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Biochemical and Biological Characterization of Wild-type and ATPase-deficient Cockayne Syndrome B Repair Protein*

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Cockayne syndrome (CS) is a nucleotide excision repair disorder characterized by sun (UV) sensitivity and severe developmental problems. Two genes have been shown to be involved: *CSA* and *CSB*. Both proteins play an essential role in preferential repair of transcription-blocking lesions from active genes. In this study we report the purification and characterization of baculovirus-produced HA-His$_6$-tagged CSB protein (dtCSB), using a highly efficient three-step purification protocol. Microinjection of dtCSB protein in CS-B fibroblasts shows that it is biologically functional *in vivo*. dtCSB exhibits DNA-dependent ATPase activity, stimulated by naked as well as nucleosomal DNA. Using structurally defined DNA oligonucleotides, we show that double-stranded DNA and double-stranded DNA with partial single-stranded character but not true single-stranded DNA act as efficient cofactors for CSB ATPase activity. Using a variety of substrates, no overt DNA unwinding by dtCSB could be detected, as found with other SNF2/SWI2 family proteins. By site-directed mutagenesis the invariant lysine residue in the NTP-binding motif of CSB was substituted with a physicochemically related arginine. As expected, this mutation abolished ATPase activity. Surprisingly, the mutant protein was nevertheless able to partially rescue the defect in recovery of RNA synthesis after UV upon microinjection in CS-B fibroblasts. These results indicate that integrity of the conserved nucleotide-binding domain is important for the *in vivo* function of CSB but that also other properties independent from ATP hydrolysis may contribute to CSB biological functions.

Nucleotide excision repair (NER)[1] is an evolutionary strongly conserved pathway responsible for the removal of a wide variety of lesions from the DNA, including the major types of UV-induced DNA injuries: cyclobutane pyrimidine dimers and (6–4) photo products. Removal of these lesions proceeds via a multi-step reaction; lesion recognition is followed by local opening of the DNA helix, dual incision in the damaged strand on each side of the lesion, release of the damage-containing fragment, gap-filling DNA synthesis, and ligation. A large number of gene products is involved in this repair reaction (reviewed in Refs. 1–3). The dramatic consequences of impaired NER are illustrated by several hereditary human diseases including xeroderma pigmentosum (XP) and Cockayne syndrome (CS). Patients with XP manifest a high sun (UV) skin sensitivity associated with a strong predisposition to skin cancer and frequently progressive neurodegeneration. Besides these common features, a significant clinical and genetic heterogeneity is observed among XP patients. Seven excision-deficient complementation groups (XP-A to XP-G) have been identified so far on the basis of cell fusion experiments (4). Patients with CS show, like XP, increased sensitivity to UV light. In addition, they suffer from severe developmental problems, which include growth retardation, skeletal and retinal abnormalities, and severe neurological deficiencies due to dysmyelination of neurons (4–6). Unlike XP patients and unlike a recently generated mouse model for CSB (7), no significant skin cancer predisposition has been noted for CS so far.

Several observations have revealed functional connections between DNA repair and basal transcription. Firstly, at least three subunits of the basal transcription factor TFIIH (XPB, XPD, and TTDA) are also required for NER (8–10). Secondly, lesions in the transcribed strand of active genes hamper or block the vital process of transcription. For some lesions such as cyclobutane pyrimidine dimers the normal (global genome) NER pathway is very slow. Therefore, a special subpathway of NER has evolved that accomplishes rapid and efficient removal of these types of damage from the transcribed strand of active genes (11, 12). This process, called transcription-coupled repair is highly conserved in evolution, because it has been described in *Escherichia coli*, yeast and mammalian cells (7, 13–16). In humans, two genes have been shown to be specifically required for transcription-coupled repair: *CSA* and *CSB*, defective in CS complementation groups A and B, respectively (17, 18). CS cells exhibit an elevated UV sensitivity correlated with a specific defect in transcription-coupled repair. Consequently, unlike normal cells, they are unable to recover RNA synthesis after UV irradiation (19, 20).

The CSB gene encodes a protein of 1493 amino acids containing a 500-amino acid region highly homologous to members of the SWI2/SNF2 subfamily of DNA-dependent ATPases (18, 21). Interestingly, SNF2-related proteins are involved in a wide variety of cellular functions, notably transcription regulation, chromatin remodelling, and all known multi-step DNA repair pathways, such as nucleotide excision repair of both transcribed and nontranscribed regions of the genome, post replication repair, and recombination repair (22, 23). Importantly,

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[1] The abbreviation used are: NER, nucleotide excision repair; CS, Cockayne syndrome; XP, xeroderma pigmentosum; HA, hemagglutinin; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; wt, wild type; PCR, polymerase chain reaction; PAGE, polyacrylamide gel electrophoresis.

we found recently that the CSB protein resides in a large molecular weight complex that includes RNA polymerase II but no transcription initiation factors (24).

The mechanism by which CSB protein mediates transcription-coupled repair is still largely unknown. Availability of a recombinant CSB protein is essential for studying CSB activity in *in vitro* assays. Here we present the generation, purification, and functional characterization of a double-tagged wt and ATPase-deficient mutant CSB protein. Surprisingly, the DNA-dependent ATPase activity was found to be partially dispensable for the *in vivo* CSB function in RNA synthesis recovery.

EXPERIMENTAL PROCEDURES

Cell Culture—The immortalized human cell lines used in this study were: HeLa, C5RO (wt), GM2965 (CS-A), CS1AN (CS-B), and XPCS1BA(XP-B). The fibroblasts were cultured in Ham's F-10 medium, supplemented with antibiotics and 12% fetal calf serum. Cell-free extract from HeLa cells was prepared as described previously (25, 26).

An insect cell line, *Spodoptera frugiperda* Sf21, was cultured at 27 °C in Hanks' medium supplemented with antibiotics and 10% fetal calf serum. Hanks' medium was prepared from Grace's insect cell culture medium (Life Technologies, Inc.) supplemented with 0.33% tissue culture yeastolate (Difco), 0.33% tissue culture lactalbumin hydrolysate (Difco), and 0.55% bovine serum albumin.

Plasmid Constructs—Previously we have generated a CSB cDNA construct (2tpSLME6) encoding a N-terminal hemagglutinin antigen (HA) epitope as well as a C-terminal histidine (His_6) tag (24). For overexpression of the HA-CSB-His_6 in the BAC to BAC Baculovirus Overexpression System (Life Technologies, Inc.), the N-terminal *Sac*I fragment and the C-terminal *Sac*I-*Xba*I fragment were cloned together in the pFASTBAC vector (Life Technologies, Inc.), giving rise to construct 2tpFBACE6. Construct 2tpFBACE6-K538R, containing a mutation changing the Lys-538 codon to Arg, was generated as described below.

Site-directed Mutagenesis—The K538R mutation in the CSB cDNA was introduced using the PCR-mediated site-directed mutagenesis method (27). For this purpose, two fragments of the CSB cDNA containing the helicase domain (1330-base pair *Kpn*I-*Sma*I fragment and 1914-base pair *Kpn*I-*Kpn*I fragment) were independently subcloned into pBluescript KS and pTZ18 vector, respectively. The primers used in the PCR reactions are: the mutagenic primer (5′ GGATTGGGCAGGACCATCCAG 3′) and the universal primers SK and M13 universal. The final PCR product was cloned into the pBluescript vector, and the internal *Asu*II fragment was used to replace the wild-type fragment in 2tpFASTBACE6. Sequencing of the entire PCR fragment ruled out PCR-derived errors and confirmed the presence of the desired mutation.

Baculovirus Infection and Purification of Recombinant Proteins—Suspension cultures of Sf21 cells at 1.5×10^6 cells/ml were infected with the recombinant baculoviruses at 27 °C for 1 h at a multiplicity of infection of 5–10. At 3 days post-infection, cells were collected and washed twice with ice-cold phosphate-buffered saline. For fractionation, the cell pellet was resuspended into 8 (packed cell) volumes of ice-cold NP lysis buffer (25 mM Tris-HCl, pH 9.0, 1 mM EDTA, 10% glycerol, 1% Nonidet P-40, 0.3 M KCl, 1 mM dithiothreitol, 0,1 mM phenylmethylsulfonyl fluoride, 0,2 μM chemostatin, leupeptin, antipain, and pepstatin A). After incubation on ice for 30 min with occasional agitation, the suspension was centrifuged at $12,000 \times g$ for 15 min to obtain the supernatant fraction and the precipitate fraction. To examine the protein remaining in the precipitate fraction, the pellets were homogenized in NP buffer by sonication. For purification, the supernatant fraction was diluted with NP buffer at pH 6,8 in a 1:1 ratio and loaded on a heparin-Sepharose (CL-6B; Amersham Pharmacia Biotech) column equilibrated with buffer A (25 mM HEPES-KOH, pH 7, 9, 0,01% Nonidet P-40, 10% glycerol, 1 mM β-mercaptoethanol, 0, 1 mM phenylmethylsulfonyl fluoride) containing 0.3 M KCl. The column was washed with the same buffer, and the adsorbed proteins were eluted with buffer A containing 0.8 M KCl. The eluate was directly loaded on a Ni^{2+}-nitrilotriacetic acid-agarose column equilibrated with buffer A containing 0.8 M KCl and 5 mM imidazole. After washing the column with 20 mM Imidazole, bound proteins were eluted with buffer A containing 0.3 M KCl and 60 mM/250 mM imidazole.

In the final step of purification, the Ni^{2+}-nitrilotriacetic acid elution fractions were incubated with a 5-fold excess of monoclonal anti-HA (12CA5) antibody O/N at 4 °C with rotation. The protein-antibody complexes were subsequently bound to protein G-Sepharose beads. After extensive washing of the beads with buffer A containing 0,1% Nonidet P-40, CSB protein was specifically eluted using the synthetic HA peptide (YPYDVPDYA) at 1 mg/ml in buffer A containing 0.2 M KCl. Proteins were stored at −80 °C. The behavior of the recombinant protein during the purification was monitored by SDS-PAGE and immunoblot blot analysis.

ATPase Assay—Standard reactions (10 μl) were carried out in buffer B (20 mM Tris-HCl, pH 7.5, 4 mM $MgCl_2$, 40 μg/ml bovine serum albumin, 1 mM dithiothreitol), 1 μCi of [γ-^{32}P]ATP (5000 Ci/mmol; Amersham Pharmacia Biotech) and the specified amounts of CSB protein. When indicated, reactions were carried out in the same buffer B supplemented with 50 μM cold ATP and 0.25 μCi of [γ-^{32}P]ATP. 150 ng of DNA cofactor/reaction was added. After 1 h at 30 °C the reactions were stopped with 5 μl of 0.5 M EDTA, and a 1-μl sample was analyzed on polyethylenimine-cellulose thin layer chromatography plates developed in 0.75 M KH_2PO_4. The extent of ATP hydrolysis was determined by image analysis and quantification on a PhosphorImager (Molecular Dynamics).

Helicase Assay—The partial duplex DNA substrates were constructed by hybridizing a 5′ ^{32}P-labeled complementary fragment to the viral (+)-strand of M13 DNA: (i) 18-base oligonucleotide; (ii) 36-base oligonucleotide; (iii) 50-base oligonucleotide with a 5′ 15-base noncomplementary overhang; and (iv) 50-base oligonucleotide with a 3′ 15-base noncomplementary overhang. Helicase substrates with a 5′-3′ and a 3′-5′ polarity were made by hybridizing a 5′-^{32}P-labeled complementary fragment to a 36-base oligonucleotide (F36), respectively: (i) 19-base oligonucleotide (F19) and (ii) 17-base oligonucleotide (F17) (see Table I). Also, a double-stranded 75-base DNA fragment was used. Free oligonucleotides were removed by gel filtration on a Sephadex G50 (Amersham Pharmacia Biotech) column. The substrates, 5 ng each, were incubated with the indicated amount of CSB protein in buffer B containing 1 mM ATP (total volume 25 μl). After incubation at 37 °C for 45 min, reactions were stopped and subjected to electrophoresis on 12% polyacrylamide gel and autoradiography as described (8).

Microneedle Injection of Recombinant Proteins and Analysis of Repair and Transcription Levels—Microneedle injection into cultured fibroblasts was performed as described previously (10, 28). Comparable amounts of purified recombinant CSB, wild type, and K538R mutant dissolved at 20 ng/μl were injected into the cytoplasm of CS1AN (CS-B) homopolykaryons (obtained after cell fusion of CS1AN (CS-B) fibroblasts with each other), multinucleated cells of GM2965 (CS-A), or XPCS1BA (XP-B) homopolykaryons. The recovery of RNA synthesis post-UV was measured. Cells were UV-irradiated with 10 or 15 J/m^2 (UV-C 254 nm) and allowed to recover for 16–20 h. RNA synthesis was measured after pulse-labeling with [^{3}H]uridine (10 μCi/ml; specific activity, 50 Ci/mmol), fixation, and autoradiography. Grains above the nuclei of injected cells (polykaryons) and noninjected neighboring cells (monokaryons) were counted and represent a quantitative measure for RNA synthesis.

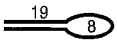
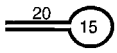
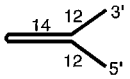
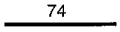
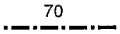
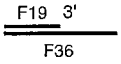
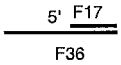
Antibodies—Anti-CSB antibodies used in this study were raised against the C-terminal 158 amino acids of CSB. Characterization of affinity-purified anti-CSB and immunoblotting procedures were performed as described (24).

DNA Substrates—DNA cofactors used in the ATPase assay are described in Table I and kindly provided by Wouter de Laat (29). HeLa polynucleosomes were a generous gift of Robert Kingston and Gavin Schnitzler.

RESULTS

Overexpression and Purification of Recombinant wt and Mutant CSB Proteins—To facilitate purification of CSB and to allow isolation of a full-length protein, we generated a double-tagged CSB (dtCSB) construct (24) in a baculovirus expression system. A 9-amino acid HA epitope was introduced at the N terminus of the cDNA sequence, and a hexameric histidine stretch (His_6) was introduced at the C terminus. It was important to verify whether the addition of tags *per se* did not interfere with the biological function of CSB. Therefore, we established by cDNA transfection a CS-B cell line stably expressing dtCSB in a mammalian expression vector. The level of expression of dtCSB protein was similar to the endogenous CSB level in normal cells. We found that the tagged protein conferred wild-type UV resistance to the CS-B cells and was incorporated into a large complex including RNA polymerase II (see Ref. 24). Thus, these findings confirm that dtCSB was

TABLE I
DNA substrates used as cofactors in ATPase assays and helicase templates
Helicase partial duplex templates were made by hybridyzing 5′ ^{32}P-labelled F19 or F17 oligonucleotides to the F36 fragment.

DNA substrate	Sequence	Structure
stem19-loop8	5'-TCGGGTCGCCAGCGCTCGG$(T)_8$CCGAGCGCTGGCGACCCGa-3'	
stem20-loop15	5'-TGGCACATCCTGGGTCGCCAGCGCTCGG$(T)_{15}$CCGAGCGCTGGCGACCCAGGATG-3'	
splayed-arm	5'-$(T)_{12}$GCCATCGCGAGTCCGGACTCGCGATGGC$(T)_{12}$-3'	
ss-oligo	5'-GATCCATCGATTCAGCTAGCGTAATCTGGAACATCGTATGGGTAAGCGCTGCCTCCGTGATGGTGATGGTGGTG-3'	
RNA-oligo	5'-CUAGGUAGCUAAGUCGAUCGCAUUAGACCUUGUAGCAUACCCAUUCGCGACGGAGGCACUACCACUACCA-3'	
F19	5'-GCGATGCGGATCCAAGTCT-3'	
F36	5'-CCTAGACTTAAGAGGCCAGACTTGGATCCGCATCGC-3'	
F17	5'-GGCCTCTTAAGTCTAGG-3'	

biologically active *in vivo* and functionally indistinguishable from the untagged wt protein.

To investigate the importance of the ATPase activity for the various CSB functions we introduced by site-directed mutagenesis a strategic amino acid substitution in the Walker-type A motif (30) by replacing the invariant lysine residue (Lys-538) in the G*X*GKT sequence by the physicochemically related arginine, yielding dtCSB (K538R). It has been shown for other ATPases that such an alteration does prevent ATP hydrolysis but still permits ATP binding (31–33). Importantly, this conservative amino acid change is not expected to drastically modify the overall protein conformation.

Both the wild-type and the mutant CSB protein were overproduced using the baculovirus/Sf21 insect cell system. Infected Sf21 cells were extracted with hypertonic buffer containing 0.3 M KCl, and the dtCSB protein was isolated from the soluble fraction. The purification was monitored by immunoblot analysis using polyclonal anti-CSB antibodies and by silver staining of SDS-PAGE. After two chromatographic steps, a heparin-Sepharose and a Ni^{2+}-nitrilotriacetic acid-agarose column that selects for the C-terminal His_6 stretch, we obtained a protein fraction consisting of a major band of about 170 kDa, corresponding to the predicted molecular weight of dtCSB (Fig. 1, *A* and *B*, *lanes 1–3*). Some degradation products were visible after immunoblot analysis using antibodies raised against the C terminus of CSB (data not shown; similar findings made by Selby and Sancar (34)). The presence of an HA epitope at the N terminus of dtCSB allowed us to select only for full-length protein during the last highly specific monoclonal affinity purification step. When the final fraction was analyzed by SDS-PAGE and silver staining, no protein species other than dtCSB were detected, indicating that the protein was purified to near homogeneity (Fig. 1*B*, *lane 4*). The same purification procedure was used for both wild-type dtCSB and dtCSB (K538R) mutant, obtaining both proteins at comparable high levels of purity, intactness, concentration, and yield (Fig. 1*C*). The three-step purification scheme described above yielded about 22 μg of protein from 10^8 infected cells. Immunoblot analysis of purified wt and mutant dtCSB and HeLa WCE using antibodies against CSB confirms the identity of the purified proteins (Fig. 1*D*, note the slight increase in size of the dtCSB as compared with the nontagged wt CSB in HeLa whole cell extract; for characterization of the antiserum, see Ref. 24).

Functionality of the Purified Recombinant dtCSB Protein in Vivo—Prior to the biochemical characterization of the recombinant dtCSB protein, it was important to verify whether overproduction in the heterologous Baculo system and/or the purification procedure had not inactivated the biological function of the protein, *e.g.* by improper folding or lack of post-translational modification.

One of the characteristic features of CS cells is their inability to recover RNA synthesis after UV exposure (18, 35). To assess the *in vivo* function of the recombinant wt CSB, the purified protein was microinjected into the cytoplasm of living CS-B (CS1AN) fibroblasts, and its ability to transiently correct the DNA repair defect of CS-B cells was analyzed. The experimental protocol is outlined in Fig. 2*A*. CS1AN (CS-B) fibroblasts were fused to generate homopolykaryons. After injection of at least 50 multi-nucleated fibroblasts, the cells were irradiated with 15J/m^2 UV light and incubated for 16–20 h to allow recovery from UV-induced inhibition of RNA synthesis. Transcription was analyzed by a 1-h pulse labeling with [^{3}H]uridine followed by autoradiography. Transcription levels were quantified by counting silver grains above the nuclei (10, 28). As shown in Table II and Fig. 2*B*, in injected CS-B polykaryons the recovery of UV-induced inhibition of transcription is resumed to levels observed in wild-type cells assayed in parallel. In contrast, the neighboring noninjected cells (monokaryons in

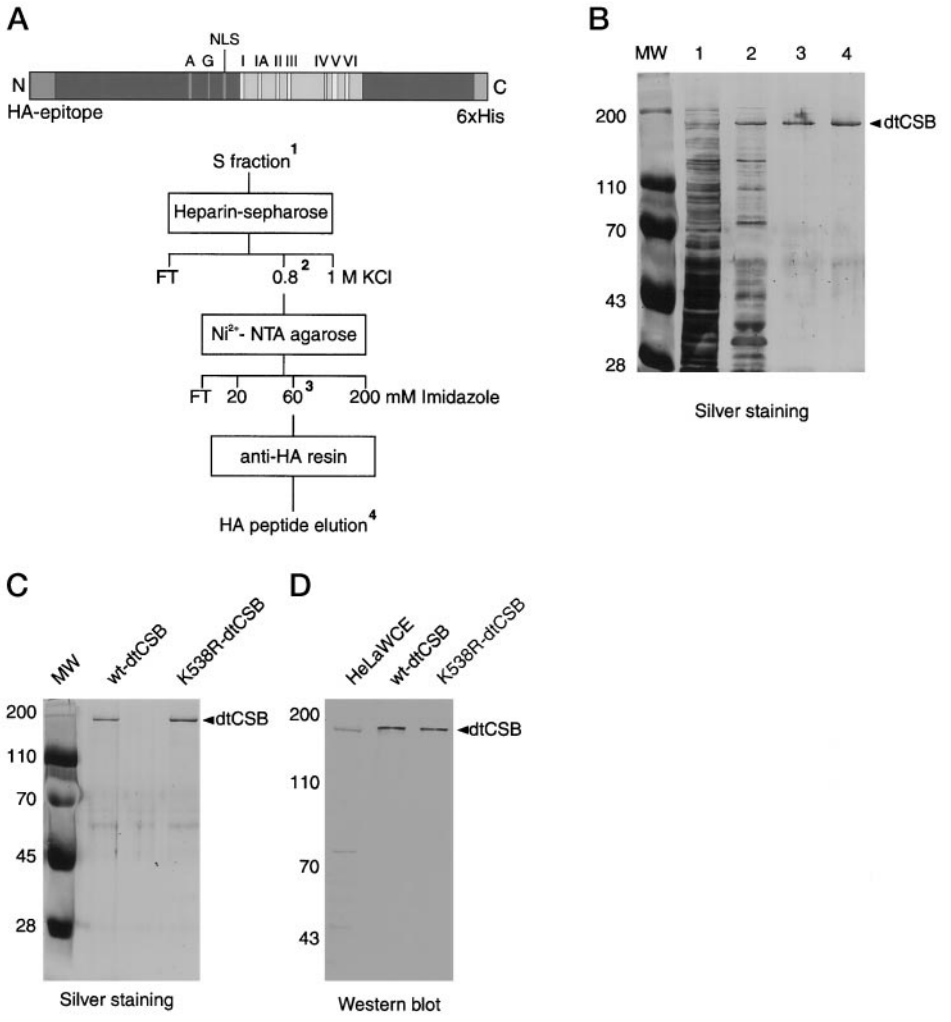


FIG. 1. **Purification of recombinant dtCSB.** *A*, dtCSB purification scheme (see "Experimental Procedures") and schematic representation of the predicted functional domains of the CSB protein: *A*, acidic amino acid stretch; *G*, glycine-rich region; *NLS*, nuclear location signal. The *lightly shaded area* represents the central CSB domain with overall high homology to the region shared between all members of the SNF2 family of DNA-dependent ATPases. Roman numbers *I–VI* within this region refer to conserved "helicase" motifs that have been identified in numerous known and putative DNA and RNA helicases. *B*, purification of recombinant wt dtCSB. Purification was monitored by SDS-PAGE. Aliquots of samples at each purification step (for numbering of the corresponding steps see *panel A*) were analyzed on a 8% SDS-PAGE gel, and protein bands were visualized by silver staining. (The faint staining at around 60 kDa is an artifact; see *panel C*). *C*, purified recombinant wild-type dtCSB and dtCSB (K538R) mutant proteins. Aliquots of the last purification step (HA elutions) of both proteins (200 ng each) were resolved by SDS-PAGE followed by silver staining. (The lane between the dtCSB samples is empty showing a faint staining artifact at the position of ~60 kDa). *D*, immunoblot analysis of HeLa whole cell extract (*WCE*, 10 μg) and purified wt dtCSB and dtCSB (K538R). Affinity-purified anti-CSB polyclonal antibodies were used (24). The positions of the molecular mass markers (*MW*) in kDa are shown.

Fig. 2*B*), exhibit a very low level of transcription, which is typical for UV-exposed CS-B fibroblasts.

When CSB was microinjected in CS-A and XP-B fibroblasts, no significant complementation of their RNA-synthesis recovery defect was observed, proving that the correcting activity is specific for CS-B cells (Table II).

These experiments demonstrate that the purified recombinant dtCSB protein is able to exert its biological function(s) *in vivo*, including transportation to the nucleus and participation in recovery of RNA synthesis, thus presumably also in transcription-coupled repair and complex formation with RNA polymerase II (24). The microinjection results confirm our earlier findings with the transfected CS-B cells stably expressing the dtCSB protein (24) and provide a solid basis for analysis of the biochemical properties of the purified protein and its mutant version.

In Vitro Biochemical Characterization of the Purified Recombinant wt and Mutant dtCSB Proteins—The presence of a SNF2 domain in the CSB protein starting with a Walker A-type nucleotide hydrolysis motif strongly suggested that CSB harbors an ATPase activity (21, 30). The mutant dtCSB (K538R) protein should serve as an optimal negative control for this assay. As shown in Fig. 3*A*, in the absence of nucleic acids, both proteins exhibited a comparable background level of activity. However, ATPase activity of wild-type dtCSB was highly stimulated by the presence of ds λ DNA in the reaction, in contrast to the mutant protein (Fig. 3*A*). The extent of ATP hydrolysis was proportional to the amount of wt dtCSB added to reactions in which ATP concentration was kept constant and the DNA cofactor was in excess (Fig. 3*B* and 5*A*). Quantification of ATP hydrolysis showed that the conservative K538R substitution resulted in a complete loss of the catalytic ATPase function of CSB (Fig. 3*B*). Because wt and mutant proteins (purified in parallel) are at the same level of purity and equally concentrated (Fig. 1*C*), this result indicates that DNA-dependent ATPase activity is an intrinsic property of CSB protein and that the K538R substitution abolishes ATP hydrolysis.

To get insight into the optimal structure of the DNA cofactor

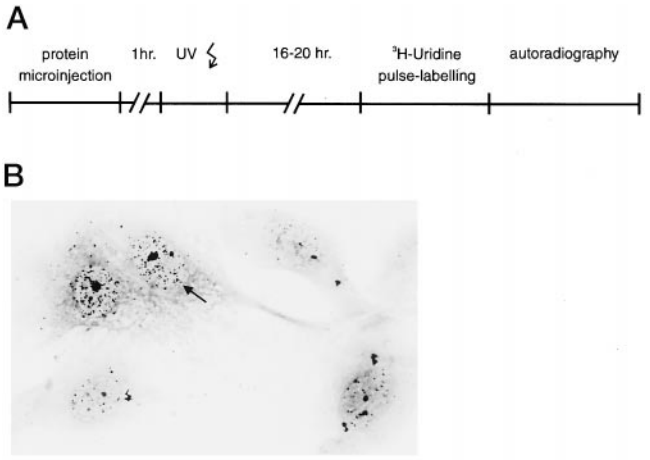


FIG. 2. **Assessment of recombinant wt dtCSB *in vivo* function using microneedle injection.** *A*, schematic representation of the microinjection RNA-synthesis recovery assay. wt dtCSB protein (0.02–0.05 $\mu g/\mu l$) purified from insect cells was injected into the cytoplasm of CS-B fibroblasts (homopolykaryons were used for the injection). RNA synthesis was assayed 16–20 h after UV irradiation by a 1-h pulse labeling with [H^3]uridine, fixation, and autoradiography (see "Experimental Procedures" for details). *B*, micrograph of a CS-B binuclear fibroblast (homopolykaryon) injected with wt dtCSB protein and assayed for RNA synthesis recovery by autoradiography. The injected binuclear CS-B cell (indicated by the *arrow*) exhibits a high level of transcription, as indicated by the high number of silver grains above both of its nuclei, whereas the noninjected surrounding CS-B fibroblasts (monokaryons) show the low level of RNA synthesis recovery typical for UV-exposed CS cells.

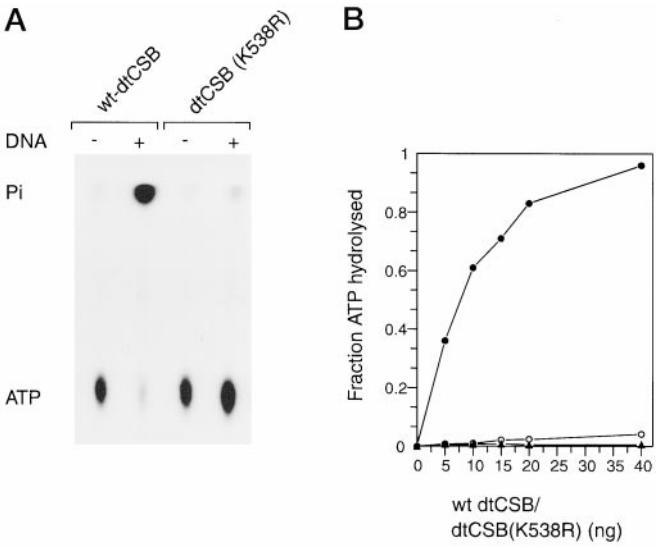


FIG. 3. **ATPase activity of recombinant wt and mutant dtCSB protein.** *A*, ATPase activity assay. Recombinant wt dtCSB and dtCSB (K538R) proteins (15 ng) purified from Sf21 insect cells were assayed for ATPase activity in the presence (+) or in the absence (−) of double-stranded λ DNA (150 ng) (see "Experimental Procedures"). Incubation was for 30 min. *B*, effect of increasing dtCSB protein concentration on ATP hydrolysis. Reactions were performed as in *A* in the presence or absence of ds λ DNA (150 ng) and the indicated protein concentration for 30 min. Release of P_i was quantified using a PhosphorImager (Molecular Dynamics). ●, wt dtCSB + λ DNA; ○, dtCSB (K538R) + λDNA; ▲, wt dtCSB and dtCSB (K538R) without DNA (data points are superimposed).

TABLE II
Effect of microinjection of recombinant dtCSB protein on RNA synthesis recovery

Cell strain	Complementation group	Injected protein[a]	UV irradiation	Grains per nucleus	Recovery of RNA synthesis
			J/m^2	*mean ± S.E.*	*% of normal*[b]
C5RO	wt		15	69 ± 3	100
C5RO	wt	wt-CSB	15	63 ± 3	91
CS1AN	CS-B		15	15 ± 1	22
CS1AN	CS-B	wt-CSB	15	62 ± 5	90
CS1AN	CS-B	K538R-CSB	15	26 ± 2	38
GM2965	CS-A		15	20 ± 1	29
GM2965	CS-A	wt-CSB	15	22 ± 2	32
C5RO	wt		10	78 ± 4	100
XPCS1BA	XP-B		10	13 ± 1	17
XPCS1BA	XP-B	wt-CSB	10	15 ± 1	19

[a] Recombinant dtCSB protein purified from insect cells (see Fig. 1). Estimated protein concentration, 0.02–0.05 $\mu g/\mu l$.

[b] As a control, basal transcription was also measured in unirradiated cells. wt and CS1AN fibroblasts showed the same RNA synthesis level under these experimental conditions: 123 ± 4 grains/nucleus in wt C5RO; 123 ± 4 grains/nucleus in CS1AN.

required for CSB ATPase activity, we tested several DNAs with different structural properties for their ability to stimulate the CSB ATPase. Various double-stranded DNAs, including ds λ DNA and supercoiled circular pBluescript DNA, elicited high activity (Fig. 4*C*). Also M13 single-stranded DNA appeared a potent activator (Fig. 4*A*, *lane 1*). Because dsDNA and ssDNA are structurally very different, it is surprising that both stimulate the CSB ATPase activity to a closely comparable extent. Therefore we decided to premelt the M13 ssDNA to reduce secondary structure. Fig. 4*A* shows that the stimulatory activity of M13 ssDNA was strongly decreased after boiling the ssDNA followed by quenching on ice. These findings indicate that unmelted M13 ssDNA is structurally ill defined and probably contains considerable double-stranded character. We conclude that the DNA structure is important for CSB ATPase activity and that the protein is primarily stimulated by dsDNA. To further investigate the substrate requirement of CSB, structurally more defined oligonucleotides were tested for stimulation of ATP hydrolysis by dtCSB. Using synthetic oligonucleotides we observed that the presence of secondary structure such as stem-loop and fork-like structures or DNA hairpins enhanced CSB ATPase activity (Fig. 4*B* and Table I), whereas single-stranded molecules of the same length and sequence, as well as RNA oligonucleotides and RNA/DNA hybrid molecules, are only very weak activators (Fig. 5*B* and Table I). In this respect, it is interesting to note the difference between the activity elicited by a single-stranded poly-$d(T)_{>80}$ molecule (Fig. 5*B*, *open circles*), which has little secondary structure, and a single-stranded oligonucleotide containing palindromic sequences, which can give rise to stem-loop structures (Fig. 5*B*, *open triangles*, for sequence see Table I). In addition, we observed that CSB DNA-dependent ATPase activity was stimulated to the same extent by both naked DNA and nucleosomal DNA (Fig. 4*C*). Presence of $MgCl_2$ was essential for catalytic activity, which was optimal at a pH value of 7.5 and at 100 mM salt (not shown).

Under the experimental conditions used, a turnover rate ranging from 27 to 33 ATP min^{-1} was calculated in the presence of dsDNA (Fig. 4*B*). The turnover number was calculated in three independent experiments, including an ATPase kinetics analysis with constant protein concentration and ds λ DNA in which ATP concentration ranged from 0.025 to 0.2 mM. When ssDNA (poly$(dT)_{>80}$) was used as cofactor, the turnover number was in the order of 3.7–6 ATP min^{-1} (Fig. 4*B*).

In conclusion, our *in vitro* data indicate that CSB is a DNA-dependent ATPase, which is specifically stimulated by double-stranded DNA and double-stranded DNA structures with a partial single-stranded character as well as nucleosomal DNA but not by true ssDNA. Moreover, the conservative amino acid substitution in the NTP binding motif impairs CSB ability of

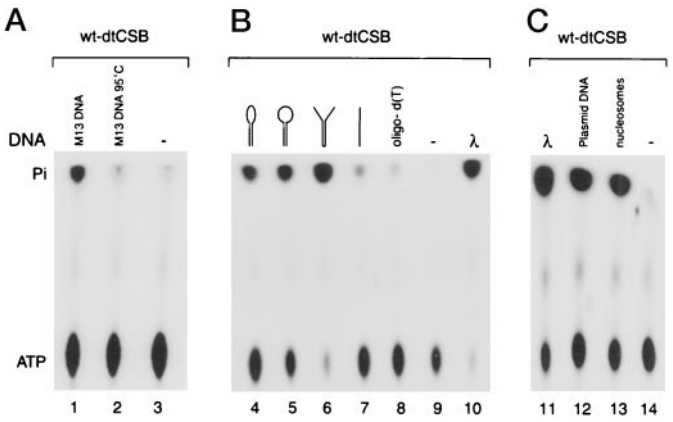


FIG. 4. **Requirement of DNA cofactors for dtCSB ATPase activity.** *A*, secondary structure in M13 ssDNA is required for dtCSB ATPase activity. wt dtCSB (10 ng) was assayed for ATP hydrolysis in standard ATPase reactions (as in Fig. 3) containing the following DNA molecules (150 ng): *lane 1*, M13 ssDNA; *lane 2*, M13 ssDNA premelted at 95 °C and subsequently quenched on ice; *lane 3*, without DNA. *B*, effect of different synthetic DNA molecules on dtCSB ATPase activity. ATP hydrolysis by wt dtCSB (15 ng) was analyzed in the presence of synthetic oligonucleotides with different secondary structures (150 ng): *lane 4*, stem 19-loop 8; *lane 5*, stem 20-loop 15; *lane 6*, splayed arm; *lane 7*, single-stranded oligonucleotide (74 nucleotides); *lane 8*, oligo$(dT)_{15}$; 9, no DNA; 10 λ DNA. See Table I for oligonucleotides sequences. *C*, nucleosomal DNA acts as cofactor for CSB ATPase activity. wt dtCSB ATPase activity was analyzed in the presence of naked and nucleosomal DNA (150 ng): *lane 11*, λ DNA; *lane 12*, double-stranded pBluescript DNA; *lane 13*, bulk HeLa polynucleosomes; *lane 14*, without DNA.

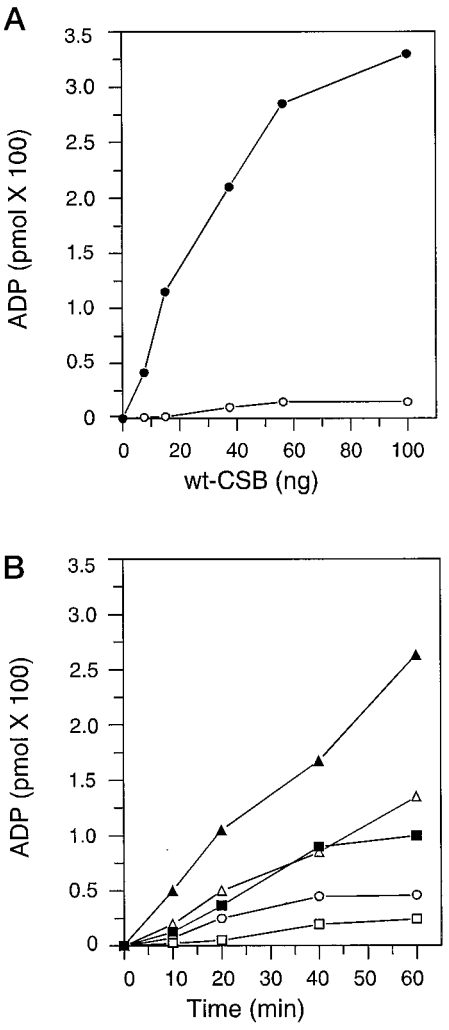


FIG. 5. **Kinetics of ATP hydrolysis.** *A*, ATP hydrolysis as a function of wt dtCSB protein concentration. Reactions contained 50 μM cold ATP supplemented with 0.25 μCi of [γ-^{32}P]ATP (see "Experimental Procedures") and were incubated for 1 h at 30 °C. ○, no DNA was added; ●, λ DNA (150 ng) was added. *B*, ATP hydrolysis as a function of incubation time. wt dtCSB protein (35 ng) was assayed for ATP hydrolysis in the presence of different nucleic acid cofactors (150 ng). Aliquots were taken after incubation for 10, 20, 40, and 60 min and analyzed for release of P_i. Reaction mixture contained synthetic oligonucleotides (74 nucleotides long): △, ssDNA; ▲, dsDNA; □, RNA; ■, RNA-DNA hybrid; ○, poly$(dT)_{>80}$. See Table I for oligonucleotide sequences. The single-stranded oligodeoxynucleotide described in Table I was annealed to its complementary sequence and used as dsDNA cofactor. The synthetic RNA oligonucleotide described in Table I was used either alone or annealed to its complementary ssDNA oligonucleotide (RNA-DNA hybrid).

hydrolysing ATP, showing that ATPase activity is not due to a contaminating polypeptide.

The highly conserved common domain of the family of SNF2/SWI2 proteins shares significant homology with seven sequence motifs identified in numerous DNA and RNA helicases that require NTP hydrolysis for disrupting the hydrogen bonds that hold the two DNA strands together (for review see Refs. 36 and 37). To test whether functional dtCSB displayed any DNA helicase activity, the protein was incubated with M13 ssDNA to which a ^{32}P-labeled 18- or 36-base primer was annealed. Release of the bound oligonucleotide from M13 DNA was analyzed by polyacrylamide gel electrophoresis (see "Experimental Procedures"). Under the assay conditions used, which were able to detect bona fide DNA helicase activity of purified TFIIH used as positive control, no displacement of the helicase templates was observed (not shown). Also, CSB failed to displace a 50-base primer with a 15-nucleotide 3′ or 5′ noncomplementary overhang (data not shown). The same negative results were obtained with a 75-base double-stranded oligonucleotide and a 36-base oligonucleotide, to which a 5′ ^{32}P-labeled 17-base or 19-base fragment (F17-F36; F19-F36; Table I) was annealed (data not shown). Absence of detectable helicase activity in the presence of DNA-dependent ATP-ase is in agreement with recent findings reported by Selby and Sancar (34) using a non-tagged, partially full-length CSB preparation.

Inactivation of the ATPase Activity Does Not Completely Impair the in Vivo Function of CSB—As shown above, the specific K538R substitution in the CSB nucleotide-binding domain abolishes its catalytic ATPase activity *in vitro*. To investigate the biological consequences of this mutation, purified dtCSB (K538R) protein was microinjected into living CS-B fibroblasts in parallel to injected wild-type dtCSB. The same type of mutation (Lys to Arg) in the ATP hydrolysis domain of the dual functional XPB DNA repair helicase was shown to possess a dominant-negative *in vivo* effect; this mutant subunit of the TFIIH repair/transcription complex completely paralyzes both repair and basal transcription in a dominant fashion in normal cells (28). Apparently, the mutant XPB protein is incorporated in the TFIIH complex but is unable to exert its function, thereby actively blocking both processes. The CSB protein too is incorporated in a RNA polymerase II complex, and thus substitution by an enzymatically dead molecule could actively disrupt the processes in which it is engaged. However, in striking contrast to the effect of mutant XPB, injection of the recombinant dtCSB (K538R) protein even partially rescued the defect in RNA synthesis recovery after UV irradiation in CS-B fibroblasts (Table II). Although the activity is much lower when compared with wt dtCSB, still a significant (approximately 2-fold) stimulation of resumption of transcription was observed in repeated experiments (Table II). This partial *in vivo* activity is in sharp contrast with the completely inactive ATP hydrolysis of (K538R) mutant protein.

DISCUSSION

Purification of the dtCSB Protein Using Tags—Here we describe the successful isolation of double-tagged CSB protein from baculovirus-infected SF21 cells. We have utilized a novel purification procedure that has a number of advantages above classical purification protocols: (i) The use of the HA and His_6 tags allows a highly selective, rapid, standardized three-step procedure. This avoids lengthy purification protocols involving a large number of column chromatography steps. In independent experiments we have found that the HA-monoclonal affinity chromatography by itself already can provide an >1000-fold purification when starting from mammalian whole cell extracts (38). The Ni^{2+}-nitrilotriacetic acid agarose column chromatography specific for the hexameric histidine tag has the benefit that it is very versatile because it can be carried out under a wide range of conditions. (ii) The strategy used in this work has also the advantage that it selects only for full-length protein avoiding inclusion of partial degradation products in the final purified protein preparation. (iii) The purification procedure is very efficient: the dtCSB approach used yielded 22 μg of intact CSB protein from 10^8 infected cells, appearing at least 15-fold more efficient compared with classical protocols in which CSB was successfully purified involving seven chromatographic steps (34). A prerequisite for the use of tags is that one should test whether the addition of tags interferes with the function of the protein. In the case of dtCSB, we demonstrate *in vivo* and *in vitro* that the tagged protein was fully functional. Microinjection and DNA transfection experiments showed that the dtCSB protein completely corrected the defect in recovery of UV-induced inhibition of RNA synthesis and UV sensitivity of CS-B cells.

Biochemical Properties of the Purified dtCSB Protein—Purified wt dtCSB possesses an *in vitro* DNA-dependent ATPase activity, as also reported recently by Selby and Sancar (34). We investigated the DNA cofactor requirement for CSB ATPase activity using structurally defined DNA substrates. The highest stimulation of CSB ATPase activity was observed when dsDNA or structured oligonucleotides with a partial double- and single-stranded character were used as DNA substrates (Figs. 4*B* and 5*B*). Unexpectedly, M13 ssDNA also exerts a strong stimulation. The notion that true dsDNA and ssDNA are physicochemically very different rendered it unlikely that both should be equally efficient in enhancing CSB-ATPase. However, the ATPase stimulation by M13 ssDNA was drastically reduced after removing secondary structure by melting. This indicates that the double-stranded character in M13 ssDNA was responsible for the stimulation and that true ssDNA is a poor activator for CSB ATPase. This was confirmed by the use of $poly(dT)_{>80}$, which is known to have little secondary structure and "single-stranded" oligonucleodides with multiple palindromic sequences. In addition to differences in protein preparations, the above observation may explain the findings of Selby and Sancar, who reported a strong stimulation of CSB-ATPase by ssDNA (34) and the yeast equivalent RAD26, which was reported by Guzder *et al.* to be stimulated preferentially by ssDNA (39). In fact, for any ssDNA-dependent ATPases for which nonpremelted ssDNA substrates were used, it should be considered a possibility that the stimulation was not derived from ssDNA but from double-stranded structures within it. The fact that the CSB ATPase activity is strongly enhanced by ds/ss DNA substrates such as Y-structures containing hairpins opens the possibility that a double-stranded to single-stranded transition is a biological relevant DNA conformation for CSB. In addition, we find that ATP hydrolysis by CSB is stimulated equally by naked dsDNA and nucleosomal DNA (Fig. 4*C*). A similar observation is made for the ySWI·SNF complex (40, 41). Specific activity of dtCSB in the presence of dsDNA was approximately 160 pmol ADP formed/μg CSB/min (K_{cat} = ~27 min^{-1}), which is within the range of activities measured for other DNA-dependent ATPases of the same family. In particular, CSB appears to be a stronger ATPase than the recombinant ySNF2 (20 pmol ADP/μg/min (42)) as well as the *Drosophila* NURF complex (K_{cat} = ~8.5 min^{-1} in the presence of nucleosomal DNA) (43).

As observed with other members of the SNF2-like family of ATPases, no overt helicase activity could be attributed to CSB, using classical helicase DNA substrates as well as DNA molecules with different types of noncomplementary overhangs (39, 42). The high homology of CSB with members of the SNF2 family suggests that these proteins may function via a similar mechanism. SNF2-like proteins have been reported to be involved in chromatin remodelling and to be able to disrupt protein-DNA interactions (44–47). On this basis, it has been proposed that they function as DNA-translocating factors that use the energy derived from ATP hydrolysis to move along the DNA, destabilizing proteins bound to the DNA (48). Similarly, we can speculate that CSB uses energy from ATP hydrolysis to induce some type of structural alteration in the DNA, involving local strand separation that diminishes the affinity of bound protein for dsDNA. It has been shown that NTP-binding proteins undergo conformational changes upon NTP binding and hydrolysis. Recently, a structural homology was found between a DNA helicase of *Bacillus stearothermophilus* and the recombination protein RecA. This suggested a common basis for the coupling of ATP binding/hydrolysis to conformational changes of the protein, affecting the affinity of the enzyme for different DNA substrates, thereby driving the catalytic reaction (49). In agreement with this idea we recently found CSB to reside in a large MW complex *in vivo*. This complex contains RNA polymerase II but is devoid of transcription initiation factors (24), and its further characterization indicates that it most likely represents an elongation mode of RNA polymerase II.[2] Direct *in vitro* association of CSB with RNA polymerase II was reported recently by Tantin *et al.* (50) and by Selby and Sancar (51). The latter study also provided evidence that CSB stimulates transcription elongation *in vitro*. The DNA structure requirements of CSB presented here are consistent with the transitions from ssDNA to dsDNA at the site of an elongating or stalled RNA polymerase. At this location the CSB protein may help destabilize nucleosome-DNA interactions, thus facilitating transcription elongation at pause sites. In addition, CSB may enable resumption of stalled transcription elongation after DNA damage removal by dissociating NER (such as TFIIH) and other repair factors and/or nucleosomes bound to the DNA.

Biological Consequences of ATPase Deficiency on the Functioning of CSB—By site-directed mutagenesis the invariant lysine 538 residue in CSB ATP binding motif was replaced with the physicochemically related arginine. The conservative K538R mutation leads to a (virtual) complete loss of dtCSB ATPase activity *in vitro* (Fig. 3, *A* and *B*), as also has been shown for a variety of other ATPases (31, 42, 52). Surprisingly, we found that the ATPase-dead CSB protein still exerts a partial function *in vivo*, as shown by microinjection experiments in CS-B fibroblasts (Table II). Interestingly, the *in vivo* results suggest that this specific mutation can partially uncouple ATP hydrolysis from other biological activities of CSB, implying that other properties of CSB are also important for its function. In this respect is relevant to note that CS-B cell line (CS1AN) used in this study carries two alleles, each leading to a severely truncated CSB protein product (18). In fact, system-

[2] E. Citterio and J. H. J. Hoeijmakers, unpublished observations.

atic mutation analysis of a large number of CSB patients has revealed that most patients produce severe CSB truncations. Our microinjection experiments suggest that having an enzymatically dead but physically intact CSB molecule is still better than having no protein at all. We suggest that the partial activity observed may be due to the fact that the enzymatically inactive CSB mutant protein may help stabilize a CSB-containing complex or otherwise permits this complex to function better when CSB is physically present. In fact, this is very similar to findings made with a comparable Lys to Arg substitution in the XPD helicase of TFIIH.[3] For the transcription function of the TFIIH complex, this protein must be physically present to permit complex formation or stability, but it does not have to be enzymatically functioning. For the NER function of the TFIIH complex, the XPD helicase activity is at least in part required. Also for other protein complexes it has been shown that absence of one subunits leads to instability of the complex (*e.g.* Ref. 53). Consistent with the above interpretations for the partial activity of mutant CSB is the notion that the conservative Lys to Arg amino acid substitution is not expected to dramatically alter the overall structure of the CSB protein. Because we observe a biological effect, the dtCSB (K538R) mutant protein probably still fits into the RNA polymerase II complex. Our finding that the same type of amino acid substitution in the XPB and XPD helicase subunits of TFIIH exerts at least some biological effects strongly suggests that also these mutant proteins are still capable of complex formation. The intriguing finding that an enzymatically inactive CSB protein still exert some function *in vivo* supports the idea that interaction of CSB with other transcription/repair proteins *per se* contributes to its function(s).

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