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Cockayne syndrome group B protein has novel strand annealing and exchange activities

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ABSTRACT

Cockayne syndrome (CS) is a rare inherited human genetic disorder characterized by UV sensitivity, severe neurological abnormalities and prageroid symptoms. The CS complementation group B (CSB) protein is involved in UV-induced transcription coupled repair (TCR), base excision repair and general transcription. CSB also has a DNA-dependent ATPase activity that may play a role in remodeling chromatin *in vivo*. This study reports the novel finding that CSB catalyzes the annealing of complementary single-stranded DNA (ssDNA) molecules with high efficiency, and has strand exchange activity. The rate of CSB-catalyzed annealing of complementary ssDNA is 25-fold faster than the rate of spontaneous ssDNA annealing under identical *in vitro* conditions and the reaction occurs with a high specificity in the presence of excess non-homologous ssDNA. The specificity and intrinsic nature of the reaction is also confirmed by the observation that it is stimulated by dephosphorylation of CSB, which occurs after UV-induced DNA damage, and is inhibited in the presence of ATPγS. Potential roles of CSB in cooperation with strand annealing and exchange activities for TCR and homologous recombination are discussed.

INTRODUCTION

Nucleotide excision repair (NER) provides a crucial cellular defense against a large variety of DNA lesions, including UV-induced pyrimidine dimers and bulky chemical adducts. NER has two major subpathways, global genome repair and transcription coupled repair (TCR) (1). The TCR pathway carries out preferential repair of DNA lesions in the transcribed strand of active genes, which is critical because these lesions obstruct transcription by RNA polymerase II (RNAPII). Transcription blocking DNA lesions act as signals of DNA damage and recruit NER proteins to damaged regions of the genome (2,3).

Cockayne syndrome (CS) is a hereditary disorder caused by mutations in *CSA* or *CSB*, which is associated with cellular deficiency in TCR. Cells from CS patients are characterized by inability to resume RNA synthesis after exposure to UV, a consequence of defective TCR (4,5). CS patients display premature aging symptoms including developmental abnormalities and neurological dysfunction (6). Approximately 80% of CS patients have mutations in *CSB*, which encodes a 168 kDa protein belonging to the SWI2/SNF2 protein family. CSB includes an acidic region, a glycine-rich region, two putative nuclear localization signal sequences and an ATPase domain characterized by seven conserved motifs (I, Ia and II–VI) (4). The SNF2-like ATPase domain is critical for CSB function *in vivo* (7,8) and is required for CSB-catalyzed DNA-dependent ATPase (9–11), ATP-dependent chromatin remodeling (12) and modulation of DNA negative supercoiling by CSB (13).

CSB is implicated in various cellular processes, including transcription by RNAPI, RNAPII and possibly RNAPIII, TCR and base excision repair of certain types of oxidative DNA damage (14). CSB stimulates elongation by actively transcribing RNAPII by interacting with transcribing RNAPII bound to DNA and nascent RNA (10,15). Several models have been proposed regarding how CSB rescues RNAPII complexes that are stalled at DNA lesions (15,16), but the details of this process remain unclear. The fact that CSB is dephosphorylated *in vivo* in cells with UV-induced DNA damage and that dephosphorylation of CSB stimulates CSB-catalyzed ATPase activity *in vitro* (17) suggests that phosphorylation of CSB may regulate its activity in cells with DNA damage.

This study reports the novel observation that CSB catalyzes the annealing of complementary single-stranded DNA (ssDNA) *in vitro* in a reaction that is sensitive to the phosphorylation state of CSB or the presence/absence of non-hydrolyzable ATP analogs. In addition, CSB has a strand

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exchange activity, which is independent of ATP. The results presented here suggest that ssDNA annealing and strand exchange activities may be important for the *in vivo* DNA repair functions of CSB.

MATERIALS AND METHODS

Recombinant proteins

Recombinant N-terminal hemaglutinin antigen (HA)- and C-terminal histidine$_6$ (His)-double-tagged CSBwt, and CSBE646Q, CSBT912/913V, CSBQ942E mutant proteins were purified from HiFive insect cells as described previously (17). Purified recombinant replication protein A (RPA) was kindly provided by Dr Mark Kenny (Albert Einstein Medical Center, Bronx, NY). *Escherichia coli* ssDNA binding protein (SSB) was purchased from Promega.

DNA strand annealing assays

The DNA strand annealing activity of purified recombinant CSB protein was measured using 1 nM complementary synthetic oligonucleotides of which 22Fork3 (5′-T_{15}GAGTGTGGTGTACATGCACTAC-3′) was labeled at the 5′ end using [γ-^{32}P]ATP and T4 polynucleotide kinase (New England Biolabs). The 22Fork4 (5′-GTAGTGCATGTACACCACACTCT_{15}-3′) was used as an unlabeled complementary oligonucleotide and 22T4 (5′-GTAGTGCATGCCCTAACCCTA-AT_{15}) was used as an unlabeled heterologous oligonucleotide in the strand annealing reaction. Annealing of a 0.5 nM [γ-^{32}P]ATP-labeled 100mer oligonucleotide (5′-GGGGTGCCTAATGAGTGAGCTAACTCACATTAATTGCGTTGCGCTCACTGCCCGCTTTCCAGTCGGGAAACCTGTCGTGCCAGCTGCATTAATGAATCGG-3′) to M13mp18 ssDNA (1 nM) was also measured. Strand annealing reactions (20 μl) were performed in the reaction buffer (20 mM Tris–HCl, pH 7.4, 5 mM $MgCl_2$, 1 mM DTT and 40 μg/ml BSA) and contained CSB as indicated in the figure legends. Reactions were incubated for 15 min at 30°C. In kinetic experiments, 200 μl reactions were initiated and 20 μl aliquots were removed at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 10 and 15 min. A control reaction mixture was set up in an identical manner to determine the level of spontaneous annealing of oligonucleotides in the absence of CSB. Reactions were terminated by the addition of 10 μl of stop buffer (35 mM EDTA, 0.6% SDS, 25% glycerol, 0.04% bromophenol blue and 0.04% xylene cyanol). Reactions products were subsequently incubated with 0.1 mg/ml Proteinase K for 30 min at 37°C. Products were electrophoresed on 12% native polyacrylamide gels (PAGE), visualized using a PhosphorImager, and quantified using ImageQuant software (Molecular Dynamics). The percent of annealed product was quantified using the following formula: Percent annealing = (the amount of annealed product/total DNA) × 100. Values were corrected for background in the no enzyme control where spontaneous annealing occurs.

Immunodepletion

Immunodepletion was conducted as described previously (18), with some modifications. Two micrograms of rabbit polyclonal anti-CSB (Santa Cruz Biotech.) or normal rabbit IgG (Santa Cruz Biotech.) antibodies were incubated for 1 h at 4°C with 40 μl Protein G agarose beads (Roche Molecular Biochemicals). After washing the beads three times with the reaction buffer, antibody-bound beads were incubated with 10 nM CSB in the reaction buffer (40 μl) for 1 h at 4°C. Beads were collected by centrifugation and the supernatant was again incubated with antibody-bound beads for 10 min at 4°C for a second immunodepletion step. Following centrifugation, supernatants were divided into two equal aliquots, one to be assayed for strand annealing activity as described above and the other to assess the efficiency of CSB depletion by western blot analysis using mouse anti-CSB antibody (kindly provided by Dr Jean-Marc Egly, Strasbourg). In control experiments, Protein G agarose beads lacking any bound antibody were incubated with CSB (10 nM), and the resulting supernatants were tested for strand annealing and western blot analysis.

In vitro phosphorylation of CSB

CSB phosphorylation and dephosphorylation reactions were performed as described previously (17), with some modifications. Briefly, purified human recombinant CSB protein (1.25 μg) was incubated with 250 U of casein kinase II (CKII; New England Biolabs) in kinase buffer (20 mM Tris–HCl, pH 7.5, 50 mM KCl, 10 mM $MgCl_2$ and 2 mM ATP) at 30°C for 15 min. For the dephosphorylation reaction, CSB protein (1.25 μg) was incubated with 625 mU of protein phosphatase I (PP1; New England Biolabs) in phosphatase buffer for 15 min at 30°C. Proteins were tested for strand annealing activity and also analyzed by SDS–PAGE and western blot using mouse monoclonal anti-phosphotyrosine (P-tyr) (Upstate) or mixed mouse monoclonal anti-phosphoserine/threonine antibodies (P-ser/thr) (Upstate) to detect phosphorylation or mouse anti-CSB antibody.

DNA strand exchange assay

The strand exchange assay was performed as described previously (19), with some modifications. Briefly, labeled fork substrate C80/G80fork 26 (1 nM each) was incubated for 15 min at 30°C with or without CSB as indicated in the figure legends. G80 or G80bub21 was used as an unlabeled complementary oligonucleotide to the labeled C80 strand of the fork substrate in the strand exchange reaction. In kinetic experiments, 120 μl reactions were initiated, and 20 μl aliquots were removed at 0, 0.5, 1, 1.5, 2, 3, 4, 5, 10, 15 and/or 30 min. A control reaction mixture was set up in an identical manner in the absence of CSB. Reactions were terminated by the addition of 10 μl of stop buffer as described above. Reactions products were subsequently incubated with 0.1 mg/ml Proteinase K for 30 min at 37°C. Products were electrophoresed on 10% native polyacrylamide gels (PAGE), visualized using a PhosphorImager, and quantified using ImageQuant software (Molecular Dynamics).

RESULTS

CSB catalyzes strand annealing of complementary ssDNA

ssDNA annealing activity was assayed by incubating two partially complementary single strand oligonucleotides (1 nM each) with increasing concentrations of purified recombinant

CSB protein. The reaction was carried out for 15 min at 30°C and the reaction products were analyzed by non-denaturing 12% PAGE. The results demonstrate that a 22 bp forked duplex reaction product, which co-migrates with a forked duplex control, forms efficiently in the presence but not the absence of CSB and the amount of reaction product is dependent on CSB concentration (Figure 1A). The amount of reaction product reached a maximum at ~10 nM CSB and did not increase further at higher CSB concentration (Figure 1A and B).

Kinetic analysis demonstrated that CSB-catalyzed ssDNA annealing is rapid in the presence of 10 nM CSB, with 50% of the ssDNA substrate being converted into forked duplex reaction product in 1.5 min (Figure 1C and D) and 84% being converted to product in 15 min. In contrast, ~5% of the substrate was converted to product in the absence of CSB in 15 min (Figure 1C and D). In addition, product formation was linear for the first 120 s of the reaction in the presence of 10 nM CSB; during this period, the rate of the reaction was 64.2 pM/s, which is 25-fold faster than the rate in the absence of CSB.

To confirm that CSB was responsible for the observed ssDNA annealing activity, and that this activity was not catalyzed by a low level contaminant in the protein preparation, ssDNA annealing reactions were carried out in the presence of polyclonal rabbit anti-CSB antibody. If the reaction is catalyzed by CSB, this immunoinactivation experiment should show loss of activity in the presence of anti-CSB antibody. As predicted, when CSB was preincubated with increasing amounts of anti-CSB antibody against the N-terminal region of CSB (1–300 amino acids), the efficiency of the ssDNA annealing reaction decreased (Figure 2A, lanes 3–7 versus lane 1). However, anti-CSB antibody did not interfere with the spontaneous ssDNA annealing reaction (Figure 2A, compare lanes 1 and 8). This result was confirmed by immunodepleting an ssDNA annealing reaction with anti-CSB antibody crosslinked to protein G agarose beads. As with the immunoinhibition experiment, immunodepletion of CSB greatly reduced the efficiency of the CSB-catalyzed ssDNA annealing reaction (Figure 2B, lanes 4 versus 2). Western blot analysis confirmed that CSB was depleted by the anti-CSB antibody (Figure 2C, lane 3). Control reactions showed that pre-immune IgG did not inhibit CSB-catalyzed ssDNA annealing (Figure 2B, lane 3). These results provide evidence that CSB carries intrinsic ssDNA annealing activity. Next, to

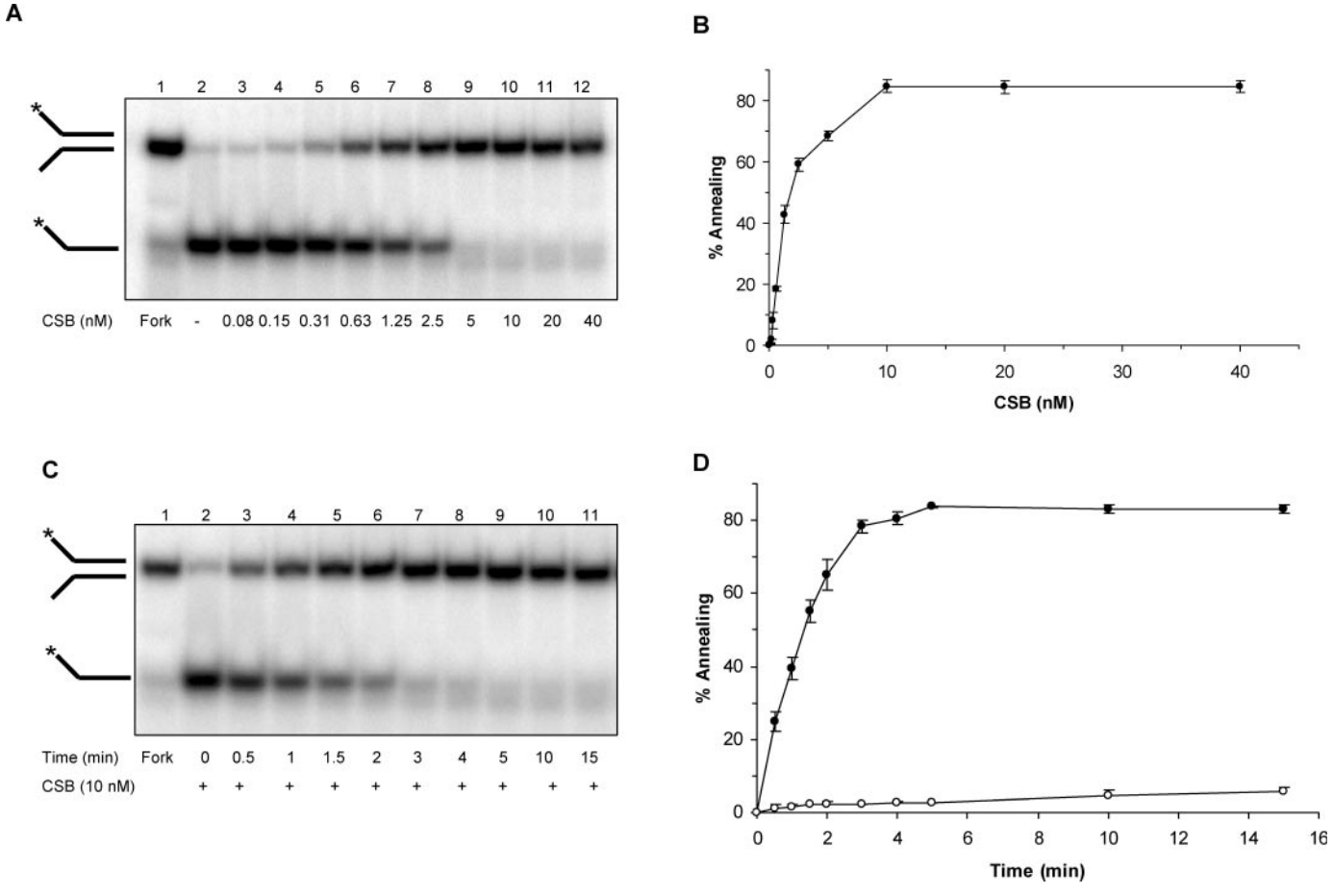

Figure 1. CSB catalyzes ssDNA annealing. (**A**) Two partially complementary oligonucleotides (1 nM each), one of which was radiolabeled at the 5′ end, were incubated with the indicated concentrations of CSB for 15 min at 30°C, and analyzed on 12% native PAGE gels. The 22 bp forked duplex co-migrates with the reaction product and was loaded as a marker. (**B**) Quantitative analysis of the data shown in (A). Values are the average of at least three independent experiments. SDs are indicated by error bars. (**C**) Kinetics of CSB mediated ssDNA annealing. Kinetics experiments were performed using the partially complementary oligonucleotides (1 nM each) and 10 nM CSB. The 22 bp forked duplex was loaded as a marker. (**D**) Quantitative analyses of data are shown in (C). Assays were carried out in the absence (open circles) or presence (closed circles) of 10 nM CSB.

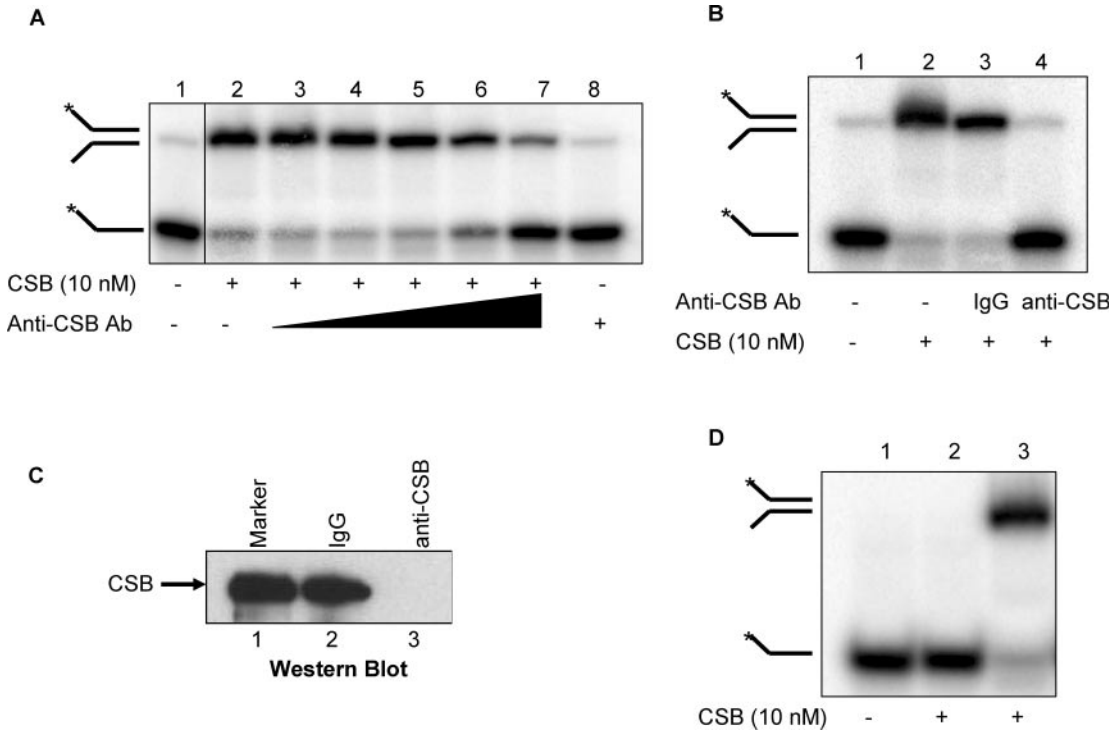

Figure 2. CSB immunodepletion inhibits CSB ssDNA annealing. (**A**) CSB was incubated with increasing amounts of CSB antibody (lanes 3–7) for 5 min at 4°C, and subsequently assayed for ssDNA annealing. Lane 2, CSB, no antibody; lane 8, CSB antibody, no CSB. (B and C) Rabbit polyclonal anti-CSB antibody or pre-immune rabbit IgG immobilized to protein G–agarose was used to immunodeplete CSB as described in Materials and Methods. Half of the supernatant from each immunodepleted sample was assayed for strand annealing (**B**) and the other half was used in a western blot with anti-CSB antibody (**C**) as described in Materials and Methods. (B) Strand annealing using samples immunodepleted with anti-CSB antibody (lane 4), pre-immune rabbit IgG (lane 3) or no antibody (lane 2). Lane 1, no enzyme control. (**D**) Strand annealing reaction was performed with non-homologous (lane 2) and homologous (lane 3) oligonucleotides. Lane 1, no protein.

test whether strand annealing activity of CSB depends on the homology of DNA substrates, non-complementary oligonucleotide was used. Since no product was seen when non-complementary oligonucleotide was used to form the annealing (Figure 2D, lane 2), the strand annealing activity by CSB depends on the homology of the DNA substrates. In Figure 2D, lane 1 shows the spontaneous annealing in the absence of CSB, and in Figure 2D, lane 3 shows the strand annealing by using complementary oligonucleotide.

The ssDNA annealing activity of CSB was also assayed using a 100 nt oligonucleotide homologous to circular M13mp18 ssDNA. In this reaction, the annealing target is very small (100 bp) and the M13mp18 DNA substrate includes a large excess (>7000 nt) of heterologous ssDNA. CSB was active in this ssDNA annealing reaction, although the efficiency was somewhat lower than for the assay shown in Figure 1 (compare Figures 1A and 3). In this reaction, the yield of reaction product was ~73% at 80 nM CSB (Figure 3).

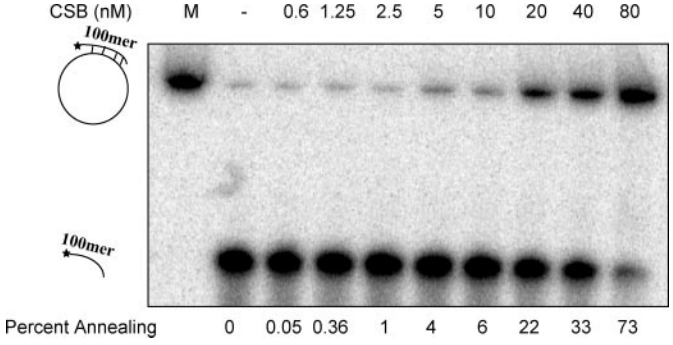

Figure 3. ssDNA annealing of a radiolabeled 100mer to M13mp18 ssDNA. (**A**) Radiolabeled 100mer (0.5 nM) and unlabeled M13mp18 ssDNA (1 nM) were incubated with the indicated concentrations of CSB for 15 min at 30°C. The reaction products were analyzed on native 10% polyacrylamide gels and detected by phosphorimage analysis. The 100 bp M13mp18 partial duplex was loaded as a marker (lane 1).

ATP binding to the CSB protein inhibits CSB-catalyzed ssDNA annealing

The DNA-dependent ATPase and ATP-dependent chromatin remodeling activities of CSB were described previously (9–12). Because ATP modulates these CSB functions, it seemed possible that ATP turnover might also modulate CSB-catalyzed ssDNA annealing. Therefore, the effect of ATP and ATPγS on the annealing activity of CSB was examined. The results show that in the absence of nucleotide, 78% of the ssDNA oligonucleotide substrates (1 nM each) were converted to forked duplex product by 5 nM CSB (Figure 4A, lane 2, and Figure 4B). However, in the presence of 3 or 4 mM ATP, the amount of reaction product was reduced by 10 or 30%, respectively (Figure 4A, lane10, Figure 4B and data not shown). ADP had no effect on CSB-catalyzed ssDNA annealing (data not shown). Previous studies indicate that double-stranded DNA stimulates the CSB ATPase more effectively than ssDNA, suggesting that poor catalytic

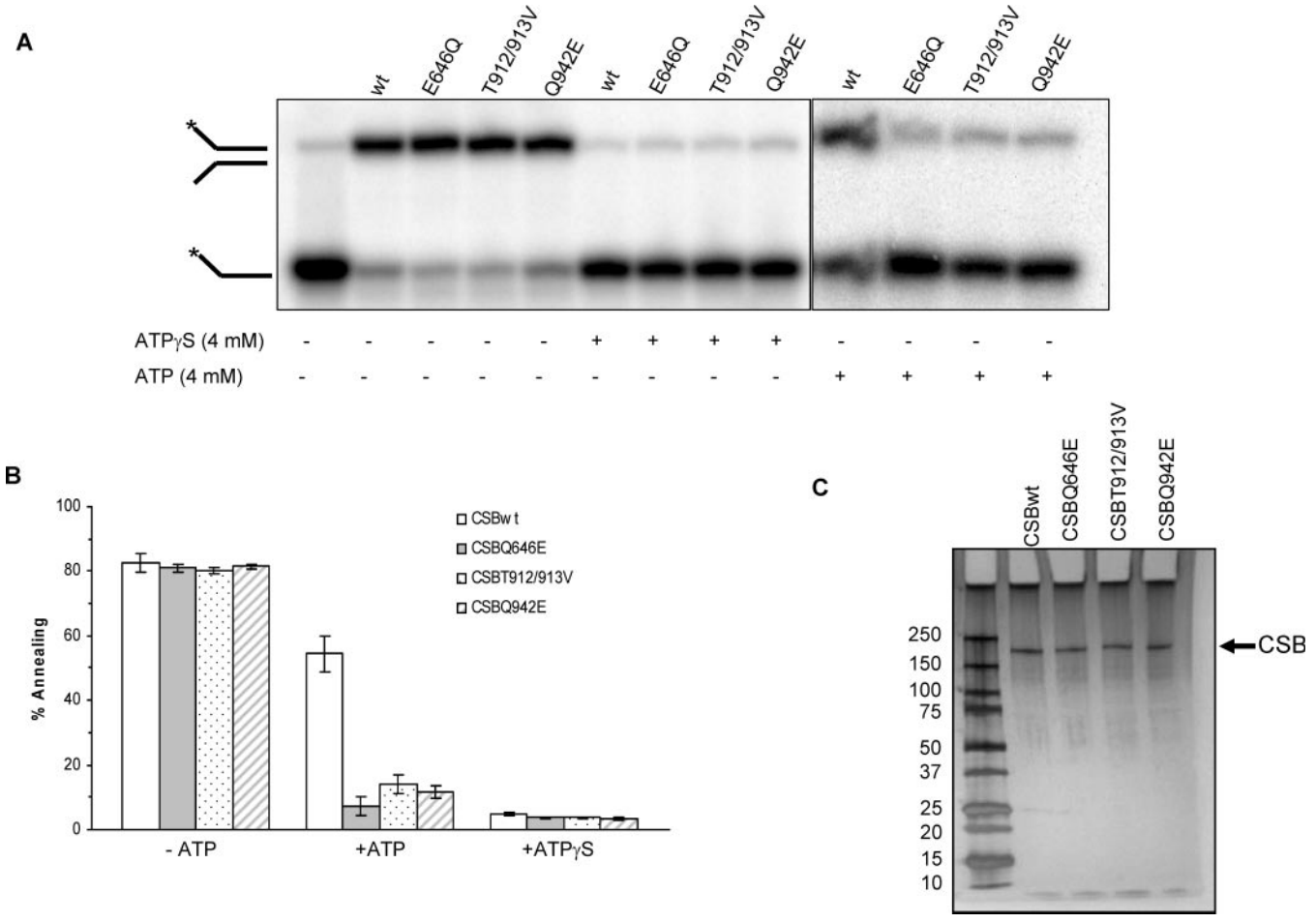

Figure 4. ATP binding inhibits CSB mediated ssDNA annealing. (**A**) Effect of ATP and ATPγS on ssDNA annealing, catalyzed by CSBwt, CSBE646Q, CSBT912/913V and CSBQ942E (5 nM each). ssDNA annealing was assayed as indicated in the legend to Figure 1. Nucleotide was included at 4 mM. (**B**) Quantification of the results in (A). Values are the average of at least three independent experiments. SDs are indicated by error bars. (**C**) A silver stain of the recombinant CSBwt, CSBE646Q, CSBT912/913V and CSBQ942E proteins (100 ng each) used in strand annealing assay after separation by SDS–PAGE.

efficiency of the CSB to hydrolyze ATP might account for the observed inhibition of CSB catalyzed strand annealing activity in the presence of ATP. Therefore, the reaction was carried out in the presence of the poorly hydrolyzable ATP analog, ATPγS. The results showed that 4 mM ATPγS dramatically inhibits the annealing activity of CSB, (Figure 4A, lane 6), reducing the rate of the reaction nearly to the background level of spontaneous strand annealing (~5%). These results suggest that the observed inhibitory effect of nucleotide on CSB strand annealing is a consequence of ATP binding rather than ATP hydrolysis.

To gain further insight into the effect of ATP and ATPγS on the mechanism of CSB mediated strand annealing, we next examine the effect of mutant CSB recombinant proteins. The following previously constructed CSB mutants were used: (i) motif II, CSBE646Q, (ii) motif V, CSBT912/913V and (iii) motif VI, CSBQ942E. Stably transfected human cell lines carrying CSB domain knockout mutants indicated that different functional motifs of CSB play distinct roles in different DNA repair pathways (8,20–22). CSBE646Q completely lacks ATPase, and similarly CSBT912/T913V and CSBQ942E are almost completely defective in ATPase activity (17). As shown in Figure 4A and B, CSBE646Q, CSBT912/T913V and CSBQ942E mutants retain the ability to catalyze ssDNA annealing in the absence of nucleotide. In addition, ATPγS inhibits ssDNA annealing by the mutants and wt CSB with equal efficiency (Figure 4A, lanes 6–9). However, ATP almost completely inhibits ssDNA annealing by the CSB mutants (Figure 4A, lanes 10 versus lanes 11–13, and Figure 4B). Previous studies demonstrated the DNA and ATP binding properties of these mutants are similar to CSBwt (17). These data suggest that CSB is incapable of catalyzing ssDNA annealing when it is bound to ATP. Figure 4C shows the purity of the purified recombinant CSBwt and CSB mutant proteins used in this study.

RPA inhibits CSB-catalyzed ssDNA annealing

RPA is a ssDNA binding protein that stimulates ssDNA annealing by Rad51 and plays several critical roles in eukaryotic DNA repair (23) and DNA replication. Thus, the effect of RPA on CSB-catalyzed ssDNA annealing was tested. As shown in Figure 5A and B, CSB-catalyzed ssDNA annealing was inhibited by RPA in a concentration dependent manner. In the presence of 10 nM CSB, 6 nM RPA reduced the amount of reaction product from 84% (Figure 5A, lane 2) to 40%

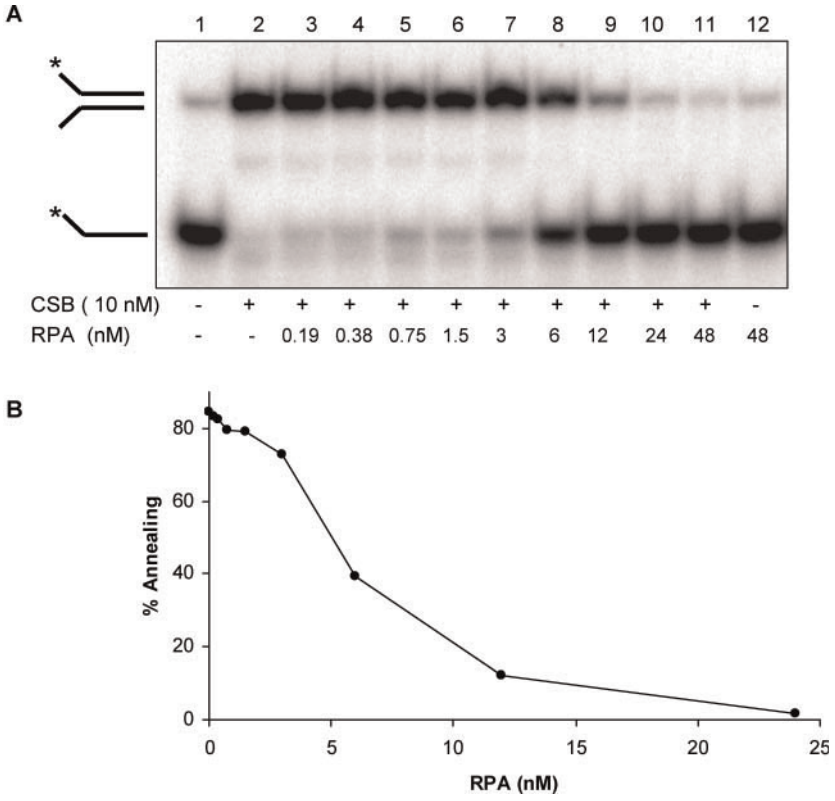

Figure 5. RPA inhibits CSB-catalyzed ssDNA annealing. (**A**) ssDNA annealing was assayed in the presence of 10 nM CSB with or without RPA. Labeled and unlabeled 37mer complementary oligonucleotides (1 nM each) were incubated with the indicated concentration of RPA in annealing reaction buffer for 2 min at room temperature. The reactions were initiated by addition of 10 nM CSB and incubated at 30°C for 15 min. Products were analyzed on 12% non-denaturing PAGE and visualized using a PhosphoImager. (**B**) Quantification of the results in (A).

(Figure 5A, lane 8). Under these conditions, there are approximately three RPA molecules per DNA binding site (given that the RPA binding footprint is ~37 nt). When the RPA concentration was increased to 12 nM, CSB-catalyzed ssDNA annealing was completely inhibited (Figure 5A, lanes 9–11). Similarly, *E.coli* ssDNA binding protein, which binds ssDNA with high affinity, inhibits CSB–catalyzed ssDNA annealing at a similar concentration (data not shown). These data suggest that RPA and *E.coli* SSB most likely inhibit CSB-catalyzed ssDNA annealing by sequestering the DNA substrate.

Phosphorylation of CSB inhibits CSB-catalyzed ssDNA annealing

CSB is phosphorylated *in vivo* and UV irradiation induces dephosphorylation of CSB (17). Recombinant human CSB protein purified from insect cells is phosphorylated at threonine residues but not phosphorylated at serine or tyrosine residues (17). In addition, CKII increases the phosphorylation of CSB at threonine and induces phosphorylation at tyrosine and serine. The effect of phosphorylation on CSB-catalyzed ssDNA annealing activity was examined by using CKII or PP1 to phosphorylate or dephosphorylate CSB *in vitro*, respectively. As shown in Figure 6A (lower panel, lane 1 versus lane 2), PP1-treatment decreases CSB phosphorylation and increases the ssDNA annealing activity of CSB (Figure 6B, lanes 2 and 3 versus lanes 7 and 8, and Figure 6D, closed triangles versus closed circles). In contrast, phosphorylation of CSB by CKII (Figure 6A, middle panel lane 3 versus lower panel lane 3) almost completely inhibits (74% decrease) CSB-catalyzed ssDNA annealing (Figure 6C, lane 6 versus lane 11, and Figure 6D, closed circles versus open circles). Mock phosphorylation or dephosphorylation assays, in which CSB was incubated in reaction buffer lacking CKII or PP1, respectively, did not change the ability of CSB to catalyze ssDNA annealing (Figure 6B and C, lanes 2–6). These results suggest that the phosphorylation state of CSB plays a role in regulating the ssDNA annealing activity of CSB.

CSB catalyzes strand exchange

In the strand exchange experiment, C80/G80fork 26 substrate and fully complementary oligomer G80 or partially complementary G80bub21 to the labeled C80 strand of the fork substrate was used (19). If strand exchange took place using G80 as a third strand, an 80 bp blunt-ended duplex would be formed (Figure 7A). Strand exchange activity was assayed by incubating the C80/G80fork 26 substrate and G80 oligonucleotide with increasing concentrations of CSB in the absence of ATP (Figure 7A). The complete conversion of the fork into the duplex product reached a maximum at ~5 nM CSB (Figure 7A and B). This reaction was repeated in the presence of 1 mM ATP and ATP did not have any effect on CSB mediated strand exchange (data not shown). CSB catalyzed almost complete conversion of the fork into the blunt-end duplex within 5 min (Figures 7C and D). These results demonstrated CSB catalyzes strand exchange in the absence of ATP.

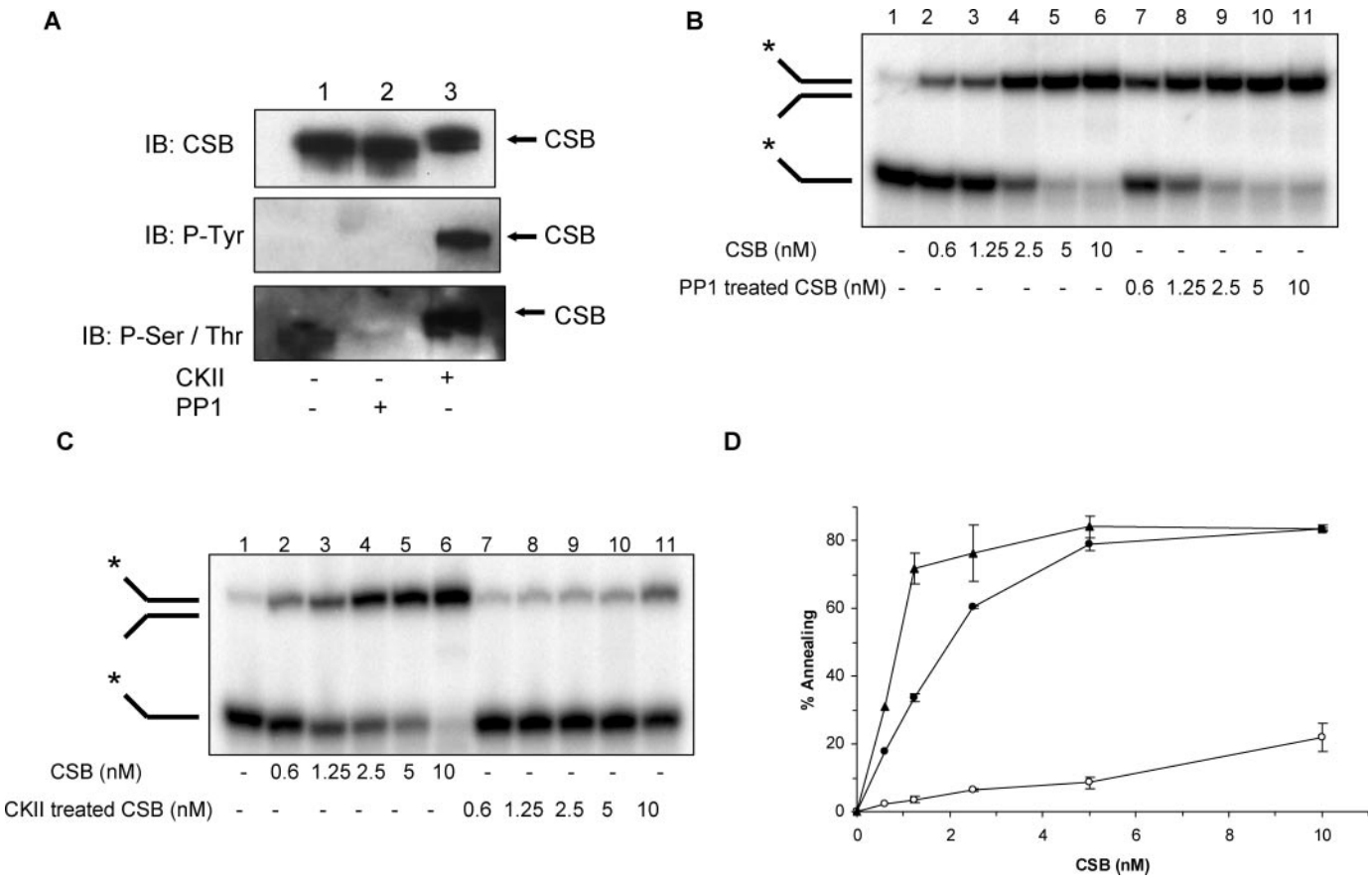

Figure 6. Phosphorylation of CSB inhibits CSB-catalyzed ssDNA annealing. (**A**) Purified recombinant CSB wt (1.25 μg) was phosphorylated with 250 U CKII or dephosphorylated with 625 mU PP1 as described in Materials and Methods. Proteins were analyzed by SDS–PAGE and immunoblotted with anti-CSB antibody (upper panel), P-tyr antibody (middle panel) or P-ser/thr antibody (lower panel). The same membrane was probed, stripped and reprobed with all three antibodies. (**B**) ssDNA annealing activity was assayed as described in Figure 1. (**C**) ssDNA annealing reactions were carried out with increasing amounts of CKII phosphorylated CSB. (**D**) Values are the average of at least three independent experiments. SDs are indicated by error bars. Dephosphorylation of CSB by PP1, closed triangles; CSB, closed circles; Phosphorylated CSB by CKII, open circles.

Using partially complementary G80bub21 as the third strand, we expected to see both annealing and exchange products. The annealing product would be the product of three-way junction structure, and the exchange product would be the blunt-ended duplex containing a 21 nt bubble. The oligomer, G80bub21, is complementary to both ends of C80, but contains a central 21 nt non-complementary stretch. The fork substrate and partially complementary G80bub21 was incubated with 5 nM CSB in a time course. As shown in Figure 8A and B, CSB mediated the pairing of the 5′ end of G80bub21 with the 3′-tail of C80 in the fork substrate to form a three-way junction structure. CSB converted 50% of the fork substrate into the three-way junction structure after 4 min (Figure 8A, lane 8, and Figure 8B). However, CSB did not catalyze the formation of the blunt-ended duplex containing a 21 nt bubble as a product of the exchange reaction under the same conditions (Figure 8A). As expected, addition of 1 mM ATP did not affect the reaction (data not shown).

DISCUSSION

This study demonstrates that CSB catalyzes annealing of complementary ssDNA *in vitro* with high efficiency and that this activity is regulated by phosphorylation/dephosphorylation of CSB. This reaction is robust, since the presence of 10 nM CSB increases the rate of the reaction 25-fold over the background rate of ssDNA annealing. Furthermore, the results strongly suggest that ssDNA annealing is an intrinsic activity of CSB since anti-CSB antibody inhibits the reaction but control antibody does not, and the strand annealing activity by CSB depends on the homology of DNA substrates. In addition ATP binding differentially inhibits ssDNA annealing by mutant and wt CSB, and the activity is altered by changes in the phosphorylation state of CSB. Together, these results strongly indicate that the CSB promotes ssDNA annealing *in vitro*.

CSB is the only member of the SWI2/SNF2 family that catalyzes ssDNA annealing efficiently. However, RAD54, which is also a member of this family, forms a complex with RAD51, and the RAD51/RAD54 heterodimer also catalyzes homologous DNA pairing with high efficiency (24). Some members of the RecQ family of helicases, which carry seven conserved helicase motifs have also been reported to catalyze ssDNA annealing (18,19,25). It is interesting to note that RPA stimulates RAD51-dependent ssDNA annealing in *Saccharomyces cerevisiae* (23), but it inhibits CSB-catalyzed ssDNA annealing (Figure 5).

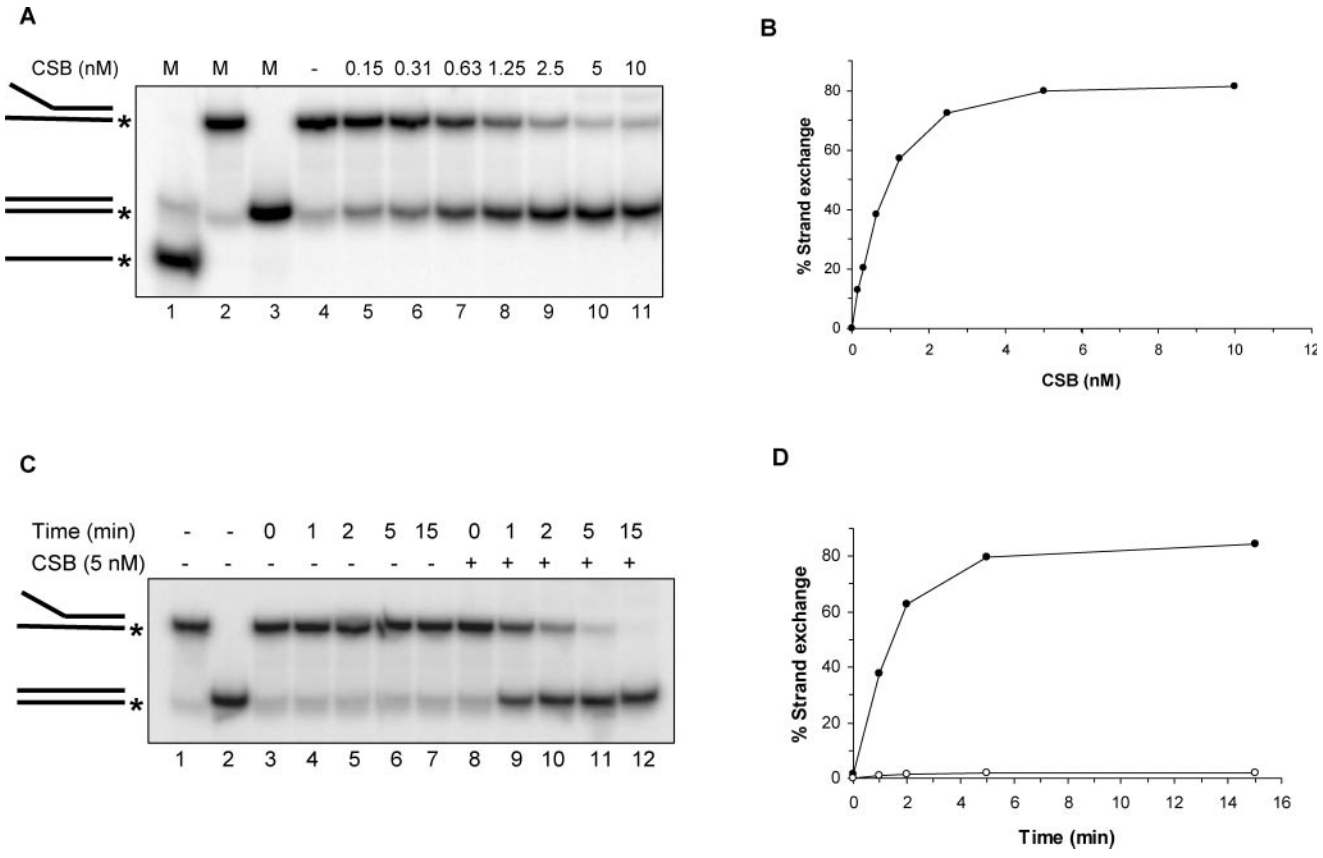

Figure 7. CSB catalyzes the strand exchange reaction with fully complementary oligomer G80. (**A**) The labeled C80/G80fork 26 substrate with or without G80 oligonucleotide was incubated with the indicated concentrations of CSB for 15 min at 30°C, and analyzed on 10% native PAGE gels. Heat denatured G80/C80 (lane 1), C80/G80fork 26 (lane 2) and G80/C80 blunt-end duplex (lane 3) were used as a marker. (**B**) Quantitative analysis of data shown in (A). (**C**) Kinetics of CSB mediated strand exchange. Kinetics experiments were performed using the fully complementary G80 and with or without 5 nM CSB. C80/G80fork 26 (lane 1) and G80/C80 blunt-end duplex (lane 2) were used as markers. (**D**) Quantitative analysis of data shown in (C). Assays were carried out in the absence (open circles) or presence (closed circles) of 5 nM CSB.

The chromatin remodeling activity of CSB requires ATP hydrolysis, but the ssDNA annealing activity of CSB does not. In fact, ATP binding inhibits ssDNA annealing by CSB. Similar observations were reported for RecQ1 and RecQ5β helicases (18,25). One possible explanation for this result is that ATP induces a conformational change in CSB that inhibits the ssDNA annealing reaction. It was reported that ATP binding but not ATP hydrolysis is required in order for CSB to introduce constrained negative supercoiling into chromatin (13). Thus, these two functions of CSB have opposite requirements for and interactions with ATP.

The present study has revealed another important activity of the CSB protein. It has the ability to promote strand exchange. It was shown recently that the WRN or BLM catalyze strand pairing and exchange reactions (19). However, an important distinction between these enzymes and CSB is that the unwinding activity is a basis for their strand exchange activity whereas CSB has strand exchange function in the absence of ATP, and cannot unwind duplex DNA. WRN or BLM can catalyze a strand exchange reaction using both partially complementary G80bub21 and fully complementary G80 as the third strand to a fork substrate by combining their strand pairing and unwinding activities. In contrast, CSB has no strand exchange function with partially complementary G80bub21 but showed its exchange activity using fully complementary G80 as the third strand in the absence of ATP.

The biological significance of the strand annealing and exchange activities is not known, although these two activities have been suggested to be involved in recombination, replication or transcription. It is known that CSB has a role in TCR and transcription after UV damage. It was demonstrated that TCR of UV damage involves homologous recombination (HR) in *S.cerevisiae* (26), in which HR proteins could bind to the transcription arrest dependent recombinogenic structure and initiate the recombination process by searching for DNA homology. However, early studies showed that cells from CS patients were hypersensitive to UV-induced sister chromatid exchange (27,28), suggesting that CSB plays a role in suppressing recombination events. Recently, Richards *et al*. (29) showed that CSB has a role in crosslink repair at G_1 phase in Chinese hamster ovary cells. DNA crosslink can be repaired by NER/TCR and HR. It is likely that CSB plays a role in crosslink repair at multiple stages: at G1 for incision and at S phase for recombination. It is also possible that CSB links TCR and HR in response to DNA damage.

Most DNA helicases function as multimers such as dimers or hexamers (30,31) suggesting that subunit multimerization may facilitate the DNA helicase and annealing reactions. CSB and several other SWI2/SNF2 family proteins possess the

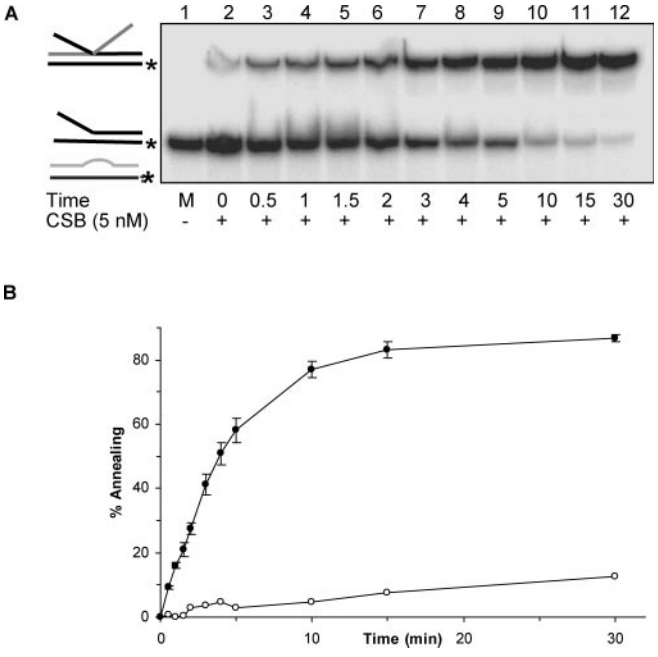

Figure 8. CSB does not catalyze the strand exchange through the three-way junction. (**A** and **B**) A partially complementary G80bub21 with or without 5 nM CSB was added to the radiolabeled C80/G80fork 26 substrate and incubated for the indicated times at 30°C. (**B**) Quantitative analyses of data shown in (A). Values are the average of at least three independent experiments. SDs are indicated by error bars. Assays were carried out in the absence (open circles) or presence (closed circles) of 5 nM CSB.

seven conserved helicase motifs associated with helicase activity, but are not catalytically active as DNA helicases *in vitro* (32). For these proteins, it is possible that the helicase-associated motifs play a role in another function that contributes to genomic stability. In fact, CSB has both DNA-dependent ATPase activity and ATP-dependent chromatin remodeling activity (9–12); the latter activity requires ATP hydrolysis and may require dimerization of CSB (13). It seems possible that the ability of CSB to facilitate annealing of complementary ssDNA plays a role in this process. Alternatively, CSB-catalyzed strand annealing may be important to prevent overly extensive shortening of the RNA transcript. This speculation is suggested by recent studies indicating that certain covalent DNA lesions in the transcribed DNA strand pose a block to RNA polymerase progression, and the nascent RNA strand can be shortened (33). Based on a recent study (34), it was suggested that CSB is stably associated with the elongating RNAPII stalled in front of the DNA damage and CSB may serve to stabilize the lesion-stalled RNAPII complex by means of limiting backtracking and shortening of the RNA transcript. Therefore, we propose that CSB could limit this process by stabilizing the RNAPII ternary complex as well as by promoting ssDNA annealing in the vicinity of the stalled transcription machinery. Further studies of the ssDNA annealing activity of CSB may provide insight into its role in this process or other steps in TCR.

An interesting observation of this study is that the phosphorylation state of CSB may regulate its ssDNA annealing activity. This result is especially intriguing, because the phosphorylation state of CSB also regulates its function in TCR (17). The results presented here show that dephosphorylation of CSB stimulates ssDNA annealing by CSB *in vitro*, and previous studies show that CSB is dephosphorylated *in vivo* in response to UV-induced DNA damage (17). Thus, the ssDNA annealing activity of CSB may be activated in cells with UV-induced DNA damage and thus it may play a critical role in enhancing genome stability.

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