of D-SSB–ssDNA complexes or alternatively a form of the

D-SSB–ssDNA complex that does not dissociate at salt concentrations below 1.7 M NaCl [as has been reported for the yeast SSB (yRP-A, Alani et al., 1992)]. Aggregation of the D-SSB–ssDNA complexes in high concentration of salt precludes resolution of these alternatives. Since the complexes are soluble during the titration, and loss of fluorescence occurs only at high salt, we do not think that it is likely that the aggregation of D-SSB–ssDNA affects the value determined for the site size.

D-SSB binds long DNA with a cooperativity parameter ω = 10–300 and an apparent affinity, $K\omega$, of approximately 10^7 M^{-1} at 225 mM NaCl. This value of cooperativity falls near that of the high salt binding mode of *E. coli* SSB (n = 65), ω_{lim} = 420 ± 80 (25 °C) (Bujalowski & Lohman, 1987; Overman et al., 1988), and is well below that of the highly cooperative gene 32 protein of phage T4, with ω = (3–10) × 10^3 (Kowalczykowski et al., 1980). These data, taken together with the observation that D-SSB stimulates DNA polymerase α, suggest that D-SSB may function in a similar manner to *E. coli* SSB. The apparent affinity, $K\omega$, for ssM13mp18 at 225 mM NaCl falls in the range of *E. coli* SSB, $K\omega > 10^7$ M^{-1} (Bujalowski & Lohman, 1989), and below that of gene 32 protein, $K\omega = 10^{10}$ M^{-1} (Kowalczykowski et al., 1980), and indicates that D-SSB also binds DNA with a high affinity.

The site size of D-SSB agrees well with the value obtained from gel mobility shift assays for the yeast and human RP-A of approximately 30 nucleotides (Kim et al., 1992). However, analysis of the interaction of ssDNA with the yeast SSB (yRP-A; Alani et al., 1992) by quenching of intrinsic protein fluorescence gave significantly different values from those that we measured for the interaction of ssDNA with D-SSB. The site size of yRP-A was found to be considerably larger (n = 90–100 for yRP-A versus n = 22 for D-SSB), and the maximum quenching is greater (Q_m = 65% versus Q_m = 35%), as is the cooperativity (ω = 10^4–10^5 versus ω = 10–300). We have found that D-SSB aggregates under conditions used by Alani et al. (1992) [yRP-A does appear to show some aggregation under these conditions (Alani et al., 1992)]. Sedimentation analysis demonstrated that D-SSB alone, or D-SSB–$(dT)_{20}$ complex, does not aggregate in our experimental conditions. The difference in site size could result from the inability of a large fraction of the D-SSB to bind DNA. However, sedimentation of the D-SSB–$(dT)_{20}$ complex demonstrates that a majority of the protein does bind to DNA (Figures 7 and 8), and therefore the difference in site size cannot be accounted for by inactive D-SSB in our preparations.

It has been suggested that there is little cooperativity in the binding of human and yeast RP-A to short polynucleotides when examined by gel shift analysis (Kim et al., 1992). Our analysis suggests that D-SSB also binds with low cooperativity. Although binding to short polynucleotides can lead to underestimation of cooperativity because of end effects, or because of different protein binding modes between oligonucleotides and long nucleotide polymers [as observed for T4 gene 32 protein (Kowalczykowski et al., 1981)], the qualitative agreement between the gel shift analysis and our fluorimetric titrations suggests that this may not be the case for the eukaryotic SSBs. Because of different experimental conditions, it is difficult to compare quantitatively our values and those of Kim et al. (1992) directly. A more careful examination of the effects of solution conditions on both the intrinsic affinity and cooperativity of binding of these SSBs is required to address this issue.

The differences observed among the binding properties of these SSBs may reflect a species specific difference in the interaction of the respective SSBs with ssDNA. A more intriguing possibility is that the differences represent differential posttranslational modification. The 36 000 molecular weight subunit of RP-A is phosphorylated, while the *Drosophila* SSB that we have isolated does not appear to have this modification. Phosphorylation, whether quantitatively or qualitatively different, may lead both to the altered solubility properties of the SSBs and their different interaction with ssDNA.

A multimeric binding species may cause a potential complication in determining site size. For example, *E. coli* SSB, a tetramer, can exhibit negative cooperativity between the sites on different subunits depending on conditions, resulting in differing apparent site size (Bujalowski & Lohman, 1989). D-SSB does not exhibit any DNA-dependent protein-protein association such as the DNA-dependent dimerization of the *E. coli rep* helicase (Chao & Lohman, 1991). Since we do not observe any multimeric protein species, and the site size is similar under several conditions, we conclude that D-SSB interacts with short nucleic acids as a monomer in solution. However, binding cooperativity suggests that there may be interactions between bound protein molecules.

D-SSB specifically stimulates its cognate DNA polymerase α, relative to the noncognate *E. coli* SSB, increasing both the length and amount of products synthesized on homopolymeric templates. The *E. coli* SSB has no effect on DNA synthesis, suggesting that the effect of D-SSB is not due to simple melting out of residual secondary structure in the template or preventing nonspecific binding of DNA polymerase α to the single-stranded regions of the template. This specificity is most likely analogous to the specific physical interaction observed between human RP-A and DNA polymerase α relative to the prokaryotic SSBs, *E. coli* SSB, and gene 32 protein (Dornrieter et al., 1992). Such specificity has also been observed between *E. coli* SSB and DNA polymerase III holoenzyme and between the T4 gene 32 protein and T4 DNA polymerase (Chase & Williams, 1986). An interaction of this kind has proven especially useful in the case of the gene 32 protein and cognate T4 replication and recombination proteins, allowing the isolation of several of these proteins (Formosa et al., 1983). Such a property may also prove useful in isolating other proteins interacting with D-SSB and investigating its role in *Drosophila* DNA metabolism.

REFERENCES

Alberts, B., & Herrick, G. (1971) *Methods Enzymol. 21*, 198–211.

Alma, N. C. M., Harmsen, B. J. M., deJong, E. A. M., Ven, J. V. D., & Hilbers, C. W. (1983) *J. Mol. Biol. 163*, 47–62.

Alani, E., Thresher, R., Griffth, J. D., & Kolodner, R. D. (1992) *J. Mol. Biol. 227*, 54–71.

Berkowitz, S. A., & Day, L. (1974) *Biochemistry 13*, 4825–4831.

Bradford, M. (1976) *Anal. Biochem. 72*, 248–254.

Brill, S. J., & Stillman, B. (1989) *Nature 342*, 92–93.

Brill, S. J., & Stillman, B. (1991) *Genes Dev. 5*, 1589–1600.

Brown, G. W., Melendy, T. E., & Ray, D. S. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 10227–10231.

Bujalowski, W., & Lohman, T. M. (1987) *J. Mol. Biol. 195*, 897–907.

Bujalowski, W., & Lohman, T. M. (1989) *J. Mol. Biol. 207*, 249–268.

Cantor, C. R., & Schimmel, P. R. (1980) in *Biophysical Chemistry*, Vol. II, p 561, W. H. Freeman & Co., San Francisco, CA.

Chao, K., & Lohman, T. M. (1991) *J. Mol. Biol. 221*, 1165–1181.

Chase, J. W., & Williams, K. R. (1986) *Annu. Rev. Biochem.* 55, 103–136.

Coverley, D., Kenny, M. K., Munn, M., Rupp, W. D., Lane, D. P., & Wood, R. D. (1991) *Nature 349*, 538–541.

Din, S., Brill, S. J., Fairman, M. P., & Stillman, B. (1990) *Genes Dev. 4*, 968–977.

Dornreiter, I., Erdile, L. F., Gilbert, I. U., von Winkler, D., Kelly, T. J., & Fanning, E. (1992) *EMBO J. 11*, 769–776.

Dutta, A., & Stillman, B. (1992) *EMBO J. 11*, 2189–2199.

Erdile, L. F., Heyer, W. D., Kolodner, R., & Kelly, T. J. (1991) *J. Biol. Chem. 266*, 12090–12098.

Fairman, M. P., & Stillman, B. (1988) *EMBO J. 7*, 1211–1218.

Formosa, T., Burke, R. L., & Alberts, B. M. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2242–2446.

Fotedar, R., & Roberts, J. M. (1992) *EMBO J. 11*, 2177–2187.

Gel Filtration Theory and Practice (1987) Pharmacia, Laboratory Separation Division, Uppsala, Sweden, 27.

Heyer, W. D., Rao, M. R. S., Erdile, L. F., Kelly, T. J., & Kolodner, R. D. (1990) *EMBO J. 9*, 2321–2329.

Kaguni, L. S., Rossignol, J. M., Conaway, R. C., & Lehman, I. R. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2221–2225.

Kelly, R. C., Jensen, D. E., & von Hippel, P. H. (1976) *J. Biol. Chem. 254*, 7240–7250.

Kenny, M., Lee, S. H., & Hurwitz, J. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 9757–9761.

Kenny, M., Schlegel, U., Furneaux, H., & Hurwitz, J. (1990) *J. Biol. Chem. 265*, 7693–7700.

Kim, C., Snyder, R. O., & Wold, M. S. (1992) *Mol. Cell. Biol. 12*, 3050–3059.

Kim, Y. T., Tabor, S., Bortner, C., Griffith, J. D., & Richardson, C. C. (1992) *J. Biol. Chem. 267*, 15022–15031.

Kowalczykowski, S. C., Lonberg, N., Newport, J. W., & Von Hippel, P. H. (1981) *J. Mol. Biol. 145*, 75–104.

Kowalczykowski, S. C., Paul, L. S., Lonberg, N., Newport, J. W., McSwiggen, J. A., & Von Hippel, P. H. (1986) *Biochemistry 25*, 1226–1240.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Lindberg, G., Kowalczykowski, S. C., Rist, J. K., Sugino, A., & Rothman-Denes, L. B. (1989) *J. Biol. Chem. 264*, 12700–12708.

Lohman, T. M., & Overman, L. B. (1985) *J. Biol. Chem. 260*, 3594–3603.

Lohman, T. M., & Bujalowski, W. (1990) in *The Biology of Nonspecific DNA-Protein Interactions* (Revsin, A., Ed) pp 131–168, CRC Press, Cleveland, OH.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Marsh, J. L., Erfle, M., & Wykes, E. J. (1984) *Gene 32*, 481–485.

McGhee, J. D., & von Hippel, P. H. (1974) *J. Mol. Biol. 86*, 469–489.

Merril, C., Goldman, D., Sedman, S. A., & Ebert, M. H. (1981) *Science 211*, 1437–1439.

Meyer-Jaekal, R. E., Baumgartner, S., Bilbe, G., Ohkura, H., Glover, D. M., & Hemmings, B. A. (1992) *Mol. Biol. Cell 3*, 287–298.

Meyer, R. R., & Laine, P. S. (1990) *Microbiol. Rev. 54*, 342–380.

Molineaux, I. J., Pauli, A., & Gefter, M. L. (1975) *Nucleic Acids Res. 2*, 1821–1837.

Moore, S. P., Erdile, L., Kelly, T., & Fishel, R. (1991) *Proc. Natl. Acad. Sci. U.S.A. 88*, 9067–9071.

Overman, L. B., Bujalowski, W., & Lohman, T. M. (1988) *Biochemistry 27*, 456–471.

Read, S. M., & Northcote, D. H. (1981) *Anal. Biochem. 116*, 53–64.

Schleif, R. F., & Wensink, P. C. (1981) *Practical Methods in Molecular Biology*, Springer-Verlag, New York.

Siegel, L. M., & Monty, K. J. (1966) *Biochim. Biophys. Acta 112*, 346–362.

Smith, M. H. (1968) in *CRC Handbook for Biochemistry Selected Data for Molecular Biology* (Sober, H. A., Ed.) pp C-3–C-49, CRC Press, Cleveland, OH.

Thommes, P., Ferrari, E., Jessberger, R., & Hubscher, U. (1991) *J. Biol. Chem. 267*, 6063–6073.

Tsurimoto, T., & Stillman, B. (1989) *EMBO J. 8*, 3883–3889.

Wobbe, C. R., Weissbach, L., Boroweic, J. A., Dean, F. B., Murakami, Y., Bullock, P., & Hurwitz, J. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 1834–1838.

Wold, M. S., & Kelly, T. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 2523–2527.