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Molecular Cloning and Characterization of *Saccharomyces cerevisiae RAD28*, the Yeast Homolog of the Human Cockayne Syndrome A (*CSA*) Gene

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Cockayne syndrome patients exhibit severe developmental and neurological abnormalities. Cells derived from these patients are sensitive to killing by UV radiation and do not support the rapid repair of the transcribed strand of transcriptionally active genes observed in cells from normal individuals. We report the cloning of the *Saccharomyces cerevisiae* homolog of the Cockayne syndrome A (*CSA*) gene, which we designate as *RAD28*. A *rad28* null mutant does not manifest increased sensitivity to killing by UV or γ radiation or to methyl methanesulfonate. Additionally, the rate of repair of the transcribed and nontranscribed strands of the yeast *RPB2* gene in the *rad28* mutant is identical to that observed in wild-type cells following exposure to UV light. As previously shown for *rad7 rad26* and *rad16 rad26* double mutants, the *rad28* null mutant shows slightly enhanced sensitivity to UV light in the presence of mutations in the *RAD7* or *RAD16* gene. Both *rad28* and *rad26* null mutants are hypermutable following exposure to UV light.

Several rare autosomal recessive human hereditary diseases are associated with the defective processing of DNA damage (for a review, see reference 14). Notable examples are xeroderma pigmentosum (XP) and Cockayne syndrome (CS). XP is characterized by defective nucleotide excision repair (NER), a ubiquitous process by which cells repair multiple forms of base damage in DNA (7, 8, 12, 13, 19, 20). The molecular etiology of CS is unknown. The disease can occur uncomplicated by other clinical syndromes (pure CS) (28, 51), or it can accompany the clinical features of XP (CS-XP complex) (6, 21). Patients with the pure form of the disease fall into two genetic complementation groups designated CS-A and CS-B (28, 51). Individuals from both groups exhibit diverse clinical features, of which the most prominent are retarded postnatal growth, a variety of neurological abnormalities, and sensitivity to sunlight (35). The latter phenotype has prompted extensive studies on the DNA repair capacity of CS cells. Such studies have demonstrated that cell lines from the majority of individuals with CS are abnormally sensitive to UV radiation at ~254 nm and to UV-mimetic chemical agents (29, 38). UV-irradiated CS cells have no obvious defect in overall NER measured in vivo and in a cell-free system that monitors NER (for a review, see reference 14).

In normal cells, NER of the transcribed strands of a number of transcriptionally active genes is approximately two to four times more rapid than in the nontranscribed strand (27, 33, 50). This phenomenon is referred to as strand-specific repair or transcription-coupled repair (15, 16, 33). The latter term derives from the notion that arrested transcription at sites of base damage initiates a signal(s) that couples the NER machinery to the transcription elongation complex, thereby facilitating the preferential repair of the template strand (4, 5, 15, 16). Direct biochemical evidence for such coupling has been provided by studies in *Escherichia coli* (for a review, see reference 44). However, there is presently no direct evidence for the coupling of NER to sites of arrested transcription in eukaryotes. Indeed, yeast cell-free extracts that support NER in vitro have been shown to do so in a transcription-independent fashion (62). A number of studies indicate that UV-irradiated CS cells are defective in strand-specific repair of transcriptionally active genes (26, 55, 56) and have led to the notion that the CS gene products may function as transcription-repair coupling factors. It has therefore been proposed that defective transcription-coupled repair of some form of spontaneous DNA damage may constitute the primary basis for the clinical features of CS, perhaps by interfering with RNA polymerase II transcription in a gene-specific fashion (17).

The UV-radiation-sensitive phenotype of CS-A and CS-B cells has facilitated the molecular cloning of the human Cockayne syndrome A (*CSA*) (18) and *CSB* (52) genes by functional complementation. The predicted amino acid sequence of the

TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype
W303-1B	*MAT*α *ho can1-100 ade2-1 trp1-1 leu2-3,112 his3-11,15 ura3-1*
MGSC104	*rad7*Δ::*LEU2*[a]
W303236	*rad16*Δ::*URA3*[a]
MGSC102	*rad26*Δ::*HIS3*[a]
MGSC106	*rad7*Δ::*LEU2 rad26*Δ::*HIS3*[a]
MGSC107	*rad16*Δ::*LEU2 rad26*Δ::*HIS3*[a]
MGSC139	*rad14*Δ::*LEU2*[a]
PB01-28	*rad28*Δ::*URA3*[a]
MGSC172	*rad7*Δ::*LEU2 rad28*Δ::*URA3*[a]
MGSC174	*rad16*Δ::*LEU2 rad28*Δ::*URA3*[a]
PB05-28	*rad26*Δ::*HIS3 rad28*Δ::*URA3*[a]
PB06-28	*rad7*Δ::*LEU2 rad26*Δ::*HIS3 rad28*Δ::*URA3*[a]
MGSC177	*rad16*Δ::*LEU2 rad26*Δ::*HIS3 rad28*Δ::*URA3*[a]
SX46	*MAT***a** *ade2 his3-532 trp1-289 ura3-52*
SX46-52	*rad52*Δ::*TRP1*[b]

[a] The remainder of the genotype is that of W303-1B.
[b] The remainder of the genotype is that of SX46.

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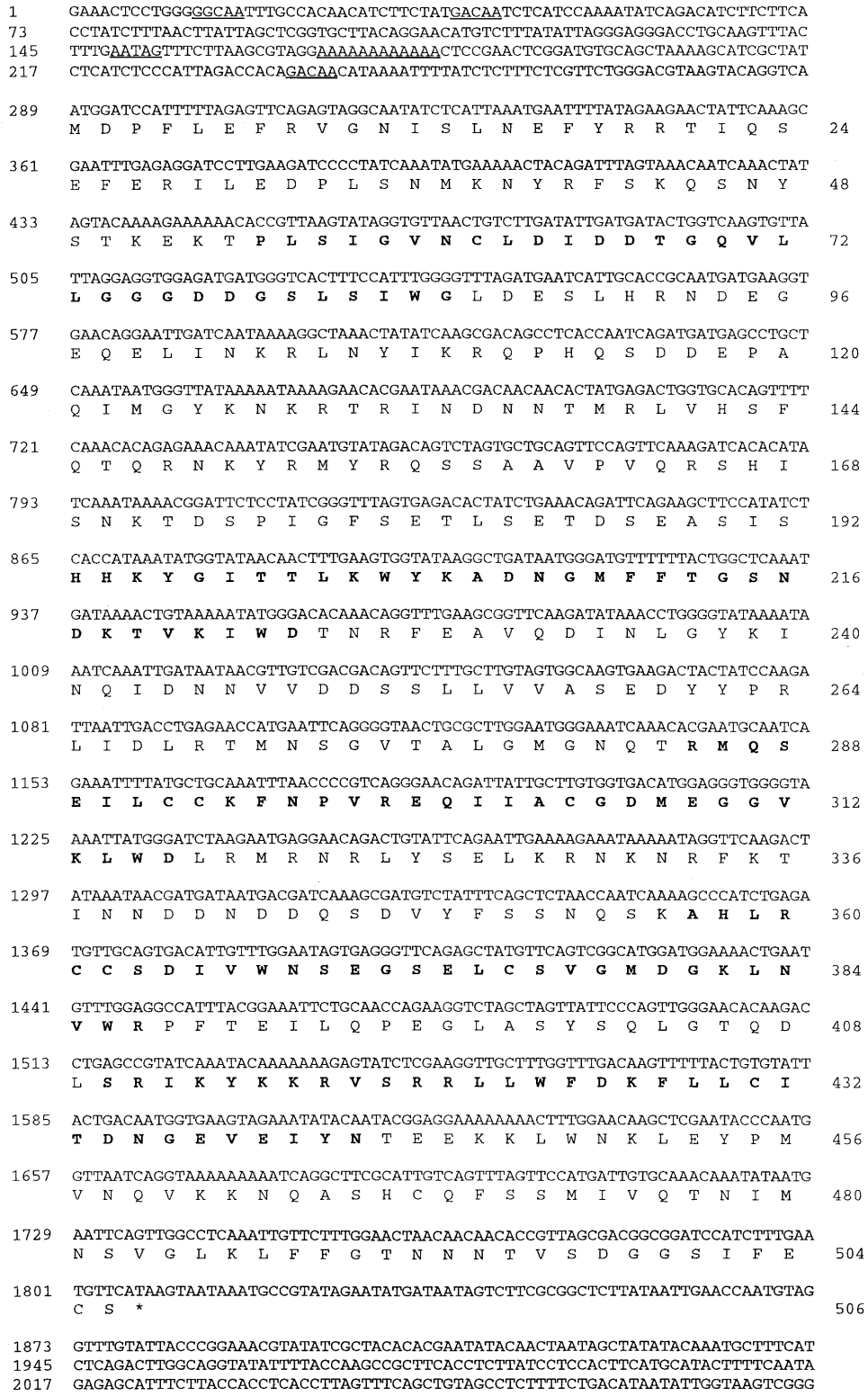


FIG. 1. Nucleotide and predicted protein sequences of *RAD28*. The numbers on the left indicate nucleotide position, and those on the right indicate amino acid position. Underlined bases are putative transcription start sites, while boldface type represents WD repeat domains.

CSB gene indicates that it is a member of the SWI-SNF superfamily of proteins, which are characterized by highly conserved consensus nucleotide-binding motifs (41, 52). Yeast Swi2-Snf2 protein is a DNA-dependent ATPase and is a subunit of a complex of ~11 polypeptides which is implicated in the remodeling of chromatin during transcriptional activation (9, 22, 41, 42). Hence, the homologous CSB protein may operate as an ATPase in a similar but distinct complex which may

TABLE 2. Spontaneous and UV-induced mutagenesis

Strain	Spontaneous mutants per 10^8 generations	UV dose (J/m^2)	Induced mutants per 10^6 cells	Fold increase vs WT[a]
W303 WT[a]	29.80	10	8.5	
		20	27.8	
W303 *rad28*	31.78	10	17.7	2.1
		20	111.8	4.0
W303 *rad26*	ND[b]	10	17.4	2.0
		20	104.1	3.7

[a] WT, wild type.
[b] ND, not determined.

also be endowed with an interactive role with chromatin. The *CSA* gene product is a WD repeat protein, examples of which have been identified in multiple aspects of cellular metabolism (36). WD repeat proteins do not possess any catalytic activity but apparently mediate protein-protein interactions. Hence, the CSA and CSB proteins may be members of a complex in which the CSA protein has an assembly role while CSB may have a catalytic function.

Many human genes involved in DNA repair and other cellular responses to DNA damage are conserved in lower eukaryotes. The yeast *Saccharomyces cerevisiae* has been a particularly informative eukaryotic model in this regard (14). With respect to genes specifically implicated in CS, the yeast homolog of the human *CSB* gene is represented by the *RAD26* gene, which was identified by amino acid sequence homology between a region of the CSB polypeptide and the translated sequence of a cloned yeast genomic DNA fragment (54). The yeast *rad26* deletion mutant demonstrates defective strand-specific repair of the *RPB2* gene (54) as well as enhanced UV sensitivity in combination with mutations in either the *RAD7* or *RAD16* gene (58). *RAD7* and *RAD16* are required for the repair of the nontranscribed strands of actively transcribed genes and of transcriptionally repressed genes (57). Here we present the isolation of the yeast homolog of the human *CSA* gene, which we designate *RAD28*, and the characterization of the DNA repair phenotype of a *rad28* deletion mutant. Like the *rad26* mutant, the *rad28* mutant is not abnormally sensitive to killing by UV radiation but augments the UV sensitivity of *rad7* or *rad16* deletion mutants. Unlike the *rad26* deletion mutant, however, the *rad28* null mutant has no detectable defect in strand-specific repair of the yeast *RPB2* gene. Both *rad28* and *rad26* mutants are hypermutable when exposed to UV light.

MATERIALS AND METHODS

General procedures. Restriction enzyme digestion, PCR, DNA purification, ^{32}P labeling of DNA probes, and gel electrophoresis were performed by standard procedures (43). PCR was carried out with *Taq* polymerase (Boehringer Mannheim). DNA ligations were performed with the Rapid DNA ligation system (Boehringer Mannheim). Alkaline DNA transfers for Southern blot analysis were performed with Hybond N^+ (Amersham) membranes. Sequence analysis was performed by dideoxy chain termination (U.S. Biochemicals). All plasmids were propagated with *E. coli* DH5α in the presence of an appropriate antibiotic. The yeast strains used are listed in Table 1. Yeast media were prepared as previously described (48).

Construction of disruption mutants. Yeast cells were transformed by standard procedures with lithium acetate. To generate the strain PB01-28 (a *rad28* null mutant), a PCR knockout strategy was utilized (3, 30, 32, 34). Amplification products were obtained with the Invitrogen PCR optimizer system. In the primer 5′-yCSApRSII (5′-ATAAAATTTTATCTCTTTCTCGTTCTGGGACGTAAG TACAGGTCAgcatcagagcagattgtactgagagtgcac-3′), uppercase bases coincide with positions 244 to 288 prior to the initiator ATG in the *RAD28* sequence (Fig. 1) and lowercase bases overlap with sequence flanking selectable markers in the pRS series of vectors (34). In the primer 3′-yCSApRSII (5′-AAGAGCCGCGA AGACTATTATCATATTCTATACGGCATTTATTACccttacgcatctgtgcggtatttc acaccg-3′), uppercase bases denote positions 1854 to 1810 immediately following the TAA stop codon and lowercase bases overlap with sequences flanking selectable markers in the pRS vectors. We amplified the *URA3* gene from vectorpRS306 and obtained an ~1.3-kb product that was transformed directly into yeast. Transformants were plated on synthetic complete medium lacking uracil, and the resulting colonies were checked for deletion of the *RAD28* open reading frame (ORF) by Southern analysis. A single *rad28* mutant was used to generate the remainder of the mutants used in the study. Plasmids that contain *rad7*, *rad16*, and *rad26* disruption constructs were digested as previously described (58), transformed into the PB01-28 mutant strain, and plated on an appropriate medium. Prior to propagating the strains on yeast peptone dextrose (YPD) medium, all mutants isolated were restreaked at least once onto synthetic complete medium lacking the amino acid used to generate the knockout allele.

Sensitivity to DNA-damaging agents. For quantitation of sensitivity to UV and γ irradiation, strains were grown to stationary phase in liquid YPD medium at 30°C and various dilutions were plated on YPD plates. The plates were exposed to the indicated doses of UV irradiation (254 nm) or γ rays (^{137}Cs source) and incubated in the dark for 3 to 4 days at 30°C. For measurement of sensitivity to methyl methanesulfonate, cells were grown to stationary phase in YPD at 30°C, harvested, washed once with water, and resuspended in 0.1 M potassium phosphate buffer (pH 7) at a concentration of 2×10^7/ml. Methyl methanesulfonate was added at increasing concentrations, and cells were incubated at 30°C with shaking for 30 min. Cells were centrifuged and washed three times with water, and dilutions were plated on YPD plates. The plates were incubated in the dark at 30°C for 3 to 4 days prior to counting colonies.

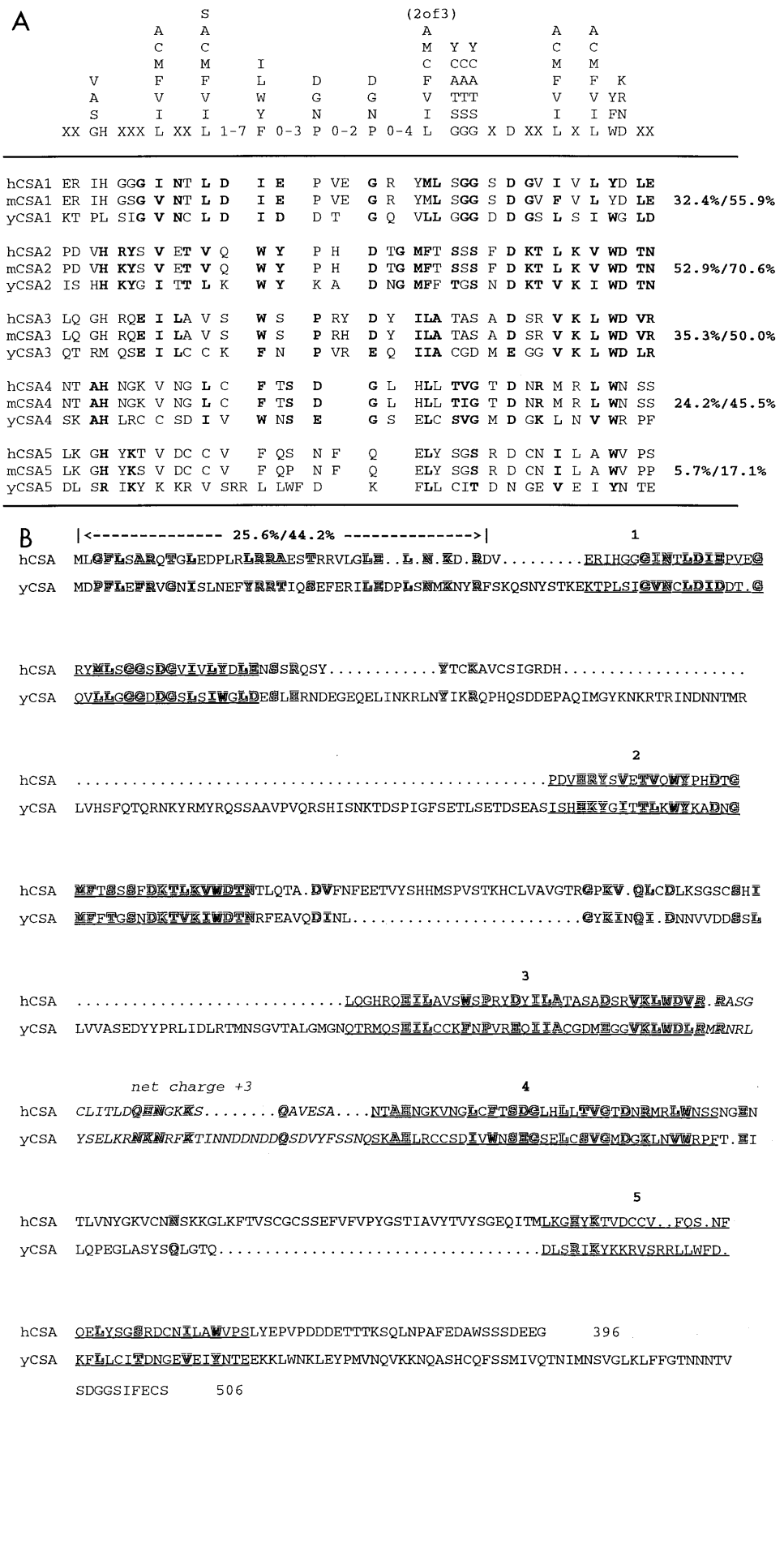
Strand-specific repair assay. Construction of strand-specific probes for the *RPB2* and *GAL7* genes was performed as described previously (57). The gene-specific repair assay was performed as described previously (58). In brief, yeast cells in suspension were irradiated with 254-nm UV light, harvested, resuspended in growth medium, and incubated in the dark at 28°C prior to DNA isolation. Genomic DNA was cut with *Pvu*I and *Pvu*II to generate a 5.2-kb *RPB2* fragment or a 4.7-kb *GAL7* fragment. The DNA was divided in half. One sample was treated with T4 endonuclease V, and the other was mock treated; both were then loaded onto denaturing agarose gels. Electrophoresis was followed by transfer to a Hybond N^+ membrane, which was hybridized with strand-specific probes. Bands were quantitated and statistically analyzed as previously described (4). The data shown in Fig. 6 are the average values at various time points from several independent experiments.

Mutagenesis studies. Spontaneous mutagenesis was measured by reversion of the *ade2-1* mutant to the ADE^+ phenotype. A total of 28 parallel 2-ml YPD cultures were inoculated with ~30 cells and grown for 3 to 4 days to stationary phase. Cells were harvested, washed in water, and plated on minimal-medium plates with or without adenine. Approximately 10^8 cells were plated on medium lacking adenine to assess spontaneous reversion. Plates were incubated at 30°C for 5 to 7 days, and the data were evaluated according to the method of the median (24, 25, 31). The spontaneous-mutation values in Table 2 represent data from a single experiment.

UV-induced mutagenesis was also measured by reversion to ADE^+ as described previously (10, 11). Inocula of ~100 yeast cells from freshly streaked colonies were grown in YPD at 30°C for 96 h to stationary phase. Cells were harvested, washed once in water, and then resuspended at 10^8 to 1.5×10^8/ml. Approximately 2×10^7 cells were plated in duplicate or triplicate on minimal plates lacking adenine. In addition, cells were plated on minimal plates containing adenine to assess survival at various UV doses. Plates were irradiated and then incubated at 30°C for at least 7 days prior to counting colonies. The calculation of the mutation frequency was corrected for spontaneous reversion and killing at each dose. The values for UV-induced mutagenesis shown in Table 2 are averages of data from three independent experiments.

RESULTS

Identification of a yeast ORF homologous to the human CSA protein. The BLASTP algorithm was used to search the National Center for Biotechnology Information genome databases with the human CSA protein sequence (1). The most significant match was with a previously uncharacterized incomplete ORF on yeast chromosome IV (accession number Z47814) derived by the Sanger Center, Cambridge, England, as part of the Yeast Genome Sequencing Project. The probability value obtained with BLASTP (which tests the null hypothesis that the two sequences are unrelated) was $6.6 \times e^{-17}$, a value consistent with the probability scores for several human proteins and their known homologs—for example, the human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I FN
XX GH XXX L XX L 1-7 F 0-3 P 0-2 P 0-4 L GGG X D XX L X L WD XX
hCSA1 ER IH GGG I NT L D I E P VE G R YML SGG S D GV I V L YD LE
mCSA1 ER IH GSG V NT L D I E P VE G R YML SGG S D GV F V L YD LE 32.4%/55.9%
yCSA1 KT PL SIG V NC L D I D D T G Q VLL GGG D D GS L S I WG LD
hCSA2 PD VH RYS V ET V Q W Y P H D TG MFT SSS F D KT L K V WD TN
mCSA2 PD VH KYS V ET V Q W Y P H D TG MFT SSS F D KT L K V WD TN 52.9%/70.6%
yCSA2 IS HH KYG I TT L K W Y K A D NG MFF TGS N D KT V K I WD TN
hCSA3 LQ GH RQE I LA V S W S P RY D Y ILA TAS A D SR V K L WD VR
mCSA3 LQ GH RQE I LA V S W S P RH D Y ILA TAS A D SR V K L WD VR 35.3%/50.0%
yCSA3 QT RM QSE I LC C K F N P VR E Q IIA CGD M E GG V K L WD LR
hCSA4 NT AH NGK V NG L C F TS D G L HLL TVG T D NR M R L WN SS
mCSA4 NT AH NGK V NG L C F TS D G L HLL TIG T D NR M R L WN SS 24.2%/45.5%
yCSA4 SK AH LRC C SD I V W NS E G S ELC SVG M D GK L N V WR PF
hCSA5 LK GH YKT V DC C V F QS N F Q ELY SGS R D CN I L A WV PS
mCSA5 LK GH YKS V DC C V F QP N F Q ELY SGS R D CN I L A WV PP 5.7%/17.1%
yCSA5 DL SR IKY K KR V SRR L LWF D K FLL CIT D N GE V E I YN TE
B
|<---------- 25.6%/44.2% ---------->|
1
hCSA ML GFL SAR Q TG L EDPLR LRRA ES T RRVLG LE . . L . N . K D . R DV . . . . . ERIHGG GIN TL DIE PVE G
yCSA MD PFL EFR V GN I SLNEF YRRT IQ S EFERI LE DP L S NMK NY R FSKQSNYSTKE KTPLSI GVN CL DID DT . G
hCSA RY ML S GG S DG V I VL Y D LE N S S R Q S Y . . . . . Y TCK AVCSIGRDH . . . . . .
yCSA QV LL G GG D DG S L S I WG LD E S L E RNDEGEQELINKRLN Y IK R QPHQSDDEPAQIMGYKNKRTRINDNNTMR
2
hCSA . . . . . PDV ERY S VE TV Q WY PH D TG
yCSA LVHSFQTQRNKYRMYRQSSAAVPVQRSHISNKTDSPIGFSETLSETDSEASISH HKY G I TTL K WY KA D NG
hCSA MF TSS SF DK TL K VW DTN TLQTA . DV FNFEETVYSHHMSPVSTKHCLVAVGTR G PK V . QL C D LKSGSC S HI
yCSA MF FTG S NDK TV K I W DTN RFEAVQ D I NL . . . . . G Y K I N Q I . D NNVVDD S S L
3
hCSA . . . . . LQGHRQ E IL AVS W S P RY D Y ILA TASA D SR V K L W DV R . RASG
yCSA LVVASEDYYPRLIDLRTMNSGVTALGMGN QTRMQS E IL CCK F N P VR E Q I I A CGDM E GG V K L W DL R MRNRL
net charge +3
4
hCSA CLITLD Q H N GK K S . . . . . Q AVESA . . . . NTA E NGKVNG L C F T S D G L H L L T V G T D N R M R L W N S S NG E N
yCSA YSELKR N K N RF K TINNDDNDD Q SDVYFSSNQSKA E LRCCSD I V W N S E G S E L C S V G M D G K L N V W RPF T . E I
5
hCSA TLVNYGKVCN N SKKGLKFTVSCGCSSEFVFVPYGSTIAVYTVYSGEQITM LKG E Y K TVDCCV . . FQS . NF
yCSA LQPEGLASYS Q LGTQ . . . . . DLS R I K YKKRVSRRLLWFD .
hCSA QE L YSG S RDCN I LA W VPSLYEPVPDDDETTTKSQLNPAFEDAWSSSDEEG 396
yCSA KF L LC I T DNGE V E I Y NTE EKKLWNKLEYPMVNQVKKNQASHCQFSSMIVQTNIMNSVGLKLFFGTNNNTV
SDGGSIFECS 506

FIG. 2. (A) Pairwise amino acid comparison of human CSA (hCSA 1 to 5), mouse CSA (mCSA 1 to 5), and Rad28 (yCSA 1 to 5) WD repeats. Shown at the top is the consensus sequence for the WD repeat. Each variable residue is indicated with an X. Boldface type indicates identity or similarity. The percentages to the right of each pair indicate identity and similarity (in that order). (B) Amino acid alignment of the human CSA (hCSA) and Rad28 (yCSA) full-length protein sequences. Periods represent gaps. Shadow type indicates amino acid identity or similarity. Underlined sequences correspond to the five WD repeat domains. The percent identity and similarity (in that order) are indicated for the N-terminal amino acids preceding repeat 1. The sequences between repeats 3 and 4 are shown in italics.

CFTR and yeast Ste6 proteins (53). When this yeast ORF was also used to search the databases, the most significant match obtained was with the human CSA ORF, with a probability score of $9.2 \times e^{-17}$. The nucleotide sequence that marked the 5′ end of the yeast ORF encoded an ATG codon. To determine if this codon (at nucleotide position 289 of Fig. 1) was indeed the translational start site, we screened a yeast genomic library with a PCR-derived internal probe and isolated a clone containing additional upstream sequence. The nucleotide and predicted amino acid sequences of the complete gene are shown in Fig. 1. We designate the gene *RAD28*, consistent with the nomenclature for the yeast *RAD26* homolog of the human *CSB* gene.

The coding region of the *RAD28* gene predicts a polypeptide of 506 amino acids with a calculated molecular mass of 58 kDa and an isoelectric point of 6.89. The predicted amino acid sequence reveals the presence of five consensus WD repeat motifs (36), the same number as in the human (18) and mouse (53a) CSA proteins. The full-length mouse CSA sequence will be published elsewhere. A comparison of individual WD repeats in the yeast Rad28 and human and mouse CSA proteins is shown in Fig. 2A. The consensus WD repeat sequence is loose and allows numerous substitutions with related amino acids (Fig. 2A). Hence, specific criteria have been established to identify related WD repeat protein sequences from different species (36). First, all members of a given family must have exactly the same number of WD repeats. Second, repeat units at the same positions are typically more closely related in sequence to each other than they are to other repeating units, both with respect to the proteins in question and with respect to functionally unrelated WD repeat proteins. WD repeat motifs 1 to 4 of the human and mouse CSA proteins and the yeast Rad28 protein demonstrate a significant level of amino acid identity or similarity, with repeat 2 showing the greatest conservation (70.6% similarity) (Fig. 2A). A comparison of the amino acid identities between repeats 1 to 4 of the human and yeast sequences showed them to be more closely related to each other than to any of the other repeats in these two proteins (data not shown). However, this was not the case for repeat 5. This is consistent with comparisons in other WD repeat protein families with proven functional identity (36). To further establish the structural relationship between the yeast Rad28 and human CSA proteins, we compared each WD repeat motif in Rad28 and CSA with 22 other WD repeat motifs from a variety of functionally unrelated proteins of this class. Pileup analysis with the Wisconsin software group GCG program confirmed the relationship of the sequences of WD repeats 1 to 3 of the yeast Rad28 and human CSA proteins (Fig. 3).

A comparison of the full-length yeast Rad28 and human CSA protein sequences reveals interesting regions of amino acid conservation in nonrepeat or intervening sequences (Fig.

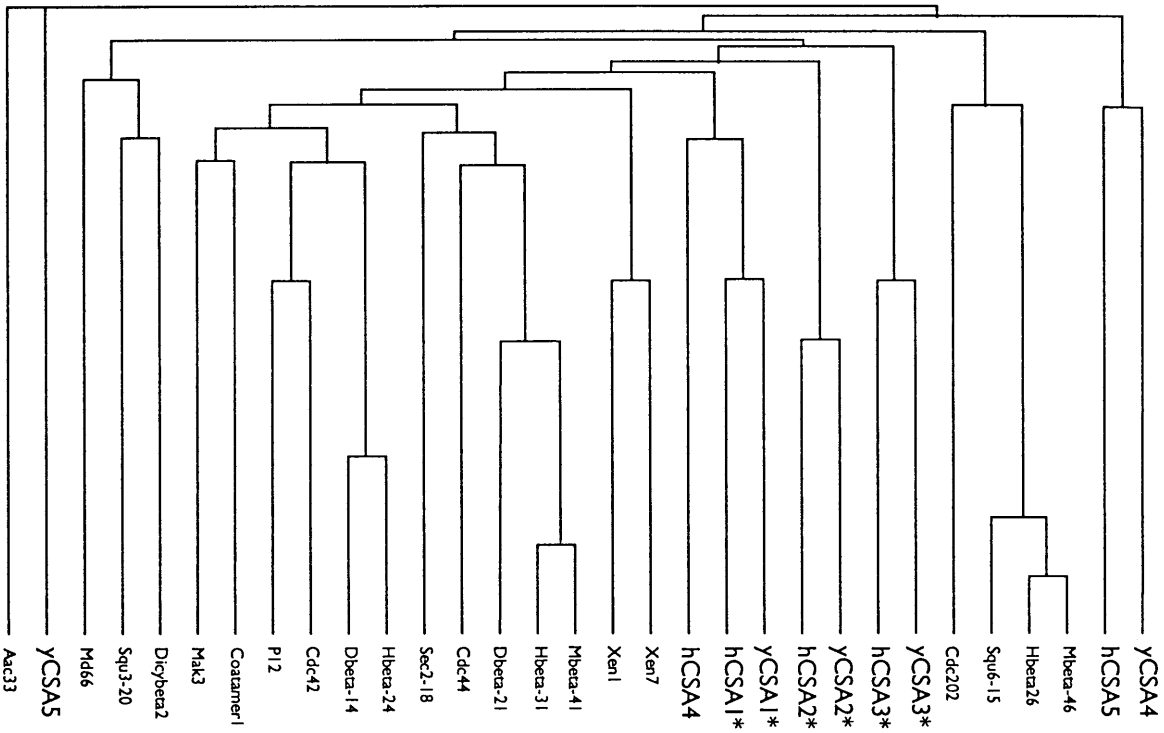

FIG. 3. Pileup comparative analysis of human and yeast CSA (hCSA and yCSA, respectively) WD repeat domains and 22 other WD repeats in unrelated WD proteins. Significance by this algorithm is associated only with the vertical distance displayed, not the horizontal distance. Asterisks indicate the positions of the closely related CSA repeats 1 to 3.

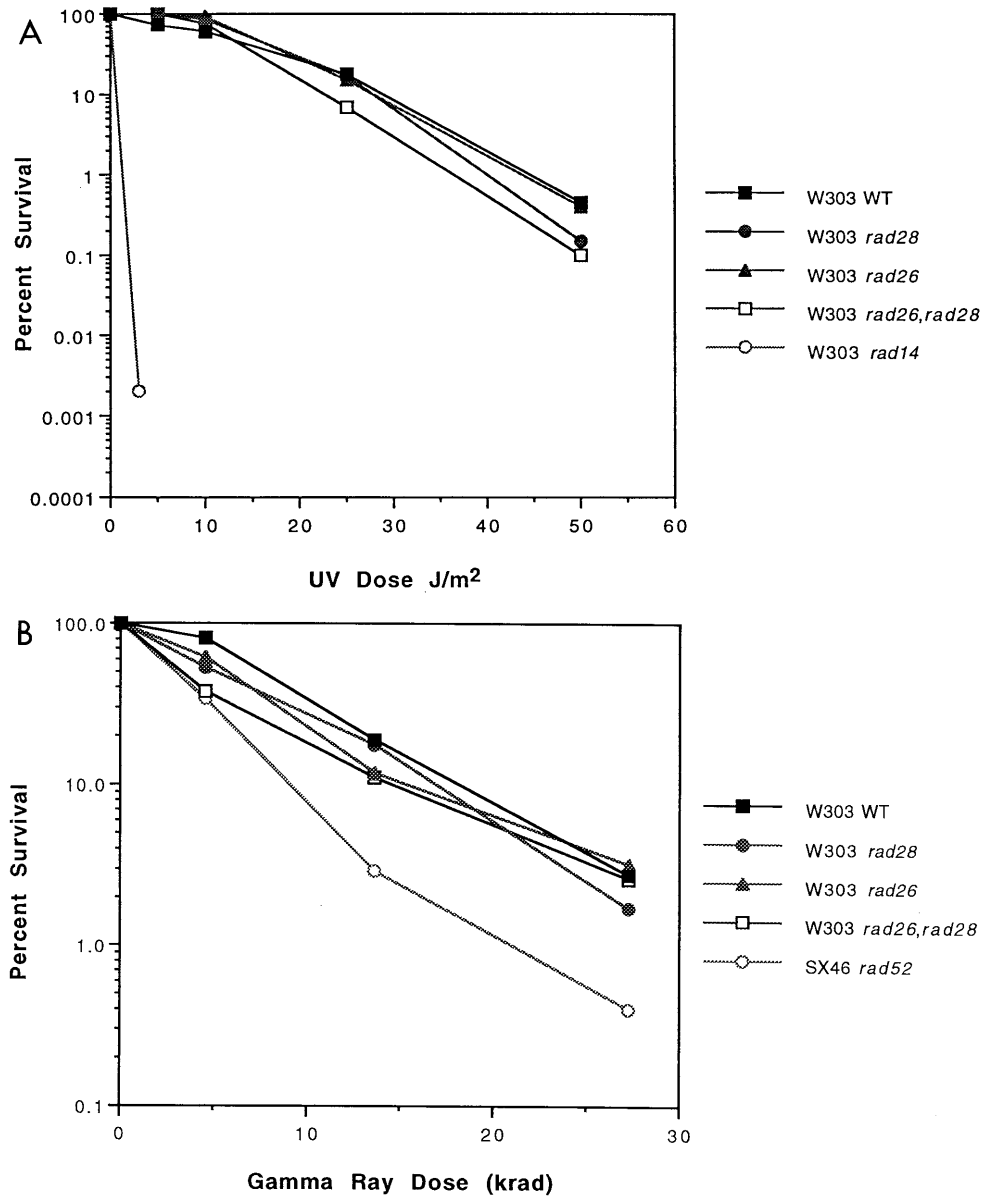

FIG. 4. Sensitivity of yeast strains to DNA-damaging agents. (A) Survival of mutants after UV irradiation; (B) survival after γ irradiation. The sensitivity profiles of strains W303-1B (wild type [WT]), PB01-28 (*rad28*), MGSC102 (*rad26*), PB05-28 (*rad26 rad28*), MGSC139 (*rad14*) (all of which are derivatives of strain W303), and SX46-52 (*rad52*) are indicated.

2B). (i) The N-terminal 43 amino acids of Rad28 and human CSA proteins share 25.6% identity and 44.2% similarity, values higher than those detected in any other nonrepeat regions. The N termini of the mammalian and yeast G_β WD repeat proteins are conserved, and this region of the mammalian protein forms an α-helix which packs with the α-helix of G_γ to form a parallel coiled-coil structure (23, 37, 49, 60). Hence, it is distinctly possible that the N termini of the Rad28 and CSA proteins also have a conserved function. (ii) The sequence between WD repeats 3 and 4 in both Rad28 and CSA proteins has the highest charge density per unit length (+3) of any of the intervening sequences (Fig. 2B). (iii) The isoelectric points of Rad28 and CSA are 6.89 and 6.32, respectively, so the two proteins are closely related in overall charge as well.

A third criterion for homology between species is the presence of amino acid identity or similarity in the immediate N- or C-terminal extensions (two amino acids flanking GH and WD in the consensus sequence shown in Fig. 2A) of some of the WD repeats. Once again, this relationship is satisfied for the C-terminal extension of repeats 1 to 3 of the human, mouse, and yeast proteins (Fig. 2A).

The entire yeast genome sequence has been completed, and an analysis of the databases has not revealed any yeast sequences with greater similarity to CSA than Rad28. Additionally, an independent comparison of the yeast genome database with those comprising human genes has designated the yeast ORF assigned accession number Z47814 as the unequivocal yeast homolog of the human *CSA* gene (GeneQuiz Consortium, European Molecular Biology Laboratory, Heidelberg,

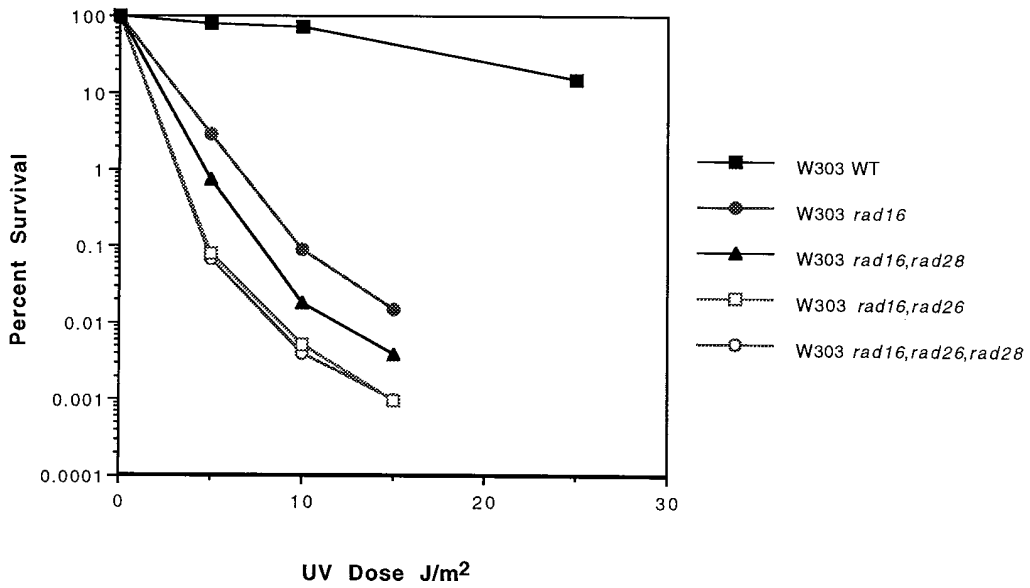

FIG. 5. UV sensitivity of various single, double, and triple mutants. The survival figures for strains W303-1B (wild type [WT]), W303236 (*rad16*), MGSC174 (*rad16 rad28*), MGSC107 (*rad16 rad26*), and MGSC177 (*rad16 rad26 rad28*) are shown.

Germany). We conclude that the yeast Rad28 and human and mouse CSA proteins are members of a single WD repeat protein subfamily.

Generation of a yeast *rad28* null mutant and characterization of sensitivity to DNA-damaging agents. In order to generate a *rad28* null mutant, we performed a PCR-based knockout strategy as described previously (3, 30, 32, 34). This method eliminated the entire coding region of *RAD28*. The mutant demonstrated normal growth kinetics at 20, 30, and 37°C on YPD plates; hence, the *RAD28* gene is not essential for viability. In contrast to the cellular phenotype of human CS-B (and CS-A) individuals, yeast *rad26* mutants are not abnormally sensitive to UV radiation (54) (Fig. 4A). Similarly, the *rad28* mutant and a *rad26 rad28* double mutant were not hypersensitive to UV radiation (Fig. 4A). Neither the *rad28* nor the *rad26* null mutant was abnormally sensitive to γ radiation (Fig. 4B) or to the alkylating agent methyl methanesulfonate (data not shown). The designation *RAD* for both the *RAD26* and *RAD28* genes is therefore a misnomer but is retained in the interests of consistency with the accepted terminology applied to yeast genes implicated in cellular responses to DNA damage.

Mutational inactivation of the yeast *RAD7* or *RAD16* gene, both of which are specifically required for NER of transcriptionally silent genes and of the nontranscribed strand of transcriptionally active genes (57), confers a modest sensitivity to UV radiation (2, 40, 47) (Fig. 5). As previously described (58), we found that this phenotype is enhanced by the simultaneous inactivation of the *RAD26* gene (Fig. 5). We observed that a *rad16 rad28* double mutant was also reproducibly more UV sensitive than an otherwise isogenic *rad16* single mutant at all doses of UV radiation tested (Fig. 5), though this enhancement of lethality was not as marked as that observed with a *rad16 rad26* double mutant. These results were independently verified in multiple experiments in the laboratories of both E.C.F. and J.B. The average survival values from several experiments in one of our laboratories (E.C.F.) with *rad7* and *rad16* single mutants and *rad7 rad26*, *rad7 rad28*, *rad16 rad26*, and *rad16 rad28* double mutants are shown in Table 3. Statistical analysis using the χ^2 and Student's *t* tests generated a *P* value of <0.05 for these experiments. If, as these results imply, *RAD26* and *RAD28* are involved in the same functional pathway, a *rad16 rad26 rad28* triple mutant should be no more UV sensitive than a *rad16 rad26* double mutant. This is indeed

TABLE 3. Comparison of the UV radiation sensitivity of *rad7* and *rad16* mutants in the presence or absence of *rad26* and *rad28* mutations

Mutant	UV dose (J/m^2)	Average survival (% ± SD)[a]
W303 *rad7*	0	100
	5	4.2 ± 0.42
	10	0.046 ± 0.063
	15	0.006 ± 0.007
W303 *rad7 rad28*	0	100
	5	0.41 ± 0.51
	10	0.023 ± 0.019
	15	0.0029 ± 0.0002
W303 *rad7 rad26*	0	100
	5	0.057 ± 0.023
	10	0.009 ± 0.011
	15	0.00073 ± 0.00002
W303 *rad16*	0	100
	5	2.9 ± 2.1
	10	0.089 ± 0.044
	15	0.015 ± 0.00004
W303 *rad16 rad28*	0	100
	5	0.735 ± 0.035
	10	0.0175 ± 0.0021
	15	0.0039 ± 0.0024
W303 *rad16 rad26*	0	100
	5	0.079 ± 0.045
	10	0.0052 ± 0.0018
	15	0.00097 ± 0.00019

[a] Average percent survival values were determined from duplicate or triplicate experiments.

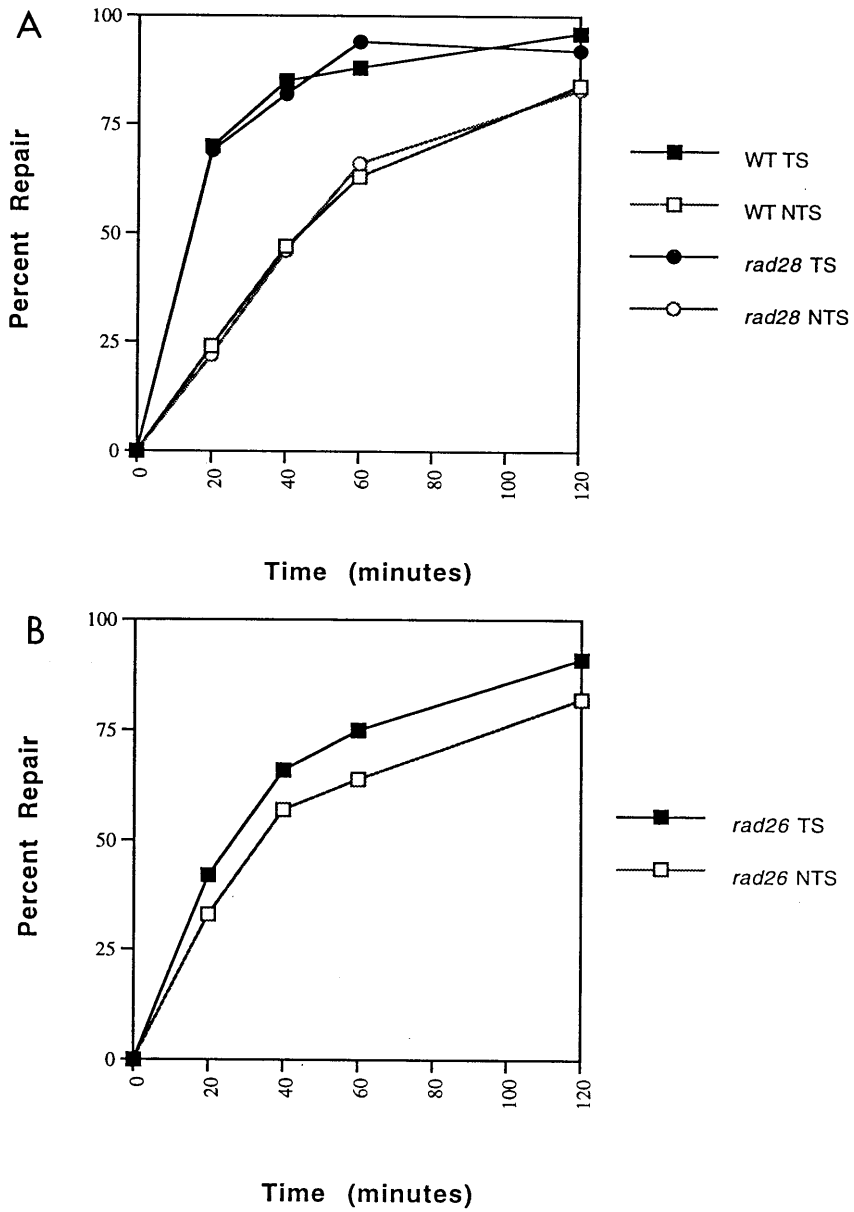

FIG. 6. Strand-specific repair of the *RPB2* gene analyzed in strains W303-1B (wild type [WT]) and PB01-28 (*rad28*) (A), strain MGSC102 (*rad26*) (B), strains MGSC104 (*rad7*) and MGSC172 (*rad7 rad28*) (C), and strains MGSC106 (*rad7 rad26*) and PB06-28 (*rad7 rad26 rad28*) (D). TS, transcribed strand; NTS, nontranscribed strand of the *RPB2* gene.

the case (Fig. 5). Identical results were obtained with the *rad7 rad26 rad28* triple mutant (data not shown).

Strand-specific repair in the yeast *rad28-CSA* mutant. Both CS-A and CS-B cells of humans fail to support an enhanced rate of removal of pyrimidine dimers from the transcribed strands of transcriptionally active genes (56). A defect in strand-specific repair of the *RPB2* gene was previously observed in UV-irradiated yeast *rad26* cells (54) and was reproduced in the present studies (compare the difference for the transcribed [TS] and nontranscribed [NTS] components of the wild-type [WT] and *rad26* curves in Fig. 6A and B). In contrast, we observed no loss of strand discrimination in the *rad28* mutant (Fig. 6A).

Inactivation of the *RAD7* (or *RAD16*) gene eliminates NER in the nontranscribed strand of the transcriptionally active *RPB2* gene (57), thereby amplifying differences in the kinetics of repair of the two DNA strands relative to that observed in wild-type cells (compare wild-type [WT] and *rad7* cells in Fig. 6A and C). Subtle defects in NER of the transcribed strand are therefore more readily detected in the *rad7* (or *rad16*) mutational background (58) (compare *rad7* and *rad7 rad26* transcribed [TS] components in Fig. 6C and D). Even with this more sensitive assay, no defect in strand-specific repair was observed in the *rad28* null mutant (compare *rad7* and *rad7 rad28* transcribed [TS] components in Fig. 6C). Furthermore, the residual transcription-dependent repair observed in a *rad7 rad26* double mutant (58) is apparently not dependent on a functional *RAD28* gene, since essentially the same kinetics of NER of the transcribed strand were observed in the *rad7 rad26*

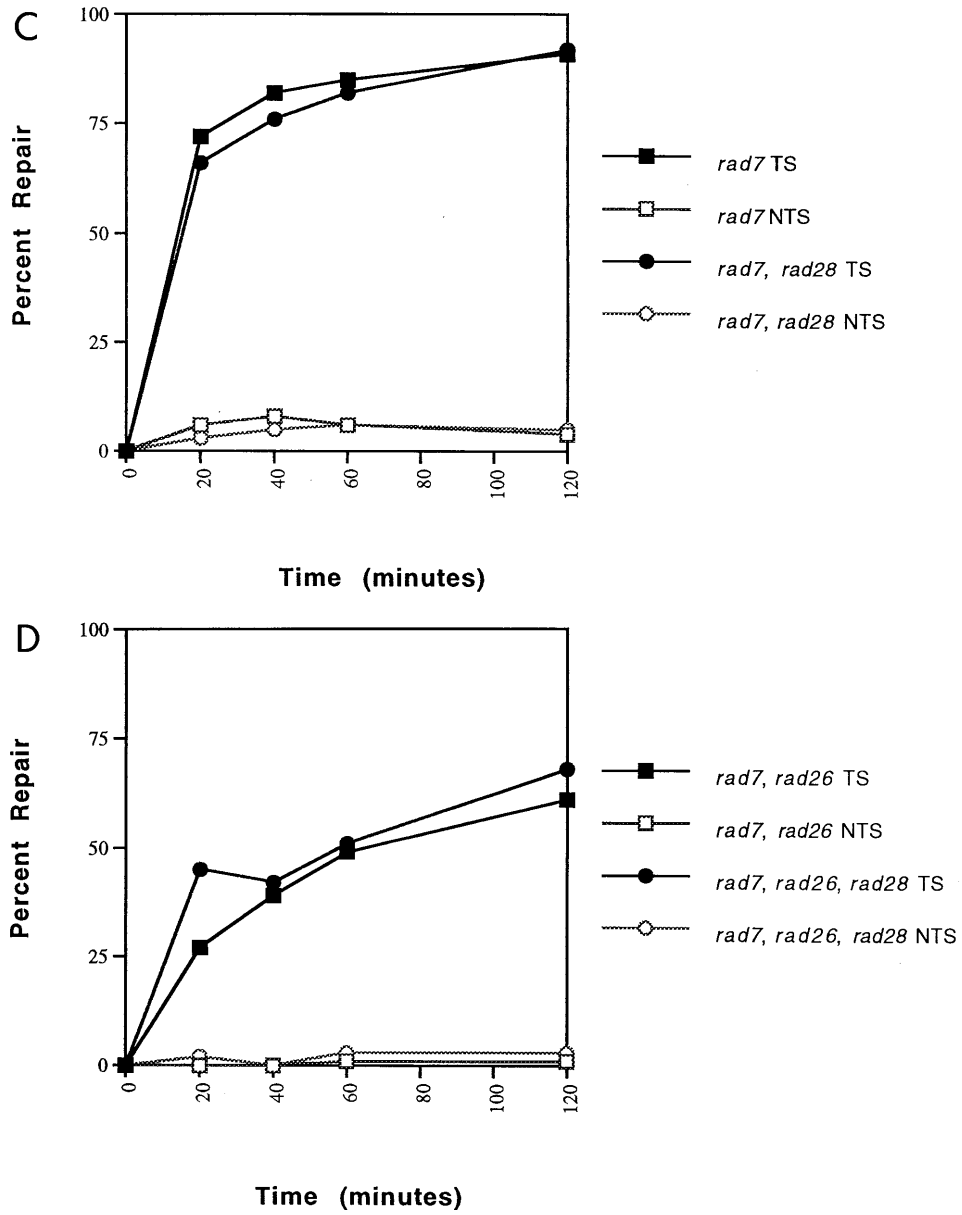

FIG. 6—*Continued.*

double mutant and the *rad7 rad26 rad28* triple mutant (Fig. 6D).

Spontaneous and UV-induced mutagenesis. CS cells have been reported to be hypermutable after exposure to UV radiation (39). Additionally, both XP and CS cells were shown to be defective in mutagenesis observed in normal cells following their transfection with triplex-forming oligonucleotides (61). Both spontaneous mutagenesis and UV-radiation-induced mutagenesis were investigated in the *rad28* and *rad26* null mutants. As shown in Table 2, no difference in spontaneous mutation rates was observed on the basis of reversion of the *ade2-1* locus to prototrophy in *rad28* and otherwise isogenic wild-type cells. (This parameter was not determined for the *rad26* mutant.) However, consistent with the phenotype of human CS cells, we observed a two- to fourfold increase in mutation frequency at the *ade2-1* locus in both *rad28* and *rad26* mutant cells following exposure to UV radiation.

DISCUSSION

The yeast gene designated *RAD28* was isolated on the basis of its amino acid sequence homology with the human CSA protein, a member of the WD repeat protein family (18, 36). Since there are many examples in nature of WD repeat proteins, it is cogent to ask whether the Rad28 and CSA proteins represent true structural and functional homologs. Several lines of evidence support our contention that they do. First, the homology identified at the amino acid sequence level obeys the requirements elaborated by Neer et al. (36) for an interspecies relationship. In particular, the homology of the first three repeats reflects the strong conservation of amino acid residues within the highly variable regions (X residues in Fig. 2A) of the consensus repeat motif, an observation made for other functionally homologous WD repeat proteins (36, 37). Each repeat in Rad28 and CSA was individually compared with the repeats

in nonequivalent positions in the two proteins. This comparison did not yield greater similarity than was found with repeats in the same position. The sequence conservation between the CSA and Rad28 protein repeats 1 to 3 was confirmed by computer-assisted comparison of them with 22 randomly selected WD repeat motifs. On the basis of comparisons in other WD repeat families (36), we anticipate that a minority of the repeats may be divergent in amino acid sequence, as is the case for repeat 5 and, to a lesser extent, repeat 4 in the Rad28 and CSA proteins.

Limited homology within interrepeat sequences in functionally related WD repeat proteins is typically observed (35a). Relationships were noted in the N-terminal regions and in the intervening sequence between repeats 3 and 4 of the Rad28 and CSA proteins. The N terminus may adopt a unique secondary structure that mediates protein-protein interactions, as is the case with the G_β WD repeat protein (23, 49, 60). Additionally, the region between repeats 3 and 4 has a high charge density, with a net +3 charge in both proteins, and may also mediate protein-protein interactions.

Neither *rad28* nor *rad26* single or double mutants manifest abnormal sensitivity to any of several DNA-damaging agents tested. These results represent a departure from the cellular phenotype of most human CS cells. However, recent studies on cells from five patients with typical clinical features of CS have failed to reveal sensitivity to UV radiation (49a). Hence, this phenotype is apparently not universal in CS. Unlike the yeast *RAD26* gene, the *RAD28* gene is not required for strand-specific repair of the *RPB2* gene after exposure of cells to UV radiation. Nonetheless, a genetic relationship between *RAD26* and *RAD28* derives from the consistent observation that both the *rad26* and *rad28* mutations enhanced the UV sensitivity of *rad7* and *rad16* yeast mutants. The extent of this enhancement is small, but it was highly reproducible in multiple experiments independently performed in both laboratories. It should be noted that the yeast mutants suffered a complete inactivation of the *RAD28* gene while most, if not all, human CS-A individuals presumably express truncated or altered polypeptides, which may have residual activity. The possibility that yeast mutants with truncated polypeptides or non-chain-terminating point mutations may demonstrate a more informative phenotype requires further investigation.

A specific requirement for the *RAD7* and *RAD16* genes for NER of transcriptionally silent DNA has been documented both in vivo (57) and in vitro (63). The observation that additional mutations in the *RAD26* or *RAD28* gene enhance lethality in response to UV-radiation-induced damage suggests that these genes operate in the repair of transcriptionally active genes, which is consistent with the observation that the *rad26* mutant is defective in strand-specific repair. The limited extent of this enhancement suggests that in yeast cells, the pathway in which the Rad26 and Rad28 proteins operate or the Rad26 and Rad28 proteins themselves make a minor contribution to protection from UV-radiation-induced lethality. This might explain why the *rad28* deletion mutant fails to manifest a defect in strand-specific repair. In this regard, it is relevant to point out that a requirement for the *RAD26* gene for strand-specific repair is not general. When tested with the yeast *GAL7* gene under induced conditions, no defect in strand-specific repair was observed in the *rad26* (58) or *rad28* mutant (data not shown). Finally, the observation that both the *rad26* and *rad28* mutants are hypermutable after exposure to UV radiation further supports the notion of a relationship between these two yeast genes and provides another correlation between the phenotype of the yeast mutants and that of human CS cells.

The discordant DNA repair phenotypes in yeast and human mutant cells prompt a reconsideration of the notion that defective strand-specific repair is a primary defect in CS. A minority of CS patients exhibit the typical clinical features of XP (6, 21). All such cases reported to date are associated with the XP-B, XP-D, and XP-G genetic complementation groups (6, 21). The fact that the human *XPB* and *XPD* genes are known to encode subunits of the RNA polymerase II transcription factor TFIIH (45, 46) has prompted an alternative view of the molecular pathology of CS. The so-called transcription hypothesis suggests that the complex and diverse clinical phenotype of this disease may arise from a primary defect(s) in RNA polymerase II transcription of a particular subset of genes, the extent and/or timing of the expression of which is critical for normal postnatal growth and development (59). Recent studies have indeed demonstrated reduced levels of RNA polymerase II transcription in vitro in extracts of CS-A and CS-B cells and in extracts of CS/XP-B and CS/XP-D cells (9a). The putative role(s) of the CSA and CSB proteins in transcription is presently unknown. The availability of the cloned yeast *RAD28* and *RAD26* genes and of mutant alleles affords opportunities to explore the transcription hypothesis in greater detail. It is possible that the Rad28 and Rad26 proteins may have functions related though not identical to those of the human CSA and CSB proteins, respectively. For example, it is known that mammalian G_β functions in cell signaling for a variety of ligand-induced G-protein-coupled receptors, while its yeast homolog, Ste4, mediates the pheromone response pathway for mating type differentiation (36). Both proteins have critical roles in signal transduction, but these roles are analogous rather than homologous. Such may be the case for the CS proteins in *S. cerevisiae* and humans.

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