Human Transcription-Repair Coupling Factor CSB/ERCC6 Is a DNA-stimulated ATPase but Is Not a Helicase and Does Not Disrupt the Ternary Transcription Complex of Stalled RNA Polymerase II*

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Transcription is coupled to repair in Escherichia coli and in humans. Proteins encoded by the mfd gene in E. coli and by the ERCC6/CSB gene in humans, both of which possess the so-called helicase motifs, are required for the coupling reaction. It has been shown that the Mfd protein is an ATPase but not a helicase and accomplishes coupling, in part, by disrupting the ternary complex of E. coli RNA polymerase stalled at the site of DNA damage. In this study we overproduced the human CSB protein using the baculovirus vector and purified and characterized the recombinant protein. CSB has an ATPase activity that is stimulated strongly by DNA; however, it neither acts as a helicase nor does it dissociate stalled RNA polymerase II, suggesting a coupling mechanism in humans different from that in prokaryotes. CSB is a DNA-binding protein, and it also binds to XPA, TFIIH, and the p34 subunit of TFIIE. These interactions are likely to play a role in recruiting repair proteins to ternary complexes formed at damage sites.

Transcribed DNA is repaired preferentially in both mammalian cells (1, 2) and E. coli (3). The preferential repair, to a large extent, is due to the high rate of repair of the template (transcribed) strand relative to the coding (nontranscribed) strand and transcriptionally inactive DNA (2). Mutations in the mfd gene in E. coli (4) and in the CSA1 and CSB genes in humans (5) abolish preferential repair. The mechanism of action of the Mfd protein and of transcription-repair coupling in E. coli has been elucidated in considerable detail (6–8). However, attempts at understanding transcription-coupled repair in humans have been frustrated by the lack of an efficient system for transcription by RNA polymerase II in vitro and by the lack of purified CSA and CSB proteins, which are known to be essential for coupling9, 10.

The CSA (11) and CSB (12) genes have been cloned and sequenced. CSA is a protein of 44 kDa and belongs in the “WD-repeat” family of proteins (11, 13). The CSB protein has a predicted size of 168 kDa, contains helicase motifs, and belongs in the SWI/SNF family of proteins (12, 14, 15). Because of size, sequence, and apparent functional similarities between CSB and Mfd, it has been generally assumed that CSB may play a role similar to that of Mfd (4, 16, 17). Hence the purification and characterization of CSB were considered essential for understanding the phenomenon of transcription-coupled repair. In this paper we describe the purification and characterization of recombinant human CSB protein. ATPase activity was detected with CSB protein, and this activity was characterized with regard to allosteric effectors. Although our CSB was active as indicated by its DNA binding and ATPase activities, it did not remove a stalled RNA Pol II. While this work was in progress Guzder et al. (18) reported that the yeast CSB homolog Rad26 protein is a DNA-dependent ATPase with no detectable helicase activity.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—Plasmids for expression of TFIIE (19–21) and TFIIH (22, 23) have been described previously. The ERCC6 gene cloned into the vector Bluescript and designated pEBlE6 (12) was provided by Dr. J. H. J. Hoeijmakers (Erasmus University). A HpaI site was placed immediately upstream of the ATG initiation codon (5′-AGAAGT to 5′-AGAGTTAACATG) by site-directed mutagenesis (24) to facilitate subcloning; this construct was designated pE6Hpa. To generate a template for in vitro transcription-translation, the CSB gene was subcloned as a HpaI-XbaI fragment from pE6Hpa into the Smal-XbaI site of the Smal-XbaI site of pMal-c2 (New England Biolabs) and was designated pMALcE6. A construct for producing a fusion of GST protein with amino acids 528-1222 of CSB was made by subcloning the internal EcoRI fragment of the CSB gene into the EcoRI site of pGEX3x (Pharmacia Biotech Inc.) and was designated pGSTE65. The same fragment of CSB was subcloned into the EcoRI site of pMAL-c2 to obtain pMALcE6.

The CSA gene was subcloned from a human cDNA library by PCR using primers 5′-AGAATTCGCCGGGATGCTGGGGTTTTTCCGCGACGCAAAACCGGT TTT and 5′-GTGAGATCCTAGATATTATCCTTTCTATCATGCTGTCCACCGCAT. These primers were made based upon the published sequence of the CSA gene (11) and place EcoRI and Smal sites at the 5′ border of the coding region and XbaI sites at the 3′ border of CSA. The PCR product was subcloned into the pCR III vector (Invitrogen) to give pCRCSA. The Smal-SpeI fragment of pCRCSA containing the entire CSA gene was subcloned into the XbaI-XbaI site of pMal-c2 to give pMALCSA.

The p2Bac vector (Invitrogen) was used to generate constructs for expression of full-length CS proteins in SF21 cells. The Smal-HindIII fragment of pCRCSA2 was inserted into the PvuII-HindIII site of p2Bac to give pBacCSA, in which the CSA gene is downstream from the 3′-end.
polyhedrin promoter. To construct a plasmid expressing both CSA and CSB genes, the HpaI-XbaI fragment of pE6Hpa was inserted into the Smal-XbaI site of pBacCSA to obtain pBacCSB, in which the CSB gene is downstream from the p10 promoter. The Baculo Gold transfection kit (Pharmingen) was used to harvest recombinant viral stocks expressed in the S. gregaria system.

**Antibody Preparation**—GST and MBP fusion proteins of CSA and CSB were made in *E. coli* DR153 (recA3 ΔdutR) and purified with glutathione-Sepharose or amyllose-agarose affinity resins (7). The CSB-MBP protein was further purified using columns of DEAE-Sepharose, Affi-Gel blue, and SP-Sepharose. To make anti-CSB antibodies, the GST-CSB(528–1222) protein was first purified by glutathione-Sepharose affinity chromatography and then by SDS-polyacrylamide gel electrophoresis. The band containing the fusion protein was excised from the gel, and the gel slice was ground into small pieces and injected into a rabbit. To make an antibody affinity column, MBP-CSB(528–1222) was covalently linked to SulfoLink coupling gel using the Pierce anti-body immobilization kit. The immunogen was passed through the column, and the anti-CSB antibodies were eluted from the column with low pH into tubes containing 1 M Tris-HCl, pH 8.0, and then dialyzed against and stored in Manley buffer (25 mM HEPES pH 7.9, 12 mM MgCl₂, 0.5 mM EDTA, 2 mM dithiothreitol, and 17% glycerol) with 100 mM KCl.

During the purification of the MBP-CSA protein, the first half of the fusion protein eluted from the amylose resin as precipitate. This insoluble material was injected into a rabbit. An affinity resin for purifying anti-CSA antibodies from the immune serum was made by linking MBP-CSA to Affi-Gel-10 (Bio-Rad) as described by the manufacturer.

**Purification of CSB**—Full-length CSB protein was purified from SF21 cells using both Coomassie Blue staining and immunoblotting to locate the fractions containing the protein. CSA was detected by immunoblotting with anti-CSA antibodies. Approximately 2 liters of SF21 cells at approximately 10⁹ cells/ml were infected with our recombinant baculovirus, which expresses CSA and CSB, and incubated at 26 °C for 42–48 h. The cells were collected by centrifugation, and CFE was prepared by the procedure of Manley et al. (25). Most of the CSA and only a small fraction of CSB were soluble. The CFE was passed through a DE52 column equilibrated with Manley buffer plus 100 mM KCl. The flow-through that contained both proteins was applied to a SP-Sepharose column equilibrated with Manley buffer plus 100 mM KCl. CSA was in the flow-through of this column, but CSB was bound quantitatively. The column was washed with 0.2 M KCl, and then a linear gradient of 0.2–1.0 M KCl in Manley buffer was applied. CSB eluted at about 0.4 M KCl. Fractions containing CSB were pooled and dialyzed to a final concentration of 0.1 M KCl in Manley buffer plus 0.01% Nonidet P-40. The sample was applied to a single-stranded DNA cellulose column equilibrated in the same buffer. The bound proteins were eluted with a linear gradient of 0.1–1.0 M KCl in Manley buffer plus 0.01% Nonidet P-40. CSB protein eluted at about 0.25 M KCl. Fractions containing CSB were pooled and dialyzed against Buffer C (20 mM Tris-HCl, pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, and 10% glycerol) to remove salt. Then, ammonium sulfate was added to 1.2 M, and the precipitate was removed by centrifugation. The supernatant was applied to a phenyl Superose 5/5 fast protein liquid chromatography column. The column was washed with loading buffer, and bound proteins were eluted with a linear gradient of 1.2 to 0.0 M ammonium sulfate in Buffer C. The CFE eluted at about 0.1 M ammonium sulfate. The combined fractions were dialyzed against Buffer C with 0.1 M KCl plus 0.01% Nonidet P-40 and then applied to a Mono Q 5/5 fast protein liquid chromatography column. The column was washed with loading buffer, and bound proteins were eluted with a linear gradient of 1.2 to 0.0 M ammonium sulfate in Buffer C. The CFE eluted at about 0.35 M KCl. Fractions were pooled, diluted to 0.12 M KCl in Buffer C plus 0.01% Nonidet P-40, and then applied to a Mono S 5/5 fast protein liquid chromatography column. The column was washed, and then a gradient of 0.1–1.0 M KCl in Buffer C plus 0.01% Nonidet P-40 was applied. CFE eluted at about 0.35 M KCl. Fractions were pooled, to 12 M KCl in Buffer C plus 0.01% Nonidet P-40, and then applied to a Mono S 5/5 column. A linear gradient of 0.1–0.8 M KCl in Buffer C plus 0.01% Nonidet P-40 was applied, and CFE eluted at about 0.45 M KCl. The pooled fractions were diluted to 0.1 M KCl in Buffer C plus 0.01% Nonidet P-40 and applied to a 0.4 M-column of Q-Sepharose. The protein bound quantitatively and was eluted with 0.55 M KCl in Buffer C plus 0.01% Nonidet P-40. Extract preparation and all chromatographic steps except the last were performed in the presence of 1 μg/ml aprotinin and 1 μg/ml pepstatin A (Boehringer Mannheim).

**Transcription Proteins**—RNA polymerase II was purified using DE52, Mono Q, hydroxyapatite, and S300 columns. yTBP and recombinant TFIIIB were gifts from Dr. D. Reinberg (Robert Wood Johnson Medical School, Piscataway, NJ). TFIE, in which the p56 subunit was fused to MBP, was purified by amylose affinity chromatography. Recombinant TFIIH subunits were purified from *E. coli* and renatured as described (22, 23). TFIIH was purified from HeLa CFE using DE52, Affi-Gel blue, SP-Sepharose, S300, and Mono S columns. The *E. coli* Mid protein was purified as described previously (6); *E. coli* DNA helicase II was the gift of Dr. S. Matson (University of North Carolina).

**Methods**—*In vitro* transcription-translation was performed with the TNT coupled reticulocyte lysate system from Promega.

ATPase, helicase, and DNA-binding assays were conducted in reaction buffer that contained 40 mM HEPES, pH 7.9, 60 mM KC1, 8 mM MgCl₂, 2.4 mM diethiothreitol, 1.1 mM EDTA, and 6.8% glycerol. ATPase assays were performed as described (26) except that the reaction conditions were not restricted to hydrolysis of 15% of the substrate. For antibody inhibition experiments, antibodies and the ATPases were mixed in the reaction buffer and incubated on ice for 1 h before mixing with ATP substrate. For gel shift analysis, DNA and protein were incubated in 10 μl of reaction buffer at 30 °C for 30 min and then separated on a 5% native polyacrylamide gel run at room temperature at 60 V in 45 mM Tris borate and 0.5 mM EDTA, pH 8.3. Helicase assay was conducted as described by Matson et al. (27), and the protein pull-down assay was as described previously (7).

To examine ternary complex stability, we used template pMLU112, which contains a “U-less cassette” as the first 112 bp of the transcription unit (28). The plasmid was transfected with a reconstituted human RNA Pol II transcription system in the absence of UTP, producing a stable, stalled RNA Pol II at the end of the cassette. The RNA was labeled by a 50-mer restriction of the cassette. Approximately 100 μl of labeled RNA was added, and the mixture was incubated for 20 min at 32 °C to cleave the DNA downstream from the stalled polymerase. Then, CSB proteins were added to the stalled polymerase complex in 31 mM HEPES, 6 mM Tris-HCl, pH 7.9, 120 mM KCl, 3.8 mM MgCl₂, 2.1 mM EDTA, 3% polyethylene glycol, 6.8% glycerol, and 208 μM each of ATP and GTP, and 2 μM CTP. After 45 min at 32 °C the transcript was chased by adding 400 μM UTP and 800 μM of cold CTP and incubating for 12 min. RNA products were resolved on a 5% denaturing polyacrylamide gel.

**RESULTS**

**Preparation of Anti-CSB and Anti-CSA Antibodies**—To assist in characterizing the CSB protein we prepared antibodies against the putative catalytic region of the protein. This was done by using as antigen a peptide containing the helicase motif region of CSB, amino acids 528–1222, fused to GST. Immune serum was purified using an affinity column ligand consisting of MBP fused to amino acids 528–1222 of CSB. Our antibody was capable of detecting native CSB among the proteins in CFE by Western analysis, although there was considerable cross-reactivity (see below). The band identified as CSB was absent when CS-B CFE was examined and when normal human CFE was examined using preimmune serum. CFE made by *in vitro* transcription-translation and by expression in SF21 cells was also detected with our antibody. Most importantly, the CSB protein expressed in SF21 cells co-migrated with native protein. In contrast, our anti-CSA antibody was of poor quality in that Western analysis of CFE did not permit identification of CSA protein among overexpressing cross-reactivity. However, the anti-CSA antibodies did allow detection of CSA overproduction in bacteria and SF21 cells by immunoblotting (data not shown).

**Purification of CSB**—Several approaches were taken to generate purified CSB protein for our investigations. The full-length protein was not appreciably expressed in *E. coli* even when fused to GST or MBP. Therefore we attempted to purify CSB from HeLa CFE. However, after three or four chromatographic steps, the protein consistently underwent extensive degradation. Next, we attempted to overproduce the protein using the baculovirus/insect cell system. CSB protein was greatly overproduced 24–48 h after infection of SF21 cells with our recombinant baculovirus. We were able to obtain about 30 mg of CSB of high purity from 2 × 10⁹ cells after a column purification procedure. As seen in Fig. 1 the final step contained a single band that was reactive with anti-CSB antibodies. Even though the expression vector contained both CSA and CSB genes, the majority of CSA protein was insoluble, and the soluble fraction separated from the CSB protein completely as described (22, 23).
in the second chromatographic step, suggesting that CSA and CSB do not make a stable complex (data not shown). van Gool (10) also found that CSA and CSB proteins readily separate upon fractionation of cell extract, although it has been reported that CSA and CSB can interact as revealed by immunoprecipitation assays (11).

### Functional Analysis of CSB

The prototype transcription-repair coupling factor (TRCF) encoded by the *E. coli* mfd gene possesses the so-called helicase motifs and is a DNA-independent ATPase and a DNA-binding protein with no detectable helicase activity (6, 8). Because the putative human coupling factor also possesses the helicase motifs, we decided to test the purified CSB for ATPase, DNA-binding, and helicase activities.

CSB exhibited ATPase activity with a $k_{cat}$ of 3.4 min$^{-1}$, which increased to 45–53 min$^{-1}$ in the presence of single- or double-stranded DNA (Table I). The ATPase activity is intrinsic to CSB and is not due to a contaminating ATPase as evidenced by the fact that anti-CSB antibodies specifically inhibited the ATPase activity of CSB without affecting ATP hydrolysis by Mfd or UvrD (helicase II), both of which also have helicase motifs (Fig. 2). CSA-MBP did not exhibit ATPase activity and did not affect the ATPase activity of CSB.

DNA binding was examined by gel shift assay using a 90-bp DNA fragment. The results in Fig. 3 show that unlike Mfd protein, CSB readily bound to DNA in the absence of nucleotide. At low concentrations of CSB, a single retarded band was detected, indicated as *B1* in Fig. 3. At higher CSB concentrations, there were apparently multiple CSB proteins bound per duplex as indicated by the appearance of discreet lower mobility bands, labeled *B2*–*B4*. The presence of ATP$\gamma$S increased the amount of DNA bound by CSB. ATP and, to a lesser extent, ATP$\gamma$S decreased the number of CSB proteins/duplex. An alternative explanation for *B2*–*B4* is the formation of intermolecular aggregates of *B1*.

### Interactions of CSB with Transcription and Repair Proteins

Helicase activity was tested using as substrate a labeled 24-mer annealed to a circular, single-stranded DNA molecule. Even though CSB binds to DNA and exhibits DNA-stimulated ATPase activity, Fig. 4 shows that CSB cannot displace the 24-mer, whereas helicase II releases it quantitatively. CSA-MBP did not exhibit helicase activity and did not confer helicase activity to CSB. Thus we conclude that CSB, like its *E. coli* and *Saccharomyces cerevisiae* counterparts Mfd (6) and Rad 26 (18), respectively, is a protein with helicase motifs but no helicase activity.

### Table I

| Addition | $k_{cat}$ (min$^{-1}$) |
|----------|-----------------------|
| None     | 3.4                   |
| ds DNA   | 45                    |
| ss DNA   | 53                    |

*a* ds, double-stranded; ss, single-stranded.
with the XPG protein (29) and that CSA interacts with the p44 subunit of TFIIH (11), suggesting that a related mechanism may be operative in humans. We wished to know if CSB exhibited binding and functional properties similar to those of Mfd, in particular binding to the damage recognition protein and inactivation of RNA Pol II stalled by a U-less cassette of pMLU112. These complexes were then digested with PvuII, which cleaves downstream of the stall site, and then incubated with combinations of CSB and CSA-MBP proteins. Then, in the even-numbered lanes, reactions were chased by incubation with UTP to allow transcription to the end of the template plus cold CTP to dilute the radiolabel, and the products were separated on the 5% sequencing gel shown. Runoff indicates the products of transcription to the end of the templates. The bands longer than stalled transcript in the absence of chase are presumably due to minor contamination of one or more of the three rNTPs with UTP.

Effect of CSB on Stalled RNA Pol II—A striking feature of transcription-repair-coupling in E. coli is the removal of stalled RNA Pol by the Mfd protein (6). Mfd removes RNA Pol stalled at a lesion, stalled by nucleotide starvation, or blocked by a DNA-bound protein (8). We tested CSB for an analogous activity. RNA Pol II was stalled 112 nucleotides into a U-less cassette by omission of UTP, and then the ternary complex was digested with downstream of the stall site, and then incubated with even the 3′-ended products of the complex were chased by incubation with UTP to allow transcription to the end of the template plus cold CTP to dilute the radiolabel, the products were separated on the 5% sequencing gel shown. Runoff indicates the products of transcription to the end of the templates. The bands longer than stalled transcript in the absence of chase are presumably due to minor contamination of one or more of the three rNTPs with UTP.

DISCUSSION

Transcription-repair-coupling occurs in many organisms, including prokaryotes and eukaryotes. Initial biochemical investigations of this phenomenon revealed that in E. coli, a TRCF encoded by the mfd gene was required and sufficient to couple the transcription and repair processes. It did so by removing
the stalled RNA Pol and delivering the repair proteins to the lesion at an accelerated rate compared to lesions in which polymerase was not stalled (6). A protein from Bacillus subtilis was found to have similar structure and function, notably, a region containing the seven so-called helicase motifs, and the ability to remove a stalled RNA Pol (33). Based on genetic data and in vivo studies, humans require two proteins for transcription-repair coupling, the CSA and CSB proteins (5). CSA is a member of the “WD-repeat” family of proteins, which so far have been found to have structural and regulatory roles but no enzymatic activity (11, 13). CSB (12) and its yeast homolog, Rad26 (34), are clearly more similar to the prokaryotic coupling factors than CSA. The properties of these four TRCFs are summarized in Table II.

Aside from having identical cellular functions, the TRCFs in Table II are similar in being relatively large and possessing the helicase motifs. Although all four proteins can bind to DNA and hydrolyze ATP, none exhibit detectable helicase activity. This property may not be unique to this class of enzymes because they have been largely identified by assays designed to detect weak protein-protein interactions. The prokaryotic interactions listed are more likely to reflect functional interactions because they have been corroborated by independent approaches. However, in no case have transcription or repair proteins been found to form a tight complex with a TRCF.

Previous studies have indicated that DNA binding and ATP hydrolysis by E. coli Mfd protein are involved in dissociation of stalled RNA Pol. It was inferred that the Mfd protein must bind nucleotide to bind to DNA and that hydrolysis of nucleotide is associated with dissociation of Mfd from DNA. It was proposed that, for dissociation of stalled RNA Pol, first Mfd-ATP binds to the ternary complex, both to the DNA and to RNA Pol. Then, hydrolysis of ATP brings about the release of Mfd from DNA together with the associated RNA Pol (7). CSB is strikingly different from Mfd in both DNA binding (ATP7-independent) and ATP hydrolysis (strongly stimulated by DNA). Thus it is not surprising that CSB, in contrast to Mfd, does not function to remove its cognate stalled RNA Pol from DNA.

A most interesting aspect of transcription-repair coupling is the reaction mechanisms and in particular the disposition of the RNA Pol stalled at the lesion. In E. coli, RNA Pol stalled at the lesion inhibits repair, and Mfd removes it. Mfd also binds to the repair enzyme, which is delivered to the transcription-blocking lesion at an accelerated rate (6, 37). In humans, RNA Pol stalled at a lesion does not inhibit repair, and CSB does not remove the stalled polymerase. CSB does, however, bind to repair proteins and may, like Mfd, function to deliver them to the transcription-blocking lesion at an accelerated rate. Such a mechanism, in which RNA Pol is not removed from the template during repair, could permit subsequent transcription by the polymerase without reinitiation at the promoter, as described by Hanawalt (16).

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