Purification, Gene Cloning, and Reconstitution of the Heterotrimeric Single-stranded DNA-binding Protein from Schizosaccharomyces pombe

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We have purified a single-stranded DNA-binding protein (SSB) from Schizosaccharomyces pombe (Sp) and have shown that it is composed of three subunits of 68, 30, and 12 kDa. The SpSSB supports T antigen-dependent unwinding of SV40 ori containing DNA, but is not functional in the SV40 in vitro replication reaction. All three genes that encode the SpSSB subunit have been isolated from the genomic DNA of the spssb1, encoding the p68 subunit, contains 609 amino acids (68.3 kDa), while that of the spssb2, encoding the p30 subunit, contains a 279 amino acids (30.3 kDa). The genomic DNA clone of the p12 subunit gene (spssb3) has 2 introns and an open reading frame of 104 amino acids (11.8 kDa). Significant homology is observed among the largest and middle subunits of eukaryotic SSBs, but there is poor homology among the smallest subunits. In addition, we have reconstituted the SpSSB complex by coexpression of all three subunits in Escherichia coli. The reconstituted complex is active in single-stranded DNA binding and the T antigen-dependent unwinding of SV40 ori DNA. Finally, we observed a cell cycle-dependent phosphorylation pattern of the p30 subunit of SpSSB, which is similar to that observed for the human and Saccharomyces cerevisiae SSB.

The mechanism by which eukaryotes initiate DNA synthesis from origins of DNA replication on chromosomes is not understood. Insight into this process has come from detailed biochemical studies using the simian virus 40 (SV49) in vitro replication system which demonstrated a pivotal role of the heterotrimeric single-stranded (ss) DNA binding protein (SSB) in DNA synthesis. SV40 DNA replication is initiated when the viral-encoded large T antigen binds as a double hexamer to the core origin, and recruits the human DNA polymerase α-prime complex and human (H) SSB (also called RP-A) to catalyze primer synthesis. Subsequently, T antigen functions as a bidirectional helicase to unwind the DNA in the presence of HSSB, and the coordinate actions of DNA polymerase α-prime and DNA polymerase δ holoenzyme at the replication forks catalyze leading and lagging strand synthesis. HSSB, in addition to its role in the initiation reaction, is also required for the elongation reaction as shown by its stimulatory effects on polymerase activity with primed templates (reviewed in Refs. 1–4).

Biochemical studies on the initiation of DNA replication on metazoan chromosomes have not been possible because origins of replication have not been defined to specific sequences. Recent studies in the budding yeast Saccharomyces cerevisiae (Sc), have led to the biochemical characterization of an origin recognition complex (ORC) that binds to the well-defined origin of DNA replication, ARS1 (S), from this organism. The ORC is a complex of six different subunits that binds to ARS1 and other ARS sequences in an ATP-dependent manner (6–8). Many factors appear to interact with the yeast ORC, and these include CDC6 and CDC46/MCM5 (9–11). Undoubtedly, other factors required for the initiation reaction would include the polymerase α-prime complex and ScSSB. Recently, some of the homologous ORC subunits have been isolated from Schizosaccharomyces pombe, Drosophila, Xenopus, and human sources, suggesting a conserved mechanism for the initiation of DNA replication in lower and higher eukaryotes (12–16).

Our primary focus is to understand the mechanisms of initiation of DNA replication in eukaryotic cells. We are using the fission yeast S. pombe (Sp) as a model system because it has certain features that are similar to higher eukaryotes, and offers both biochemical and genetic analyses of DNA replication mechanisms. Origins of replication, as defined by an ARS assay and two-dimensional mapping techniques, have been isolated from S. pombe and characterized (17–19). These origins are in the range of >500 bp, larger than ARS elements from S. cerevisiae (~100 bp) (18, 19). The availability of these ARS elements will facilitate a biochemical study of an initiation complex from S. pombe. To this end, our objective is to isolate the ORC complex from S. pombe, along with SpSSB and the DNA polymerase α-prime complex, as a step toward analyzing the initiation of DNA synthesis from S. pombe origins. In this study, we report the purification of the single-stranded DNA-binding protein complex from S. pombe. The protein is heterotrimeric and shows regions of conservation to SSBs isolated from other eukaryotes. We demonstrate the overexpression and reconstitution of the active complex from Escherichia coli.
MATERIALS AND METHODS
Yeast and E. coli Strains

S. pombe mutants used in this study are all isogenic derivatives of 972h- strain (kindly provided by Dr. T. Wang, Stanford University). The genotypes of cdcl10-129, cdcl22-45, cdcl25-22, and cdcl-33 were described previously (20). E. coli strains DH5α, DH10, Y10808*, XL-1 blue, and BL21(DE3) were used for plasmid construction, cDNA library maintenance, hosts for genomic library, and sources for making single-stranded DNA for sequencing and expression of recombinant protein, respectively.

Replication Proteins and Other Reagents

SV40 T antigen and human proteins used for the SV40 DNA replication assay were described previously (21). Enzymes used in DNA manipulations were from New England Biolabs, Perkin-Elmer, and Boehringer Mannheim. Restriction enzymes obtained from Clonetech, while the pDB20 based S. pombe cDNA library was kindly provided by Dr. L. Guarente, Massachusetts Institute of Technology (22). pBluescript SK- was used for cloning and sequencing, was from Stratagene. SV40-origin containing plasmid pSV01ΔEP (23) and pET19b-GST, used for SpSSB expression in E. coli (24), were described previously.

Purification of SpSSB from S. pombe Cells

SpSSB from S. pombe was purified according to the procedure described for the isolation of the S. cerevisiae SpSSB with some modifications (25). All steps were carried out at 4 °C. S. pombe 972h- wild type cells (500 g, wet weight) were resuspended in 1 liter of buffer A (25 mM Tris-HCl, pH 7.5, 1 mM EDTA, 10% glycerol, 0.2 mM leupeptin, 0.1 mM aprotinin, and 50 μM EGTA) with 1 mM NaCl and 10 mM sodium bisulfite. Disruption of cells was carried out using a ratio of 1 volume of glass beads to 2 volumes of cell suspension with a Bead-Beater (Biospec Products: total volume, 360 ml). Disruption was carried out using 8 cycles of vortexing for 30 s followed by 1 min of cooling in ice. The lysate was centrifuged at 16,200 g for 35 min. The supernatant (800 ml, 20 g protein) was adjusted to 0.5 mM NaCl with buffer A and applied to a 40 ml (2.3 × 8 cm) Affi-Gel blue column (Bio-Rad), pre-equilibrated with buffer A + 0.5 mM NaCl. This column was washed with 20 column volumes of buffer A + 0.8 mM NaCl and the SpSSB eluted with 2 column volumes of buffer A + 2 mM NaCl with 40% ethylene glycol. The eluted protein peak was pooled (76 ml, 170 mg protein), diluted to 0.5 mM NaCl with buffer A, and applied to a 20-ml (2.3 × 45 cm) ssDNA cellulose column (Sigma), pre-equilibrated with buffer A + 0.5 mM NaCl. This column was washed with 20 column volumes of buffer A + 0.8 mM NaCl and 2 column volumes of buffer A + 2 mM NaCl with 40% ethylene glycol. The peak of protein obtained in the latter was washed (5.6 ml, 1 mg protein) and dialyzed against 2 liters of buffer A.

Characterization of S. pombe SSB

To purify SpSSB, we separated by SDS-PAGE and individual subunits excised and used as antigens for the preparation of antisera against the SpSSB p68 and p30 subunits in rabbits. Affinity purification of antibodies from these sera was performed using the purified proteins.

SV40 T Antigen-dependent Unwinding Assay

The lysate was centrifuged at 16,200 g, using 8 cycles of vortexing for 30 s followed by 1 min of cooling in ice. Beater (Biospec Products: total volume, 360 ml). Disruption was carried out using 8 cycles of vortexing for 30 s followed by 1 min of cooling in ice. Purification was carried out using 8 cycles of vortexing for 30 s followed by 1 min of cooling in ice.

Polymerase Chain Reaction (PCR)

All PCR reactions were performed in 50-μl reaction mixtures containing 100 pmol each of primer, 2 units of Ultima DNA polymerase (Perkin-Elmer) or Taq DNA polymerase (Boehringer), and substrate DNA using conditions specified by the manufacturer.

Cloning of the SpSSB Genes

p68 Subunit Gene (ssb1 gene) — Two degenerate oligonucleotides were used to amplify the region of the gene by PCR. The primer sequences used were as follows: T29a (32 mer; 5'-AAYGTICARAAYGARTAGYTARTIGYATGGYAAG-3') and T35b (30 mer; 5'-CATTCAAGCCTGGATCCTTATTCTTTAATGATTTGCTCTT-3'). The PCR product of 0.9 kb was cloned and found to encode the predicted amino acid sequence. This fragment was then used to probe a S. pombe cDNA library for the isolation of ssb1 gene. From 2 × 105 colonies screened, two positive clones were identified. These clones were subcloned into pBluescript SK+ vector and both strands were completely sequenced.

p30 Subunit Gene (ssb2 gene) — A 0.4-kb product was obtained after PCR with two degenerate oligonucleotides (26 mer each): T13a (5'-AAAYCAYCIAYCARATHGARGAYGG-3') and T24b (5'-RTGATIGCCTGAATCCTCTGATTCTTTAATGATTTGCTCTT-3'). The PCR product was sequenced (Microchemistry, Sloan-Kettering Institute). Seven distinct peptide sequences for the p68 subunits, two distinct sequences for p30 subunits, and four distinct sequences for the p12 subunit of the SpSSB were obtained. These peptide sequences are underlined in Fig. 3 (A, B, and D).

Construction of Plasmids Expressing the SSB Genes

The three SpSSB genes were coexpressed in E. coli using the pET system developed by Studier et al. (26). A description of this procedure is summarized in Fig. 5. The plasmid that expressed the p68 subunit was made as follows. The SpSSB p68 cDNA was amplified by PCR using two primers. One primer 68A (26 mer; 5'-TTTGGACATCATGGTACGGCAGGATTACCG-3') has an Ncol site that contains the start codon, and the other, 68D (29 mer; 5'-TAAACCCGTTGACATTGATTGACGAACG-3') has a Kpn1 site.
site after the stop codon (both sites are underlined). The PCR amplified 1.8-kb fragment was digested with both Ncol and KpnI and ligated to the Ncol-KpnI-digested pET19b-GST vector. The resulting plasmid was devoid of the glutathione S-transferase from the parental pET19b-GST vector. The cloned SpSSB p68 subunit cDNA was expressed from the authentic start codon of the cDNA. This plasmid is referred to as pET19b-SpSSB p68. The construction of p12 subunit gene expression plasmid, pET19b-SpSSB p12, was described above.

The plasmid that expressed the p30 subunit gene was made as follows: Two primers (30-mer each) 30A (5'-TACGATCCAAGCCTTGGCT-TATGATTTTGCCG-3') containing a BamHI site and 30D (5'-CTTGAAATCTGACCGCTAAGTAAAATG-3') containing an EcoRI site were used to generate a 0.2-kb product by PCR of the N-terminal region of the p30 gene. This fragment was digested with both BamHI and EcoRI and cloned into the same sites of pBluescript SK+ (data not shown). The 0.7-kb EcoRI-HindIII fragment containing the adjoining C-terminal region of the SpSSB p30 cDNA was ligated to the EcoRI and HindIII digested plasmid to construct pBS-SpSSB p30. For formation of pET19b-SpSSB p30, the 0.9-kb Ncol-HindIII fragment from pBS-SpSSB p30 containing the entire region of SpSSB p30 gene was ligated to the Ncol-HindIII site of pET19b-GST.

For the construction of pET19b-SpSSB p12/p30 (see Fig. 5), the pET19b-SpSSB p30 plasmid was digested with both Xbal and HindIII and both ends were blunt-ended with T4 DNA polymerase. This 0.9-kb Xbal-HindIII fragment containing the entire SpSSB p30 cDNA was ligated to the Acc65I-digested and T4 DNA polymerase-treated pET19b-SpSSB p12. We use the isoschizomer Acc65I instead of KpnI, since the constructed plasmid retained a KpnI site. pET19b-rSpSSB (containing all three subunits) was made in a similar way (also see Fig. 5). A 1.2-kb Xbal-HindIII fragment from the pET19b-SpSSB p12/p30, following treatment with T4 DNA polymerase, was ligated to the Acc65I site (filled in with T4 DNA polymerase) of the pET19b-SpSSB p68. All recombinant plasmids were sequenced to insure that no mutations had occurred during these manipulations.

Purification of Recombinant (r) SpSSB from E. coli

E. coli BL21(DE3) containing pET19b-rSpSSB was grown in 12 liters of L broth containing 100 μg/ml ampicillin at 37 °C to an A600 of 0.5. Isopropyl β-D-thiogalactoside (0.4 mM) was added and the cells were incubated for 3 h. Induced cells were collected by centrifugation and stored frozen at −20 °C until needed. Purification of rSpSSB was purified from E. coli following the purification procedure used for the isolation of the SpSSB from S. pombe cells, except that a phosphocellulose column was used instead of Q-Sepharose to remove a contaminating cell lysate. The protein that had ssDNA binding activity. Induced cells were collected by centrifugation and stored frozen at −20 °C until needed. Purification of rSpSSB was purified from E. coli following the purification procedure used for the isolation of the SpSSB from S. pombe cells, except that a phosphocellulose column was used instead of Q-Sepharose to remove a contaminating cell lysate. The protein that had ssDNA binding activity. Induced cells were collected by centrifugation and stored frozen at −20 °C until needed.

RESULTS

The SSB purified from S. pombe is a Heterotrimeric Complex—We assumed that the chromatographic properties of the Sp SSBS were similar to those of HSSB and ScSSB. For this reason, the purification procedure used for the isolation of the ScSSB (25), as described under “Materials and Methods,” was used to purify SpSSB. An analysis of the protein composition of fractions obtained after each chromatographic step is shown in Fig. 1A. To determine the subunit structure of the SpSSB, the Q-Sepharose fraction was subjected to glycerol gradient sedimentation by SDS-PAGE analysis (Fig. 1B). As shown, SpSSB contained three polypeptides of p68, p32, and p12 kDa that cosedimented at 5.6 S. This subunit structure and sedimentation coefficient are similar to those observed for HSSB and ScSSB (27–29). Multiple bands migrating slightly slower than the SpSSB p30 subunit were observed which corresponded to phosphorylated derivatives of the p30 subunit (see below). The 50- and 20-kDa protein bands (Fig. 1B, lane 1) were identified by antibodies against SpSSB p68 and p30 subunits, respectively, since they were recognized by antibodies against SpSSB p68 and p30 subunits, respectively (data not shown).

SpSSB Supports Unwinding of SV40 ori DNA by T Antigen—Previous studies demonstrated that the HSSB and ScSSB support the unwinding of SV40 ori DNA by T antigen (25, 30). Since SpSSB has a similar subunit structure as HSSB and ScSSB, we examined whether it would support the unwinding reaction. T antigen was incubated with a 32P-labeled fragment containing the SV40 core origin and the glycerol gradient fractions. After incubation the reactions were deproteinized and subjected to PAGE analysis. As shown in Fig. 2A, SpSSB glycerol gradient fractions 11–13 (Fig. 1B) supported efficient unwinding reactions, consistent with the notion that the SpSSB is a functional homolog of HSSB and ScSSB. The omission of SSBS (lane 2), T antigen (lane 3), or ATP (lane 4) resulted in no single-stranded DNA formation. As described below, SpSSB binds ssDNA in a manner analogous to HSSB.

SpSSB Does Not Support the SV40 Monoplymerase Reaction—It established that of a number of SSBS examined, only the human, mouse, and Drosophila SSBS support SV40 DNA replication (31, 32). We analyzed whether SpSSB would function in the SV40 monoplymerase system. The reaction was assembled as described previously (27) in the presence of in-
Cloning of the Genes Encoding SpSSB—We obtained a genomic clone containing a 3-kb insert of the SpSSB p12 subunit gene (ssb1) (Fig. 3C). Partial sequence of this clone showed that the middle 1-kb HindII fragment included peptide sequences obtained from tryptic peptides of the p12 subunit. The complete nucleotide sequence of this fragment was determined and is shown in Fig. 3D. This sequence contains the entire coding region as well as 461 and 143 bp of upstream and downstream untranslated sequences, respectively. We found that the genomic DNA sequence, corresponding to the second peptide sequence in Fig. 3D, has a 63-bp gap that contained a perfect match to the consensus sequence for a 5′-splice site (GTANG), branch (CTRAY), and a 3′-splice site (AG) of S. pombe introns (33). We also found another 101-bp sequence with perfect match to intron consensus sequences. The authenticity of the introns was confirmed by direct isolation of the cDNA sequences spanning this region. The nucleotide sequences of cDNAs obtained by PCR using an S. pombe cDNA library were devoid of the regions corresponding to the predicted introns. The ORF, determined from the cDNA encoding the small subunit, contained 104 amino acids, was calculated to be an 11.8-kDa protein, and contained all four peptide sequences obtained from the p12 subunit.

Amino Acid Sequence Comparison among the SpSSB Subunits and the Homologous Subunits of Other Eukaryotes—The amino acid sequences of the SSB subunits from a variety of eukaryotes have been reported (34–41). We have used the Megalign program to determine the regions of homology (identity and homology) among the respective SSB subunits and the SpSSB subunits.

The amino acid sequence of the p68 subunit of SpSSB showed significant identity and homology to the corresponding subunit of S. cerevisiae (34.7%, 60.9%), human (36.6%, 63.7%), Xenopus laevis (38.2%, 63.9%), Caenorhabditis elegans (21.9%, 50.7%), and Trypanosoma Cruithia fasciculata (21.7%, 42.1%) (Fig. 4A). The C-terminal 2/3 region (amino acids 179–609 of SpSSB p68) showed the highest degree of conservation. The identity and homology of this region of SpSSB compared to the other SSBs are S. cerevisiae (44.2%, 69.9%), human (40.6%, 69.1%), Xenopus (41.7%, 68.6%), C. elegans (26.2%, 55.9%), and C. fasciculata (29.5%, 56.7%).

Significant identity and homology was observed for the p30 homologs of SSB of S. cerevisiae (27.2%, 49.5%), mouse (23.6%, 49.2%), human rpa2 (23.9%, 50.5%), rpa4 (19.3%, 43.4%), and C. fasciculata (21.7%, 41.7%) (Fig. 4B).

In contrast to the largest and middle subunits, poor identity was detected when the smallest subunit of HSSB, ScSSB, and SpSSB were compared. SpSSB showed identity and homology to ScSSB (19%, 42.1%), and HSSB (19.4%, 50%). The HSSB and ScSSB showed much less identity and homology using the Megalign program (11.8%, 37%) (Fig. 4C).

Coexpression of the Three SpSSB Genes in E. coli Results in the Formation of a Stable HeterotrimERIC Complex—We constructed expression plasmids for each SpSSB subunit using the T7 RNA polymerase expression system in E. coli (26). Each cDNA was cloned into a plasmid containing the T7 RNA polymerase promoter (Fig. 5). DNA sequencing established that the ATG start codon was maintained for each subunit and that there were no amino acid changes made in any of the coding sequences (see “Materials and Methods”). Each subunit of
SpSSB expressed individually in E. coli was found to be insoluble and not amenable for biochemical analysis. Attempts to solubilize the proteins using various refolding protocols were unsuccessful (data not shown).

It was reported that active HSSB complex is produced from coexpression of the three genes on the same plasmid in E. coli (42). For this reason, an expression plasmid was constructed that contained all three subunits of SpSSB (Fig. 5). The resulting plasmid (pET19b-rSpSSB) contained a single T7 RNA polymerase promoter and the cDNA for each subunit was preceded by a Shine-Delgano ribosome binding site. This plasmid was used to transform BL21(DE3) and the resulting transformant was examined for the expression of recombinant proteins following SDS-PAGE (Fig. 6, lanes 1 and 2). When the three subunits of SpSSB were expressed together, they formed an active complex as determined by gel filtration. The active complex was isolated and characterized further.
subunits were expressed together, approximately 50% of the recombinant protein was soluble (data not shown).

Purification of the recombinant SpSSB expressed in E. coli was carried out using the purification procedure used to isolate native SpSSB from S. pombe cells with modifications (see "Materials and Methods"). Fig. 6 shows the SDS-PAGE analysis of the material obtained during the isolation. The soluble protein fraction from induced cells carrying pET19b-rSpSSB was successfully chromatographed through Affi-Gel blue and ssDNA cellulose columns. To remove contaminating ssDNA binding activity presumably contributed by E. coli, the pooled fraction after elution from ssDNA cellulose was chromatographed through a phosphocellulose column. The recombinant SpSSB had the same sedimentation coefficient as the native SpSSB measured by glycerol gradient centrifugation. No partial assembled SpSSB subunits were observed in other fractions in this gradient (data not shown). We conclude that recombinant SpSSB is a stable complex with a structure similar to SpSSB isolated from S. pombe (Fig. 6, lanes 7 and 8).

The Recombinant and Native SpSSBs Show Equivalent Activities—We analyzed the binding of native and recombinant SpSSB, as well as HSSB, to ssDNA using a gel mobility shift assay. The protein preparation used for the following assays was that described in Fig. 6, lanes 7–9. A ssDNA of 45 nucleotides was used as the substrate for the binding reaction. With the addition of increasing amounts of protein, two complexes (C1 and C2) were detected with the native and recombinant SpSSB and HSSB (Fig. 7A). The efficiency of binding for the three SSBs was essentially similar (Fig. 7B). From this, we estimated that a monomer of SpSSB and HSSB occupied approximately 16–22 nucleotides of ssDNA, consistent with previous reports that HSSB occupied 20–30 nucleotides of ssDNA (29, 43, 44).

Since HSSB and ScSSB bind preferentially to ssDNA (25, 27), competition experiments were carried out to measure the binding efficiency of SpSSB to ssDNA and dsDNA. The levels of the slow migrating complexes C1 and C2 were quantitatively decreased by the addition of competitor ssDNA, whereas dsDNA had no effect (Fig. 8). SpSSB was more sensitive to competition by ssDNA than by dsDNA. Similar results were obtained for HSSB in this assay.

In addition, the native and recombinant SpSSB were equally active in the unwinding of SV40 origin containing DNA (data not shown). Based on these results, we conclude that the recombinant and native SpSSBs have identical biochemical properties.

Cell Cycle-dependent Phosphorylation of SpSSB—We initially observed that the p30 subunit of the purified SpSSB migrated as multiple bands following SDS-PAGE (Fig. 1, A and B). The possibility existed that these were either degradation products of the largest subunit or alternatively, modified derivatives of intact p30. It is known that the middle subunit of HSSB and ScSSB is phosphorylated in a cell cycle-dependent manner, being phosphorylated at G1/S through to M, when dephosphorylation occurs (45). The SpSSB preparation, isolated from S. pombe cells, was treated with alkaline phosphatase and subjected to SDS-PAGE. As shown in Fig. 9A, modified species were no longer present, and only the faster migrating species was detected (Fig. 9A, compare lanes 7 and 8). This suggests that the p30 subunit of SpSSB exists in different phosphorylated states in vivo. To determine whether phosphorylation occurred in a cell cycle-dependent manner, we used cell division cycle (cdc) mutants of S. pombe that arrested at known stages of the cell cycle at the nonpermissive temperature (20). Cells were grown to early log phase and shifted to
thenonpermissive temperature for 4 h for cell cycle arrest, and then extracts were prepared and analyzed by SDS-PAGE and immunoblotting using affinity-purified p30 antibodies. At the same time, cells were processed for FACS analysis to monitor DNA content to confirm their duration in the cell cycle. With the cdc10-129 mutant, the DNA content was G1 indicating a G1 arrest. In the cdc22-m45 and cdc25-22 mutants, their DNA contents were consistent with a S and G2 arrest, respectively. The data from the immunoblot experiments demonstrated that the predominant fast migrating species of p30 subunit was observed only in the G1 extract, whereas multiple slow migrating species were observed in the S and G2 extracts (Fig. 9A, lanes 2–5). To confirm that phosphorylation of the p30 subunit occurred as cells traversed the G1 to S boundary, cdc10-129 cells were synchronized by first arresting their growth at the nonpermissive temperature followed by release at the permissive temperature. Extracts were prepared at various times and FACS analysis was used to monitor the DNA content of cells. As shown in Fig. 9B, the p30 subunit was poorly phosphorylated between 0 and 40 min; phosphorylated derivatives, however, accumulated after incubation from 40 to 240 min. The majority of cells after incubation at the permissive temperature for 220 min were in the M phase (data not shown). The FACS analysis indicated that cells progressed through the G1/S boundary between 40 and 80 min. We conclude that the p30 subunit of SpSSB is hypophosphorylated in G1 and becomes phosphorylated at the G1/S boundary and is maintained in this state through the M phase. These data are similar to the cell cycle-dependent phosphorylation of the middle subunit of the human and S. cerevisiae SSBs (45).

DISCUSSION
We have purified to homogeneity the SSB from S. pombe and shown that it is a heterotrimeric complex of 68, 30, and 12 kDa. Homologous SSBs isolated from a variety of widely divergent eukaryotes, including human, mouse, S. cerevisiae, Drosophila,
Xenopus, and the trypanosome C. fasciculata, have a similar heterotrimeric structure. All of these multimeric SSBs characterized have strong affinity for ssDNA and supports the SV40 T antigen-dependent unwinding of SV40 origin containing DNA (25, 46–48). The binding of ssDNA appears to be an important factor governing the T antigen-mediated unwinding reaction. This notion is supported by the observation that E. coli SSB, adenovirus DNA-binding protein, and herpesvirus ICP8 also support the unwinding reaction. However, other SSBs, such as the T4 gene product 32 and the T7 gene product 2.5 do not (49–51). These findings suggest that the sequestration of ssDNA generated by the T antigen may not be the complete explanation for the role of a DNA-binding protein in the unwinding reaction.

At present, the most stringent assay available for determining the biological activity of the multisubunit DNA binding protein is its ability to support the SV40 T antigen-dependent replication of SV40 ori DNA. This reaction involves the direct interaction of T antigen with HSSB and the interactions between these two proteins and the DNA polymerase α-primase complex (21, 31, 52, 53). In light of the marked species specificity involved in the parvovirus replication systems, it is not surprising that the evolutionarily divergent ScSSB, SpSSB, and the C. fasciculata SSB are unable to support this replication system. However, the SSB from mouse and Drosophila can support the SV40 replication reaction (25, 31, 32, 48). The data suggest that the mouse and Drosophila SSBs have functional features that are conserved with HSSB, whereas the others do not.

We have isolated and determined the nucleotide sequence of each of the genes encoding the p68, p30, and p12 subunit of SpSSB. The p12 gene contains two introns. Coexpression of these subunits in E. coli resulted in the formation of an active heterotrimeric complex, as assayed by ssDNA binding and the T antigen-dependent unwinding reaction following by glycerol gradient sedimentation analysis. An active heterotrimeric complex was similarly observed for the reconstituted HSSB (42). It is not clear how coexpression of these subunits lead to the formation of an active complex, but it is likely that a coordinated folding event of all three subunits is important.

The amino acid sequence of the largest subunit of SSB is highly conserved among the various species analyzed here. All have a C4-type zinc finger motif, as previously reported, which spans amino acids 477–498 of SpSSB. It is noteworthy that the C-terminal two-thirds region of the largest subunit (amino acids 179–609 of SpSSB) shows the highest degree of conservation. Interestingly, the C. fasciculata largest subunit equivalent lacks a segment that corresponds to approximately one-third of the N-terminal region present in the p68 homologs of human, frog, nematoda, and the two yeasts (40). As described above, C. fasciculata SSB is heterotrimeric, has ssDNA binding activity, and supports the unwinding reaction, indicating that the well conserved C-terminal two-thirds region is ample for these functions. Gomes and Wold (54) concluded from a deletional analysis of the HSSB p70 subunit that amino acids 1–411 of HSSB define the ssDNA binding domain (core is 169–249). They also concluded that the conserved zinc finger motif is not involved in ssDNA binding. In addition, the 507-amino acid C-terminal end region of HSSB p70 is involved in complex formation with p34 and p14 (54). These results are consistent with idea that the conserved C-terminal regions are
required for DNA binding and for complex formation with the middle and/or smallest SSB subunits. Based on the homology, we anticipate that it may also be true for *S. pombe* SSB, but it remains to be determined by further analysis.

Significant homology was found among the p30 subunit amino acid sequences of *S. pombe* and those of the other species. Lee and Kim (55) reported that N-terminal and C-terminal deletion derivatives of the p34 subunit of HSSB formed a heterotrimeric complex with the p70 and p14 subunits. The N-terminal deletion lacked amino acids 2–30, and the C-terminal deletion lacked 33 amino acids from the end of the protein. The HSSB complex containing the p34 N-terminal deletion was as active as native HSSB in supporting the SV40 in vitro DNA replication reaction, whereas the C-terminal deletion did not.

**FIG. 5.** Construction of recombinant SpSSB expression plasmid. cDNA encoding the subunits of SpSSB were cloned into pET19b vectors as described under “Materials and Methods.” Restriction enzyme sites used for various constructs are indicated. Open triangles, closed bars, and double triangles indicate T7 promoter, ribosome binding site, and T7 terminator, respectively.

**FIG. 6.** SDS-PAGE (15% gel) analysis of purified recombinant *S. pombe* SSB. Numbers to the left indicate the size and position of marker proteins. The position of the p68, p30, and p12 subunits are indicated on the right. The fractions and amounts of protein analyzed were: lane 1, preinduced E. coli cell lysate (10 μg); lane 2, induced E. coli cell lysate (15 μg); lane 3, soluble fraction of cell lysate (10 μg); lane 4, pool of the Affi-Gel blue column eluate (5 μg); lane 5, pool of the ssDNA cellulose eluate (4 μg); lane 6, pool of the phosphocellulose eluate (1.7 μg); lane 7, purified native SpSSB (peak fractions of the glycerol gradient, 3 μg); lane 8, purified recombinant SpSSB (peak fractions of the glycerol gradient, 3 μg); lane 9, purified HSSB (3 μg).

**FIG. 7.** Analysis of the binding of native and recombinant SpSSB, and HSSB to ssDNA by a gel mobility shift assay. A, the reaction mixture (15 μl) contained 5 fmol of the 32P-labeled 45-nucleotide oligonucleotide and the indicated amounts of SpSSB or HSSB. Two complexes (C1, C2) were observed as indicated. B, the amount of C1 and C2 products formed in A was quantitated by PhosphorImager BAS1000 (Fuji) and plotted. ○, native SpSSB; ●, recombinant SpSSB; □, HSSB.

**FIG. 8.** Influence of dsDNA and ssDNA as competitors on the binding of SSBs. 50 ng of SpSSB or HSSB were incubated with 5 fmol of the 32P-labeled 45-nucleotide oligonucleotide and indicated amounts of ssDNA (42 nucleotides) or dsDNA (1-kb ladder) as competitors. The ratio of bound complex was quantitated by the PhosphorImager BAS1000 (Fuji) and plotted. ○, native SpSSB competed with ssDNA; ●, native SpSSB competed with dsDNA; □, recombinant SpSSB competed with ssDNA; ■, recombinant SpSSB competed with dsDNA; Δ, HSSB competed with ssDNA; ▲, HSSB competed with dsDNA.

The reason for the lack of function of the latter, is that it did not support the T antigen catalyzed unwinding of origin containing DNA (55). The alignment of the amino acid sequence of the middle subunit (Fig. 4B) reveals three conserved regions: region 1 (amino acid 52–113 of SpSSB p30 subunit), region 2 (126–167), and region 3 (257–274). The nonfunctional C-terminal deletion of the HSSB p34 subunit lacks region 3, and the
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**In addition to replication, the trimeric SSB is also required for DNA recombination (61) and nucleotide excision repair in cell-free systems (62–64). Recently, the human repair proteins XPA and XPG were reported to bind to HSSB (65–68). Mutations in the ScSSB p70 subunit gene (raf1) show defective phenotypes in cell growth, repair, and recombination in yeast cells (69–71). SSB may be involved in transcription since Sc-SSB was identified as a factor that binds to sequence elements involved in regulating transcription (72, 73), and HSSB also binds to the acidic domain of transcriptional factor VP16 and p53 (74–76). Since DNA replication, repair, and recombination are highly conserved in eukaryotes, the conserved region among the trimeric SSBs may be essential for interaction with factors involved in these processes.**

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In contrast to the conserved regions of the p68 and p30 subunits, the p12 subunit showed the least homology among the known SSBs. Gene disruption experiments show that each of the three SSB genes is essential for cell viability in *S. cerevisiae* (36). Antibodies against each of the three subunits of HSSB inhibited the SV40 replication reaction, suggesting that each subunit has a specific function(s) in DNA replication (35, 39, 60).
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