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Deficient repair of the transcribed strand of active genes in Cockayne's syndrome cells

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ABSTRACT

Removal of ultraviolet light induced cyclobutane pyrimidine dimers (CPD) from active and inactive genes was analyzed in cells derived from patients suffering from the hereditary disease Cockayne's syndrome (CS) using strand specific probes. The results indicate that the defect in CS cells affects two levels of repair of lesions in active genes. Firstly, CS cells are deficient in selective repair of the transcribed strand of active genes. In these cells the rate and efficiency of repair of CPD are equal for the transcribed and the nontranscribed strand of the active ADA and DHFR genes. In normal cells on the other hand, the transcribed strand of these genes is repaired faster than the nontranscribed strand. However, the nontranscribed strand is still repaired more efficiently than the inactive 754 gene and the gene coding for coagulation factor IX. Secondly, the repair level of active genes in CS cells exceeds that of inactive loci but is slower than the nontranscribed strand of active genes in normal cells. Our results support the model that CS cells lack a factor which is involved in targeting repair enzymes specifically towards DNA damage located in (potentially) active DNA.

INTRODUCTION

Cockayne's syndrome (CS) is an autosomal recessive disease characterized by growth retardation, skeletal and retinal abnormalities, progressive neural degeneration, and severe photosensitivity. Cells derived from CS patients are hypersensitive to the lethal and mutagenic effects of ultraviolet light (UV), suggesting a defect in DNA excision repair (1). Initial studies failed to show an abnormal UV response of CS cells with respect to repair replication, incision and removal of cyclobutane pyrimidine dimers (CPD) from the genome overall (2). Recently however, we have shown that CS cells are deficient in preferential repair of nuclear matrix associated DNA (3) and concomitantly have a defect in the removal of CPD from transcriptionally active DNA (4).

In normal human cells CPD are removed more rapidly and efficiently from transcribed genes than from inactive sequences or the genome overall (4,5,6). Further analysis showed that this preferential repair of transcriptionally active genes was partly caused by a faster repair of the transcribed strand compared to the nontranscribed strand of active genes (7,8). This fast repair of the transcribed strand, a phenomenon called transcription coupled repair, is superimposed on the preferential repair of active genes in general (9). Transcription coupled repair of active genes has also been reported in rodent (10,11) and yeast cells (12) and in *Escherichia coli* (13).

Despite the ubiquitous nature of preferential repair, the molecular mechanism of this process has remained largely obscure. Recent evidence however, has provided strong arguments that preferential repair of transcribed sequences involves a process directed by specific cellular factors. A protein was purified from *E.coli* cells which specifically stimulates DNA repair from the transcribed strand in an *in vitro* DNA repair assay (14,15). Also chromatin structure could play a role in the mechanism of preferential repair. CPD in the nontranscribed strand of active genes in normal human cells are still more efficiently repaired than in inactive sequences (8). Moreover, the faster repair of the adenosine deaminase (ADA) housekeeping gene compared to inactive sequences, is not abolished in the absence of transcription (16). This preferential repair could be attributed to the higher accessibility of the poised chromatin at these loci, due to the more open chromatin configuration. Also the spatial localization of a gene may be important: active genes are attached to the nuclear matrix where the enzymes required for repair may also be localized. In humans the deficiency of CS cells in preferential repair indicates the existence of genes

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which function in this separate repair pathway. The absence of preferential repair in CS cells correlates well with the inability of these cells to restore UV induced inhibition of RNA-synthesis (1) and may underlie the enhanced mutability observed in these cells (17). Interestingly, it was recently reported that the ERCC6 gene is able to complement the DNA repair defect in CS cells from complementation group B (18). From the amino acid sequence, it was deduced that the CS-B protein has a DNA helicase domain that is very similar to that found in several transcription factors, suggesting a function of this protein in recognizing or scanning for DNA damage located on transcribed strands.

In order to gain more insight into the factors governing the rate and the extent of repair of CPD in active genes, we decided to study in more detail the repair pattern in CS cells. The data known so far do not distinguish between repair of the individual strands of active genes. The inefficient repair of active genes observed in CS cells could be explained in two ways, i.e. either the absence of preferential repair of the transcribed strand, or repair within active genes still being strand specific but with both strands repaired at a lower level.

This study presents the results of experiments measuring the removal of CPD from the complementary strands of the human adenosine deaminase (ADA) and dihydrofolate reductase (DHFR) genes. We have used both primary and SV40-transformed cell lines derived from normal donors and CS patients. Furthermore, to examine the possibility that the repair defect in CS cells not only affected the initial rate but also the extent of CPD removal from active genes, repair of both active genes was compared to that of two X-chromosomal nontranscribed genes, namely the 754 gene (19) and the tissue specific gene coding for coagulation factor IX (20). The results show that the removal of CPD from active DNA in CS cells is similar for both strands, indicating a defect in preferential repair of the transcribed strand. The slower repair of both strands of active genes in CS compared to the nontranscribed strand of active genes in normal cells points towards an impaired mechanism of targeting repair enzymes towards specific chromatin compartments.

MATERIALS AND METHODS

Cell lines and culture conditions

Primary normal human (VH16) and Cockayne's syndrome (CS1AN, complementation group B) fibroblasts as well as SV40-transformed normal human (1BR.3gn2) and Cockayne's syndrome (CS3BE.S3.G1, complementation group A) fibroblasts (21) were cultured in Ham's F10 medium (without hypoxanthine and thymidine) supplemented with 15% fetal calf serum and antibiotics. Cells were prelabeled with ^{3}H-thymidine (0.05 μCi/ml, 80 Ci/mmol) and 1 μM thymidine. Prior to irradiation, cells were grown in 94 mm petri dishes with regular medium changes until UV irradiation.

Removal of CPD from specific DNA sequences

CPD removal was analyzed essentially as described previously (4). Cells were irradiated with 10 J/m^2 UV and incubated in medium for up to 24 h in the presence of bromodeoxyuridine (to allow for separation of parental and replicated DNA). After incubation cells were lysed and high molecular weight DNA was purified by phenol and chloroform extractions and ethanol precipitation. The DNA was restricted with either BclI, KpnI or EcoRI (Pharmacia) and centrifuged to equilibrium in CsCl density gradients. Gradients were fractionated and fractions containing parental density DNA were pooled, dialyzed against a Tris−EDTA buffer (pH 8.0) and ethanol precipitated. Equal amounts of DNA were either treated or mock treated with the CPD specific enzyme T4 endonuclease V (22) and electrophoresed in 0.6% alkaline agarose gels. The DNA was transferred to Hybond N$^+$ membranes (Amersham) by vacuum Southern blotting (Pharmacia-LKB Vacugene 2016) and hybridized with ^{32}P-labelled gene specific probes. After autoradiography the films were scanned using a Video densitometer (Biorad) and the number of CPD was calculated from the relative band densities in the lanes containing DNA either treated or not treated with T4 endonuclease V using the Poisson expression.

DNA probes

A genomic Factor IX probe (23) a 440 bp HinfI fragment covering exons 2 and 3, and a genomic 2.0 kb HindIII fragment of 754 (19), were radioactively labelled by random primer extension (24). Two ADA cDNA PstI fragments, containing either exon 1−5(partly) or exon 12 (25) and a 690 bp HindIII−EcoRI genomic fragment from intron V of the DHFR gene (26) were subcloned in M13 SSEV18/19 vectors (27). The orientation of all DNA fragments was confirmed by sequence analysis. The SSEV vector contains a polylinker which, in the single stranded form, is able to form a stem-loop structure. This stem-loop structure contains an EcoRI site in the stem and thereby allows for the separation of single stranded cloned inserts from vector sequences. Isolation and purification of inserts was performed essentially as described by Biernat *et al.* (27). The purified fragment was radioactively labelled by filling in the 3′ recessed end of the EcoRI site in the stem using the Klenow fragment of DNA polymerase I.

RESULTS

Removal of CPD was measured in restriction fragments of the transcriptionally active human ADA and DHFR genes and the inactive 754 and coagulation factor IX genes. Repair was analyzed in a 20 kb BclI and an 18.5 kb EcoRI fragment of the ADA gene and in a 20 kb KpnI fragment of the DHFR gene. Analysis of the 754 gene and Factor IX gene occurred in a 14 kb EcoRI and a 17 kb KpnI fragment respectively. Maps of all loci examined including relevant restriction sites have been published previously (4,6,8,16). Removal of CPD in these defined DNA fragments was measured using T4 endonuclease V, which specifically incises DNA at the site of a dimer. The presence of CPD was visualized as the disappearance of the fragment when analyzed by Southern blotting of alkaline gels and hybridization with a specific probe. With increasing time after UV-irradiation, removal of CPD was seen as the reappearance of the fragment of interest. It should be noted that the EcoRI fragment of the ADA gene also covers a transcription unit on the non-ADA template strand, which extends up to ADA exon 7 (28). Consequently this fragment contains two transcribed strands, which in normal cells results in a higher rate of repair of CPD compared to the 5′ located BclI fragment of the ADA gene (8).

CPD removal in SV40-transformed cells

In Fig. 1 autoradiograms are shown from an experiment in which removal of CPD was measured in the ADA BclI (Fig. 1A) and DHFR KpnI fragment (Fig. 1B) of normal 1BR.3gn2 cells. The

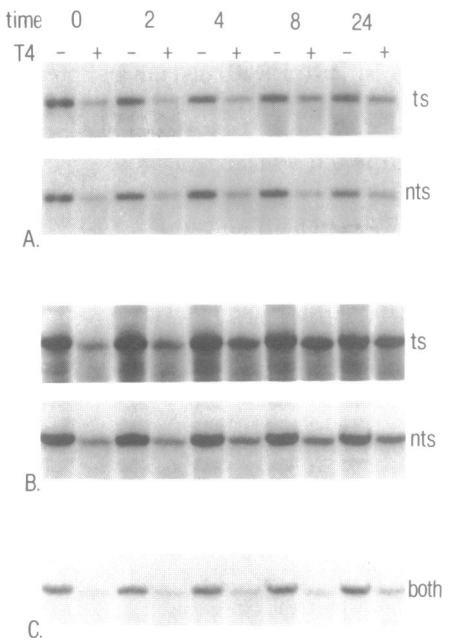

Figure 1. Autoradiograms showing removal of CPD from the ADA BclI (**A**), DHFR KpnI (**B**) and Factor IX KpnI (**C**) fragments in SV40-transformed, normal 1BR.3gn2 cells. The ADA and DHFR fragments were analyzed with strand specific probes recognizing the transcribed (ts) or nontranscribed (nts) strand. The Factor IX gene was analyzed with a probe recognizing both strands.

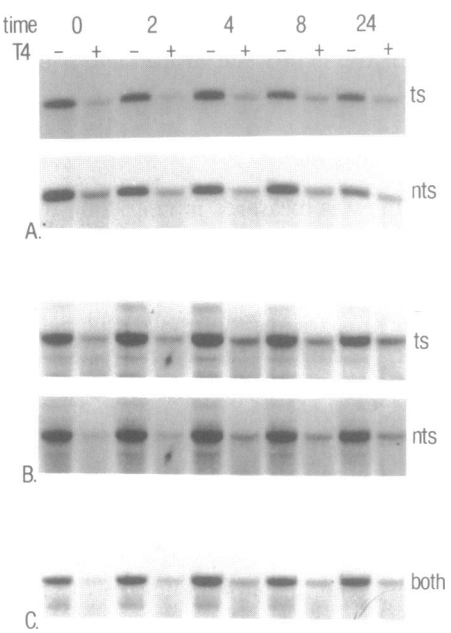

Figure 2. Autoradiograms showing removal of CPD from the ADA BclI (**A**), DHFR KpnI (**B**) and Factor IX KpnI (**C**) fragments in SV40-transformed CS3BE.S3.G1 cells. The gene fragments were analyzed as described in Fig. 2.

filters were consecutively hybridized with probes specific for the transcribed and the nontranscribed strand. It is clearly shown that in both the ADA and the DHFR gene of normal 1BR.3gn2 cells, dimer removal was faster from the transcribed than from the nontranscribed strand, especially at early times after UV-

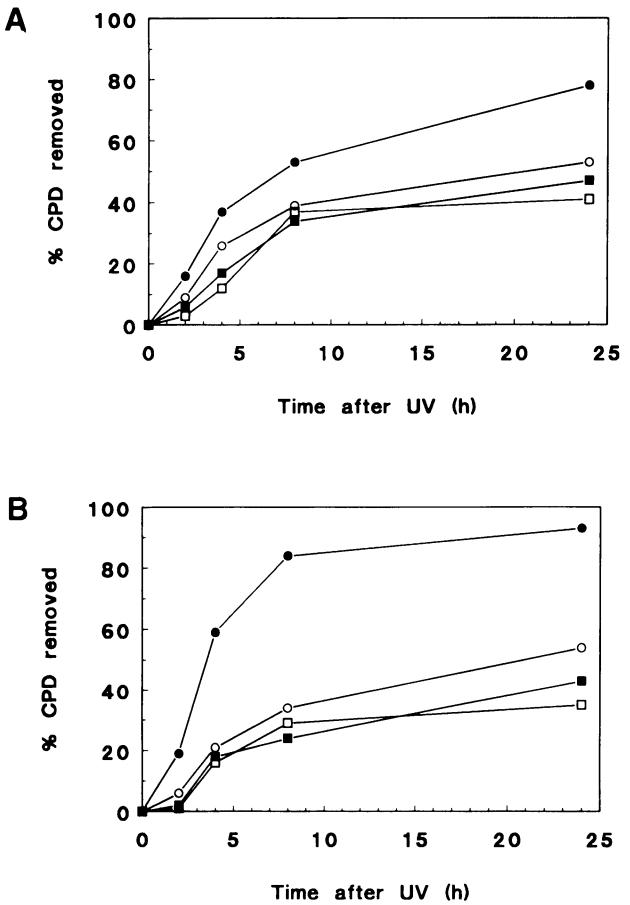

Figure 3. Removal of CPD from the transcribed strand (closed symbols) and the nontranscribed strand (open symbols) of the 20 kb ADA BclI fragment (panel **A**) and the 20 kb DHFR KpnI fragment (panel **B**). (●,○) 1BR.3gn2; (■,□) CS3BE.S3.G1.

irradiation. Fig. 2 shows the results of a similar experiment with SV40-immortalized CS3BE cells. The rate of repair in these cells was much slower than that seen in normal cells and, moreover, the rate was similar for both strands (Fig. 2A and 2B).

The results of these and other independent experiments were quantified by densitometric scanning of the autoradiograms and are presented in Fig. 3. Normal 1BR.3gn2 cells performed preferential repair of the transcribed strand: in the DHFR gene about 50% of the CPD was removed within 4 h after UV irradiation and 80% within 8 h (Fig. 3B). The transcribed strand of the ADA gene was repaired somewhat more slowly with 40 and 60% of the CPD being removed after 4 and 8 h, respectively (Fig. 3A). In S-phase cells the DHFR gene is transcribed at a relatively high frequency (29). This may account for the faster repair of the transcribed strand of the DHFR gene in these cells compared to the transcribed strand of the ADA gene for which no differences in expression in various phases of the cell cycle have been detected (30). The nontranscribed strand of either gene in the SV40-transformed cell lines was repaired at a clearly slower rate than the transcribed strand: only 20% repair was observed after 4 h and within 8 h after treatment 35% of the CPD was removed. In CS cells, the transcribed as well as the nontranscribed strand was repaired slowly (Fig. 3A,B). Within 4 h after UV irradiation only 15% repair was seen, with 30% of the CPD being removed after 8 h. In addition, the extent of

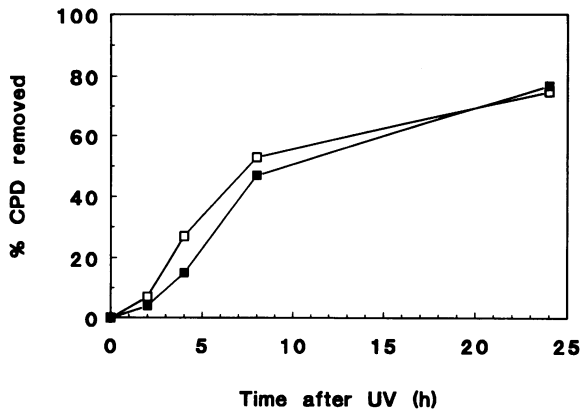

Figure 4. Removal of CPD from the transcribed strand (closed symbols) and the nontranscribed strand (open symbols) of the 20 kb ADA BclI fragment in primary CS3BE fibroblasts.

repair in CS cells appeared less than that observed in normal cells with only 40–45% CPD removed after 24 h (Fig. 3A,B).

To study the effect of the repair deficiency in CS cells on the extent of repair in more detail, we also analyzed removal of CPD in two inactive X-chromosomal genes, namely the 754 gene and the gene coding for coagulation factor IX. Repair was determined in a 14 kb EcoRI fragment of the 754 gene and an internal 17 kb KpnI fragment of the Factor IX gene. Both normal human and CS cells performed slow and incomplete repair of these genes measured over a 24 h time period (See fig. 1C and 2C for Factor IX; 754 data not shown). The results indicate that there were no clear differences in the rate and extent of repair of the inactive genes between normal and CS cells. Both cell types showed about 10% CPD removal after 4 h, which increased to only 35% after 24 h. It is obvious that the repair of CPD in these immortalized cells measured over a 24 h time period is too slow to draw firm conclusions on differences in repair between active and inactive genes in CS cells. However the tendency is that the active genes are repaired somewhat faster than the inactive genes.

CPD removal in primary fibroblasts

Repair was also analyzed in primary fibroblasts which, in contrast to the exponentially growing SV40-transformed cells, were in stationary phase at the time of UV irradiation. Removal of CPD was measured in the 5′ BclI fragment and the 3′ EcoRI fragment of the active ADA gene and in the inactive 754 gene. The repair values shown for normal fibroblasts include the results published previously (8,16) to which new experimental data were added.

As can be seen in Fig. 4 which shows a representative experiment, in primary CS3BE cells both strands of the BclI fragment were repaired at a similar rate, i.e. about 20% repair within 4 h and 50% repair within 8 h after UV treatment. To make an accurate comparison of the repair of active genes in normal cells with that in CS cells we plotted the average data of a large number of experiments. Two sets of data demonstrate that repair of active genes in CS cells is slower than in normal cells: (i) repair of CPD in the nontranscribed strand of the ADA gene in normal cells as determined in the BclI fragment is higher than the average of both strands in CS cells (Fig. 5A); (ii) repair of CPD in both strands of the BclI fragment and in the ADA template strand of the EcoRI fragment of the ADA gene in normal human cells, lacking transcription of the ADA gene by a deletion

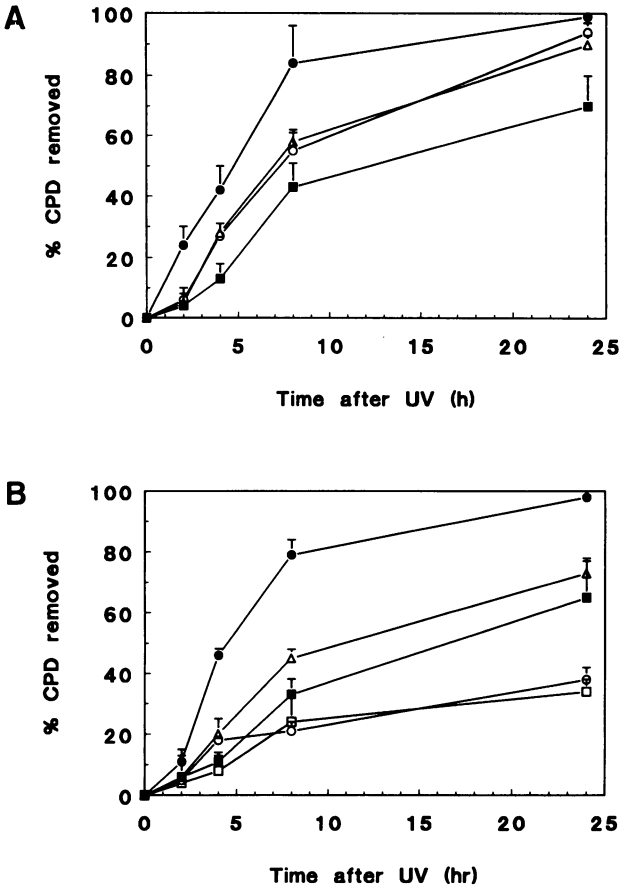

Figure 5. Removal of CPD from the 20 kb BclI (panel A) and the 18.5 kb EcoRI (panel B) fragment of the ADA gene and the 14 kb EcoRI fragment of the 754 gene (panel B) in primary fibroblasts. Data are shown from normal cells (VH16, 7 independent experiments), CS cells (CS1AN, 5 independent experiments) and from normal cells having a promoter deletion in the ADA gene ('del.ADA', previously published results (17)). Panel A: (●) VH16, TS; (○) VH16, NTS; (■) CS1AN, BS; (△) DEL.ADA, BS. Panel B: (●) VH16, ADA, BS; (△) DEL.ADA, ADA-template strand; (■) CS1AN, ADA, BS;(○) VH16, 754, BS; (□) CS1AN, 754, BS. Bars represent standard errors of the mean (SEM). BS = both strands; TS = transcribed strand; NTS = nontranscribed strand.

of the promoter, is more rapid than in the same sequences in CS cells (Fig. 5A,B)

To compare the repair kinetics of active and inactive genes in CS, we plotted the data obtained for EcoR1 digested DNA (Fig. 5B). In this way an estimation can be made of the repair in the ADA and 754 genes from the same membrane thus eliminating possible differences due to DNA loading. It is clear that in CS cells in spite of the absence of transcription coupled repair the active ADA gene is faster and more efficiently repaired than the inactive 754 gene.

Generally, the repair values for primary fibroblasts were higher than for the SV40-transformed cells, which was observed in our previous study as well (4). A lower level of UV-induced excision repair in transformed cells has been observed by other investigators as well (31). However, this is not likely to be a cell cycle effect since we found previously comparable repair efficiencies in confluent and exponentially growing primary fibroblasts (6). In CS cells the 24 h value is subject to significant variability within the various experiments. For example in the current study we found up to 60–70% repair after 24 h, which is similar to data obtained by Mellon and Hanawalt (pers.

comm.). However, previous experiments have shown lower repair values only for the 24 h time point (4). We do not have an explanation for this variability, although we can exclude an effect of the passage number of the cells. Notwithstanding this observation, the repair pattern in the first eight hours after UV-irradiation, which were shown previously to be the most important for selective repair of the transcribed strand, is similar in both primary and immortalized cells.

DISCUSSION

We have analyzed the removal of cyclobutane pyrimidine dimers from the active ADA and DHFR genes in normal and CS fibroblasts using strand specific probes. The results show that in SV40-transformed normal cells the transcribed strand of the ADA BclI fragment, located entirely within the gene, is repaired faster than the nontranscribed strand. At early times after UV treatment, the difference in the rate of repair is twofold. These results further extend the observation of transcription coupled repair in the ADA gene of primary human fibroblasts (8). Similar results were observed for the DHFR KpnI fragment, in agreement with data obtained by Mellon *et al.* (7), who also showed selective repair of the transcribed strand of the DHFR gene in an established human cell line. Repair of the 754 gene and the Factor IX gene, which are not expressed in fibroblasts (19,32,33), is slower. The levels of repair are comparable in both loci (4) and are likely to be representative for X-chromosomal inactive genes. However, it is important to point out that the slower repair of the inactive genes might be characteristic for X-chromosomal inactive genes and that differences in repair of active housekeeping genes and inactive autosomal loci may be less pronounced.

CS cells show a marked deficiency in selective repair of CPD in the transcribed strand. In the gene fragments analyzed in this study, there is no significant difference between the rate and extent of repair in both strands. The results clearly show that the CS defect lies in the targeting of DNA repair enzymes towards DNA lesions in the transcribed strand of active genes.

In spite of the lack of transcription coupled repair, the rate of repair of active genes in CS cells still exceeds that of inactive genes. In normal cells, repair of the nontranscribed strand of active genes is much more efficient than repair of inactive loci. This seems to point at a mechanism in which the rapid repair of the transcribed strand is superimposed on the already efficient repair of active genes (9). Additional evidence for this comes from our recent studies using a cell line in which, due to a promoter deletion, the ADA gene is transcriptionally inactive. Repair of this gene was still considerably more efficient than that of the inactive 754 and Factor IX loci although the strand specificity for CPD removal from the ADA gene was lost (16). In a previous study (4) we suggested that CS cells are defective in preferential repair of active genes and that repair of active genes occurred at a level characteristic for inactive X-chromosomal genes. Indeed data obtained with immortalized CS cells in the current study do not reveal distinct differences between active and inactive genes. However the large number of experiments with primary CS cells indicate undoubtedly a difference in repair between active and inactive genes, the active being repaired more efficiently than the inactive, as observed in normal cells.

Yet the defect in CS may not solely affect the preferential repair of the transcribed strand of active genes. We consistently found that repair of CPD in active genes in CS is slower than repair in the nontranscribed strand of active genes in normal cells. No clear differences are observed for repair of inactive genes between normal and CS cells. This is consistent with the observation that the repair capacity of CS cells measured in the genome overall is very similar to that in normal cells (2). However, we want to make two remarks here: (i) the rate and extent of repair of the inactive loci both in normal and CS cells are low, which makes it difficult to detect small differences (Fig. 5B); (ii) we cannot exclude that the difference in repair of the active genes between normal and CS cells simply reflects heterogeneity of repair which may reside within the normal population as well, although we did not observe significant differences in the repair capacity of fibroblasts derived from different individuals (4,6,16). Taken together the efficiency of CPD repair in normal and CS cells can be ranked as follows: normal cells, TS active genes > normal cells, NTS active genes > CS cells, BS active genes > normal and CS cells, BS X-chromosomal inactive genes.

Our results suggest that the defect in CS specifically concerns preferential repair of active DNA and extends beyond a defect in transcription coupled repair. It could be that human cells possess mechanisms which are actively engaged in targeting repair complexes to regions of active chromatin. Once operating in such domains, the blockage of transcription by CPD could form an additional signal for efficiently targeting repair enzymes towards the transcribed strand. We propose that CS cells are defective in this process of targeting, leading to the absence of transcription coupled repair and impairment of preferential repair of active genes in general. Eventually, active domains could be repaired by slowly operating systems governing repair in bulk DNA, since this repair pathway is not defective in CS cells. This hypothesis also predicts that it is not merely the greater accessibility of active chromatin, which determines preferential repair.

In what way does the CS gene product play a role in preferential repair of the transcribed strand of active genes? From recent studies on the genes involved in CS, it has become clear that ERCC6, which is the gene defective in CS-B, bears similarity to specific domains in several transcription factors (18). The putative DNA helicase activity of the ERCC6 protein could be used to induce local unwinding of DNA strands at the site of DNA damage. Perhaps the gene products defective in CS function in a complex with RNA polymerase(s) to detect DNA damage in front of the transcription machinery. Alternatively, the CS proteins could be involved in scanning of the transcribed strand for DNA damage which could stall transcription. It remains to be established whether the defect in CS cells bears any resemblance with the recently identified 'transcription coupling factor' in *E.coli*, which was shown to specifically stimulate repair of the transcribed strand *in vitro* (14,15).

The deficiency in both the rate and extent of repair of active genes in CS cells may be of critical importance to the biological effects of UV irradiation. CS cells do remove a considerable part of the damage from the genome overall within 24 h. However, due to the delayed and incomplete repair of active genes, these cells do not have the ability to timely restore the functional expression of essential genes. This deficiency is causing UV sensitivity. Furthermore, inadequate repair of active regions may cause an increase in UV induced mutations in growing CS cells (17). The role of (6–4) photoproducts in this regard remains to be established. Removal of these adducts from the genome overall in CS cells occurs at a similar rate as in normal cells

(D.L.Mitchell, pers. comm.). We are currently investigating the removal of these adducts from specific genes in CS cells.

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