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Preferential repair of ionizing radiation-induced damage in the transcribed strand of an active human gene is defective in Cockayne syndrome

(strand-selective DNA repair/UV damage/xeroderma pigmentosum/human repair disorders)

STEVEN A. LEADON* AND PRISCILLA K. COOPER†

*Department of Radiation Oncology, University of North Carolina, Chapel Hill, NC 27599-7512; and †Life Sciences Division, Lawrence Berkeley Laboratory, University of California, Berkeley, CA 94720

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ABSTRACT **Cells from patients with Cockayne syndrome (CS), which are sensitive to killing by UV although overall damage removal appears normal, are specifically defective in repair of UV damage in actively transcribed genes. Because several CS strains display cross-sensitivity to killing by ionizing radiation, we examined whether ionizing radiation-induced damage in active genes is preferentially repaired by normal cells and whether the radiosensitivity of CS cells can be explained by a defect in this process. We found that ionizing radiation-induced damage was repaired more rapidly in the transcriptionally active metallothionein IIA (MTIIA) gene than in the inactive MTIIB gene or in the genome overall in normal cells as a result of faster repair on the transcribed strand of MTIIA. Cells of the radiosensitive CS strain CS1AN are completely defective in this strand-selective repair of ionizing radiation-induced damage, although their overall repair rate appears normal. CS3BE cells, which are intermediate in radiosensitivity, do exhibit more rapid repair of the transcribed strand but at a reduced rate compared to normal cells. Xeroderma pigmentosum complementation group A cells, which are hypersensitive to UV light because of a defect in the nucleotide excision repair pathway but do not show increased sensitivity to ionizing radiation, preferentially repair ionizing radiation-induced damage on the transcribed strand of MTIIA. Thus, the ability to rapidly repair ionizing radiation-induced damage in actively transcribing genes correlates with cell survival. Our results extend the generality of preferential repair in active genes to include damage other than bulky lesions.**

Damage produced by UV light and certain chemical carcinogens is repaired more rapidly in transcriptionally active DNA compared to the genome as a whole (1, 2) due to a faster repair of damage in the transcribed strand (3, 4). Strand-specific repair of UV damage occurs in *Escherichia coli* (5, 6) and *Saccharomyces cerevisiae* (7–9) as well as mammalian cells and hence appears to be a highly conserved pathway. Recent studies with *S. cerevisiae* strains carrying a temperature-sensitive mutation in one of the subunits of RNA polymerase II have directly demonstrated its dependence on transcription (8, 9). Evidence that the presence of an RNA polymerase stalled at a lesion on the transcribed strand serves as a signal efficiently directing repair to that strand has recently been elucidated in *E. coli*, in which strand-selective repair is known to depend on the product of the *mfd* gene (10). The Mfd protein appears to displace RNA polymerase stalled at a lesion, bind the UvrA subunit of the excision nuclease, and stimulate the repair of the transcribed strand (11). Preferential repair of UV damage in transcriptionally active DNA in human cells has been shown to be specifically defective in cells of patients with the hereditary disease Cockayne syndrome (CS; 12), but whether it occurs by a mechanism similar to that in *E. coli* is not yet known. Complementation analysis has indicated the existence of three genes in CS (13), one of which (complementation group C) is genetically identical to complementation group B of the clinically distinct UV-sensitive disease xeroderma pigmentosum (XP). More recently, CS has been shown to overlap with some members of XP groups D and G as well, thus defining an "XP/CS complex" syndrome (14, 15) and implying the potential involvement of at least five different genes in CS.

The generality of transcription-associated repair for damage other than that induced by UV has not been well established. It has been demonstrated for bulky adducts produced by certain chemical carcinogens (4, 16, 17), suggesting that nucleotide excision repair of bulky lesions in general may be targeted preferentially to the transcribed strand. In contrast, dimethyl sulfate-induced N-methylated purines were found to be removed equally rapidly from both strands of an active hamster gene and from a transcriptionally silent downstream sequence (18). Since such damage is primarily removed by specific glycosylases, this result has been interpreted to suggest that repair initiated by means other than nucleotide excision is not subject to targeting to transcribed strands. Transcription-associated strand-selective removal of thymine glycol formed in the yeast *GAL7* gene by treatment with hydrogen peroxide has recently been demonstrated (8), but since this damaged base can be recognized *in vitro* not only by a specific glycosylase but also by the *E. coli* UvrABC complex (19), it is not yet clear whether its preferential repair in yeast is carried out by the same nucleotide excision repair complex as UV-induced pyrimidine dimers. Whether ionizing radiation-induced damage, which shows little overlap with UV damage in the spectrum of lesions induced, is preferentially repaired in active genes has not previously been reported.

There are very few mammalian mutant cells that are hypersensitive to the lethal effects of both UV- and ionizing radiation-induced damage, consistent with the idea that to a large extent the repair processes for these two types of damage are separable. However, one human mutant cell type that does exhibit such cross-sensitivity is CS (20–22). The radiosensitivity of CS cells raises the question of whether ionizing radiation-induced damage in active genes is preferentially repaired by normal cells but not CS cells. In this study we compare repair of γ-ray damage in transcriptionally active and inactive members of the human metallothionein (MT) gene family in normal, CS, and XP group A fibroblast

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Abbreviations: CS, Cockayne syndrome; MT, metallothionein; XP, xeroderma pigmentosum.

cells using an approach that detects the resynthesis step of excision repair (23, 24). We show that CS but not XP-A cells are defective in a normal ability to perform strand-specific repair of ionizing radiation-induced damage and that the extent of the defect in strand-specific repair correlates with the degree of hypersensitivity to cell killing.

MATERIALS AND METHODS

Cell Culture Conditions and Treatment. Primary human skin fibroblasts obtained from the NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, were as follows: GM38A from a normal individual; GM739 from CS group B patient CS1AN and GM1856 from group A patient CS3BE; and GM5509A from XP group A patient XP12BE. Normal human lung fibroblasts WI38 (CCL 75) were obtained from the American Type Culture Collection. Cells were grown in minimal essential medium containing 10% fetal bovine serum, 2 mM glutamine, and antibiotic/antimycotic solution (GIBCO). For repair analysis, cells were prelabeled for 3 days in medium containing 1 μCi of [^{3}H]thymidine per ml (Amersham; 1 Ci = 37 GBq) with 5 μg of unlabeled thymidine per ml and then grown for an additional 4 days in nonradioactive medium (80–90% confluent). Cells were incubated for 1 hr prior to irradiation in medium containing 10 μM BrdUrd and 1 μM FdUrd, washed with phosphate-buffered saline (PBS), and irradiated either with a ^{60}Co γ-source at dose rates of 1.2–1.4 Gy/min or at 254 nm using a germicidal lamp at an incident dose rate of 1 J/m^2 per sec. Cultures were either harvested immediately or allowed to repair in medium containing BrdUrd and FdUrd. For harvest, cultures were washed twice with PBS and lysed in 10 mM Tris/10 mM EDTA/0.5% SDS.

Colony-Forming Ability. For survival studies, irradiated cell cultures were trypsinized and reseeded at appropriate densities into four to six 100-mm culture dishes per experimental condition. At the end of 7–9 days, cells were stained with crystal violet and colonies that contained >50 cells were counted as survivors. Plating efficiencies for control cultures were 15–32% for GM38A, 9–22% for WI38, 7–14% for CS1AN, 9–20% for CS3BE, and 18–29% for XP12BE.

Repair Analysis. Repair analysis of UV- and ionizing radiation-induced DNA damage was carried out as described (4) using a monoclonal antibody against BrUra (supplied by M. Vanderlaan, Lawrence Livermore National Laboratory, Livermore, CA).

Probes. Strand-specific probes for the MT genes were prepared using the plasmid pZMTIIA, which contains most of the coding region of the human MTIIA gene (4). Linearizing the plasmid with *Hin*dIII and using T7 RNA polymerase and ribonucleotides produces an RNA probe specific for the transcribed strand, while using *Bam*HI and SP6 RNA polymerase generates an RNA probe specific for the nontranscribed strand. Nick translation produces a DNA probe that will hybridize to both strands.

RESULTS

Cell Survival. Normal human fibroblasts GM38A and two CS cell strains CS3BE and CS1AN (from complementation groups A and B, respectively) were irradiated and their colony forming ability was assessed. The two CS strains showed similar levels of hypersensitivity to the lethal effects of UV irradiation (Fig. 1*A*). In contrast, CS1AN was markedly hypersensitive to the lethal effects of γ-irradiation compared to the normal cells, while CS3BE was only slightly more sensitive (Fig. 1*B*). The D_0 value (inverse of the slope of exponential portion of survival curve) for the normal GM38A cells was 1.74 and for CS3BE and CS1AN was 1.30 and 1.04, respectively. Because the known defect in CS cells

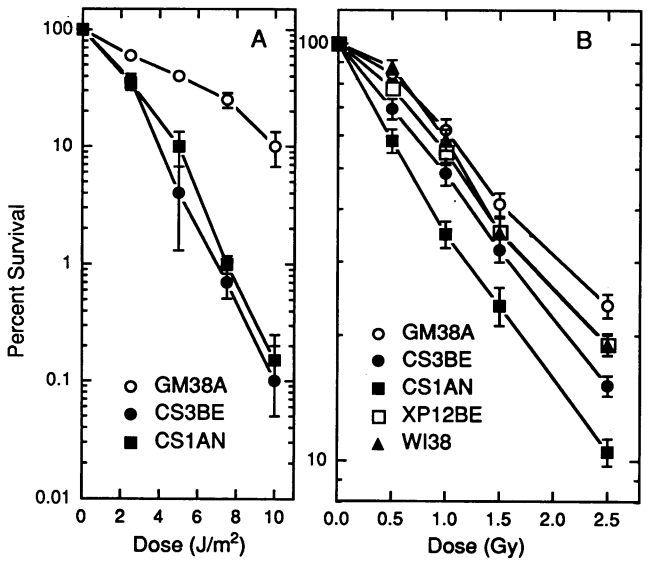

FIG. 1. Sensitivity of CS cells to UV and ionizing radiation. Survival curves for cells exposed to either UV (*A*) or γ-rays (*B*) are shown. Irradiated cultures were reseeded into four to six 100-mm dishes. Points indicate the means of two to five experiments; bars indicate standard errors.

is in preferential repair of UV damage, we wished to compare their observed radiosensitivity to that of cells from an XP complementation group A individual (XP12BE) that are completely defective in UV repair (25). In comparison to GM38A, the XP12BE cells are somewhat more sensitive to killing by γ-rays, with a D_0 value of 1.45 (Fig. 1*B*). However, their survival curve is coincident with that of a second normal fibroblast strain, WI38 (D_0 value of 1.44) and does not overlap with that of CS3BE. We conclude that there is no indication of radiosensitivity for the XP-A cells but that both CS strains are significantly more sensitive to killing by γ-rays than either of the two normal strains tested, with CS1AN showing the greatest hypersensitivity.

Strand Selectivity of Repair of UV Damage in Normal and CS Cells. We used our assay for repair in specific DNA sequences (23, 24) to confirm the reported defect in the preferential repair of UV-induced damage in CS cells, which had previously been demonstrated by a different method (12). Our approach involves the physical separation of DNA regions containing BrdUrd substitution in repair patches from all other DNA using a monoclonal antibody against BrdUrd. We applied this method to examine repair of UV damage in transcriptionally active and inactive MT genes of GM38A normal cells and the two CS strains. Cultured human fibroblasts labeled in their DNA with [^{3}H]thymidine were irradiated with UV (10 J/m^2) and either harvested immediately or allowed to repair in the presence of BrdUrd for 6 hr, a time that we have previously shown to reflect the initial repair rates for UV damage and during which the rate of repair is independent of the size of the restriction fragments probed (24). Purified restriction enzyme-digested unreplicated DNA was allowed to react with a monoclonal antibody that binds BrdUrd in DNA, and total repair was assessed by the amount of ^{3}H-labeled DNA bound by the antibody. Gene-specific repair was determined by electrophoresing on agarose gels equal amounts of DNA from the bound (i.e., containing repair patches) and free (i.e., not containing repair patches) fractions and then quantitating by densitometry the intensity of hybridization to specific restriction fragments of a probe for the MT genes. This value was compared to the total amount of DNA in each fraction to determine the percentage of each strand of the two genes in the bound

fraction. Repair of damage in the MT genes was examined using the plasmid pZMTIIA, which contains a portion of MTII cDNA. Because of the extensive homology (at least 80%) of the multiple members of this gene family to the coding region of MTII, this probe hybridizes to 12–15 different fragments when genomic DNA is digested with *Eco*RI (26). Our studies focused on two members of this gene family: the active MTIIA gene, located on a 5.9-kbp fragment, and the inactive MTIIB gene, which, due to a polymorphic *Eco*RI site in its 5′ flanking region, is on two fragments of 4.8 and 4.6 kbp (26, 27). The size of the transcriptional unit of MTIIA is ≈1 kbp, while MTIIB is ≈0.5 kbp.

Hybridization of probes for the transcribed and nontranscribed strands to the restriction fragments from the bound and free fractions is shown in Fig. 2. Quantitation of the hybridization (Table 1) shows that there is significantly more of the transcribed strand of the active MTIIA gene (15%) in the repaired (bound) fraction in the normal GM38A cells compared to the nontranscribed strand (5%), indicating a more efficient repair of damage on the transcribed strand of the gene compared to the nontranscribed strand. In contrast, there is no more of the transcribed strand of the MTIIA gene in the repaired fraction for either CS strain than there is of the nontranscribed strand, and both of these amounts are similar to the average response of the entire genome (4–6% of total DNA in the repaired fraction). This result confirms by a different method the previously published report (12) of a defect in preferential repair in the same two CS strains and extends it to show that this defect is due to a complete failure to direct repair to the transcribed strand of active genes.

Visual inspection of the autoradiogram in Fig. 2 shows that, for all three cell strains, an equal amount of each strand of the allele of the inactive MTIIB gene that is contained on the 4.6-kb fragment is in the bound and free fractions, and the percentage bound is similar to the average response of the entire genome (Table 1). This result verifies our previous observations that UV-induced damage in the transcription-

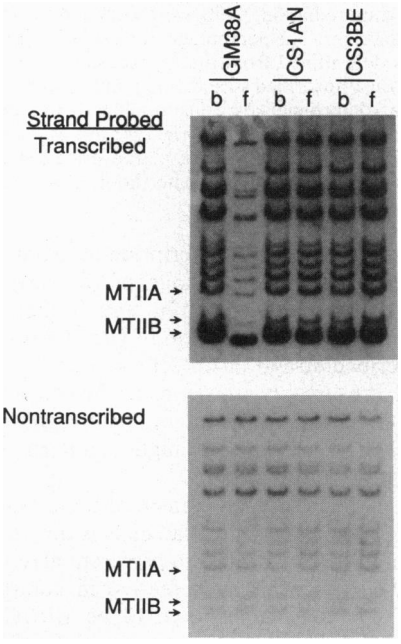

FIG. 2. Southern analysis of strand-selective repair in the MT genes of normal and CS cells following UV. Cells were exposed to UV (10 J/m²) and allowed to repair in the presence of 10 μM BrdUrd. Genomic DNA, digested with *Eco*RI, was allowed to react with the antibody to BrdUrd. DNAs from the bound (b) and free (f) fractions were electrophoresed on 0.7% agarose gels and transferred to GeneScreen*Plus*. Positions of two MT genes detected by probes from pZMTIIA following a 6-hr repair period are identified by arrows.

Table 1. Repair in the MT genes of normal and CS cells following UV (10 J/m²)

		Total amount in each fraction after 6 hr					
		MTIIA			MTIIB		
Cell line	Strand probed	Bound	Free	% bound	Bound	Free	% bound
GM38A	T	20	110	15	20	620	3
	N	20	390	5	10	200	5
CS1AN	T	20	450	4	20	510	4
	N	10	230	4	20	290	6
CS3BE	T	20	480	4	30	540	5
	N	20	400	5	20	350	5

The percentage of total DNA bound was from 4% to 6% for all three cell lines. T, transcribed; N, nontranscribed.

ally inactive MTIIB gene is not preferentially repaired. However, for the GM38A cells, more of the 4.8-kb MTIIB fragment is in the repaired fraction when using a probe for the transcribed strand (Fig. 2). This indicates that damage in the 4.8-kb fragment is preferentially repaired, in contrast to our previous results with human fibrosarcoma cells (4, 24). The most likely explanation for the repair differences between the two fragments containing MTIIB is the presence of transcriptionally active DNA in the additional 5′ flanking region associated with the 4.8-kb fragment. Since repair patches produced anywhere on a DNA fragment are detected in our assay, this would result in damage in the 4.8-kb fragment appearing in the preferentially repaired population even though the MTIIB gene itself is inactive. We have therefore confined our analysis of repair in the MTIIB gene to the 4.6-kb fragment in this study.

Strand Selectivity of Repair of γ-Ray Damage in Normal, XP-A, and CS Cells. Using the same approach, we examined whether damage produced by ionizing radiation is preferentially repaired in active genes. Normal GM38A cells, cells of the two CS strains, and XP12BE cells were exposed to 10 Gy of γ-rays and allowed to repair for 30 or 60 min in the presence of BrdUrd as before. These shorter repair times in comparison to the UV experiments were chosen based on our previous work showing essentially complete removal of radiation-induced thymine glycols in human cells in 2 hr following irradiation (23). Hybridization to the restriction fragments from the bound and free fractions from cells after 1 hr of repair is shown in Fig. 3. The percentage of the MTIIA gene and 4.6-kb MTIIB fragment, and of each strand of those genes, in the bound fraction for each repair point is indicated in Fig. 4. In the normal GM38A cells, repair in the active MTIIA gene is much faster than in the inactive MTIIB gene (Fig. 4*A*). This more efficient repair arises from a more rapid repair on the transcribed strand of the gene (15% in 1 hr) and a slower rate, like that of the genome overall, on the nontranscribed strand (3%). Both strands of the MTIIB gene are repaired with kinetics similar to total DNA (4% in 1 hr). In the CS3BE cells, which are intermediate in sensitivity to ionizing radiation, a more efficient repair on the transcribed strand of the MTIIA gene is also observed, but at a considerably reduced rate (Fig. 4*B*). As in the normal cells, the nontranscribed strand of the MTIIA gene of CS3BE and both strands of the MTIIB gene showed repair rates similar to total DNA. However, in the CS1AN cells, which are the most radiosensitive, there is a complete absence of strand-selective repair in the MTIIA gene (Fig. 4*C*). Thus, the defect in preferential repair of ionizing radiation-induced damage in active genes in CS1AN mimics its defect in repair of UV damage, while CS3BE appears to have a partial defect in preferential repair of radiation damage in contrast to its complete lack of preferential repair of UV damage. A defect in nucleotide excision repair in itself does not result in loss of preferential

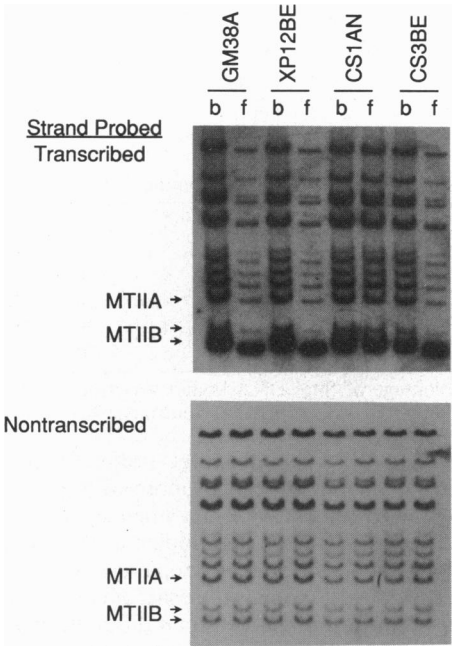

FIG. 3. Southern analysis of strand-selective repair in the MT genes of normal and CS cells following ionizing radiation. Cells were exposed to 10 Gy of γ-rays and allowed to repair in the presence of 10 μM BrdUrd. Genomic DNA, digested with *Eco*RI, was allowed to react with the antibody to BrdUrd. DNAs from the bound (b) and free (f) fractions were electrophoresed on 0.7% agarose gels and transferred to GeneScreen*Plus*. Positions of two MT genes detected by probes from pZMTIIA following a 1-hr repair period are identified by arrows.

repair of ionizing radiation-induced damage in active genes, as shown by the essentially normal rapid repair of the MTIIA gene in XP12BE cells, with 12% of the transcribed strand found in the repaired fraction after 1 hr (Fig. 4*D*).

DISCUSSION

In this study, we demonstrated that in normal human fibroblasts and excision-repair-defective fibroblasts from patients of XP complementation group A, DNA damage produced by ionizing radiation is preferentially repaired in the transcriptionally active MTIIA gene compared to the nontranscribed MTIIB pseudogene or to the genome overall. We show that this preferential repair is due to a much faster initial repair on the transcribed strand, as has previously been found for UV damage and certain chemical carcinogens (3, 4). Our observation extends the generality of transcription-associated repair to include damage other than bulky lesions recognized by nucleotide excision repair, at least as it is defined by its defect in XP-A.

The approach we used for measuring repair in specific DNA sequences recognizes the repair patch itself and therefore does not provide information on the type of damage being repaired. Ionizing radiation damage includes a large variety of altered bases as well as both single strand and double strand breaks (28, 29), and restitution of most or all of these would be expected to give rise to a repair patch. We recently demonstrated transcription-associated removal of thymine glycols induced in yeast by treatment with hydrogen peroxide (8). Since this product of oxidative damage is also formed by ionizing radiation and rapidly removed from human cells (23), we presume that repair of thymine glycols and perhaps other similar oxidatively damaged bases is one component of the observed strand-selective preferential repair reported here, but this remains to be directly demonstrated. Most models for transcription-associated repair assume a requirement for blockage of transcription by the lesion to be preferentially repaired, and, in fact, blockage of RNA polymerase II transcription *in vitro* by thymine glycol in the transcribed strand has recently been directly demonstrated (30). Presumably, scissions in the transcribed strand would also fulfill this requirement, so the possibility exists that repair of strand breaks might similarly be directed preferentially to active genes.

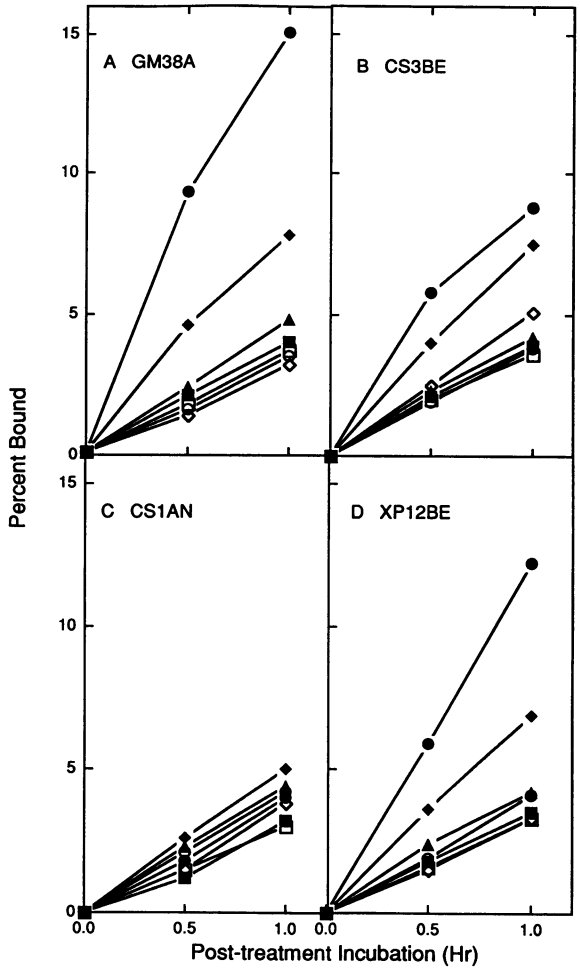

FIG. 4. Kinetics of strand-selective repair in the MT genes following ionizing radiation. Cells were exposed to 10 Gy of γ-rays and allowed to repair. The percentage of total DNA (▲) bound by the antibody was determined from the ^{3}H prelabel. The percentage of both strands, the transcribed strand, and nontranscribed strand of the MT genes was determined by densitometry of the autoradiogram. MTIIA: both strands (◆); transcribed strand (●); nontranscribed strand (○). MTIIB: both strands (◇); transcribed strand (■); nontranscribed strand (□). Points indicate the means of three to seven experiments.

Although the detailed mechanism of the coupling of transcription and repair in mammalian cells is not known, one of the genes required for targeting of preferential repair has been identified. The gene that is defective in complementation group B of CS has been shown to be *ERCC6* (31), and mutations in both alleles of this gene have been demonstrated in CS1AN, the most radiosensitive of the CS strains in our study. The gene defective in CS complementation group A has not yet been identified, nor have separate roles of the CS-A and CS-B gene products in transcription-associated repair of UV damage been defined. Since the sole representatives of group A and group B mutants studied thus far are equally devoid of the ability to target repair of UV damage to

active genes (Table 1 and ref. 12), both gene products are presumably required for this process. At this point we do not know if our finding of a partial defect in preferential repair of γ-ray damage in the group A mutant CS3BE is characteristic of group A mutants in general or is a property of the particular mutation(s) in CS3BE. In the former case, this would be evidence of separable roles for the two gene products; in the latter case, analysis of the CS3BE mutation once the gene has been identified may provide insight into the function of the affected domain. In either case, CS group A mutants may prove useful in dissecting the interaction of proteins required for targeting with particular repair proteins that may differ for UV and γ-ray damage.

In addition to CS group A and B genes, there is evidence for the potential involvement of three other genes in CS, since certain XP group B, D, and G individuals also display clinical symptoms of CS (14, 15). The XP-B/CS and XP-D genes have been identified as *ERCC3* and *ERCC2*, respectively (32, 33), and both encode proteins with putative helicase functions whose homologs have vital functions that are separable from their roles in repair (reviewed in ref. 15). The ERCC3 protein has recently been shown to be a component of transcription factor BTF2 (34), presumably defining the vital function of ERCC3. However, its role as a transcription factor is unlikely to explain the absolute requirement for ERCC3 in overall excision repair activity, which is undetectable after UV in XP-B (25). Since it seems likely that the clinical features of Cockayne's group B patients stem from a defective ability of ERCC6 to function as a transcription repair coupling factor (11), the most conservative interpretation is that the CS symptoms in XP/CS also arise from a defect in transcription-associated repair rather than from a general defect in transcription. However, the conjunction of XP and CS symptoms poses a paradox, since the XP defect is an inability to repair bulky lesions throughout the genome, while the defect in CS has been presumed to be in a subset of that response—i.e., its targeting to actively transcribing DNA. Our finding that the CS defect is not limited to repair of bulky lesions but includes ionizing radiation damage that is repaired normally in XP-A suggests a possible explantation for the XP/CS complex. We propose the following model: (*i*) the genes that are defective in XP groups B, D, and possibly G have dual roles both in the removal of bulky lesions from the genome overall and in transcription-associated repair, with the latter role associated with a vital function; (*ii*) most viable mutations in these genes affect the former repair function only but rarely observed mutations affect both; (*iii*) CS symptoms occur in those rare XP cases in which a mutation affects both hypothesized functions and arise from inability to target to active genes the rapid repair of metabolically generated oxidative DNA damage similar to the preferentially repaired component of radiation damage. A prediction of the model is that cells from XP/CS complex individuals should also be hypersensitive to killing by ionizing radiation and defective in its preferential repair.

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