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The genetic defect in Cockayne syndrome is associated with a defect in repair of UV-induced DNA damage in transcriptionally active DNA

(preferential DNA repair/human disorders/pyrimidine dimer)

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ABSTRACT **Cells from patients with Cockayne syndrome (CS) are hypersensitive to UV-irradiation but have an apparently normal ability to remove pyrimidine dimers from the genome overall. We have measured the repair of pyrimidine dimers in defined DNA sequences in three normal and two CS cell strains. When compared to a nontranscribed locus, transcriptionally active genes were preferentially repaired in all three normal cell strains. There was no significant variation in levels of repair between various normal individuals or between two constitutively expressed genes, indicating that preferential repair may be a consistent feature of constitutively expressed genes in human cells. Neither CS strain, from independent complementation groups, was able to repair transcriptionally active DNA with a similar rate and to the same extent as normal cells, indicating that the genetic defect in CS lies in the pathway for repair of transcriptionally active DNA. These results have implications for understanding the pleiotropic clinical effects associated with disorders having defects in the repair of DNA damage. In particular, neurodegeneration appears to be associated with the loss of preferential repair of active genes and is not simply correlated with reduced levels of overall repair.**

Cockayne syndrome (CS) is an autosomal recessive disorder characterized by growth retardation, skeletal and retinal abnormalities, progressive neurological degeneration, and severe photosensitivity. At the cellular level, CS is characterized by an increased sensitivity to the killing effects of UV-irradiation and, in response to UV-irradiation, CS cells show elevated levels of sister chromatid exchanges and reduced host-cell reactivation of irradiated viruses and are hypermutable (1). These responses indicate that CS cells are defective in their ability to repair DNA damage. Analysis of cultured CS cells indicated an apparently normal capacity for removing UV-induced DNA damage. The rate of removal of T4 endonuclease-sensitive sites, levels of repair replication, postreplication repair, incision frequencies, and ligation of repair patches after UV-irradiation fell within the normal range (2). This is in contrast to the UV-sensitive disorder xeroderma pigmentosum (XP) in which UV sensitivity has been correlated with the inability to excise UV-induced DNA damage or to seal daughter-strand gaps left after DNA synthesis on a damaged template (1).

Despite the apparently normal ability of CS cells to remove DNA damage, CS cells were unable to restore normal levels of RNA and DNA synthesis after UV-irradiation, a defect also seen in excision-defective XP cells (3). After low fluences of UV-irradiation, normal cells showed a depression of RNA synthesis but recovery to unirradiated levels was rapid and occurred before the removal of the bulk of pyrimidine dimers. As pyrimidine dimers are blocks to the transcription machinery (4) and as recovery of RNA synthesis occurred before the removal of these blocks in the genome overall, Mayne and Lehmann (3) proposed that an excision pathway existed in normal cells to preferentially remove damage from transcriptionally active chromatin. Further it was proposed (3) that, as CS cells were unable to restore RNA synthesis to unirradiated levels despite a normal removal of damage in the bulk of the DNA, the CS defect might lie in this special excision pathway that allowed preferential repair of damage in transcriptionally active DNA.

Evidence for the existence of a special repair pathway for the removal of pyrimidine dimers from transcriptionally active DNA has since come from studies of excision repair at the gene level. In UV-irradiated Chinese hamster ovary cells, single-copy and amplified copies of the housekeeping gene dihydrofolate reductase (DHFR) were shown to be repaired rapidly and efficiently when compared to the low level of repair seen in the bulk DNA (5, 6). These studies have been extended to human cells for the amplified DHFR gene and the single-copy c-*abl* and actin genes. In each case, pyrimidine dimers in the transcriptionally active gene were repaired at a faster rate than the bulk DNA (7, 8).

Direct evidence for a repair defect in CS cells came from our studies (9) on the distribution of UV-induced repair sites in chromatin loops associated with the nuclear matrix. In normal cells after low fluences of UV-irradiation, DNA associated with the nuclear matrix was preferentially repaired relative to the loop DNA. Transcriptionally active DNA has been shown to be enriched at the nuclear matrix and this result is consistent with an excision pathway that preferentially removes damage in transcriptionally active DNA. In CS cells, nuclear matrix-associated DNA was less efficiently repaired than loop DNA (9), suggesting that CS cells are deficient in repair of transcriptionally active DNA.

In this report we examine the repair of transcriptionally active and inactive genomic DNA directly by measuring the removal of pyrimidine dimers in defined DNA sequences. We have chosen the constitutively expressed housekeeping genes for adenosine deaminase (ADA) (10) and DHFR (11) and compared their levels of repair to the nontranscribed locus 754 and the genome overall. The X chromosome locus 754 is located in the proximity of the Duchenne muscular dystrophy locus and is not transcribed in HeLa or primary human cells (12). We have used both primary and simian virus 40 (SV40)-transformed skin fibroblasts from normal individuals and from individuals from two complementation groups of CS.

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Abbreviations: ADA, adenosine deaminase; CS, Cockayne syndrome; DHFR, dihydrofolate reductase; XP, xeroderma pigmentosum; SV40, simian virus 40.

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In normal human primary cells, we demonstrate that during the first 24 hr after UV-irradiation the ADA and DHFR genes are repaired at a faster rate and to a greater extent than the nontranscribed chromosomal locus 754. Similar results were found for SV40-transformed normal cell lines, confirming previous reports (7) of preferential repair of active genes in transformed human cell lines. In contrast, CS cells are able to remove UV-induced pyrimidine dimers from their DNA but they are unable to repair the DHFR and ADA genes as rapidly and efficiently as normal cells. Instead, the level and rate of repair of both active genes are similar to those of nontranscribed DNA.

MATERIALS AND METHODS

Cell Culture and Prelabeling of Cells. Primary skin fibroblasts derived from a normal (VH16) and a CS donor (CS3BE) were cultured in Ham's F10 medium supplemented with 15% (vol/vol) fetal calf serum and antibiotics. SV40-transformed human fibroblast lines from normal [MRC5V1 (13) and 1BR.3gn2 (14)] and CS donors [CS3BE.S3.G1 and CS1AN.S3.G2 from complementation groups A and B, respectively (14)] were cultured in Eagle's minimal essential medium supplemented with 10% fetal calf serum and antibiotics. Cells were prelabeled with [^{3}H]thymidine 0.1 μCi/ml; (300–370 GBq/mmol; 1 Ci = 37 GBq) and 1 μM thymidine (Sigma) for 3–7 days. For stationary-phase cells, cultures were set up and left for 10 days. The medium was changed every 3 days.

UV-Irradiation and Incubation of Cells After UV-Irradiation. Cells were irradiated in monolayer with a Philips TUV lamp (254 nm) at a dose rate of 0.21–0.35 J·m^{-2}·sec^{-1}. After irradiation, cells were harvested immediately or incubated in medium containing 10 μM bromodeoxyuridine and 1 μM fluorodeoxyuridine.

Isolation and Purification of DNA. Cells were lysed in 150 mM NaCl/1 mM EDTA/0.5% SDS/10 mM Tris·HCl, pH 8.0, and incubated with proteinase K (100 μg/ml) at 37°C for a minimum of 1 hr. DNA was further purified by phenol extraction and digestion with RNase. Ethanol-precipitated DNA was resuspended in 1 mM EDTA/10 mM Tris·HCl, pH 8.0 (TE), and digested to completion with *Bcl* I, *Eco*RI, or *Hin*dIII (Pharmacia) at 2.5 units/μg of DNA for 16 hr at 50°C (*Bcl* I) or 37°C (*Eco*RI or *Hin*dIII). Nonreplicated DNA was then isolated using CsCl gradients. Gradients were fractionated and radioactivity in samples was measured in a scintillation counter to determine the amounts and positions of parental and hybrid density DNA. Pooled fractions of parental DNA were dialyzed against TE and the DNA content was determined by absorption measurement at 260 nm. The DNA was concentrated by butanol extraction and precipitated with ethanol.

T4 Endonuclease V Digestion. DNA samples were resuspended at 1 μg/μl of TE and 15-μg portions of each sample were incubated in 10 mM Tris·HCl, pH 8.0/10 mM EDTA/75 mM NaCl with either T4 endonuclease V (15) or enzyme buffer. (Under our experimental conditions, T4 endonuclease V completely digested 1 μg of plasmid DNA containing an average of four pyrimidine dimers per kilobase pair of DNA.) The reaction was terminated by the addition of alkaline loading buffer to give a final concentration of 50 mM NaOH/1 mM EDTA/2.5% (vol/vol) Ficoll/0.025% bromocresol blue.

Electrophoresis and Southern Blot Analysis. Equivalent amounts (based on radioactivity) of T4 endonuclease V-treated or untreated DNA were loaded into adjacent wells in an alkaline 0.6% agarose gel and electrophoresed for 16 hr at 20 V in buffer containing 30 mM NaOH and 1 mM EDTA. DNA was transferred to a nylon membrane (NEN and Amersham) in a solution containing 0.6 M NaCl and 0.4 M NaOH. Membranes were prehybridized (16 hr) and hybridized (48 hr) in 40% (vol/vol) formamide at 42°C as described by the manufacturer. DNA probes were labeled by random-primer extension using [α-^{32}P]dATP. After hybridization washes were at 65°C with a final wash in 1× SSPE/1% SDS (1× SSPE = 0.15 M NaCl/10 mM sodium phosphate, pH 7.4/1 mM EDTA). Membranes were exposed to Fuji or Kodak XARS films using intensifying screens at −70°C. Intensities of bands were quantified using a Bio-Rad video densitometer model 620. The average number of T4 endonuclease V-sensitive sites were calculated from the band densities as described (5).

DNA Probes. The two *Pst* I fragments covering exons 1–5 (BA) and 12 (BE), isolated from the ADA cDNA clone pLL and inserted into pUC19 were a gift from T. M. Berkvens (10). The 2.0-kilobase-pair (kb) *Hin*dIII fragment of the 754 locus, cloned into pAT 153, was a gift from B. Bakker (University of Leiden, The Netherlands) (12). A 1.8-kb *Eco*RI genomic fragment from the human DHFR gene was a gift of V. Bohr (National Institutes of Health) (11).

Measurement of T4 Endonuclease V-Sensitive Sites in the Genome Overall. T4 endonuclease V sensitive sites were determined as described by Van Zeeland *et al.* (16).

RESULTS

Removal of Pyrimidine Dimers from Defined DNA Sequences. Repair of damage in defined DNA sequences was measured using the method of Bohr *et al.* (5, 17). DNA was extracted from cells at various times after UV-irradiation and digested with a restriction enzyme and then with T4 endonuclease V. T4 endonuclease V incised the DNA at the site of pyrimidine dimers and led to the loss of gene-specific bands when the DNA was separated on alkaline agarose gels and analyzed by Southern blotting. With increasing time after UV-irradiation and subsequent repair of the damage, the intensity of the band returned. Thus the intensity of the band gave a direct measure of the amount of damage left in that piece of DNA and, therefore over time, a measure of the rate of repair in defined fragments of DNA.

By using this method, we compared the removal of pyrimidine dimers from two transcriptionally active genes (DHFR and ADA) and a nontranscribed locus (754). Fig. 1 shows the genomic organization of these genes. Removal of pyrimidine dimers was measured in the 19.9-kb *Bcl* I fragment and the

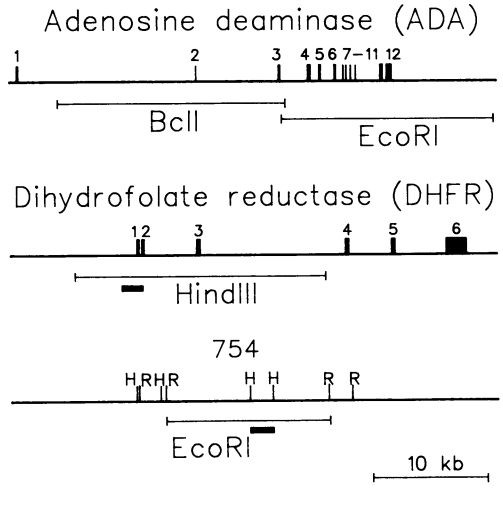

FIG. 1. Genomic organization of the ADA, DHFR, and 754 loci. Genomic DNA maps indicating exons, relevant enzyme restriction sites, and location of DNA probes. Solid lines in the DHFR and 754 maps indicate genomic probes. ADA probes were cDNA fragments comprising exons 1–5 (for the *Bcl* I fragment) and exon 12 (for the *Eco*RI fragment), respectively. H, *Hin*dIII; R, *Eco*RI. Scale is in kb.

18.5-kb *Eco*RI fragment of the ADA gene. The *Bcl* I fragment forms the 5′ end of the gene and is entirely within the transcription unit. The *Eco*RI fragment extends beyond the 3′ end of the gene and contains 9 kb of flanking DNA. Pyrimidine dimer removal was also measured in the 22-kb *Hin*dIII fragment at the 5′ end of the DHFR gene and in the 14-kb *Eco*RI fragment of the 754 locus (12).

Repair in defined DNA sequences was measured using both primary and SV40-transformed skin fibroblasts. Cells were derived from three normal individuals and two CS patients. The CS cells represented two complementation groups, indicating the involvement of independent genetic defects (18).

Removal of Pyrimidine Dimers from Defined DNA Sequences in Primary Human Fibroblasts. Cultures of stationary-phase primary cells were UV-irradiated (10 J/m^2) and allowed to repair for various times up to 24 hr after treatment. Under these conditions, no replicating DNA was detected on CsCl gradients, confirming that the cells were in stationary phase. Fig. 2 is an autoradiogram of DNA isolated from both normal and CS cells and probed with the *Eco*RI fragment of the ADA gene. The intensity of the band representing the ADA gene immediately after irradiation (t = 0 hr) was diminished after treatment with T4 endonuclease V, indicating the presence of pyrimidine dimers. The frequency of dimers in the ADA gene was estimated as 0.75 dimer per 10 kb for normal and CS cells and compared well with 0.80 dimer per 10 kb measured in the genome overall. In Fig. 2*A*, the intensity of the ADA band after treatment with T4 endonuclease V had significantly returned within 4 hr and by 24 hr had reached an intensity similar to the untreated band, indicating the near complete removal of pyrimidine dimers in the ADA gene of normal primary cells. In contrast, the CS ADA band (Fig. 2*B*) showed only limited repair and did not return to full intensity during 24 hr, indicating that CS cells were unable to perform normal levels of repair of the ADA gene.

Fig. 3 summarizes data for the ADA, DHFR, and 754 loci obtained from autoradiograms similar to the one shown in Fig. 2. Fig. 3*A* shows the rate of removal of pyrimidine dimers from the 19.9-kb *Bcl* I ADA fragment, the 18.5-kb *Eco*RI ADA fragment, and the DHFR gene in normal and CS primary cells. For normal cells, the rate and extent of repair for both ADA fragments and the DHFR gene were very similar. Repair was rapid at early times with 10–20% of pyrimidine dimers being removed within the first 2 hr and more than 60% of the pyrimidine dimers being removed by about 8 hr. Repair reached 80–90% by 24 hr. In contrast, in CS cells little or no repair was seen in these actively transcribed loci during the first 4 hr after irradiation. Repair reached only 20–30% at about 8 hr and 30–50% at 24 hr.

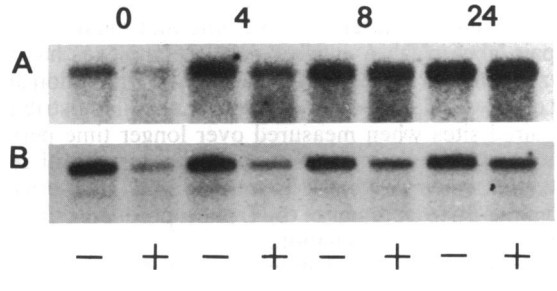

FIG. 2. Autoradiograms of DNA isolated at various times after UV-irradiation. DNA was isolated from normal VH16 (*A*) and CS3BE (*B*) cells incubated for 0, 4, 8, and 24 hr after a UV fluence of 10 J/m^2 and digested with *Eco*RI. DNA samples were treated (+) with T4 endonuclease V or were untreated (−) and separated on an alkaline 0.6% agarose gel. After transfer to nylon membranes, the filters were probed with the ADA BE fragment (exon 12).

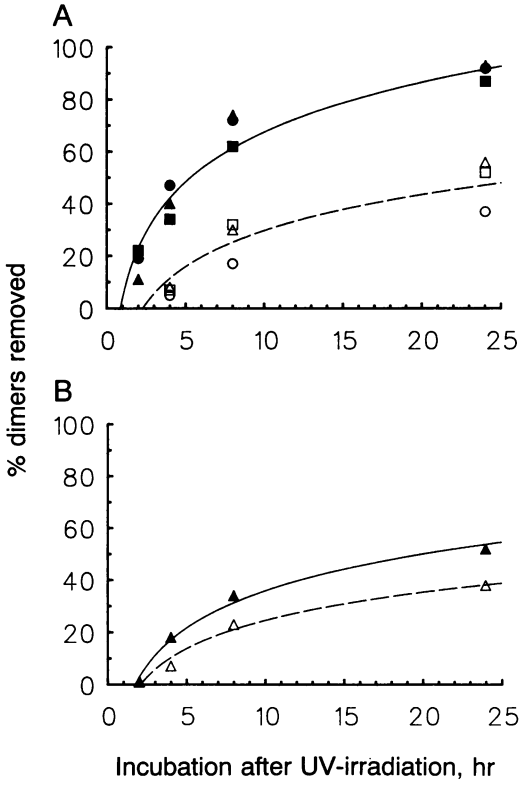

FIG. 3. Removal of dimers from defined DNA sequences in primary human skin fibroblasts from a normal individual and a CS patient. The proportion of dimers removed from primary fibroblasts is shown for various times after a UV fluence of 10 J/m^2. Normal cells were VH16 (solid symbols). CS cells were CS3BE (open symbols). (*A*) ADA *Eco*RI [▲ (n = 6) and △ (n = 3)], ADA *Bcl* I [● (n = 2) and ○ (n = 1)]. DHFR *Hin*dIII [■ (n = 2) and □ (n = 1)]. (*B*) 754 *Eco*RI [▲ (n = 3) and △ (n = 2)].

The rate of repair of the 754 locus is shown in Fig. 3*B*. In normal cells, the rate and extent of repair of the 754 locus was significantly less than that in the ADA and DHFR genes. No repair occurred in the first 2 hr after treatment and only 50% of the pyrimidine dimers were removed within 24 hr. In CS cells the 754 locus was repaired at a similar rate to that in normal cells. Moreover, this repair level was comparable with that observed in the active ADA and DHFR genes in CS cells.

Repair of pyrimidine dimers in the genome overall in normal cells was determined by alkaline sucrose gradient centrifugation (16). Average repair values from two independent experiments were 23%, 33%, and 67% after 4, 8, and 24-hr, respectively.

Removal of Pyrimidine Dimers from Defined DNA Sequences in Transformed Human Cells. The rate of removal of pyrimidine dimers from defined DNA sequences was measured in SV40-transformed derivatives of two normal and two CS cell lines. The repair of the ADA, DHFR, and 754 loci after a UV fluence of 10 J/m^2 is shown in Fig. 4. The kinetics and extent of repair for these loci were comparable for SV40-transformed and primary cells. The DHFR and ADA genes were preferentially repaired relative to the 754 locus in normal cells but not in the CS cells.

Repair was also examined after UV fluences of 7.5 and 15 J/m^2 in normal and two CS cell lines from two complementation groups. Fig. 5 shows the rate and extent of repair in the ADA *Eco*RI gene fragment after various UV fluences. Normal cells showed a consistently fast and efficient repair of the ADA gene, indicating that preferential repair was not saturated at UV fluences up to 15 J/m^2. Compared to normal

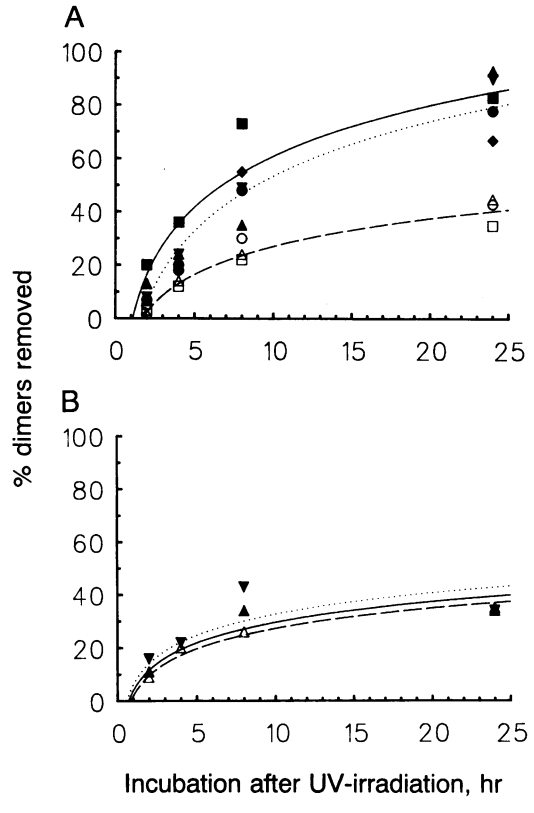

FIG. 4. Removal of dimers from defined DNA sequences in SV40-transformed human fibroblasts from normal individuals and a CS patient. The proportion of dimers removed from SV40-transformed fibroblasts is shown for various times after a UV fluence of 10 J/m^2. Normal cells were MRC5V1 (solid line) and 1BR.3gn2 (dotted line). CS cells were CS3BE.S3.G1 (dashed line). (*A*) ADA *Eco*RI, ADA *Bcl* I, DHFR *Hin*dIII. (*B*) 754 *Eco*RI. For MRC5V1: ▲, ADA *Eco*RI (n = 2) or 754 *Eco*RI (n = 1); ■, DHFR *Hin*dIII (n = 1). For 1Br.3gn2: ▼, ADA *Eco*RI (n = 1) or 754 *Eco*RI (n = 1); ●, ADA *Bcl* I (n = 1); ◆, DHFR *Hin*dIII (n = 1). For CS3BE.SE.G1: △, ADA *Eco*RI (n = 3) or 754 *Eco*RI (n = 2); ○, ADA *Bcl* I (n = 3); □, DHFR *Hin*dIII (n = 2).

cells, CS cells exhibited a reduced repair of the ADA gene over the whole dose range employed.

DISCUSSION

In this study, we have demonstrated that UV-induced pyrimidine dimers are preferentially repaired in the transcriptionally active ADA and DHFR genes when compared to a nontranscribed locus (754) or to the genome overall. These results were obtained using cells from three normal individuals. The analysis was made on single-copy sequences, and for ADA and locus 754 we were able to obtain results from the same experiment (using the same nylon membrane for hybridization) for both genes, providing an internal control. We did not detect significant differences in repair between the two active genes, nor were there differences between the different normal human cells. These results confirm and extend previous observations on preferential repair of active genes in human cells (for review, see ref. 19).

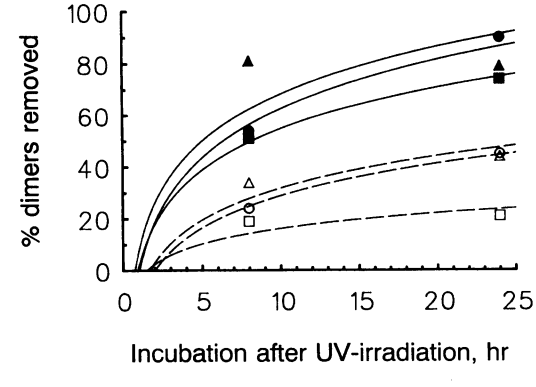

FIG. 5. Removal of dimers from the ADA *Eco*RI gene fragment after various UV fluences. The proportion of dimers removed from SV40-transformed fibroblasts after 8 and 24 hr is shown after various UV fluences. For 7.5 J/m^2: ▲, MRC5V1 (——); △, CS3BE.S3.G1 (– – –). For 10 J/m^2: ●, 1BR.3gn2(—); ○, CS3BE.S3.G1 (– – –). For 15 J/m^2: ■, MRC5V1 (——); □, CS1AN.S3.G2 (– – –).

In primary normal cells exposed to UV-irradiation (10 J/m^2) repair of active genes was very rapid with 40% of pyrimidine dimers being removed within 4 hr. Repair was almost complete (80%) within 24 hr. In SV40-transformed normal cells repair appeared to be somewhat slower. In both cell types, the 754 locus was repaired at a slower rate with only 20% of pyrimidine dimers being removed within 4 hr and about 50% after 24 hr. The repair level after 24 hr is less than observed in preliminary experiments reported earlier (20) and is lower than that of the genome overall (about 65%). There were no apparent differences in repair between the 5′ and 3′ ends of the ADA gene despite the inclusion of 9 kb of flanking DNA in the 3′ *Eco*RI fragment. This result may be due to an overlapping transcript originating in the flanking DNA (21). Alternatively, repair domains may extend beyond the actual coding sequence.

Cells from patients with CS are hypersensitive to UV-irradiation but are able to remove pyrimidine dimers from the bulk of their DNA at normal rates (2). We demonstrate here that cells from two CS patients belonging to two complementation groups are unable to repair transcriptionally active DNA as rapidly and as efficiently as normal cells. Virtually no removal of pyrimidine dimers from the ADA and DHFR genes occurred during the first 4 hr after irradiation. At later times CS cells are able to repair pyrimidine dimers in the ADA and DHFR genes, but they do so at a similar rate and extent seen for the inactive 754 locus. In CS, the rate and extent of repair of all three loci 24 hr after treatment is similar to that seen for the 754 locus in normal cells. I. Mellon and P. C. Hanawalt (personal communication) found similar results with CS1AN cells for the c-*abl* gene.

In a previous report (9), we demonstrated that repair synthesis in CS cells was depleted in DNA associated with the nuclear matrix when compared to normal and XP-C cells. As active genes are associated with the nuclear matrix, these results suggested that repair of active genes may be deficient even when compared to inactive DNA or the genome overall. The present data do not reveal striking differences in repair between active and inactive DNA. However, the repair level of the genome overall, which is similar in normal and CS cells, may exceed that of active genes in CS cells, especially during the first 4 hr after UV. We note that the distribution of repair label in DNA–nuclear matrix complexes has been analyzed only during the first 2 hr after UV-irradiation and it is not clear whether CS cells will exhibit a similar distribution of repaired sites when measured over longer time periods. Furthermore, repair incorporation is the sum of all repair events that incorporate [^{3}H]thymidine and may originate predominantly from repair of (6–4) photoproducts during the first few hours after irradiation.

The finding of a reduced ability of CS cells to perform fast and efficient repair of active genes over a dose range of 7.5 to 15 J/m^2 provides an explanation for the lack of recovery of transcription and is consistent with increased levels of cell killing and mutagenesis in CS cells after UV irradiation. From these data, we propose that the gene(s) defective in CS are essential for efficient repair of transcriptionally active DNA after UV-irradiation. The defect relates to a reduced repair

rate and also affects the extent of repair of pyrimidine dimers from active genes when measured over a 24-hr period. Mellon *et al.* (22) demonstrated that preferential repair of the amplified DHFR gene in a normal human cell line was due to selective repair of the transcribed strand over the nontranscribed strand. The data presented here do not distinguish between repair of both strands. Experiments using strand-specific probes may reveal whether CS cells are deficient in preferential repair of transcribed strands. Further experiments are also needed to determine whether the reduced repair is limited to transcriptionally active DNA or whether the CS defect extends to other domains of DNA as well.

The clinical manifestations of CS may be a consequence of the failure to perform preferential repair. This proposal is supported by clinical and cellular observations at XP. In XP, some but not all patients suffer from severe and progressive neurodegeneration. The incidence of neurodegeneration correlates with the level of survival of fibroblast cultures after UV-irradiation (24). Andrews *et al.* (24) proposed that neurodegeneration results from the failure to repair DNA damage leading to premature death of neurons. If neurodegeneration was simply due to a general failure to repair damage in neurons, this correlation should extend to CS. However, in general, the survival of CS cells is higher than that of XP cells from patients with neurodegeneration yet virtually all CS patients suffer from severe and progressive degeneration. Excision-defective XP cells from complementation groups C and D have very similar levels of overall excision repair of UV-induced DNA damage. However, in general, group C cells are more resistant to UV-irradiation and show enhanced recoveries of RNA and DNA synthesis (3). We (20, 23) have demonstrated that XP group C cells are able to perform normal levels of repair in active genes despite being deficient in repair of the 754 locus and the genome overall. The repair capabilities of XP-C cells appear to be the reverse of those in CS. The data presented here and our previous results (20, 23) on XP-C cells suggest that the key factor in neurodegeneration is not survival, but the ability to perform preferential repair of transcriptionally active genes. XP-C cells are able to perform preferential repair and patients virtually never suffer from neurodegeneration. In contrast, it is likely that cells from XP-A and -D patients have lost both their ability to repair the bulk of DNA and to repair transcriptionally active genes preferentially. Patients from XP groups A and D have a very high incidence of neurological degeneration. The nervous system may be a potentially sensitive target for the loss of this pathway as central nervous system neurons do not divide, have very high levels of transcription, and transcribe a larger fraction of their genome than other cell types. In addition, the interconnecting nature of the nervous system amplifies the consequences of losing a single cell where the loss of one "link in the chain" leads to the effective loss of the whole chain.

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