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Purification of DNA polymerase II stimulatory factor I, a yeast single-stranded DNA-binding protein

(DNA replication/*Saccharomyces cerevisiae*/DNA repair)

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ABSTRACT **Incidental to the purification of yeast DNA polymerase II was the observation that various chromatographic fractions contained activities that stimulated synthesis by this polymerase. In this paper we report the purification and initial characterization of one such factor, stimulatory factor I (SFI). SFI, which is associated with an apparent complex of three polypeptides of 66, 37, and 13.5 kDa, binds preferentially to single-stranded DNA, possibly explaining its ability to stimulate DNA polymerase II. Single-stranded DNA-binding activity is associated with the 66-kDa polypeptide.**

The replication of genomic DNA is an intricate and highly regulated process that involves the coordination of a variety of protein–protein and protein–DNA interactions (1). The complexities of eukaryotic DNA replication have recently begun to yield somewhat to the power of enzyme purification, and this is especially true for yeast and for simian virus 40 (SV40) DNA replication. While the DNA polymerases were well-characterized by traditional enzymology, the fractionation of cell extracts into purified components that can reconstitute SV40 origin-specific DNA replication *in vitro* has resulted in the identification of a number of replication proteins in addition to DNA polymerases (2). One of these proteins is proliferating-cell nuclear antigen (PCNA), whose role in SV40 replication appears to be as an accessory protein of DNA polymerase δ. Another of these putative replication proteins is a multisubunit complex that binds to single-stranded DNA. This protein has been called HeLa single-stranded DNA-binding protein (SSB), replication factor A (RF-A), and replication protein A (RP-A) (3–6). RP-A has been shown to participate in SV40 replication at the stage of large tumor (T)-antigen-mediated unwinding of the SV40 origin of replication, but an additional role in elongation has not been ruled out (6). Although genetic proof of a role for RP-A in cellular DNA replication is lacking, the biochemical evidence in the viral system is very compelling.

Yeast presents the opportunity for extensive genetic manipulations not presently possible with higher eukaryotes. Research in this lab has focused on identifying and isolating the proteins involved in yeast DNA replication with subsequent isolation of corresponding genes and mutants. This approach has been particularly successful in the study of the DNA polymerases. DNA polymerase I (metazoan DNA polymerase α), polymerase II, and polymerase III (metazoan DNA polymerase δ) have been purified. The genes for DNA polymerases I and III have been cloned and mutants have been identified that indicate that polymerase I, polymerase II, and polymerase III are the products of separate genes and that polymerase I and polymerase III are essential for replication (7–9). The availability of genetically and biochemically well-defined DNA polymerases makes feasible a strategy for identifying additional replication proteins, namely, the purification and characterization of polymerase accessory proteins. In this paper, we describe one such auxiliary protein. During the purification of DNA polymerase II from yeast, a PCNA-independent DNA polymerase containing a 3′→5′ exonuclease, several stimulatory factors were identified (10). One of these factors, SFI, has been purified to near homogeneity and has been shown to possess single-stranded DNA binding activity and DNA polymerase stimulatory activity.

MATERIALS AND METHODS

Materials. Nucleotides were purchased from Pharmacia, [α-^{32}P]dNTPs from Amersham, [*methyl*-^{3}H]dTTP from New England Nuclear, phosphocellulose P11 and DEAE-cellulose DE52 from Whatman, and Bio-Gel HTP hydroxylapatite from Bio-Rad. Pre-packed Mono Q HR 5/5 and Superose 12 FPLC columns were from Pharmacia. The SSB of *Escherichia coli* was the generous gift of Louis Romano (Department of Chemistry, Wayne State University, Detroit).

DNA Polymerase Assays. DNA polymerase activity and stimulation of this activity were assayed in reaction mixtures (60 μl) containing 40 mM Tris·HCl (pH 7.5); 2 mM 2-mercaptoethanol; 8 mM $MgCl_2$; 6 μg of bovine serum albumin; 100 μM each dATP, dGTP, and dCTP; 50 μM [^{3}H]dTTP (205 cpm/pmol); 2 μg of poly(dA)$_{400}$·oligo(dT)$_{10}$, 40:1; and 0.02 unit of yeast DNA polymerase II, as described by Budd *et al.* (10). Yeast SFI was added as indicated. Reaction mixtures were incubated at 37°C for 20 min.

Single-Stranded DNA-Binding Assays. DNA-binding activity was assayed by retention of protein–DNA complexes by nitrocellulose filters, essentially as described by Jong *et al.* (11). Reaction mixtures (10 μl) containing 20 mM Tris·HCl (pH 7.9), 1 mM EDTA, 2 mM 2-mercaptoethanol, 50 mM NaCl, 5% (vol/vol) glycerol, and 80 pmol of heat-denatured pUC.HSOd4 (2.9 kilobase pairs; ref. 12) labeled by nick-translation with [α-^{32}P]dCTP to 1.5 × 10^{8} cpm/μg. Positive controls contained 1.6 μg of *E. coli* SSB. Incubations were for 30 min at 30°C. After incubation, reaction mixtures were passed over nitrocellulose filters that had been prewashed with 1.5 ml of buffer, as described (13). The filters were then washed and dried, and radioactivity was determined by liquid scintillation counting.

Purification of SFI. *Phosphocellulose chromatography.* The protein in the phosphocellulose flowthrough (4800 ng) from the purification of yeast DNA polymerase II (10) was precipitated with $(NH_4)_2SO_4$ (0.39 g/ml) and stored at −20°C. The precipitate was dissolved in 25 mM Tris·HCl, pH 7.5/1.25 mM EDTA/0.01% Nonidet P-40/1 mM dithiothreitol/10% glycerol (buffer A) containing 1 mM phenylmethylsulfonyl fluoride, 1 mM benzamidine, 20 μg of pepstatin A per

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Abbreviations: SV40, simian virus 40; T antigen, SV40-encoded large tumor antigen; SFI, stimulatory factor I; SSB, single-stranded DNA-binding protein; RP-A, replication protein A; PCNA, proliferating-cell nuclear antigen.

ml, 10 μg of leupeptin per ml, 10 μg of soybean trypsin inhibitor per ml, and 0.5 mM diisopropyl fluorophosphate. This solution was dialyzed against buffer A containing 175 mM NaCl and loaded onto a 50-ml (2.1 cm^2 × 23 cm) phosphocellulose column equilibrated with buffer A containing 175 mM NaCl. The column was washed with 40 ml of this buffer and the flowthrough and wash were collected, pooled, and designated fraction I (4800 mg of protein).

DEAE-cellulose chromatography. Fraction I was loaded onto a 400-ml (25 cm^2 × 16 cm) DE52 column equilibrated with buffer A containing 175 mM NaCl. The column was washed with 400 ml of this buffer and eluted with 400 ml of buffer A containing 1 M NaCl. Fractions active in stimulation were pooled and concentrated by dialysis against dry polyethylene glycol (fraction II, 184 mg of protein).

Mono Q FPLC. Fraction II was dialyzed against buffer A with 200 mM NaCl. Samples containing 25 mg of protein were injected onto a 1-ml Mono Q HR 5/5 column equilibrated with buffer A with 200 mM NaCl. The column was washed with 2 ml of this buffer and eluted with an 8-ml linear gradient of NaCl (200–600 mM) in buffer A. Stimulatory activity was eluted at about 300 mM NaCl. Active fractions were pooled (fraction III, 46 mg of protein).

Superose FPLC. Fraction III was dialyzed against buffer A with 200 mM NaCl and concentrated using a Centricon 10 microconcentrator (Amicon). Aliquots (250 μl) were injected onto a 240-ml Superose 12 column and eluted in buffer A with 200 mM NaCl. Active fractions were pooled to yield fraction IV (24 mg of protein).

Hydroxylapatite chromatography. Fraction IV was dialyzed against 40 mM potassium phosphate, pH 7.5/1 mM dithiothreitol/10% glycerol (buffer B) and loaded onto a 5-ml (0.7 cm^2 × 8 cm) hydroxylapatite column. The column was washed with 5 ml of buffer B and eluted with buffer B at 100 mM potassium phosphate. Active fractions were pooled and dialyzed into 25 mM Tris·HCl, pH 7.5/0.1 mM EDTA/0.1 mM dithiothreitol/2.5 mM NaCl/10% glycerol (buffer C). Fraction V (10 mg of protein) was stored frozen at −70°C. It is important to note that after we discovered that the protein binds single-stranded DNA and a purification was carried out during which both stimulatory activity and DNA binding were assayed, we found that 100 mM phosphate had an inhibitory effect on the single-stranded DNA-binding activity of the yeast SFI, reducing binding by 50%.

Glycerol gradient centrifugation. Fraction V (0.15 ml), representing one-fifth of the total activity in fraction V, was layered onto a 5-ml gradient of 10–30% glycerol in buffer C. Protein standards were run in separate tubes. Gradients were centrifuged at 45,000 rpm for 20 hr at 4°C in a Beckman SW 50.1 rotor. Fractions (0.2 ml) were collected from the bottom of the tube and assayed for stimulatory activity. Active fractions were pooled and stored frozen at −70°C (fraction VI, 0.46 mg). SFI remains active when stored at this temperature for at least 4 months and also has survived several freeze–thaw cycles.

RESULTS

Purification of SFI. During purification of DNA polymerase II, two fractions that stimulated yeast DNA polymerase II synthetic activity on primer–templates with long regions of single-stranded DNA were identified (10). One of these, SFI, appeared in the flowthrough of the phosphocellulose column employed at the beginning of the DNA polymerase II purification. This flowthrough has been fractionated in order to identify the protein or proteins responsible for the stimulatory activity.

The purification procedure is described in *Materials and Methods*. In brief, to ensure that no residual polymerase activity remained, the phosphocellulose flowthrough was again subjected to phosphocellulose chromatography. The protein that failed to bind to the column was passed over a DEAE-cellulose column, and activity was eluted with 1 M NaCl. Active fractions were further fractionated on a Mono Q column, followed by gel filtration with a Superose column. The sample was then loaded onto a hydroxylapatite column and eluted with 100 mM potassium phosphate. This was followed by glycerol gradient sedimentation.

The active fractions obtained from glycerol gradient sedimentation showed two major bands at 66 and 37 kDa and a faint band at 13.5 kDa when analyzed by SDS/PAGE (Fig. 1). This 13.5-kDa band is more easily seen in Fig. 3*B*. The sedimentation coefficient of the purified material in the active fractions of the gradient was 4.7 S. This is larger than what would be expected for each polypeptide alone and suggests that the SFI activity is probably a complex of these three proteins.

SFI Binds Single-Stranded DNA. The size distribution of the polypeptides and the sedimentation profile of the stimulatory activity in the glycerol gradient are similar to those of human RP-A, a multisubunit protein required for SV40 DNA replication *in vitro* that can stimulate DNA polymerase α in the presence of T antigen (3, 5–6). While no known enzymatic activity is associated with RP-A, one distinguishing property of RP-A is its ability to bind single-stranded DNA. As shown in Fig. 2*A*, like human RP-A, yeast SFI also binds single-stranded DNA.

In the presence of equimolar amounts of labeled substrate and unlabeled single-stranded competitor DNA, the retention of radiolabeled DNA decreased by 40% (Fig. 2*A*). A 25-fold excess of single-stranded competitor DNA decreased retention of radiolabeled DNA by 90%. In contrast, addition of an equal amount of double-stranded competitor DNA did not decrease retention of radiolabeled DNA, while addition of a

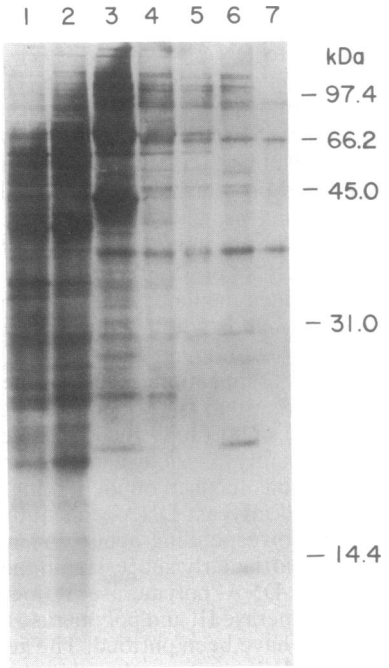

FIG. 1. SDS/15% PAGE of fractions from the purification of yeast SFI. Lane 1, phosphocellulose resuspension; lane 2, phosphocellulose flowthrough (fraction I); lane 3, DEAE-cellulose (fraction II); lane 4, Mono Q (fraction III); lane 5, Superose (fraction IV); lane 6, hydroxylapatite (fraction V); lane 7, glycerol gradient (fraction VI). Molecular mass markers were phosphorylase *b* (97.4 kDa), bovine serum albumin (66.2 kDa), ovalbamin (45 kDa), carbonic hydrase (31 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). SDS/PAGE was performed according to Laemmli (14). Protein bands were visualized by staining with silver (15).

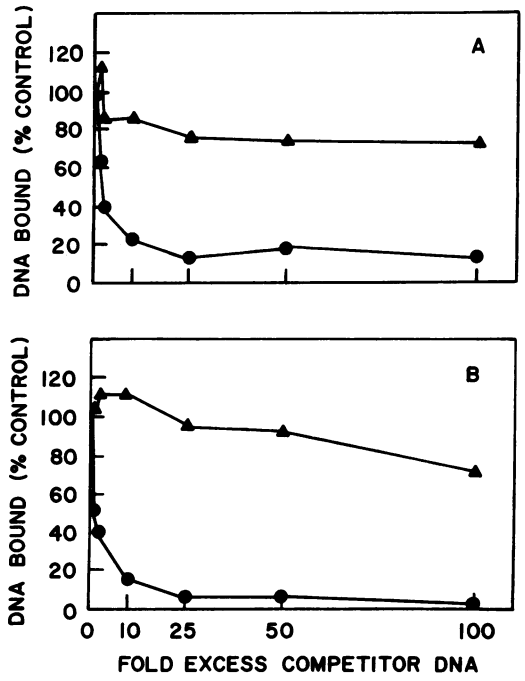

FIG. 2. Preferential binding of SFI and its 66-kDa subunit to single-stranded DNA. Binding to single-stranded DNA was assayed as described in *Materials and Methods*. Reaction mixtures (10 μl) contained 80 pg of single-stranded DNA and a 1-, 2.5-, 5-, 25-, 50-, or 100-fold excess of competitor DNA. Competitor DNA was pUC.HSOd4 linearized with *Hin*dIII. It was thermally denatured for use as a single-strand competitor. (*A*) SFI. Each reaction contained 0.85–0.95 μg of total protein (fraction VI). Maximal binding was 6.67 pg of DNA. (*B*) The 66-kDa protein. Each reaction mixture contained ≈0.3 μg of total protein. Maximal binding was 15.7 pg. Each point is the average of four to six determinations. ●, Single-stranded competitor; ▲, double-stranded competitor.

25-fold excess of this double-stranded competitor DNA decreased retention of the radiolabeled single-stranded substrate by only 25%. Consequently, yeast SFI exhibits a specific affinity for single-stranded DNA.

To verify that SFI and the DNA-binding activity were actually the same, a purification was carried out in which fractions (beginning with fraction III) were assayed both for the ability to stimulate DNA polymerase II and for single-stranded DNA-binding activity. These two activities copurifed on all columns and coincided in glycerol gradient sedimentation (Fig. 3*A*).

Stimulatory activity of the purified protein was titrated, and the optimum extent of stimulation of polymerase II by the purified SFI protein (90 ng) was only 3-fold. Since the crude SFI (fraction I) increased synthesis 25-fold (10), there may have been additional stimulatory factors in fraction I that were removed during purification. HeLa SSB also requires additional factors to stimulate DNA polymerases (3).

The Single-Stranded DNA-Binding Activity Is Associated with the 66-kDa Subunit. Since the 70-kDa subunit of human RP-A binds single-stranded DNA in the absence of the other subunits (3, 16), it was of interest to see whether the DNA-binding activity from yeast also localizes to the 66-kDa polypeptide. While establishing the conditions for hydroxylapatite chromatography, we observed that during elution of SFI from hydroxylapatite by a linear gradient of 40–100 mM potassium phosphate the 66-kDa polypeptide began to be eluted just prior to the other two polypeptides, as revealed by SDS/PAGE analysis of individual fractions. The effect led to substantial loss of stimulatory activity, presumably due to separation of the essential protein components. However, the fractions containing the 66-kDa subunit were active in single-stranded DNA binding. This observation was exploited to separate the 66-kDa subunit from the other proteins. A fraction eluted from hydroxylapatite chromatography between 40 and 80 mM phosphate, in which the 66-kDa polypeptide of SFI was the major protein band in an SDS/polyacrylamide gel, was loaded onto a glycerol gradient. The fractions were assayed for DNA binding and the peak activity fractions were pooled and analyzed by SDS/PAGE. This fraction contained essentially homogeneous 66-kDa protein

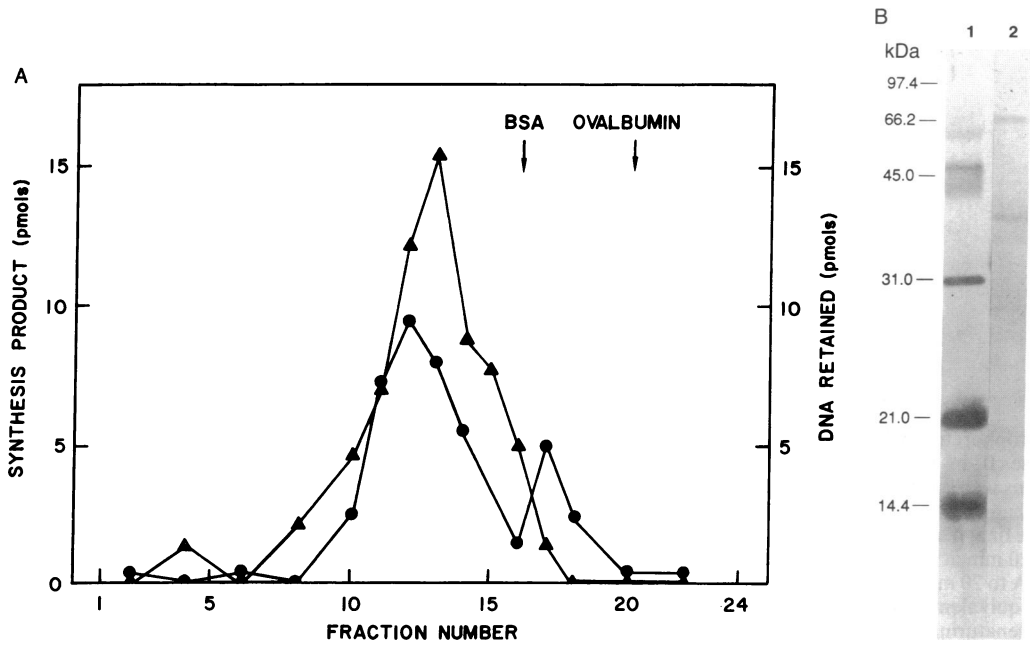

FIG. 3. (*A*) Glycerol gradient sedimentation profile of SFI. A 150-μl aliquot (450 μg of protein) of the hydroxylapatite activity pool was layered onto a 10–30% glycerol gradient and spun at 45,000 rpm for 20 hr at 4°C in a Beckman SW 50.1 rotor. Fractions were assayed for both polymerase stimulatory activity (●) and the ability to bind single-stranded DNA (▲). Stimulation is plotted as DNA synthesis minus that of polymerase II alone. The activities coincide in the gradient. BSA, bovine serum albumin. (*B*) Proteins in fraction 13 were detected by silver staining after SDS/15% PAGE (lane 2). Molecular mass standards are shown (lane 1) and are described in the legend to Fig. 1.

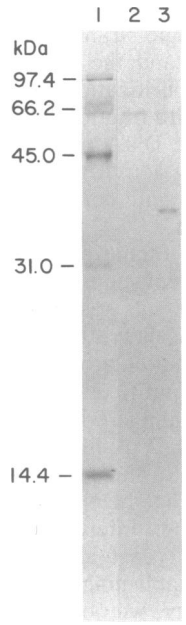

FIG. 4. SDS/PAGE analysis of the 66-kDa subunit. SDS/15% PAGE was used to examine glycerol gradient sedimentation of hydroxylapatite fractions containing the 66-kDa protein (lane 2) or the entire complex (lane 3). Markers (lane 1) are described in the legend to Fig. 1.

(Fig. 4, lane 2), suggesting that this polypeptide binds DNA in the absence of the others. The fractions eluted from hydroxylapatite between 80 and 100 mM potassium phosphate were also further purified on a glycerol gradient. The DNA-binding fractions contained the 66-kDa and 37-kDa polypeptides (Fig. 4, lane 3), but the 13-kDa protein was not recovered in stoichometric amounts when hydroxylapatite was eluted with this gradient.

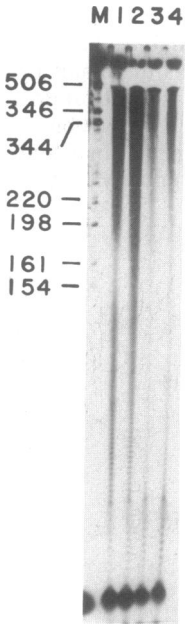

FIG. 5. Electrophoretic analysis of products synthesized by yeast DNA polymerase II in the presence or absence of SFI. DNA synthesis reactions were as described in *Materials and Methods* with the following changes: [α-^{32}P]dTTP (4100 cpm/pmol) replaced [^{3}H]dTTP and the final reaction volume was 20 μl. Reaction mixtures were incubated 20 min at 37°C. After the reactions were stopped by addition of EDTA to 20 mM, the samples were phenol-extracted and denatured, and equivalent amounts of radioactivity from each were loaded onto a denaturing 8% polyacrylamide gel. Each reaction mixture contained 0.02 unit of DNA polymerase II activity with SFI added at 0 ng (lane 1), 90 ng (lane 2), 225 ng (lane 3), or 450 ng (lane 4). Size markers (lane M; lengths indicated in nucleotides) were prepared by digestion of YIp5 with *Hin*fI followed by end-filling using *E. coli* DNA polymerase I Klenow fragment and [α-^{32}P]dTTP (4100 cpm/pmol).

The availability of pure, native 66-kDa protein allowed us to compare the DNA-binding properties of the apparent SFI complex with those of the 66-kDa polypeptide. Comparison of Fig. 2*A* and *B* shows that the 66-kDa polypeptide binds single-stranded DNA with essentially the same specificty as the complete SFI complex, and further suggests that the DNA-binding activity of the complex resides in this polypeptide. We have been unable to detect either ATPase or helicase activity in either the SFI complex or the purified 66-kDa protein.

Titration of SFI and Characterization of Mode of Stimulation of Yeast DNA Polymerase II. Partially purified preparations of DNA polymerase II have been shown to be highly processive and insensitive to the processivity factor PCNA (17). Since other polymerases are not processive in the absence of auxiliary factors and since the polymerase II preparations used in those studies were not pure, we reinvestigated the processivity of polymerase II. A nearly homogeneous preparation of DNA polymerase II, prepared previously in this laboratory (10), was used to titrate stimulatory activity, and polymerase processivity was examined in the absence and presence of SFI. With poly(dA)$_{400}$·oligo(dT)$_{10}$ such that there was one primer per template, synthesis reactions were carried out with increasing amounts of SFI and the products were loaded on a denaturing 8% polyacrylamide gel. The size of the newly synthesized DNA was visualized by autoradiography (Fig. 5). Under these conditions (pH 7.5, 8 mM Mg^{2+}), highly purified DNA polymerase II is a processive enzyme, since product length is limited only by the length of the template (lane 1). The presence of yeast SFI leads to an increase of all size products (lane 2) and an excess of SFI slightly inhibits overall synthesis (lanes 3 and 4). Thus, SFI has no obvious effect on processivity of DNA polymerase II over stretches of DNA up to 400 base pairs but instead may stimulate synthesis by reducing nonproductive binding and/or promoting more efficient primer utilization.

DISCUSSION

We have purified a stimulatory factor of yeast DNA polymerase II to near homogeneity. The most purified fraction contains three polypeptides with apparent molecular masses of 66 kDa, 37 kDa, and 13.5 kDa. These proteins may associate to form a complex that is responsible for the observed activities. The yeast stimulatory factor, SFI, is relatively stable and may be isolated by glycerol gradient centrifugation. The yeast polymerase stimulatory activity is coincident with single-stranded DNA-binding activity in glycerol gradient fractions (Fig. 3*A*). Further, the apparent yeast complex (Fig. 2*A*) displays specificity for single-stranded DNA over double-stranded DNA. The binding activity associated with human RP-A is contributed by the 70-kDa subunit (3, 16). We have been able to disrupt the yeast complex and isolate the 66-kDa polypeptide. The 66-kDa protein has an intrinsic and specific single-stranded DNA-binding activity (Fig. 2*B*). The subunits of the human RP-A complex may be more tightly associated than those of the yeast complex, since human RP-A may be isolated intact in the presence of 6 M urea (5). The ability to separate the native 66-kDa subunit and study it in the absence of the other subunits should be extremely valuable in characterizing the roles of the individual subunits. For all of their similarities, however, the yeast SFI complex was not recognized by antibodies to the human RP-A complex (M. Wold, personal

communication). The functional yeast analog of mammalian PCNA has also been found to be immunologically distinct from mammalian PCNAs (18).

SFI is of interest in that it is a unique yeast SSB. There have been several reports of DNA-binding proteins purified from yeast. They display a range of biological activities with some (SSB_{14}, SSB_{20}, SSB_{26}, and SSB_{35}; subscripts indicate molecular mass in kilodaltons) facilitating DNA aggregation and homologous strand exchange (19, 20) and others (DBP I, DBP II, and DBP III) affecting DNA topoisomerization (21). SSB2 and SSB_{mt}, a mitochondrial DNA-binding protein, have unknown functions (11). Gene disruptions have disclosed that yet another protein, SSB1, is nonessential (22). This protein has been localized to the nuceolus and sequencing revealed that SSB1 has extensive homology with proteins involved in RNA binding (23). Yeast SSB1 is now thought to be involved in RNA metabolism. A 34-kDa yeast protein that binds specifically to single-stranded DNA in a cooperative fashion and stimulates strand-exchange reactions has recently been reported (24). This protein is distinguished from previously described abundant yeast SSBs in that it is eluted from DNA-cellulose at a much higher ionic strength. The ability of the 34-kDa SSB to stimulate *E. coli* RecA-mediated strand exchange makes this protein appear more like the *E. coli* SSB and the gene *32* protein of phage T4, which are required for recombination and replication, than any SSB described before SFI (24).

Thus, the eukaryotic replicative SSB has remained elusive because of the profusion of SSB-like proteins in eukaryotes. It was known that two viruses, adenovirus type 2 and herpes simplex virus, encoded SSBs that were essential for viral replication (2, 25, 26). The identification of a potential human cellular counterpart of these viral SSBs, however, by the demonstration that an SSB was required for SV40 DNA replication *in vitro*, provided a more direct clue as to how to distinguish an SSB that might be involved in replication among the numerous yeast SSBs. The greatest difference between SFI and all of the yeast SSBs just enumerated is its subunit composition. RP-A is the only other SSB with this composition that has been described in any eukaryote.

The apparent multisubunit structure of SFI may reflect other molecular functions for this protein besides binding to single-stranded DNA. Our results, though still incomplete in the absence of direct genetic information, suggest a role for SFI as a DNA polymerase accessory protein. Indeed, HeLa SSB has been shown to stimulate DNA polymerase α (3). Our result with the purified 66-kDa polypeptide of SFI indicates that DNA-binding activity alone is not sufficient for maximal stimulation of polymerase, implying a role for the smaller polypeptides in protein–protein interactions. Availability of the yeast proteins now allows cloning of the genes encoding the subunits to determine whether yeast SFI is required for DNA replication *in vivo* and to delineate the specific roles of the individual polypeptides. This is especially important for the 37- and 13.5-kDa proteins, for which there is no known biochemical function or prokaryotic counterpart.

Note Added in Proof. While this manuscript was in press, work describing a yeast SSB with a subunit structure similar to SFI and to human RP-A appeared (27). We have found that antibody to the 66-kDa subunit of SFI does not crossreact with the large subunit of the SSB purified as described by Brill and Stillman (27).

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