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The Cockayne Syndrome Group A Gene Encodes a WD Repeat Protein That Interacts with CSB Protein and a Subunit of RNA Polymerase II TFIIH

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Summary

The hereditary disease Cockayne syndrome (CS) is characterized by a complex clinical phenotype. CS cells are abnormally sensitive to ultraviolet radiation and are defective in the repair of transcriptionally active genes. The cloned *CSB* gene encodes a member of a protein family that includes the yeast Snf2 protein, a component of the transcriptional regulator Swi/Snf. We report the cloning of the *CSA* cDNA, which can encode a WD repeat protein. Mutations in the cDNA have been identified in CS-A cell lines. CSA protein interacts with CSB protein and with p44 protein, a subunit of the human RNA polymerase II transcription factor IIH. These observations suggest that the products of the *CSA* and *CSB* genes are involved in transcription.

Introduction

Cockayne syndrome (CS) is a rare autosomal recessive disease characterized primarily by postnatal failure of growth and progressive neurological dysfunction (reviewed by Friedberg et al., 1995). A recent comprehensive review of the clinical features of 140 cases (Nance and Berry, 1992) indicates that growth failure is a cardinal clinical feature in all cases and generally manifests in the first year of life. Body weight tends to be affected more severely than body length, leading to a characteristic appearance of so-called cachectic dwarfism. All CS patients develop neurological symptoms or signs, of which the most common are mental retardation and microcephaly (Nance and Berry, 1992). The majority of CS patients also suffer ophthalmic changes, in particular pigmentary degeneration of the retina and cataracts. Dental abnormalities are common, and about 75% of patients manifest abnormal sensitivity to sunlight. The mean age of death in 34 cases was ~12 years, but some patients have survived into their teens and twenties (Nance and Berry, 1992). Laboratory tests are not diagnostic. However, increased ventricular size, cerebral atrophy, or both, as well as calcification of the basal ganglia, are frequently observed by computerized tomography or magnetic resonance imaging of the brain. Histopathology of the brain at autopsy typically reveals patchy demyelination of subcortical matter (Nance and Berry, 1992).

The observation of photosensitivity in many cases of CS prompted studies of radiation sensitivity at the cellular level. Lymphoblastoid and fibroblast cell lines from all known CS patients have increased sensitivity to killing by ultraviolet (UV) radiation at ~254 nm (Schmickel et al., 1977; Andrews et al., 1978; Hoar and Waghorne, 1978; Wade and Chu, 1979; Marshall et al., 1980; Lehmann et al., 1993). Conventional measurements of nucleotide excision repair, the repair mode primarily involved in the repair of DNA damage produced by UV radiation, indicate no defect in CS cells (reviewed by Friedberg et al., 1995). However, in contrast with normal human cells, which perform nucleotide excision repair of the template (transcribed) strand of transcriptionally active genes at a faster rate than the coding (nontranscribed) strand, CS cells fail to show this kinetic difference and are considered to be defective in strand-specific repair of transcriptionally active genes (Mullenders et al., 1992; van Hoffen et al., 1993). This phenotype may also be reflected in the observation that CS cells are defective in the reactivation of the expression of a plasmid-borne chloramphenicol acetyltransferase (*CAT*) reporter gene when this gene is inactivated by irradiation of the plasmid prior to transfection of cells (Schweiger et al., 1987; Barrett et al., 1991).

CS cells in culture suffer a significant delay in the recovery of bulk DNA and RNA synthesis following exposure to UV radiation (Lehmann et al., 1979; Lehmann, 1982; Mayne and Lehmann, 1982; Sugita et al., 1987; Lehmann et al., 1993). The delayed kinetics of the recovery of RNA synthesis have been exploited for complementation analysis in heterodikaryons derived by fusion of cells from different CS patients. This analysis has defined two genetic complementation groups, designated CS-A and CS-B (Tanaka et al., 1981; Lehmann, 1982). A human gene (originally called *ERCC6*) was isolated by correction of the UV sensitivity of a Chinese hamster ovary cell line designated UV61 (Troelstra et al., 1990). Subsequent studies showed that the *ERCC6* gene corrects the cellular phenotypes of

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CS-B cells, but is without effect on the UV sensitivity of CS-A cells, or cells from the nucleotide excision repair–defective disorder xeroderma pigmentosum (XP) (Troelstra et al., 1992). Sequence analysis of the *ERCC6* gene from CS-B patients has revealed the presence of mutations (Troelstra et al., 1992; D. Mallery and A. R. L., unpublished data). In light of these observations and in keeping with recent nomenclatural recommendations (Lehmann et al., 1994), the *ERCC6* gene is now referred to as *CSB*.

The molecular basis of the complex clinical phenotype of CS is unknown. Here we report the molecular cloning of the human *CSA* gene by functional complementation of CS-A cells. A *CSA* cDNA uniquely and specifically corrects UV sensitivity and defective *CAT* gene reactivation in CS-A cells. Additionally, mutations have been identified in *CSA* cDNAs of all CS-A cell lines examined, including an identical mutation in two CS-A siblings. The *CSA* gene is expected to encode a polypeptide of 396 amino acids with a calculated molecular mass of ~44 kDa. The predicted amino acid sequence of the putative CSA protein indicates that it is a WD repeat (WD-40 repeat) protein (reviewed by Neer et al., 1994). In vitro translated CSA protein specifically interacts with CSB protein and with p44 protein, a subunit of the RNA polymerase II basal transcription factor TFIIH (Drapkin et al., 1994; Roy et al., 1994; Mu et al., 1995; Aboussekhra et al., 1995). These observations, coupled with the observation that the amino acid sequence of the predicted CSB polypeptide is homologous with a component of the yeast Swi/Snf transcriptional activation complex, suggest that CS cells may be defective in RNA polymerase II transcription.

Results

Functional Complementation of CS-A Cells

A human cDNA library constructed from size-fractionated cDNA cloned in an episomal plasmid vector (Legerski and Peterson, 1992) was screened for functional complementation of the UV radiation–sensitive phenotype of CS-A cells. No UV-resistant colonies were obtained after the screening of ~400,000 hygromycin-resistant colonies obtained by electroporation with a fraction enriched for cDNAs >4 kb in size. A single UV-resistant colony was observed after screening ~200,000 hygromycin-resistant colonies with a fraction enriched for cDNA inserts in the 2–4 kb size range. The UV-resistant clonal line was expanded, and the plasmids were rescued to Escherichia coli. Restriction analysis revealed that all the plasmids were identical and contained a cDNA insert of ~2.2 kb. This plasmid is designated pCSA5 and was purified for further analysis.

To verify that the increase in resistance to UV radiation was effected by a plasmid-borne element rather than by mutational reversion in the mass culture caused by exposure of the cells to UV light, plasmid pCSA5 was retransfected into CS-A cells. These cells express the Epstein–Barr virus (EBV) nuclear antigen 1 (EBNA1) protein. Hence, the EBV-based vectors used in these studies replicate autonomously as episomal plasmids, circumventing the need to analyze numerous clonal lines to identify a particular line that expresses the cDNA. A population of ~2000 independent transfectants not previously exposed to UV light was analyzed for complementation of the UV sensitivity of CS-A cells. Survival was quantitated by colony-forming ability and expressed as a percentage relative to unirradi-

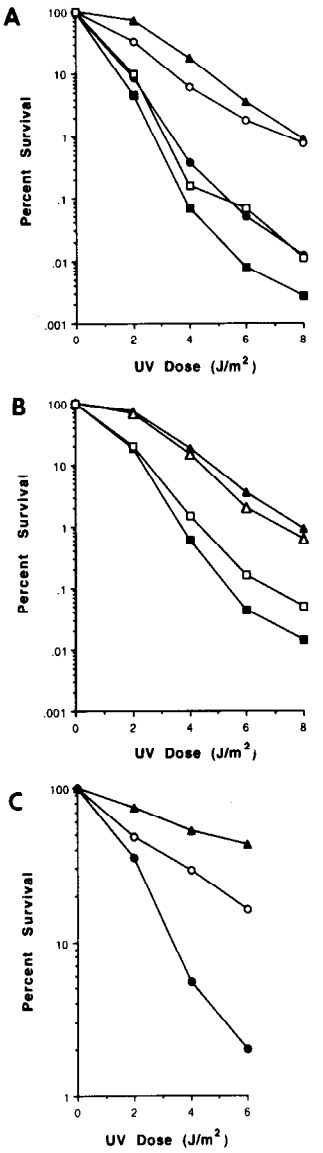

Figure 1. Complementation of UV Radiation Sensitivity of CS-A Cells, But Not CS-B or XP-C Cells, by Plasmid pCSA5

(A) Survival after exposure to UV radiation of the repair-proficient line HT1080 (closed triangles), the CS-A line CSA/EN12 (closed circles), CSA/EN12 cells carrying plasmid pCSA5 (open circles), the CS-B line CSB/EN7 (closed squares), and CSB/EN7 carrying pCSA5 (open squares).

(B) Survival after exposure to UV radiation of the repair-proficient line HT1080 (closed triangles), an XP-C line XPC/EN12 (closed squares), XPC/EN12 cells carrying plasmid pXPC3, which expresses the human *XPC* cDNA (open triangles), and XPC/EN12 cells carrying pCSA5 (open squares).

(C) Survival after exposure to UV radiation of the normal lymphoblastoid line GM1953 (closed triangles), the CS-A lymphoblastoid line GM2964 carrying the vector (closed circles), and GM2964 cells carrying plasmid pCSA5 (open circles).

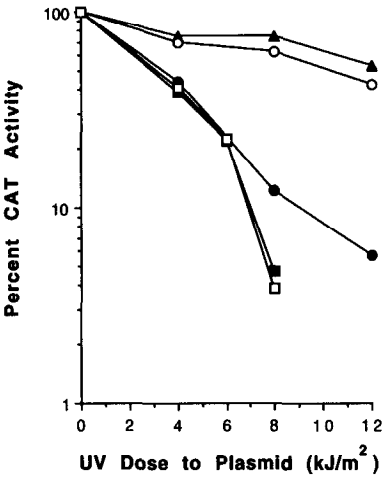

Figure 2. Complementation of *CAT* Reactivation Ability of CS-A But Not CS-B Cells by Plasmid pCSA5

CAT activity is expressed as a percentage of the activity observed in irradiated cells versus unirradiated controls. Repair-proficient line HT1080 (closed triangles); CS-A line CSA/EN12 (closed circles); CSA/EN12 cells carrying plasmid pCSA5 (open circles); CS-B line CSB/EN7 (closed squares); CSB/EN7 cells with plasmid pCSA5 (open squares).

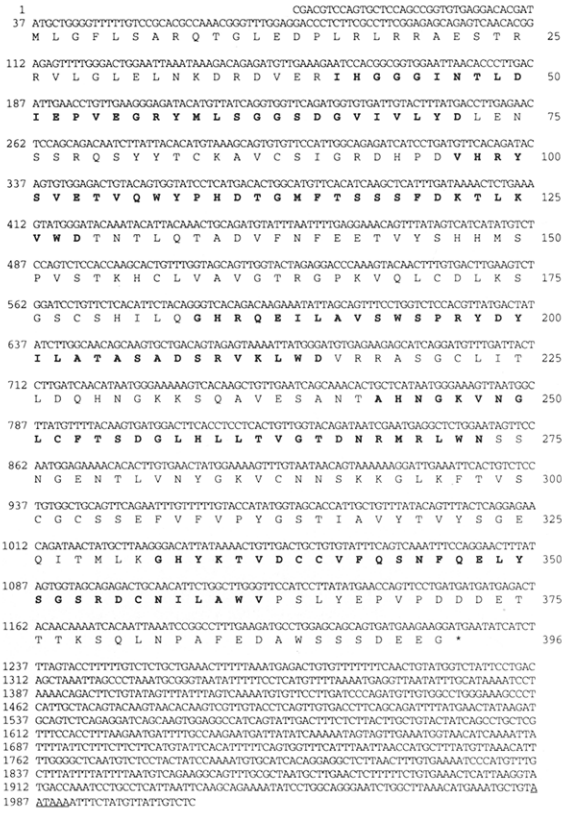

Figure 3. Nucleotide and Predicted Amino Acid Sequence of the *CSA* cDNA

The cDNA insert of clone pCSA5 was sequenced on both strands. The amino acids in core regions of the WD repeat motifs are shown in bold. The polyadenylation signal AATAAA in the 3′ untranslated region is underlined.

ated controls. Once again, plasmid pCSA5 conferred enhanced radiation resistance to CS-A cells (Figure 1A).

In experiments designed to determine the genetic specificity of this phenotypic complementation, the plasmid was also transfected into CS-B cells and cells from XP genetic complementation group C (XP-C). The latter cells are known to be hypersensitive to UV radiation because of a defect in nucleotide excision repair (reviewed by Friedberg et al., 1995). The plasmid did not enhance the UV radiation resistance of either of these cell types, verifying that the UV resistance is specific for CS-A cells (Figures 1A and 1B). To exclude the possibility that the UV resistance was an artifact of the particular cell line used, plasmid pCSA5 was also electroporated into the CS-A lymphoblastoid cell line GM2964 derived from a different CS-A individual. Once again, we observed enhanced UV resistance compared with that in the presence of the vector alone (Figure 1C). This result simultaneously demonstrates that the cloned cDNA complements the CS cellular phenotype in both lymphoblastoid and fibroblast cell lines. The level of phenotypic correction observed with the lymphoblastoid line was not as complete as that observed with SV40-transformed fibroblasts. This may in part reflect limitations of the survival procedure used with the lymphoblastoid cells, which were grown in suspension culture (see Experimental Procedures).

To confirm that the enhanced resistance to UV radiation correlates with an increased capacity for DNA repair, we measured the ability of cells to reactivate the expression of an inactivated (by exposure to UV radiation) *CAT* reporter gene. CS-A cells carrying plasmid pCSA5 showed significantly enhanced levels of CAT activity compared with those in the parental cell line (Figure 2). Transfection of CS-B cells with the plasmid did not result in reactivation of *CAT* gene expression (Figure 2), once again demonstrating the genetic specificity of the complementation with plasmid pCSA5.

Sequence Analysis of the *CSA* cDNA

The cDNA insert of clone pCSA5 (designated as the *CSA* cDNA) was sequenced on both strands (Figure 3). The first ATG at nucleotide 37 is preceded by G at the −3 position, where a purine residue has been shown to be important for the initiation of translation (Kozak, 1986). An open reading frame that could encode a 396 amino acid polypeptide terminates at nucleotide position 1224, followed by an extended 3′ untranslated region. The poly(A) addition signal AATAAA begins at nucleotide position 1986, and a poly(A) tail begins at nucleotide 2011 (Figure 3). The calculated size of the predicted CSA protein is 44 kDa, with a calculated isoelectric point of 6.32 as determined by the Peptidesort program of Genetics Computer Group. No significant matches were found by searching the GenBank, EMBL, or dbEST databases with the *CSA* cDNA sequence by using the BLASTN program. However, by use of the BLASTX program, significant scores were observed with a number of WD repeat proteins (reviewed by Neer et al., 1994), including the Xenopus laevis β-Trcp

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                                                              {2 of 3}
                           A  A                                A           A  A
                           C  C                                M  Y Y      C  C
                           M  M       I                        C  CCC      M  M
WD-Repeat           V      F  F       L       D       D        F  AAA      F  F  K
Consensus           A      V  V       W       G       G        V  TTT      V  V YR
Regular             S      I  I {1,7} Y {0,3} N {0,2} N {0,4}  I  SSS      I  I FN
Expression          GH XXX LXXL   X   F   X   P   X   P   X    L  GGGX D XXLX L WD

CSA repeat 1        IH GGG INTL   D   I   E   P   VE  G   R   YML SGGS D GVIV L YD
CSA repeat 2        VH RYS VETV   Q   W   Y   P   H   D   TG  MFT SSSF D KTLK V WD
CSA repeat 3        GH RQE ILAV   S   W   S   P   RY  D   Y   ILA TASA D SRVK L WD
CSA repeat 4        AH NGK VNGL   C   F   TS  D       G   L   HLL TVGT D NRMR L WN
CSA repeat 5        GH YKT VDCC   V   F   QS  N   F   Q       ELY SGSR D CNIL A WV
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Figure 4. Alignment of the Conserved Regions of the WD Repeats in the Predicted CSA Protein Sequence

The WD repeat regions of the CSA sequence are aligned by use of published criteria (Neer et al., 1994). The regular expression pattern is shown at the top of the figure, where X connotes any amino acid, and curly brackets indicate the range over which that amino acid can be repeated.

protein, the Saccharomyces cerevisiae Tup1 protein, the human Miller–Dieker lissencephaly protein, and the human β-transducin protein. Further analysis of the predicted CSA polypeptide sequence revealed the presence of five WD repeat motifs (Figure 4). In the first repeat, the consensus amino acid sequence G/S/A/V, H, which marks the beginning of the repeated motif, is replaced by I, H (Figure 4), and in repeat 5, the consensus sequence W/F/Y, D/N/R/K, which denotes the end of the motif, is replaced by W, V (Figure 4). The length of the core between the GH and WD sequences in each repeat of the predicted CSA protein is 34 or 35 amino acids, consistent with the range of 23–41 amino acids observed in other members of this class of proteins (reviewed by Neer et al., 1994). Also consistent with the structure of this class of proteins, two of the five repeats (repeats 2 and 3) have no mismatches with the consensus core repeat sequence (Figure 4), while the remaining three repeats (repeats 1, 4, and 5) each have three or fewer mismatches (Figure 4) (reviewed by Neer et al., 1994).

Mutational Analysis of the *CSA* Gene

Polymerase chain reaction (PCR) primers were prepared that facilitated amplification of the *CSA* coding region in two overlapping segments. The primer pairs used for the amplification of the 5′ and 3′ halves of *CSA* cDNAs were designated M1–M2 and M3–M4, respectively. First-strand cDNAs prepared from the CS-A primary fibroblast cell lines CS5BR and CS6BR, the normal primary human fibroblast cell line CCD-42Sk (CRL1513), or HeLa cells were used as substrates in the PCR reaction. Agarose gel electrophoresis of the PCR products derived from CS5BR cDNA by using the M1–M2 primer pair showed a single band that comigrated with the PCR band derived from HeLa cDNA. This band was gel purified and directly sequenced with an automated sequencer and was found to be completely congruent with the wild-type sequence. Electrophoresis of the PCR products derived from the CS5BR cell line by using the M3–M4 primer pair revealed two *CSA*-specific bands, both of which migrated faster than the single band derived from the normal human fibroblast cell line or from HeLa cells (Figure 5A). The faster-migrating band was gel purified and directly sequenced. This analysis revealed a 279 bp deletion in the *CSA* coding region, starting at nucleotide position 880 and ending at nucleotide position 1158 (Figure 5B). The slower-migrating CS5BR band was contaminated by a nonspecific PCR product. Hence, this band was cloned into the vector pBSII, and two independently isolated clones were sequenced. This analysis demonstrated the presence of an identical 81 bp deletion in the *CSA* coding region in both clones, starting at nucleotide position 1078 and also ending at nucleotide position 1158 (Figure 5B). The same two deletions were identified in *CSA* cDNA isolated from the cell line CS6BR, derived from a sibling of the CS5BR individual, but not in two other CS-A cell lines examined (data not shown).

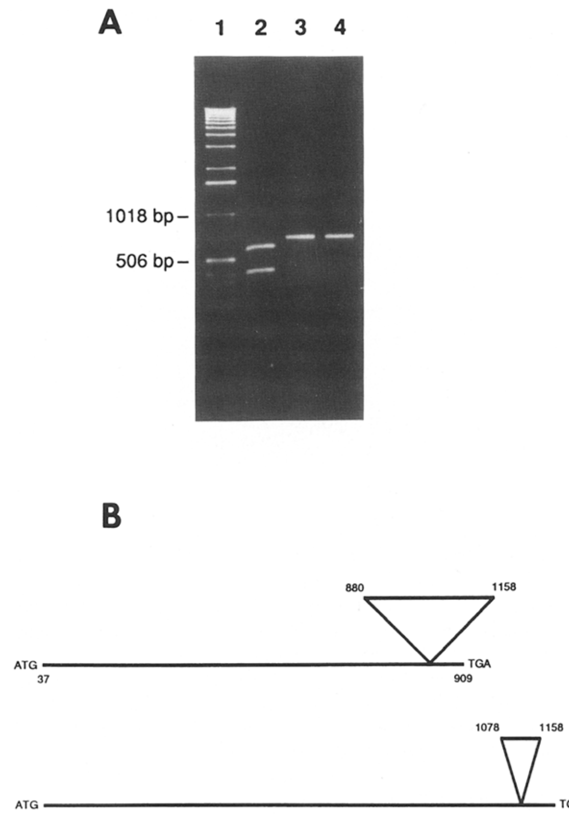

Figure 5. Mutational Analysis of the *CSA* Gene in the Primary Fibroblast Cell Line CS5BR

(A) PCR products derived from cDNAs by using the M3–M4 primer pair (see text for details). Lane 1, DNA size markers; lane 2, PCR products from CS5BR cells; lane 3, PCR products from CCD-42Sk cells (normal diploid human fibroblasts); lane 4, PCR products from HeLa cells.

(B) Schematic representation of deletions identified in *CSA* cDNAs of CS5BR and CS6BR cells.

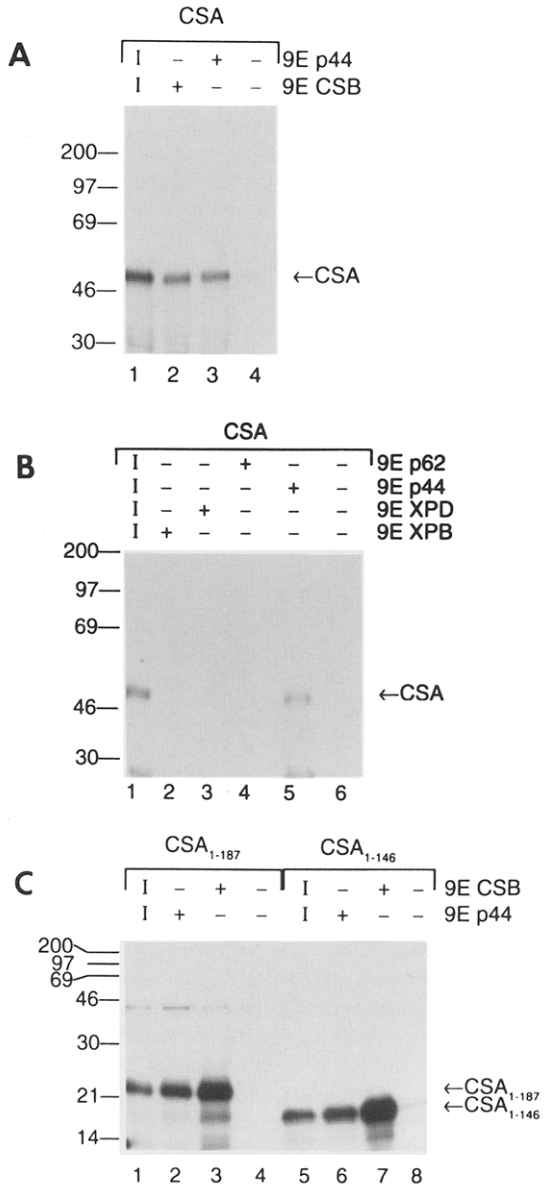

Figure 6. Interaction of CSA with CSB and p44 Proteins

(A) CSA protein was synthesized and radiolabeled in the presence of [^{35}S]methionine during in vitro translation and precipitated by ammonium sulfate (lane 1; I, input), or mixed with either in vitro translated epitope-tagged CSB protein (lane 2) or epitope-tagged p44 protein (lane 3), and precipitated in the presence of monoclonal antibodies to the 9E10 epitope. Control precipitation in the absence of in vitro translated protein is shown in lane 4. Equal volumes of the immunoprecipitates were analyzed by SDS–PAGE.

(B) Similar experiment as described in (A) but using in vitro translated epitope-tagged XPB protein (lane 2), XPD protein (lane 3), p62 protein (lane 4), and p44 protein (lane 5). Control without epitope-tagged proteins is lane 6.

(C) Identical experiment to that in (A) using two radiolabeled truncations of the CSA polypeptide, CSA1–187 (lanes 1–4) and CSA1–146 (lanes 5–8).

CS5BR and CS6BR are known to be the offspring of a consanguineous marriage (A. R. L., unpublished data) and are therefore likely to carry the same mutation in both alleles of the *CSA* gene. The observation that the deletions identified in both cDNAs from both siblings end at the identical nucleotide position (nucleotide 1158) strongly suggests that this nucleotide marks an exon/intron junction and that the truncated cDNAs represent abnormally spliced products missing either one (81 bp) or two (279 bp) upstream exons. These abnormal splice products presumably derive from a homozygous mutation in a splice donor site. Neither deletion creates a frame shift. Hence, the two abnormal transcripts potentially encode internally deleted proteins with predicted sizes of 303 and 369 amino acids.

Mutations were also identified in *CSA* cDNAs in two other CS-A primary fibroblast cell lines examined and in the lymphoblastoid line GM2964, the UV sensitivity of which was shown to be corrected by the *CSA* cDNA (see above). One of these mutations changes codon 322 from TAC (encoding tyrosine) to the stop codon TAA. The details of this mutational analysis will be presented elsewhere (L. L. et al., unpublished data). On the basis of these results, we conclude that the cDNA that complements the cellular phenotypes of CS-A cells is derived from the *CSA* gene.

CSA Protein Interacts with Human CSB Protein and with the p44 Subunit of TFIIH

In light of the possibility that the products of the *CSA* and *CSB* genes are involved in the same biochemical or regulatory pathway, or both, we asked whether these polypeptides physically interact. The cloned *CSB* gene was engineered to express the 9E10 epitope, which is specifically recognized by a monoclonal antibody to the human c-Myc protein (see Experimental Procedures). Following the synthesis of unlabeled epitope-tagged CSB protein and of radiolabeled CSA protein by in vitro transcription and translation of the cloned *CSA* and *CSB* cDNAs, the two proteins were observed to coprecipitate in the presence of antibody to the epitope (Figure 6A, lane 2). Only background levels of precipitated CSA protein were observed in the presence of the antibody but in the absence of epitope-tagged CSB protein (Figure 6A, lane 4). The interaction between CSA and CSB proteins was confirmed in vivo by the use of the two-hybrid genetic system (Chien et al., 1991). When yeast cells were transformed with a plasmid carrying the *CSA* gene fused to the DNA-binding domain in the yeast *GAL4* gene, together with one carrying the *CSB* gene fused to the *GAL4* activation domain, 18 Miller units of β-galactosidase were detected. Transformation with either plasmid alone yielded 0.5–1.0 units of activity.

Radiolabeled CSA protein also coprecipitated with epitope-tagged p44 protein, the 44 kDa subunit of the human RNA polymerase II basal transcription factor TFIIH (Drapkin et al., 1994; Roy et al., 1994; Mu et al., 1995; Aboussekhra et al., 1995) (Figure 6A, lane 3). This interaction was independently confirmed in other experiments (Figure 6B, lane 5), in which it was additionally demonstrated that CSA protein did not coprecipitate with epitope-tagged p62, xeroderma pigmentosum D (XPD), or XPB proteins, other known components of TFIIH (Drapkin et al., 1994; Roy et al., 1994; Mu et al., 1995; Aboussekhra et al., 1995) (Figure 6B).

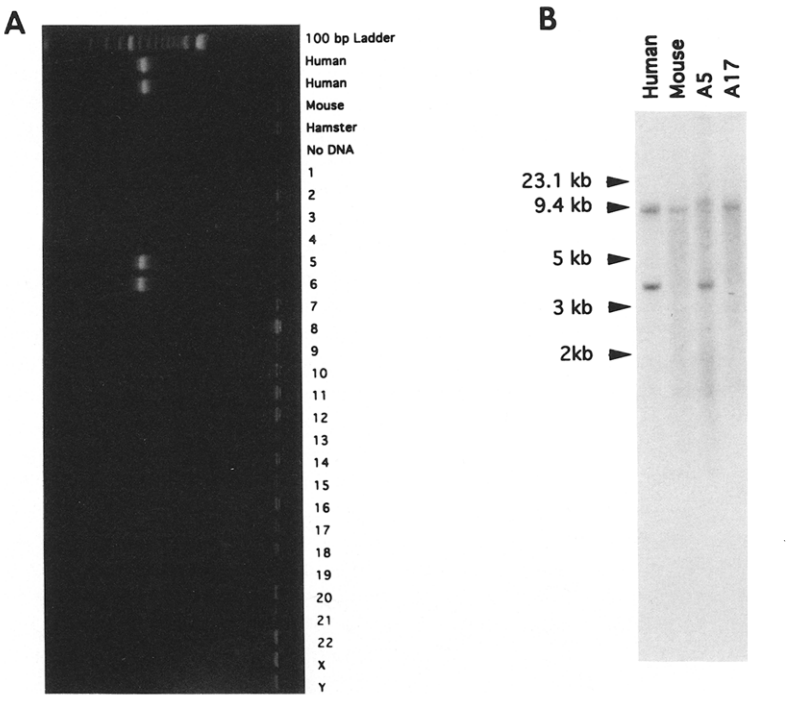

Figure 7. Mapping of the *CSA* Gene to Human Chromosome 5

(A) Agarose gel of the PCR amplification products from DNA in the human/rodent somatic cell hybrid panel number 2. The numbers refer to chromosomes in the normal human karyotype.

(B) Southern blot hybridization of EcoRI-digested DNA from human, mouse, hybrid-A5 (a mouse/human hybrid containing human chromosome 5), and hybrid-A17 (a mouse/human hybrid containing human chromosome 17) with gel-purified *CSA* cDNA.

To define regions of the CSA polypeptide required for these interactions, we expressed runoff transcripts and translated them in vitro to generate C-terminal deletions of the CSA protein. Truncations of the CSA protein carrying just the N-terminal 146 or 187 amino acids (CSA(1–146) and CSA(1–187)) both interacted with p44 and CSB proteins (Figure 6C). Thus, the N-terminal 146 amino acid residues of the CSA protein are apparently sufficient for its interactions with both the p44 subunit of the TFIIH complex and the CSB protein. This region of the polypeptide includes the first two of the five WD repeats (see Figure 3).

Chromosomal Mapping of the *CSA* Gene

PCR primers designed to produce human-specific products from the 3′ untranslated region of the *CSA* gene were used to amplify DNA from the NIGMS human/rodent somatic cell hybrid mapping panel number 2. The 724 bp PCR product was identical to that in the *CSA* cDNA, indicating that there are no introns in the region of the *CSA* gene amplified. Following electrophoresis, amplification products were observed in the human control lanes and in the lanes for the chromosome 5 and 6 human/rodent somatic cell hybrids (Figure 7A). The chromosome 6 hybrid (GM10629) has been previously reported to give a positive signal for the primer set C9, which maps to chromosome 5p14-p12, indicating that this hybrid also carries portions of chromosome 5 (Dubois and Naylor, 1993). Southern blot hybridization was performed on restriction-digested genomic DNA from human, mouse, hybrid-A5 (a mouse/human hybrid containing only human chromosome 5), and hybrid-A17 (a mouse/human hybrid containing human chromosome 17) with gel-purified *CSA* cDNA (Figure 7B). A hybridizing band of 3.2 kb was detected uniquely in human DNA and in the hybrid-A5 DNA, but not in the mouse or hybrid-A17 DNA (Figure 7B). A second 9 kb band hybridized to both the mouse and the hybrid-A17 DNA (Figure 7B). To confirm the specificity of the 3.2 kb band for human DNA, a second Southern blot hybridization was performed, using the 724 bp PCR product (nucleotides 1218–1942 of the *CSA* cDNA) as a probe. This exclusively yielded the 3.2 kb hybridizing bands in the human and hybrid-A5 lanes (data not shown). Additionally, fluorescent in situ hybridization to human metaphase preparations using biotinylated cDNA as a probe produced a signal exclusively in the pericentromeric region of human chromosome 5 (data not shown). Collectively, these data localize the *CSA* gene to human chromosome 5. More refined mapping of the *CSA* gene on chromosome 5 will be reported elsewhere (L. D. M. et al., unpublished data).

Discussion

The increased sensitivity to UV radiation manifested by CS cells in culture invited a functional strategy for gene cloning. Screening a human expression library in an episomal-based plasmid yielded a cDNA that corrected the UV-sensitive phenotype in both lymphoblastoid and fibroblast lines derived from two different CS-A individuals. This cDNA did not complement the UV sensitivity of cells either from genetic complementation group B or from an individual with the nucleotide excision repair–defective disease XP. This specificity, coupled with the reactivation of defective expression of a UV-inactivated plasmid-borne reporter gene, strongly suggests that the cDNA derives from the *CSA* gene. Direct confirmation of this suggestion stems from the demonstration of mutations in three different CS-A cell lines, and of truncated cDNAs in two other CS-A lines that likely represent abnormal splice products conse-

quent to a splice donor site mutation. The *CSA* gene maps to human chromosome 5 and hence is unlinked to the *CSB* gene, which was previously shown to map to the region 10q11.2 (Troelstra et al., 1992).

Sequencing the *CSA* cDNA revealed that the gene can encode a polypeptide of 44 kDa with the signature features of a WD repeat protein. WD repeat proteins are associated with multiple and diverse aspects of cellular metabolism, including signal transduction, vesicular trafficking, regulation of cytoskeletal assembly, cell cycle regulation, RNA processing, and gene regulation (reviewed by Neer et al., 1994). In general, their functions appear to be regulatory in nature; no WD repeat proteins have been shown to have catalytic functions (reviewed by Neer et al., 1994). Structural models suggest that WD repeat proteins are endowed with the potential for interactions with other proteins, consistent with the fact that many such proteins are components of multiprotein complexes (reviewed by Neer et al., 1994).

The molecular basis for the well-established sensitivity of CS cells to UV radiation is not understood. Measurements of nucleotide excision repair in UV-irradiated cells indicate normal repair of base damage in the genome overall. However, both CS-A and CS-B cells have been shown to be defective in the repair of cyclobutane pyrimidine dimers in transcriptionally active genes compared with transcriptionally silent regions of the genome (Venema et al., 1990). More refined studies indicate that there is a particularly pronounced defect in nucleotide excision repair of the template (transcribed) strand of transcriptionally active genes (Mullenders et al., 1992; van Hoffen et al., 1993). These results have fostered the notion that the primary defect in CS cells is in the preferential repair of transcriptionally active genes. This model has been reinforced by the observation that the translated amino acid sequences of the human *CSB* (*ERCC6*) gene and the E. coli *mfd* gene (which is believed to be required for the coupling of nucleotide excision repair to sites of arrested transcription) both contain consensus helicase motifs (reviewed by Selby and Sancar, 1994). Hence, it has been suggested that the *CSB* gene product may function as a transcription repair coupling factor in human cells (reviewed by Selby and Sancar, 1994).

It is conceivable that defective repair of transcriptionally active genes might alter levels of gene expression. However, on the basis of present knowledge, it is difficult to imagine how this could selectively affect certain genes, thereby resulting in the specific clinical and cellular phenotypes of CS. Defective repair of UV radiation–induced damage in CS cells is not confined to the template strand of transcriptionally active genes. The rate of removal of pyrimidine dimers from the coding (nontranscribed) strand is also somewhat reduced compared with the rate in normal cells (Mullenders et al., 1992; van Hoffen et al., 1993). Additionally, defective repair of rRNA genes has been observed in CS cells (Christians and Hanawalt, 1994). Hence, it is suggested that the *CSA* and *CSB* gene products may play a role in the modulation of chromatin structure during general transcription, thereby facilitating repair in such genes (Christians and Hanawalt, 1994).

The notion that the CSA and CSB proteins are involved in transcription in as yet unidentified ways is supported by several observations. Recent studies have shown that the *XPD* and *XPB* genes encode polypeptides that are subunits of the RNA polymerase II basal transcription factor TFIIH (Drapkin et al., 1994; Roy et al., 1994; Mu et al., 1995; Aboussekhra et al., 1995). These (and other components of TFIIH) are bifunctional proteins that participate both in RNA polymerase II transcription initiation and in nucleotide excision repair (Drapkin et al., 1994; Roy et al., 1994; Mu et al., 1995; Aboussekhra et al., 1995). It has been suggested that mutations in these proteins may inactivate their function in nucleotide excision repair, perturb their role in RNA polymerase II transcription initiation, or both (Bootsma and Hoeijmakers, 1993; Friedberg et al., 1994; Vermeulen et al., 1994). Hence, a transcription defect, rather than a defect in DNA repair, may be the primary pathogenetic determinant that results in the complex clinical phenotype of CS associated with mutational inactivation of the *XPB* or *XPD* genes.

The observation that the clinical features of CS also accompany mutational inactivation of the *CSA* and *CSB* genes invites speculation that the polypeptides encoded by these genes also participate in RNA polymerase II transcription. Studies in one of our laboratories (that of E. C. F.) have demonstrated reduced levels of transcription in vitro from several plasmid-borne RNA polymerase II promoters (but not RNA polymerase I or III promoters) in extracts of lymphoblastoid cells and fibroblasts from all CS-A and CS-B individuals examined (G. Dianov et al., unpublished data). Similar results have been obtained with extracts of XP cells from genetic complementation group B (G. Dianov et al., unpublished data). The further observation that the predicted polypeptide product of the *CSA* gene is a WD repeat protein that interacts with the 44 kDa subunit of TFIIH is also provocative with regard to a role of the *CSA* gene product in RNA polymerase II transcription. In this regard, it is of interest that the Drosophila melanogaster coactivator $dTAF_{II}80$, which associates with the basal transcription factor TFIID, has also been shown to be a WD repeat protein (Dynlacht et al., 1993), as has the Tup1 protein of S. cerevisiae, which is known to repress the transcription of a variety of genes in yeast (Keleher et al., 1992).

CSA protein also interacts with CSB protein. The sequence of a region of ~500 amino acids located in the middle of the 1493 amino acid CSB polypeptide shows significant homology with a number of proteins known to be involved in the regulation of gene expression, including the Snf2 protein of S. cerevisiae, a component of the multiprotein Swi/Snf transcriptional activation complex (Troelstra et al., 1992). Given the assumption that abnormal basal transcription, activated transcription, or both are indeed primary defects in CS cells, it is not difficult to imagine that such defects might interfere with the efficiency of DNA repair that is coupled to transcription, thereby resulting in a secondary phenotype of defective repair of transcriptionally active genes when cells are exposed to UV radiation.

The observation that some individuals with the group G form of XP also manifest typical clinical features of CS

requires that the transcription hypothesis accommodate some role of the *XPG* gene in transcription. To date, we know of no direct evidence that speaks directly for or against such a role. However, recent coimmunoprecipitation studies have demonstrated an interaction between in vitro translated XPG and CSB proteins (N. I. and E. C. F., unpublished data). Additionally, the yeast homolog of XPG protein (Rad2) interacts with Ssl1 protein, the yeast homolog of human p44 protein, which, as shown in the present study, interacts with CSA protein. Finally, it has been suggested that mutations in XPG protein might alter RNA polymerase II transcription by perturbing an interconversion between the form of TFIIH required for RNA polymerase II basal transcription and that required for nucleotide excision repair (Svejstrup et al., 1995).

Experimental Procedures

Cell Lines, Transfections, and UV Selection

The SV40-transformed CS-A fibroblast line CS3BE.S3.G1 was provided by L. V. M. (Brumback et al., 1978; Mayne et al., 1986). The EBV-transformed lymphoblast line GM2964 is from an atypical case of CS with late onset of clinical symptoms and unusually prolonged survival; it was obtained from the Human Genetic Mutant Cell Repository (Kennedy et al., 1980; Otsuka et al., 1984). The CS-A fibroblast cell lines CS5BR and CS6BR were identified and characterized by A. R. L. and M. S., and the SV40-transformed CS-B cell line CS1AN.S3.G2 was isolated by L. V. M. (Mayne et al., 1986). The normal EBV-transformed lymphoblast line GM1953 was from the Human Genetic Mutant Cell Repository; HT1080 (CCL121) and CCD-42Sk (CRL1513) were from the American Type Culture Collection; and the XP-C cell line XP4PA-SV was provided by Dr. A. Sarasin.

Fibroblast lines were maintained in Eagle's minimal essential medium (MEM) supplemented with 10%–20% fetal bovine serum (FBS), 2× nonessential amino acids, 2 mM L-glutamine, 100 U/ml penicillin, 100 U/ml streptomycin sulfate, and 0.25 μg/ml amphotericin B (GIBCO). Lymphoblast lines were maintained in RPMI 1640 media supplemented with 20% heat-inactivated FBS, 2 mM L-glutamine, 100 U/ml penicillin, and 100 U/ml streptomycin sulfate (GIBCO). Selection for integration of pSV2neo was with 250 μg/ml G418 (GIBCO), and selection for episomal vectors carrying the *hph* gene was with 177 U/ml hygromycin B (Calbiochem).

The SV40-transformed fibroblast lines CS3BE.S3.G1, CS1AN.S3.G2, and XP4PA-SV were cotransfected with plasmids pSV2neo (Southern and Berg, 1982) and pCMVEBNA (Swirski et al., 1992) by calcium phosphate coprecipitation. Clonal lines showing increased stable transfection with plasmid pDR2 (Murphy et al., 1992) were designated CSA/EN12, CSB/EN7, and XPC/EN12, respectively. Transfection with episomal plasmids was by electroporation.

To screen for complementation of CS-A cells with a human cDNA library in an EBV-based vector, 5 × 10^6 CSA/EN12 cells/ml in serum-free MEM at room temperature were electroporated at 750 V/cm with 50 μg of the cDNA library and 350 μg of sonicated herring testes DNA as carrier, at a capacitance of 800 μF. After a 10 min recovery period, cells were plated with 10 ml of growth media containing 250 μg/ml G418. After electroporation (48 hr later), plates were fed with 10 ml of growth media containing 177 U/ml hygromycin B to select for the presence of the cDNA library and were refed with hygromycin-containing medium every 2 days. Approximately 4000 hygromycin-resistant colonies were obtained per electroporation. On days 6, 10, and 14, plates were irradiated with 4 J/m^2 UV radiation at a dose rate of 1 J/m^2/s to select for cDNAs that complemented the UV sensitivity of CS cells. After electroporation (18–21 days), surviving colonies were picked, expanded, and tested for resistance to UV light by irradiating one of two 60 mm dishes at 4 J/m^2.

UV Radiation Survival

Survival of fibroblast lines was quantitated by colony-forming ability. Dilutions were plated in triplicate and allowed to attach for 6–16 hr. Medium was removed, and the plates were irradiated at a dose rate of 1 J/m^2/s. Growth medium was replaced, and plates were incubated until colonies were visible (~14 days), with feeding as necessary. Plates were fixed and stained with crystal violet, and colony-forming ability was determined. The UV sensitivity of lymphoblast cell lines was quantitated by postirradiation viability (Otsuka et al., 1984). Cells (2 × 10^6) were washed and resuspended in 3 ml of phosphate-buffered saline (PBS) and irradiated at a dose rate of 1 J/m^2/s in a 35 mm tissue culture dish (in triplicate). The cells were transferred to a 25 cm^2 flask containing 12 ml of growth medium. After 3 days, the number of viable cells was determined by trypan blue exclusion.

CAT Reactivation Assay

pRSVcat DNA was irradiated at a dose rate of 3.6 J/m^2/s. Plasmid DNA (10 μg) was electroporated with 400 μg of carrier DNA into 10^6 cells and plated into 10 ml of growth medium (in triplicate). Later (48 hr), plates were washed with PBS, and cells were scraped into a microfuge tube. Samples were pelleted, resuspended in 0.25 M Tris (pH 7.7), and frozen in dry ice–EtOH, followed by thawing at 37°C, three times. Debris was pelleted for 5 min at 4°C and the supernatant transferred to a new tube. Protein was quantitated by use of Bio-Rad protein reagent. *CAT* reactions were carried out at 55°C with 20 μg of total protein, 0.2 μCi 1-deoxy[dichloroacetyl-1-^{14}C]chloramphenicol (Amersham), and 2.8 mg/ml acetyl coA (Sigma) in 100 μl of 0.25 M Tris (pH 7.7). Reactions were terminated by extraction with 1 ml of cold ethyl acetate. Acetylated products were separated by thin-layer chromatography and quantitated by scintillation counting.

Plasmid Rescue, Subcloning, and Sequencing

Episomal plasmids were rescued by alkaline lysis and transformed into E. coli DH5α. An XbaI fragment with the cDNA insert and part of the polylinker region of pEBS7 (Peterson and Legerski, 1991) was subcloned from pCSA5 into a kanamycin-resistant derivative of pUC, pBGS19 (Spratt et al., 1986) for sequence analysis. The cDNA insert was sequenced on both strands with Sequenase version 2.0 (United States Biochemical Corporation).

RNA Isolation and cDNA Synthesis

Poly(A)$^+$ RNA was extracted from cells with a Fast Track Kit (Invitrogen). First-strand cDNAs were prepared from 0.5 μg of poly(A)$^+$ RNA by priming with oligo(dT) as described (Peterson and Legerski, 1991). After first-strand synthesis, cDNAs were recovered by phenol–chloroform extraction, precipitated with ethanol, and resuspended in 50 ml of TE.

PCR Amplification and Sequencing

First-strand cDNA (10 μl) was used for PCR amplification by Taq polymerase (Promega) in buffer supplied by the manufacturer. Final $MgCl_2$ concentration in the reaction was 1.5 mM. Amplification was performed for 30 consecutive cycles of 94°C for 15 s, 52°C for 30 s, and 72°C for 2.0 min. Two pairs of primers (M1–M2 and M3–M4) that divided the *CSA* coding region into two overlapping segments were used. The sequences of the primers and their positions relative to the A residue of the ATG initiation codon, starting with the upstream pair, were as follows. M1, 5′-GCCGGTGTGAGGACACGATA-3′, −19 to +1; M2, 5′-AGCACTTGCTGTTGCCAAGA-3′, 621–602; M3, 5′-CTCACATTCTACAGGGTCAC-3′, 536–555; M4, 5′-AAAGTTTCAGCAGAGACAAA-3′, 1230–1211. PCR products were purified by agarose gel electrophoresis and sequenced by the dideoxy method on an Applied Biosystems Model 373A automated sequencer.

Plasmid Constructs for In Vitro Transcription

Plasmid constructs were generated for in vitro expression of TFIIH components either with or without the 9E10 epitope tag by using the vector pGEM4Z-9E10 as described (Bardwell et al., 1994). This vector contains a sequence encoding 10 amino acids representing the 9E10 epitope of c-Myc protein, followed by the polylinker in the SP6 orientation. Cloning in-frame in these sites followed by in vitro transcription from the SP6 promoter generated mRNA that was translated to yield 9E10 epitope–tagged fusion proteins. Cloning in the opposite orientation resulted in constructs that produced nontagged mRNA following transcription from the T7 promoter. Constructs were verified by restriction mapping.

The *CSA* gene was cloned into vectors for in vitro transcription by

PCR with the following primers: 5′-TGGAATTCATGCTGGGGTTTTTGTCCGC-3′ (CSA forward) and 5′-TTGGAATTCTATTCATCCTTCTTCATCACT-3′ (CSA reverse). EcoRI restriction sites for cloning in-frame are underlined. Synthesis of nontagged *CSA* mRNA was from the T7 promoter. To transcribe the full-length *CSA* gene, the construct was linearized at the 3′ end beyond the termination codon. Digestion of the template using SspI and NdeI generated runoff transcripts that were translated in vitro to yield polypeptides with C-terminal deletions CSA(1–187) and CSA(1–146), respectively.

In Vitro Transcription and Translation

In vitro transcription with the Ambion SP6 or T7 mMessage mMachine kit and purification of mRNA were as described (Bardwell et al., 1994). In vitro translation of purified mRNA using rabbit reticulocyte lysate (Novagen) was carried out in the presence of [^{35}S]methionine (Amersham) at 30°C. Translation of epitope-tagged mRNA and control translations were performed under identical conditions, except that the reactions did not contain labeled methionine. All translation products were partially purified by ammonium sulfate precipitation as described (Bardwell et al., 1994) and used for immunoprecipitation.

Immunoprecipitation

Immunoprecipitation reactions were carried out in buffer A consisting of 20 mM Tris (pH 7.5), 75 mM KCl, 5 mM sodium bisulfite, 4 mM $MgCl_2$, 0.5 mM EDTA, 1 mM DTT, 0.1% Tween 20, 12.5% glycerol, and the protease inhibitors pepstatin (1 μg/ml), leupeptin (1 μg/ml), and PMSF (1 mM). Immunoprecipitation was carried out as described (Bardwell et al., 1994). Partially purified in vitro translated proteins were resuspended in buffer A at 3 × translation volume. Aliquots (60 μl) of each of the two interacting proteins were mixed with 80 μl of buffer A and incubated at 30°C for 30 min. Reactions were incubated in the presence of 9E10 antibody–protein A–agarose complexes for 1 hr at room temperature on a rocking platform. Protein complexes were pelleted and washed at least three times with buffer A. Following resuspension and heat denaturation to disrupt complexes, radioactivity in aliquots was measured by liquid scintillation counting. Samples were also analyzed by SDS–PAGE using equal volumes of test and control reactions on 10% or 15% gels, followed by fluorography. Protein–protein interactions were evaluated by comparing the amount of radioactivity immunoprecipitated by antibody in the presence of epitope-tagged protein with that precipitated nonspecifically in the absence of epitope-tagged protein. Radioactivity at least 3-fold above background was considered positive after multiple independent experiments.

Protein–Protein Interactions In Vivo

Interaction of CSA and CSB proteins in vivo was evaluated with the yeast two-hybrid system as described (Bardwell et al., 1993). The *CSA* gene was amplified by PCR from pGEM4Z-CSA by using primer 4216 (5′-GGCCATGGGCATGCTGGGGGTTTTTGTCCGC-3′) containing an NcoI site (underlined) and primer 4217 (5′-TTGGTCGACTATTCATCCTTCTTCATCACT-3′) containing a SalI site (underlined). The amplified product was digested with NcoI and SalI and cloned into plasmid pAS1 (Durfee et al., 1993). The *CSB* gene was amplified by PCR from plasmid pGEM4Z-CSB by using primer 5091 (5′-CTCGGGATCCGTATGCCAAATGAGGGAATCCCCCAC-3′) containing a BamHI site (underlined) and primer 2388 (5′-AAGGGATCCTGTTTAGCAGTATTCTGGCTTGAGTTT-3′) containing a BamHI site (underlined). The amplified product was digested with BamHI and cloned into pACT (Durfee et al., 1993).

Chromosome Mapping

PCR amplifications were performed in a Perkin-Elmer 9600 thermal cycler using AmpliTaq polymerase and the conditions recommended by the supplier (Cetus). Sequences for primers were 5′-GAGCAGCAGTGATGAAGAAGG-3′ (forward) and 5′-AGGCAGGATTTGGTCATACC-3′ (reverse). Resulting PCR products were electrophoresed on 2% agarose gels. Standard methods were employed for the isolation of genomic and plasmid DNA. Southern blot hybridization used EcoRI- and HinDIII-digested genomic DNA blotted onto Zeta-Probe Genomic Tested membrane according to the recommendations of the manufacturer (Bio-Rad).

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