

The ATPase Domain but Not the Acidic Region of Cockayne Syndrome Group B Gene Product Is Essential for DNA Repair

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Cockayne syndrome (CS) is a human genetic disorder characterized by UV sensitivity, developmental abnormalities, and premature aging. Two of the genes involved, *CSA* and *CSB*, are required for transcription-coupled repair (TCR), a subpathway of nucleotide excision repair that removes certain lesions rapidly and efficiently from the transcribed strand of active genes. CS proteins have also been implicated in the recovery of transcription after certain types of DNA damage such as those lesions induced by UV light. In this study, site-directed mutations have been introduced to the human *CSB* gene to investigate the functional significance of the conserved ATPase domain and of a highly acidic region of the protein. The *CSB* mutant alleles were tested for genetic complementation of UV-sensitive phenotypes in the human CS-B homologue of hamster UV61. In addition, the *CSB* mutant alleles were tested for their ability to complement the sensitivity of UV61 cells to the carcinogen 4-nitroquinoline-1-oxide (4-NQO), which introduces bulky DNA adducts repaired by global genome repair. Point mutation of a highly conserved glutamic acid residue in ATPase motif II abolished the ability of *CSB* protein to complement the UV-sensitive phenotypes of survival, RNA synthesis recovery, and gene-specific repair. These data indicate that the integrity of the ATPase domain is critical for *CSB* function in vivo. Likewise, the *CSB* ATPase point mutant failed to confer cellular resistance to 4-NQO, suggesting that ATP hydrolysis is required for *CSB* function in a TCR-independent pathway. On the contrary, a large deletion of the acidic region of *CSB* protein did not impair the genetic function in the processing of either UV- or 4-NQO-induced DNA damage. Thus the acidic region of *CSB* is likely to be dispensable for DNA repair, whereas the ATPase domain is essential for *CSB* function in both TCR-dependent and -independent pathways.

INTRODUCTION

Cockayne syndrome (CS) is an autosomal recessive human disorder with diverse clinical symptoms that include severe mental and physical growth retardation, microcephaly, progressive neurological and retinal degeneration, skeletal abnormalities, and a hypersensitivity to sunlight (Friedberg, 1996). Genetic analysis of fused heterodikaryons have identified two complementation groups involved in CS, desig-

nated *CSA* and *CSB* (Tanaka *et al.*, 1981; Lehmann, 1982). CS cells demonstrate a reduced rate of nucleotide excision repair (NER) of active genes and, more specifically, of the transcribed strand of such genes (Venema *et al.*, 1990; van Hoffen *et al.*, 1993; Evans and Bohr, 1994). However, no defect in global NER is observed in CS. Defective transcription-coupled repair (TCR) in CS cells is found not only after UV exposure but also after exposure to certain forms of oxidative stress, suggesting that TCR is responsible for processing at least some types of oxidative DNA damage as well (Leadon and Cooper, 1993; Cooper and Leadon, 1994; Cooper *et al.*, 1997).

A characteristic feature of CS cells is the lack of recovery of RNA synthesis after UV irradiation (Lehmann *et al.*, 1979; Lehmann, 1982; Mayne and Lehmann, 1982). The *CSB* gene

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Abbreviations used: CPD, cyclobutane pyrimidine dimer; CS, Cockayne syndrome; NER, nucleotide excision repair; 4-NQO, 4-nitroquinoline-1-oxide; nt, nucleotide; RNAPII, RNA polymerase II; TCA, trichloroacetic acid; TCR, transcription-coupled repair.

was originally cloned by its ability to complement the delay in RNA synthesis recovery displayed in the hamster mutant cell line UV61 isolated from the repair-proficient parental cell line AA8. Transfection of the human *CSB* gene into UV61 effectively restored RNA synthesis and UV resistance to normal levels (Troelstra *et al.*, 1990). Gene-specific repair studies have demonstrated that the human *CSB* gene corrects the TCR defect in the hamster CS-B mutant UV61 (Orren *et al.*, 1996). Thus the hamster system has been extremely useful to demonstrate that the human *CSB* gene is directly implicated in TCR. Subsequent studies confirmed that the *CSB* gene complements the UV sensitivity and the delay in RNA synthesis recovery of the human CS-B cell line CS1AN.S3.G2 (Troelstra *et al.*, 1992). By sequence homology, the *CSB* gene product belongs to superfamily 2 and, more specifically, the SWI/SNF family of proteins, which have roles in transcription regulation, chromosome stability, and DNA repair (Eisen *et al.*, 1995). All proteins belonging to the SWI/SNF family contain seven sequence motifs similar to those found in DNA and RNA helicase families. The *CSB* protein is a DNA-stimulated ATPase but fails to exhibit DNA unwinding activity as measured by the conventional strand displacement assay (Selby and Sancar, 1997a; Tantin *et al.*, 1997). It is quite possible that *CSB* may play a role in chromatin remodeling as has been suggested for the SWI/SNF complex and related proteins in the gene family (Pazin and Kadonaga, 1997). However, some members of SWI/SNF subfamilies, such as MOT1, appear to function independently of chromatin (Pazin and Kadonaga, 1997). The mechanism of how ATP hydrolysis is coupled to the diverse roles of these proteins in chromatin regulation, stability, and repair remains to be determined.

The mechanism of TCR in mammalian cells and the precise role of *CSB* in this pathway remain to be elucidated. Using a biochemical approach to address the role of *CSB* protein in TCR, Tantin *et al.* (1997) have demonstrated that hydrolysis of the ATP β - γ phosphoanhydride bond is required for the formation of a stable RNA polymerase II (RNAPII)-*CSB*-DNA-RNA complex. However, *in vitro* studies indicate that *CSB* protein does not release RNAPII stalled at a T<>T lesion from the DNA (Selby and Sancar, 1997A). Furthermore, *CSB* protein has no effect on repair of a lesion protected by a stalled RNAPII (Selby *et al.*, 1997). Most recently, it was shown that the RNAPII-*CSB*-DNA-RNA quaternary complex recruits a molecular complex containing the TFIIH core subunits p62 and XPB (Tantin, 1998). This finding suggests that *CSB* may facilitate repair of active genes by recruiting proteins involved in an early step of DNA damage recognition (Tantin, 1998). More studies are necessary to understand the role of *CSB* in the mechanism of TCR.

TCR may not be the only pathway defective in CS. There is some evidence for a defect in basal transcription. The transcription defect has been observed in human CS-B lymphoblastoid cells and fibroblasts without any exposure to stress such as UV light (Balajee *et al.*, 1997). The reduced transcription in CS-B cells is complemented in chromatin by the addition of normal cell extract and in intact cells by transfection with the *CSB* gene (Balajee *et al.*, 1997). In a reconstituted system, purified *CSB* protein enhances the rate of transcription by RNAPII, suggesting that *CSB* protein may indirectly stimulate TCR by facilitating the process of

transcription (Selby and Sancar, 1997b). Conceivably *CSB* may serve dual roles as a transcription elongation factor and a repair coupling factor at the site of the RNAPII-blocking lesion *in vivo*. Direct involvement of *CSB* protein in transcription is supported by the finding that 10–15% of RNAPII is tightly associated with *CSB* protein in whole-cell extracts (van Gool *et al.*, 1997). Thus, the CS phenotype may arise from a combined deficiency in repair and transcription. It is possible that the biological functions of *CSB* in different DNA metabolic pathways may be mediated by distinct functional domains of the protein.

Limited information is available addressing the functional significance of specific domains in the *CSB* protein. *In vitro* data suggest that ATP hydrolysis is required for the formation of a stable quaternary complex between *CSB* and a stalled RNAPII on a DNA template (Tantin *et al.*, 1997). However, it was recently shown that a *CSB* mutant protein totally defective in ATP hydrolysis was able to partially rescue the delay in RNA synthesis recovery after exposure to UV light (Citterio *et al.*, 1998). These data suggest that *CSB*-catalyzed ATP hydrolysis plays a role in TCR but may not be essential for the pathway to proceed.

In addition to the ATPase domain, *CSB* contains a negatively charged region located in the amino terminus before the ATPase and helicase motifs. Approximately 60% of the residues in a 39-amino-acid stretch are acidic. Acidic domains are found in a number of nuclear proteins that associate with chromatin or histones, suggesting that this domain may play a role in chromatin remodeling or transcriptional activation (Lapeyre *et al.*, 1987; Ptashne, 1988; Sung *et al.*, 1988; Wen *et al.*, 1989). The fact that *CSB* belongs to a gene family containing members implicated in chromatin remodeling (for review, see Pazin and Kadonaga, 1997) suggests that the acidic region may be important to *CSB* function. In addition to a putative role in chromatin remodeling, acidic regions have been implicated in the function of transcriptional activator proteins (Ma and Ptashne, 1987; Hope *et al.*, 1988). In fact, acidic transcriptional activation domains have been shown to directly interact with the RNA-Pol II transcription machinery. For example, the acidic activation domain of VP16 directly binds to TATA box-binding factor TFIID (Stringer *et al.*, 1990) as well as RNAPII initiation factor TFIIB (Gupta *et al.*, 1996). The functional significance of the strikingly rich acidic region in *CSB* protein is presently unknown but it may play a role in the repair and/or transcription functions of *CSB*.

To gain a better understanding of the functional importance of different domains in the *CSB* protein in DNA repair, site-specific mutations were introduced in motif II of the ATPase domain and the acidic region of *CSB* protein (Figure 1). We have characterized the sensitivity of the CS-B mutant cell lines to two DNA-damaging agents, UV light and the chemical carcinogen 4-nitroquinoline-1-oxide (4-NQO). The lesions induced by these agents are processed by different repair pathways. The most abundant UV-induced lesions, cyclobutane pyrimidine dimers (CPDs), are repaired by TCR (Mellon *et al.*, 1986). In contrast, 4-NQO induces both alkali-labile single-strand DNA breaks and UvrABC excinuclease-cleavable bulky adducts, which are repaired without any strand bias in mammalian cells (Snyderwine and Bohr, 1992). Genetic characterization of the ATPase mutant indicates that replacement of the highly conserved glutamic acid

in motif II with glutamine abolishes the genetic function of the CSB protein in survival, RNA synthesis recovery, and gene-specific repair upon treatment with UV light. In contrast, mutations in the acidic region of CSB do not impair the ability of CSB protein to repair UV-induced damage. Similarly, the ATPase mutant failed to complement the sensitivity of the CS-B mutant cell line to 4-NQO, whereas the acidic mutants confer resistance to 4-NQO. These results provide genetic evidence that the ATPase activity of CSB protein is essential in TCR as well as in a TCR-independent pathway.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

AA8 and UV61, the hamster cell lines used in this study, and their repair characteristics have been previously described (Mitchell and Nairn, 1989). AA8 is a repair-proficient wild-type cell line. UV61 cells, derived from AA8, belong to rodent complementation group 6 and are homologous to human CS-B cells based on genetic complementation by the human *CSB* gene (Troelstra *et al.*, 1990; Orren *et al.*, 1996). UV61 cell lines transfected with the mammalian expression vector pcDNA3.1 (Invitrogen, San Diego, CA; abbreviated pc3.1) or pc3.1 containing the wild-type human *CSB* gene are designated UV61/pc3.1 and UV61/pc3.1-CSBwt, respectively. UV61 cells transfected with pc3.1-CSB containing various mutations in the *CSB* gene were designated UV61/pc3.1-CSBE646Q, UV61/pc3.1-CSBAC377, and UV61/pc3.1-CSBACMUT1, respectively. All the cell lines were routinely grown in Ham's F-10 and Dulbecco's minimal essential medium (1:1) supplemented with 10% fetal bovine serum and antibiotics. UV61 cell lines transfected with pc3.1, which contains the neomycin resistance gene, or pc3.1 derivatives were grown in media containing 400 μ g/ml geneticin (Life Technologies, Gaithersburg, MD).

DNA, Nucleotides, and Enzymes

pcBLsE6, a plasmid containing the entire human *CSB* cDNA, was kindly provided by Dr. Jan Hoeijmakers (Rotterdam University, Rotterdam, The Netherlands). Nucleotides were purchased from Boehringer Mannheim (Indianapolis, IN). Primers used for site-directed mutagenesis, sequencing, reverse transcription, and PCR were purchased from Life Technologies. Restriction endonucleases *Sall*, *XhoI*, and *PvuI*, phage T7 DNA polymerase, calf intestinal alkaline phosphatase, phage T4 polynucleotide kinase, and DNA ligase were purchased from New England Biolabs (Beverly, MA). Restriction endonuclease *KpnI* was from Boehringer Mannheim. T4 Endo V was purified from an overproducing strain. Ready to Go T4 DNA ligase was from Amersham Pharmacia Biotech (Piscataway, NJ). The reaction conditions were essentially the same as suggested by the supplier.

Site-directed Mutagenesis and DNA Constructions

pcBLsE6, a bacterial phagemid containing the *f1* origin of replication and the entire human *CSB* gene, was used for site-directed mutagenesis by published procedures (Kunkel *et al.*, 1991). A description of the mutations introduced in the *CSB* gene is shown in Figure 1. The oligonucleotide 5'-TTGTGTCCTGGTCCAAGATC-3' was used to replace the negatively charged Glu⁶⁴⁶ in the CSB protein to a neutral glutamine (E646Q). The oligonucleotide 5'-CTCTGCCCTCCACTGTGGGGAAATACTC-3' was used to precisely delete 10 consecutive acidic residues, 377–386, in the CSB protein (AC377). The oligonucleotide 5'-CACCTCGTCAGCTGCTCCGCTGCCTCCTC-3' was used to replace Glu³⁸¹, Glu³⁸², Glu³⁸⁴, and Asp³⁸⁵ with neutral alanine residues (ACMUT1).

The *CSB* gene, containing the mutant or wild-type sequence, was cloned into the mammalian expression vector pc3.1 to yield pc3.1-

CSB. Briefly, pcBLsE6 was digested to completion with *Sall* and a 4.7-kb linear DNA fragment containing the entire *CSB* gene was excised from a 0.8% agarose gel and purified using Gene-Clean II kit (BIO 101, La Jolla, CA). pc3.1 was digested with *XhoI* to completion, and the 5.4-kb linear DNA fragment was excised from a 0.8% agarose gel and purified using Gene-Clean II. The 5' phosphates were removed from the linear 5.4-kb DNA fragments using calf intestinal alkaline phosphatase, and the 4.7-kb DNA fragment containing the *CSB* gene was ligated into pc3.1 using Ready to Go T4 DNA ligase. Site-directed mutations in the *CSB* gene in each construct were verified by sequencing. The entire *CSB* coding sequence of pc3.1-CSBwt and pc3.1-CSBE646Q was sequenced to verify that the coding sequences of the two plasmids were identical with the exception of the site-specific mutation.

Construction and Selection of UV61 Stable Transfectant Cell Lines

CsCl-purified pc3.1-CSB plasmids (100 μ g) were linearized with *PvuI* (200 U) at 37°C for 16 h. The DNA sample was treated with proteinase K (200 μ g/ml) in 1 M lithium acetate and 0.1% SDS at 50°C for 2 h, phenol extracted, and ethanol precipitated. UV61 cells were transfected with pc3.1 and pc3.1-CSB plasmids using liposomes (*N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium-methyl sulfate; Boehringer Mannheim) according to manufacturer's procedure. Briefly, 250,000 cells were seeded in a 3-cm² dish and allowed to grow until 50% confluent. To these cells were added 40 μ g of *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium-methyl sulfate and 10 μ g linearized plasmid in 1 ml of media. The cells were incubated with the liposome-DNA mixture for 6 h at 37°C. The media were then replaced, and cells were grown for an additional 48 h at 37°C. The cells were trypsinized and transferred to a 10-cm² dish, and geneticin was added to a final concentration of 400 μ g/ml for selection of antibiotic-resistant cells. After 10 d of selection, the surviving cells were trypsinized and seeded for isolation of clones (50 cells per 10-cm² dish). Individual colonies were isolated and reseeded for UV and 4-NQO experiments.

Analysis of CSB Expression

Whole-cell lysate and cytoplasmic and nuclear extracts of hamster CS-B transfectant cell lines were tested by immunoblot to detect CSB protein using an affinity-purified rabbit polyclonal antibody against a recombinant fragment of the human CSB protein (amino acids 528-1222) graciously provided by Drs. C. Selby and A. Sancar (University of North Carolina, Chapel Hill, NC) (Selby and Sancar, 1997a). Although the CSB antibody effectively reacted with native CSB protein expressed in human cell lines GM0637D and HeLa by immunoblot analysis (our unpublished results), we were unable to detect CSB protein in the UV-resistant hamster cell line UV61/pc3.1-CSBwt. The lack of detection of CSB protein in the hamster transfectant cell line may be accounted for by several explanations. The level of CSB protein may be quite low in the hamster CS-B transfectant cell line but at a sufficient level to complement the TCR deficiency by the wild-type CSB protein. Alternatively, the CSB protein may be poorly recognized by the CSB antibody when the human protein is present in a preparation (whole-cell lysate or cytoplasmic or nuclear fraction) in a background of hamster proteins.

Two approaches were used to examine the integrity and relative amounts of CSB transcripts from UV61 transfectant cell lines. To evaluate the expression of intact *CSB* transcript in isolated clones of UV61 transfectants, RNA was isolated and evaluated by RT-PCR using *CSB*-specific primers. The first-strand synthesis was performed according to the method of Mallery *et al.* (1998) with the following modifications. RNA was extracted from 10⁷ cells using RNA STAT-60 (Tel-Test, Friendswood, TX) according to manufacturer's protocol. cDNA synthesis was performed by incubating 5 μ g of total RNA, 1.5 μ g oligo(dT)₁₂₋₁₈ primer (Life Technologies), and

500 U of Superscript II (Life Technologies) at 44°C for 60 min. Samples were subsequently processed using the manufacturer's recommended procedure for Superscript II. For PCR amplification of cDNA products, 5 U of AmpliTaq Gold (Perkin Elmer, Norwalk, CT) were used according to the manufacturer's procedures. The 4.7-kb cDNA product was amplified as six overlapping fragments ranging in size from ~0.6 to 1.5 kb using primers and annealing conditions previously described (Mallery *et al.*, 1998). DNA products were verified by electrophoresis on a 1.2% agarose-1× Tris acetate-EDTA gel and analysis by ethidium bromide staining (our unpublished results). For sequencing, PCR products were purified using a QIAquick PCR purification kit (Qiagen, Hilden, Germany) and analyzed using an Applied Biosystems (Foster City, CA) automatic sequencer. Comparison of sequence analysis of cDNA obtained from UV61/pc3.1-CSBE646Q, UV61/pc3.1-CSBAC377, or UV61/pc3.1-CSBACMUT1 with UV61/pc3.1-CSBwt verified the presence of the engineered mutation at the defined position (our unpublished results). The entire cDNAs of UV61/pc3.1-CSBwt and UV61/pc3.1-CSBE646Q were sequenced to verify that the only difference between the wild-type and E646Q mutant CSB genes was the engineered mutation (our unpublished results).

To quantitate the relative levels of CSB expression in UV61/pc3.1-CSBwt and UV61/pc3.1-CSBE646Q, we performed relative quantitative RT-PCR experiments (QuantumRNA module; Ambion, Austin, TX). The QuantumRNA module provides a method for comparing relative transcript abundances standardized by coamplification of a highly conserved fragment of 18S rRNA as an invariant internal standard. The CSB transcript and 18S rRNA were analyzed during the exponential phase of amplification to determine an accurate level of relative expression. Initial experiments were performed to determine the optimal conditions for the linear range of amplification of the CSB fragment. Once this was established, the amplification efficiency of the 18S rRNA standard was optimized to the efficiency of the amplification of the CSB transcript by adjusting the ratio of 18S:competimers. Competimers are modified 18S rRNA primers blocked at their 3' ends to prevent extension by DNA polymerase (QuantumRNA module). PCR cycling of the CSB fragment and 18S RNA standard was performed in a single tube. For CSB cDNA amplification, the primers 5'-GGTGTAGGTGGCTGTGGGAATT-3' (3F) and 5'-GTATCTCGTAAGACACACATGCACAC-3' (3R) were used, which produce a 671-bp product from the coding sequence of CSB mRNA (Mallery *et al.*, 1998). The RNA used for cDNA synthesis was isolated from UV61 transfectant cell lines by the procedure described above. cDNA synthesis was performed by incubating 2.5 µg of total RNA, 250 ng of random decamer primer (Ambion), and 500 U of Superscript II at 42°C for 50 min. For PCR amplification of cDNA products, 5 U of AmpliTaq Gold (Perkin-Elmer) were used according to the manufacturer's procedures. In addition to the standard reaction components, PCR mixtures contained 20 pmol of CSB primers 3F and 3R, 20 pmol of 18S RNA primer:competimer (9:1), and [α -³²P]dCTP (0.1 µCi/µl) in a total volume of 50 µl. Reaction products were amplified by 31 cycles (94°C, 1 min; 67°C, 1.5 min; and 72°C, 3 min). Ten microliters of the reaction products were mixed with 10 µl of 2× denaturing gel loading buffer (95% formamide) and denatured at 95°C for 3 min. The samples were loaded onto an 8 M urea-6% polyacrylamide gel. Radiolabeled DNA species in polyacrylamide gels were visualized using a PhosphorImager and quantitated using the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). The ratio of the counts of the upper CSB band (671 nucleotides [nt]) and the lower 18S RNA standard (488 nt) band was determined.

The results of the relative RT-PCR experiments are shown in Figure 2. The ratios of CSB transcript to 18S RNA standard (671-nt CSB fragment:488-nt 18S RNA internal standard) for UV61/pc3.1-CSBwt and UV61/pc3.1-CSBE646Q were determined to be 1.68 ± 0.05 and 1.37 ± 0.15 , respectively. The results demonstrate that the wild-type and mutant CSB transcripts are expressed with nearly the same efficiency in the UV61 transfectant cell lines.

UV and 4-NQO Survival Assays

Chinese hamster cell AA8 and UV61 transfectants were trypsinized, and 300 cells were seeded per 10-cm² dish and allowed to grow overnight. For UV treatment, the cells were washed once with PBS and then irradiated at the indicated doses of UV light (254 nm). The cells were grown for 6 d, washed once in PBS, and fixed with methanol for 10 min. The fixed cells were then stained with methylene blue and washed once in PBS, and blue colonies were counted to determine the clonogenic survival of cells. For 4-NQO treatment, the cells were washed once with PBS and incubated in media lacking serum but containing 4-NQO at the indicated concentrations for 1 h at 37°C. Cells were washed once with PBS, and the media were replaced with media supplemented with 10% fetal bovine serum and antibiotics. The cells were then allowed to grow for 6 d and treated as described above to determine colony-forming units.

RNA Synthesis Recovery

UV61 transfectant cell lines were grown in the presence of [¹⁴C]thymidine (0.02 µCi/ml) for 3 d to uniformly label the DNA. For UV experiments, the cells were washed with PBS and irradiated with UV light (254 nm) at the indicated doses. For 4-NQO experiments, the cells were washed with PBS and exposed to the indicated concentrations of 4-NQO in serum-free media for 60 min. Cells were restored to complete media and incubated for 16 h. Cells were subsequently pulse labeled with 5 µCi/ml [³H]uridine for 60 min at 37°C, washed once with PBS, and lysed in 10 mM Tris, pH 8.0, 1 mM EDTA buffer containing 0.5% SDS and 100 µg/ml proteinase K for 2 h at 37°C. Trichloroacetic acid (TCA) was added to the cell lysate at a final concentration of 10%, and the samples were then spotted onto glass fiber discs (Whatman, Maidstone, United Kingdom). The filters were sequentially washed in 5% TCA, 70% ethanol, and acetone. The TCA-precipitable radioactivity was then scintillation counted.

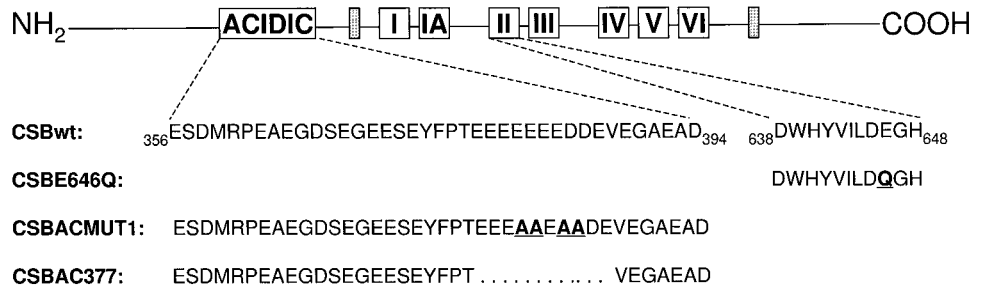
Gene-specific Repair

Treatment of cells in culture with UV light and subsequent isolation of genomic DNA was essentially performed according to previously published techniques (Bohr and Okumoto, 1988). In brief, cells were either untreated or irradiated with UV light (254 nm) at a dose of 20 J/m². The cells were harvested (0 h) or incubated in fresh medium containing bromodeoxyuridine and fluorodeoxyuridine and harvested after 8 or 24 h. Cells were lysed by a proteinase K-SDS treatment, and total genomic DNA was extracted after an NaCl salting out protocol (Miller *et al.*, 1988). Genomic DNA was treated with RNase A (100 g/ml) and *Kpn*I restriction enzyme as described previously (Bohr and Okumoto, 1988). The resulting restriction fragment (14 kb) resides entirely within the 5' end of the *DHFR* gene (May *et al.*, 1993). Unreplicated DNA was isolated from replicated DNA (containing bromodeoxyuridine) in CsCl density gradients, dialyzed against Tris, pH 8.0, 1 mM EDTA buffer, and ethanol precipitated. All DNA concentrations were measured spectrophotometrically at 260 nm.

The presence of CPDs in DNA samples was measured in an enzymatic assay as described previously (Bohr *et al.*, 1985). In brief, unreplicated DNA samples (10 µg) from the different time points were incubated with T4 endonuclease V in a reaction buffer containing 10 mM Tris, pH 8.0, 100 mM NaCl, and 10 mM EDTA for 20 min at 37°C, and the reactions were stopped by the addition of alkaline loading dye containing 10 mM EDTA and 500 mM NaOH. In parallel, comparable reactions minus T4 endonuclease V were performed to determine the original distribution of restriction fragments.

T4 endonuclease V-treated and untreated DNA samples were electrophoresed under alkaline conditions on a 0.5% agarose gel. The DNA was transferred to a Hybond N+ membrane (Amersham Pharmacia Biotech) by Posiblot (Stratagene, La Jolla, CA) using standard protocols (Bohr and Okumoto, 1988). The membrane was

Figure 1. Site-directed mutations introduced in the ATPase motif II and the acidic region of CSB. The CSB protein contains the ATPase and helicase motifs conserved in superfamily 2 as well as a negatively charged domain in which 22 of 39 residues are acidic (56%). The E646Q mutation in CSB changes the highly conserved glutamic acid in ATPase motif II to glutamine (CSBE646Q). The ACMUT1 mutation replaces acidic residues at positions 381, 382, 384, and 385 with alanines (CSBACMUT1). The AC377 mutation deletes 10 consecutive acidic residues (378–387) from the CSB protein (CSBAC377).



baked at 80°C for 2 h before treatment with prehybridization buffer (0.342 M Na₂HPO₄ + 0.088 M NaH₂PO₄, pH 7.2, 7% SDS, and 2 mM EDTA) for a minimum of 2 h. Double-stranded DNA probes were synthesized with a random-primed labeling kit (Amersham Pharmacia Biotech) using a 3.4-kb *DHFR* gene template as described (May *et al.*, 1993). Membranes were hybridized with the probe at 68°C overnight and washed to remove nonhybridized probe. The blots were visualized using a PhosphorImager and quantitated using the ImageQuant software. By comparison of untreated and T4 endonuclease V-treated DNA samples, the average number of CPDs per fragment was determined from the zero class of the Poisson distribution. Percent repair is calculated as the frequency of lesions remaining at a given time point compared with the CPD frequency at time zero. In some experiments, negative values for repair were obtained as observed previously (Orren *et al.*, 1996), indicating no detectable repair.

RESULTS

To evaluate the relative biological importance of the ATPase and acidic domains of CSB protein, site-specific mutations were introduced into these motifs, and the CSB mutant alleles were transfected into the hamster CS-B homologue UV61. Specifically, a point mutation in the ATPase domain of CSB, designated CSBE646Q, replaced the highly conserved glutamic acid found in a large number of ATPases and helicases with a neutral glutamine (Figure 1). In addition, two mutations were engineered in a stretch of 10 consecutive acidic residues in a negatively charged region of CSB located in the amino terminus before the helicase motifs. CSBAC377 is a CSB deletion mutant in which the entire 10-acidic-residue stretch is removed from the protein (Figure 1). CSBMUT1 is a mutant in which four acidic residues in the acidic core are replaced with neutral alanines (Figure 1). The CSB mutant alleles were cloned behind a cytomegalovirus strong promoter in the mammalian expression vector pc3.1. The CSB expression plasmids were transfected into the hamster CS-B homologue UV61, and isogenic stable transfectants were obtained for studies. Quantitation of relative amounts of CSB transcript from the cell lines UV61/pc3.1-CSBwt and UV61/pc3.1-CSBE646Q demonstrated very similar levels of expression (Figure 2). However, the inability to detect CSB protein in the hamster transfectant cell lines implies that we cannot dismiss the possibility that CSB protein levels may differ between the cell lines because of translation and degradation effects. We examined repair and transcription of CS-B transfectants by genetic complementation assays after treatment with the DNA-damaging

agents UV light and 4-NQO. The phenotypes measured to assess DNA damage sensitivity included 1) viability, 2) RNA synthesis recovery, and 3) gene-specific DNA repair.

UV Sensitivity of CS-B Transfectants

We determined the relative UV sensitivity of various CS-B-transfected clonal cell lines compared with AA8 by clonogenic survival assays. As shown in Figure 3, CSBE646Q, containing a point mutation in motif II of the ATPase domain, was severely compromised in its ability to complement the UV-sensitive phenotype exhibited by the vector alone (UV61/pc3.1), whereas the wild-type CSB allele restored UV resistance. In contrast to CSBE646Q, the acidic

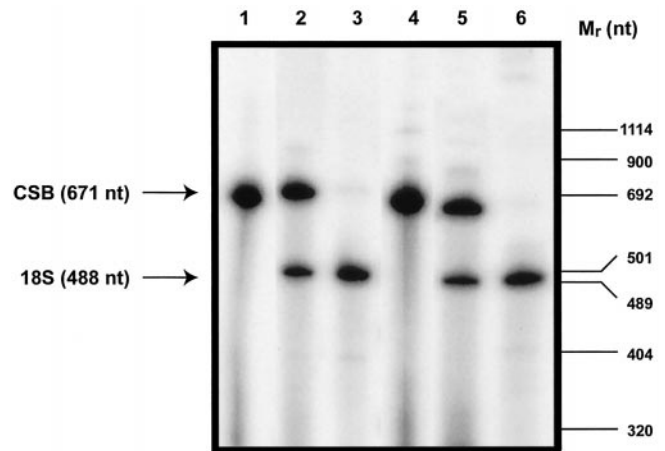


Figure 2. Relative quantitative RT-PCR of CSB transcripts isolated from UV61/pc3.1-CSBwt and UV61/pc3.1-CSBE646Q. CSB expression in hamster transfectant cell lines was determined relative to the internal 18S rRNA standard as described in MATERIALS AND METHODS. RT-PCR products were electrophoresed on an 8 M urea-6% polyacrylamide gel. The RT-PCR products from the CSB mRNA and the 18S rRNA using site-specific CSB and 18S primers respectively are as follows: lanes 1–3, UV61/pc3.1-CSBwt using CSB primers (lane 1), CSB primers plus 18S RNA primers (lane 2), and 18S RNA primers (lane 3); lanes 4–6, UV61/pc3.1-CSBE646Q using CSB primers (lane 4), CSB primers plus 18S RNA primers (lane 5), and 18S RNA primers (lane 6).

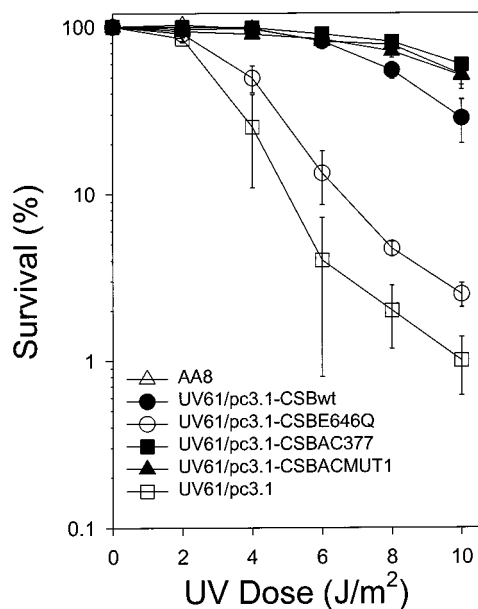


Figure 3. UV sensitivity of AA8 and isogenic clonal populations of UV61 transfectant cell lines. Cells were irradiated with UV light as described in Materials and Methods. Data (percent survival) are expressed as the number of UV-irradiated cells forming colonies as a fraction of the colonies formed by unirradiated cells and represent the average of at least three independent experiments.

mutants *CSBACMUT1* and *CSBAC377* fully complemented the UV sensitivity to a level comparable with UV61/pc3.1-CSBwt.

The viability studies clearly show that replacement of the highly conserved glutamic acid in the ATPase domain of CSB with a neutral glutamine dramatically impairs the function of the CSB protein. Thus the integrity of the ATPase domain of CSB is critical for cellular resistance to UV light. On the contrary, deletion of 10 consecutive acidic residues in the highly negatively charged motif of CSB does not impair the genetic pathway conferring UV resistance. These data suggest that the acidic region, which resides outside the ATPase and helicase motifs, is dispensable, whereas the ATPase domain of CSB is important for cell survival after UV-induced DNA damage.

RNA Synthesis Recovery after UV Treatment

To further characterize the molecular defects giving rise to the increased cell killing of CS-B mutant cell lines by UV light, we examined RNA synthesis recovery in clonal populations of UV61 cell lines transfected with wild-type or site-specific mutant *CSB* genes (Figure 4). Recovery of RNA synthesis was an early hallmark of the CS phenotype (Mayne and Lehmann, 1982). This assay is generally thought to reflect TCR. RNA synthesis was measured by [³H]uridine incorporation during a 60-min pulse 16 h after exposure of cells to UV light. Cells were either unirradiated or irradiated with 3, 6, 9, or 12 J/m². After a dose of 3 J/m², transcription levels in UV61/pc3.1 and UV61/pc3.1-CSBE646Q were reduced to approximately one-half the level of the parental cell

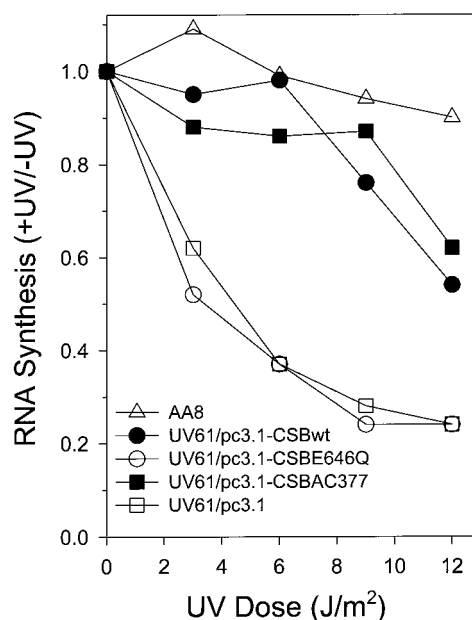


Figure 4. RNA synthesis recovery of UV61 transfectant cell lines after UV irradiation with increasing dose. Cells unirradiated or UV irradiated with the indicated dose were pulse labeled with [³H]uridine 16 h after irradiation, and acid-insoluble radioactivity was determined.

line AA8. In contrast, UV61 cell lines transfected with either pc3.1-CSBwt or pc3.1-CSBAC377 showed a level of transcription only slightly reduced compared with AA8. At a UV dose of 6 J/m², the RNA synthesis of UV61/pc3.1-CSBE646Q and UV61/pc3.1 was reduced to 0.37, but UV61/pc3.1-CSBwt remained close to 1 (0.98). UV61/pc3.1-CSBAC377 also retained a high level of RNA synthesis (0.86), only slightly reduced compared with UV61/pc3.1-CSBwt. Similar results were obtained at a UV dose of 9 J/m². At 12 J/m², the recovery of RNA synthesis was compromised for UV61/pc3.1-CSBwt and UV61/pc3.1-CSBAC377 but still nearly threefold greater than either UV61/pc3.1 or UV61/pc3.1-CSBE646Q. These results correlate well with the viability studies. The failure of the ATPase point mutant to complement the RNA synthesis inhibition in UV61 cells strongly suggests that ATPase activity is instrumental for CSB protein to genetically function in the TCR pathway. In contrast, deletion of 10 consecutive acidic residues in the acidic region of CSB does not impair RNA synthesis recovery, indicating that the acidic domain of CSB is not likely to be important for its repair function.

Gene-specific Repair of UV-induced DNA Damage

To directly measure the ability of CSB mutants to function in preferential repair of UV-induced DNA damage, gene-specific repair experiments were performed. We measured the induction and removal of CPDs in the *DHFR* housekeeping gene in AA8 and UV61 transfectant cell lines. Quantitative Southern blots were used to measure the amount of CPDs in the *DHFR* gene 8 and 24 h after irradiation (Figure 5). Seventy-one percent of the CPDs in the *DHFR* gene in AA8

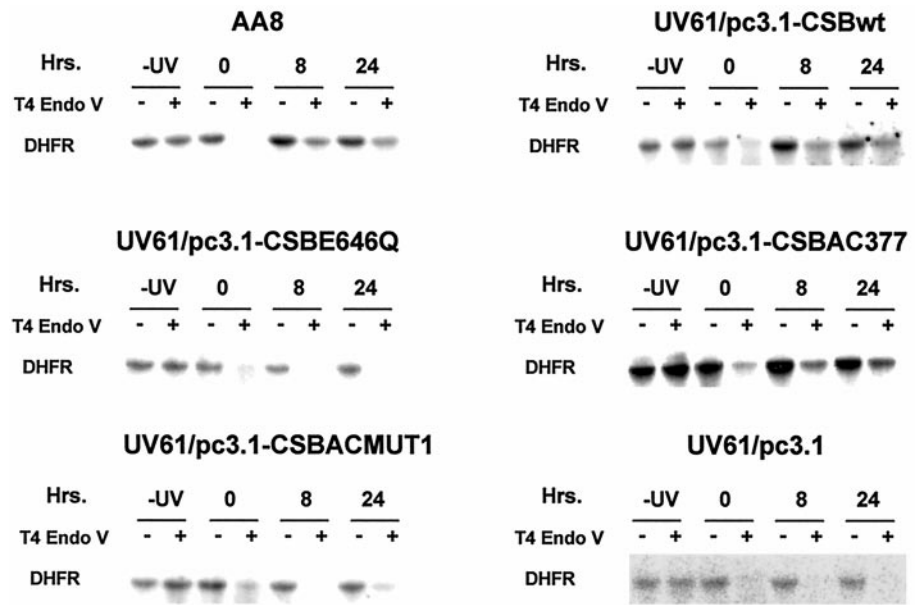


Figure 5. Formation and removal of CPDs in the *DHFR* gene in AA8 and UV61 transfectant cell lines. Genomic DNA (10 μ g) was isolated from either unirradiated (-UV) or UV irradiated (20 J/m²) cells at 0, 8, and 24 h after exposure as described in MATERIALS AND METHODS. The DNA was subsequently digested with *KpnI* and either treated with T4 endonuclease V (+) or untreated (-). DNA was electrophoresed through an alkaline agarose gel (0.5%) and quantitatively transferred to a nylon membrane. The membrane was hybridized to ³²P-labeled denatured double-stranded DNA probe for the *DHFR* gene. Hybridization of gene-specific ³²P-labeled *DHFR* probes to membranes was detected by PhosphorImager analysis.

were repaired by 24 h compared with 43% repair in UV61/pc3.1-CSBwt (Table 1). Both acidic mutant cell lines UV61/pc3.1-CSBAC377 and UV61/pc3.1-CSBMUT1 demonstrated repair to the level of the wild-type CS-B transfectant, indicating that these CSB mutant proteins are competent in TCR. In contrast, no repair of CPDs was detected in UV61/pc3.1-CSBE646Q or UV61/pc3.1 at 8 or 24 h, indicating that the E646Q mutant CSB protein was completely nonfunctional in TCR (Table 1). The gene-specific repair analysis demonstrates that the ATPase point mutation has rendered CSB protein defective in removing CPDs. The repair deficiency observed in UV61/pc3.1-CSBE646Q is consistent with the increased UV sensitivity and prolonged transcription inhibition after UV damage. In contrast, both the AC377 and ACMUT1 acidic mutants retained the ability to preferentially repair UV-induced DNA damage in an active gene. We thus do not observe any phenotypic defect in TCR with the acidic mutations.

4-NQO Survival Studies

The genotoxin 4-NQO introduces a number of types of DNA damage, including bulky lesions, oxidative damage such as

8-hydroxyguanine, and strand breaks (Friedberg *et al.*, 1995). However, unlike UV-induced CPDs, the 4-NQO-induced bulky adducts recognized by UvrABC excinuclease are repaired without strand bias (Snyderwine and Bohr, 1992) and thus without transcription coupling. To characterize the functional significance of the ATPase and acidic domains of CSB in this TCR-independent pathway, the respective CSB mutant alleles were examined for their abilities to complement the 4-NQO-sensitive phenotype of CS-B mutant cell line UV61 as previously demonstrated by clonogenic survival assays (Wade and Chu, 1979). Clonal populations of the CS-B transfectant cell lines were evaluated for survival as a function of 4-NQO dose (Figure 6). The ATPase point mutant *CSBE646Q* failed to complement UV61 in resistance to 4-NQO, whereas the wild-type *CSB* allele, introduced by the same plasmid during transfection, exhibited complementation. The acidic mutant *CSB* allele *CSBAC377* complemented the 4-NQO sensitivity to a level similar to that of the wild-type *CSB* allele. We conclude that the *CSBE646Q* allele fails to complement the 4-NQO-sensitive phenotype, reflecting a phenotype similar to that observed with UV light.

RNA Synthesis Recovery after 4-NQO Treatment

To further characterize the sensitivity of CS-B cell lines to the DNA-damaging agent 4-NQO, recovery of RNA synthesis after exposure of cells to 4-NQO was measured (Figure 7). At all doses tested, UV61/pc3.1 and UV61/pc3.1-CSBE646Q exhibited closely matched reduced levels of [³H]uridine incorporation compared with the 4-NQO-resistant lines UV61/pc3.1-CSBwt and UV61/pc3.1-CSBAC377. The peak reduction in RNA synthesis of UV61/pc3.1-CSBE646Q, ~50% of the control, was attained at a minimal 4-NQO concentration of 0.6 μ g/ml. At this concentration, both UV61/pc3.1-CSBwt and UV61/pc3.1-CSBAC377 exhibited levels of RNA synthesis ≥ 1 . These results demonstrate that the E646Q replacement inactivated the genetic function of CSB to restore transcription after 4-NQO-induced DNA

Table 1. Induction and removal of CPD in the *DHFR* gene

Cell line	Initial CPD	Repair (%)	
		8 h	24 h
AA8	1.55 \pm 0.05	35.5 \pm 4.95	71.5 \pm 10.6
UV61/pc3.1-CSBwt	1.53 \pm 0.06	20.2 \pm 10.7	46.3 \pm 15.28
UV61/pc3.1-CSBAC377	1.72 \pm 0.13	26.1 \pm 7.64	51.9 \pm 8.46
UV61/pc3.1-CSBACMUT1	1.45 \pm 0.19	27.1 \pm 10.14	37.9 \pm 12.10
UV61/pc3.1-CSBE646Q	1.35 \pm 0.12	-17.1 \pm 6.42	-16.4 \pm 9.04
UV61/pc3.1	1.39 \pm 0.06	5.7 \pm 5.41	-4.6 \pm 6.8

Mean \pm SD are calculated from three independent experiments.

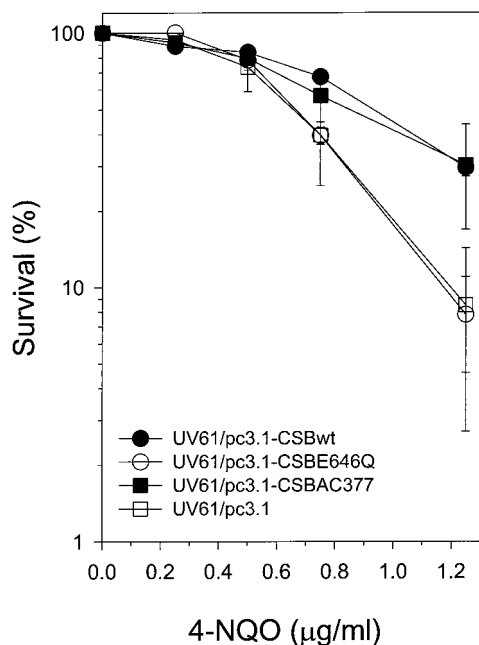


Figure 6. 4-NQO sensitivity of AA8 and isogenic clonal populations of UV61 transfectant cell lines. Cells were treated with 4-NQO as described in MATERIALS AND METHODS. Data (percent survival) are expressed as the number of 4-NQO-treated cells forming colonies as a fraction of the colonies formed by untreated cells and represent the average of at least three independent experiments.

damage. On the contrary, the CSBAC377 mutant protein with a deletion of the core 10 acidic residues in the largely negatively charged region of CSB facilitated RNA synthesis recovery, suggesting that the acidic domain is not important for processing DNA damage adducts induced by either 4-NQO or UV.

DISCUSSION

The results in this study demonstrate that the single amino acid substitution of glutamine for an invariant glutamic acid in motif II of the ATPase domain severely reduced the function of CSB protein in cellular pathways conferring resistance to UV light. Specifically, the CSBE646Q mutant was seriously compromised in its ability to complement the UV sensitivity of the hamster CSB homologue UV61 by a colony-forming survival assay. Moreover, the CSB ATPase point mutant failed to function in RNA synthesis recovery and gene-specific repair. The genetic data suggest that ATP hydrolysis catalyzed by CSB protein is an important step in TCR of UV-induced DNA damage *in vivo*. Abrogation of CSB function in the TCR pathway by the ATPase point mutation resulted in UV-induced cell killing and failure to resume RNA synthesis.

The genetic results presented here suggest that CSB functions in TCR by a step involving ATP hydrolysis. This notion is consistent with biochemical data that demonstrate that hydrolysis of the ATP β - γ phosphoanhydride bond is required for the interaction of CSB with a stalled RNAPII on a

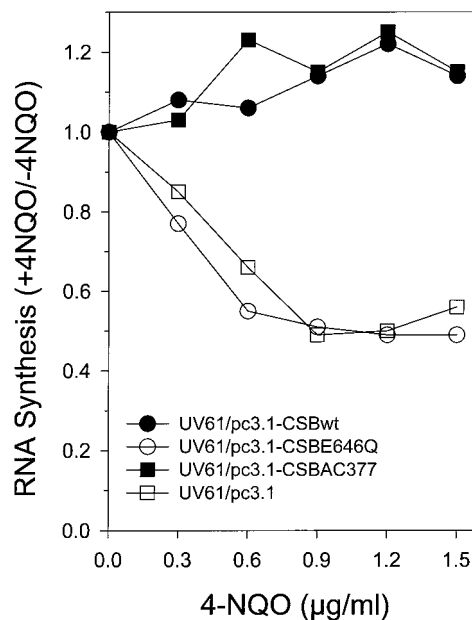


Figure 7. RNA synthesis recovery of UV61 transfectant cell lines after 4-NQO treatment with increasing dose. Cells unexposed or exposed to 4-NQO at the indicated dose were pulse labeled with [3 H]uridine 16 h after treatment, and acid-insoluble radioactivity was determined.

DNA template (Tantin *et al.*, 1997). The ability of the RNA-Pol II-CSB-DNA-RNA quaternary complex to recruit a molecular complex containing the TFIIH core subunits p62 and XPB provides a biochemical basis to facilitate repair of active genes (Tantin, 1998). However, the precise role of CSB-catalyzed ATP hydrolysis in TCR remains to be elucidated. It is quite likely that the enzymatic turnover of ATP may be important in the role of CSB protein as a repair-coupling factor. Alternatively, ATP hydrolysis may be essential to the function of CSB as a transcription elongation factor. Stimulation of the efficiency and rate of RNAPII transcription would boost the number of encounters of RNAPII with sites of DNA damage, which may serve as the damage-signaling function in mammalian TCR. Thus CSB would stimulate the rate of lesion removal from the transcribed strand by increasing the efficiency and rate of RNAPII transcription. Either possibility would explain the genetic deficiency of the CSBE646Q mutant in TCR.

The functional significance of ATP hydrolysis by CSB protein has also been examined in the immortalized human CS-B fibroblast cell line CS1AN (Citterio *et al.*, 1998). In these studies, replacement of the invariant lysine in motif I of the ATPase domain with an arginine (K538R) abolished the ATPase activity of the protein. Purified mutant and wild-type CSB proteins were microinjected into the cytoplasm of CS1AN fibroblasts, and recovery of RNA synthesis was measured 16–20 h after exposure to UV light (15 J/m²). The authors observed 90% recovery of RNA synthesis by cells injected with the wild-type CSB protein compared with basal transcription of unirradiated cells (Citterio *et al.*, 1998). In contrast, the level of RNA synthesis of cells injected with the K538R mutant protein was restored to 38% of that

of the control. CS1AN cells that were not injected with any CSB protein, mutant or wild-type, exhibited only 22% of the level of RNA synthesis of unirradiated cells. The 1.7-fold difference in RNA synthesis recovery after UV exposure between CS1AN injected with K538R mutant protein and CS1AN not injected with CSB protein suggested that the catalytically inactive mutant CSB protein retained partial function in vivo. Citterio *et al.* (1998) proposed that the enzymatically inactive mutant CSB-K538R protein possibly functions to stabilize a CSB-containing protein complex (involving RNAPII?), enabling the complex to retain residual activity. A previous demonstration of a stable association of a fraction of RNAPII (10–15%) with wild-type CSB in cell-free extracts (van Gool *et al.*, 1997) is consistent with this proposal.

The results in this study clearly show that the E646Q substitution in motif II of the ATPase domain severely reduced the ability of the CSB protein to complement the UV-sensitive phenotypes of survival, RNA synthesis recovery, and gene-specific repair of the hamster cell line UV61. The slightly higher levels of cell survival of UV61/pc3.1-CSBE646Q compared with UV61/pc3.1 may suggest that the ATPase point mutant CSB protein may weakly function to confer UV resistance; however, the difference between UV61/pc3.1-CSBwt and UV61/pc3.1-CSBE646Q is an order of magnitude at the higher doses of UV light (8 and 10 J/m²), suggesting that the E646Q mutation has greatly diminished the biological function of CSB protein. In addition, the CSBE646Q mutant completely failed to complement the UV-sensitive phenotypes of RNA synthesis recovery and gene-specific repair of the UV61 cell line, consistent with the notion that the mutant CSB protein fails to function in TCR.

Based on the UV survival data (Figure 3), it is possible that the CSBE646Q mutant protein retains residual biological activity. It was previously shown that mutations in *swi2* that reduce ATP hydrolysis impair cellular transcription of *SWI/SNF*-dependent genes and abolish chromatin remodeling in vitro by the reconstituted complex (Laurent *et al.*, 1993; Richmond and Peterson, 1996). A double point mutation of acidic residues D894 and E895 of SWI2, which are conserved in motif II of the ERCC6 and SWI/SNF subfamilies, resulted in a complete loss of transcription activity for yeast SWI2/SNF2 (Richmond and Peterson, 1996). The SWI2 D894AE895A mutant protein could be incorporated into a multiprotein complex in vivo, although the complex was less stable in vitro. Richmond and Peterson (1996) concluded that *swi2* mutations in ATPase motif II do not grossly impair SWI/SNF complex assembly. More recently, it was reported that a yeast *swi2K798A* allele containing a point mutation in motif I of the ATPase domain supported a wild-type growth rate in contrast to the reduced viability of the *swi2* deletion mutant allele (Biggar and Crabtree, 1999). These genetic data suggest that there may be residual ATP-independent activity of the SWI2 protein. Likewise, it is possible that the ATPase mutant CSB protein is able to weakly complement the UV-sensitive phenotype.

The dramatic loss of RNA synthesis recovery after UV treatment of the E646Q mutant versus the significantly compromised but residual function of the K538R mutant (Citterio *et al.*, 1998) may be explained by a number of reasons, some of which will be considered here. First, we used a hamster cell line that was originally used to identify the human CSB gene by cross-complementation, whereas Citterio

et al. (1998) used an SV40-transformed human CS-B cell line. It is certainly possible that the difference in genetic background may influence the amount, stability, or activity of the CSB protein. Second, the site-directed mutations introduced to the CSB protein are directed at two spatially separated sequence motifs and may differentially impact CSB function in vivo. Crystal structure data (Subramanya *et al.*, 1996; Korolev *et al.*, 1997) and mutational analysis (Sung *et al.*, 1988; George *et al.*, 1994; Brosh and Matson, 1995) have implicated both motifs I and II in nucleotide binding and/or hydrolysis. Motif I, which contains the signature NTP-binding sequence GSGKS, is responsible for binding the triphosphate tail of the ATP. Motif II, which contains the DEXH sequence, is involved in binding Mg⁺² via the acidic residues and is required for hydrolysis of ATP in these enzymes. Thus the conserved residues of both domains play an important role in ATP hydrolysis. It should be pointed out, however, that the phenotypic difference between the CSBK538R and CSBE646Q mutant cell lines may reflect the differential effect of the point mutations on the CSB protein. In both studies the recovery of RNA synthesis of CS-B cells with the ATPase mutant CSB protein present was significantly reduced compared with cells containing the wild-type CSB protein, indicating that mutations in either domain seriously affect the function of CSB in vivo. These data are consistent with the notion that CSB exerts its effect on the TCR pathway by a step involving ATP hydrolysis.

A third consideration to explain the lack of genetic complementation by the CSBE646Q allele is that the mutant protein is rendered unstable by the amino acid replacement. Contrary to this notion, a number of studies in which similar motif II mutations have been made to ATPases and helicases have shown that the stability of the mutant proteins did not change (Pause and Sonenberg, 1992; Jindal *et al.*, 1994; Brosh and Matson, 1995; Richmond and Peterson, 1996; Washington *et al.*, 1996). In addition, biochemical analysis of the mutant proteins has demonstrated that properties other than ATP hydrolysis or unwinding activity have remained intact (Pause and Sonenberg, 1992; Jindal *et al.*, 1994; Brosh and Matson, 1995; Richmond and Peterson, 1996; Washington *et al.*, 1996). For example, motif II point mutants retain nucleotide binding (UvrD, eIF-4A, and NS-1), DNA binding (UvrD and T7 primase and helicase), and protein interaction (T7 primase and helicase and Swi2). These findings indicate that replacement of the highly conserved acidic residues in motif II with neutral amino acids does not grossly impair the overall structure of these proteins. These results suggest that the lack of genetic function of the CSBE646Q allele is not likely to be a consequence of protein destabilization because of the single amino acid substitution.

In contrast to the critical importance of the ATPase domain of CSB, genetic characterization of UV61/pc3.1-CSBAC377 suggests that the acidic region is not important in TCR. The CSBAC377 mutant is characterized by a deletion of 10 consecutive acidic residues in the "core" of a negatively charged domain in the CSB protein. The possibility exists that the remaining 11 acidic residues of the region fulfill the functional requirement of CSB in TCR. Similarly, the replacement of four acidic residues in the core of the acidic region with neutral alanine residues did not impact the function of CSB protein. Perhaps the acidic domain of CSB has some other specialized function not detected by the

genetic assays used in this study. An interesting area to explore is the transcriptional status of various CS-B mutant cell lines. The presence of an acidic region in the domain responsible for activation of the transcriptional machinery in a large class of transcriptional activators suggests an important role in transcription (Ma and Ptashne, 1987; Hope *et al.*, 1988). In addition, functional domains of the CSB protein, such as the acidic region, may be important in processing of DNA damage other than UV-induced photoproducts. For example, the molecular functions required of CSB protein to process oxidative DNA damage have not been addressed in this study. Protein-protein interactions, mediated by the acidic domain of CSB, may be essential in one of these pathways. Further studies using a structure-function approach should prove useful in understanding the requirements of CSB protein in DNA repair of various types of DNA damage as well as the process of transcription. In a recent study, Mallery *et al.* (1998) have analyzed the location of mutations in the CSB gene in CS patients. Their findings demonstrate that the site or nature of the mutation (truncation vs. amino acid substitution) did not correlate well with the severity of the clinical features. These data suggest fairly complex relationships among genotype, molecular phenotype, and clinical phenotype of CS.

In this study we examined some repair characteristics of UV61 transfectant cell lines exposed to the chemical carcinogen 4-NQO. 4-NQO has been referred to as a "UV-mimetic" agent because it elicits similar repair mechanisms to correct DNA damage induced by the compound (Kondo and Kato, 1968; Ikenaga *et al.*, 1975) and because XP cells are sensitive to it (Takebe *et al.*, 1972). NER is responsible for repairing bulky 4-NQO adducts in a manner similar to UV-induced pyrimidine dimers. CS and XP cells have been previously shown to exhibit a hypersensitivity to 4-NQO compared with normal cells, as demonstrated by clonogenic survival assays (Wade and Chu, 1979). However, Snyderwine and Bohr (1992) demonstrated that the gene-specific repair characteristics operating for UV dimers and 4-NQO adducts are different from one another. UV dimers are repaired faster in actively transcribing genes than inactive regions. In contrast, 4-NQO adducts, recognized by UvrABC excinuclease, are repaired without any bias toward the transcriptionally active genes. In addition, there is no preferential repair of these 4-NQO adducts in the transcribed strand over the nontranscribed strand. Collectively these data indicate that 4-NQO adducts are processed by a pathway that is different from TCR of UV-induced DNA damage. Aside from the bulky adducts, several types of oxidative lesions are introduced to DNA in cells exposed to 4-NQO (Gallegue-Zouitina *et al.*, 1985; Kohda *et al.*, 1987). Defective TCR of oxidative base damage has been shown in CS cells (CS-B) (Leadon and Cooper, 1993) and XP-G/CS (Cooper and Leadon, 1994; Cooper *et al.*, 1997), suggesting that oxidative damage may also play a role in the hypersensitivity of CS cell lines to 4-NQO (Wade and Chu, 1979). Therefore, it was interesting to determine what effect mutations in the CSB gene would have on the sensitivity of CS-B transfectant cell lines to 4-NQO. Our results demonstrate that the E646Q (ATPase) point mutation inactivates CSB function as determined by clonogenic survival assays and RNA synthesis recovery after 4-NQO treatment. The requirement for an intact ATPase domain of CSB to complement the deficiencies

in survival and RNA synthesis after treatment with either UV light or 4-NQO suggests that ATP hydrolysis is important in a TCR-dependent pathway (operational for UV-induced CPDs) as well as a TCR-independent pathway. We propose that the molecular function of CSB to confer resistance to 4-NQO is likely to be a mechanism that allows the cell to overcome transcription inhibition by DNA adducts.

It was originally suggested by this laboratory that specific alterations to DNA structure, such as lesion size or degree of distortion caused by the adduct, may explain the lack of TCR of some adducts (Snyderwine and Bohr, 1992). 4-NQO-induced adducts recognized by UvrABC excinuclease, such as UV-induced pyrimidine 6-4 pyrimidone photoproducts, are efficiently removed by general global repair. Other forms of DNA damage, such as UV-induced CPDs, are predominantly repaired by TCR. Despite the existence of a global repair pathway for bulky 4-NQO adducts, RNA synthesis of CS-B cells fails to resume after 4-NQO treatment. Recent studies have also demonstrated a lack of TCR repair of *N*-acetoxy-2-acetylaminofluorene (N-AAF) adducts, yet the drug is extremely cytotoxic and inhibits transcription (van Oosterwijk *et al.*, 1996, 1998). Similar repair kinetics of *N*-(deoxyguanosine-8-yl)-2-acetylaminofluorene in CS cells compared with normal cells suggested that the elevated drug sensitivity and RNA synthesis inhibition of CS cells by NA-AAF are not consequences of defective TCR. It is conceivable that the lesions induced by 4-NQO or NA-AAF titrate out a critical factor required for transcription initiation and/or elongation. Recent work has suggested that proteins involved in RNAPII transcription may be titrated or hijacked by DNA damage (Vichi *et al.*, 1997; You *et al.*, 1998; Cullinane *et al.*, 1999). Also, a model was proposed in which CS proteins act as repair-transcription uncoupling factors allowing the basal transcription factor TFIIF to convert from a repair mode to a transcription initiation mode (van Oosterwijk *et al.*, 1996, 1998). Presumably, an ATPase-defective CSB protein fails to recycle TFIIF from global genome repair to transcription. Further molecular studies will be necessary to clearly address the functions of CS proteins in TCR-dependent and -independent pathways.

These studies demonstrate that the integrity of the ATPase domain of CSB is essential for the biological function of the protein in UV-resistant phenotypes of survival, RNA synthesis recovery, and TCR. Likewise, the ATPase domain plays a pivotal role in the genetic pathway responsible for the cellular resistance to DNA damage introduced by the chemical carcinogen 4-NQO. Further structure-function studies should provide insight to the molecular functions of CSB in pathways relating to DNA damage, repair, and transcription.

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