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Transcription-coupled repair removes both cyclobutane pyrimidine dimers and 6-4 photoproducts with equal efficiency and in a sequential way from transcribed DNA in xeroderma pigmentosum group C fibroblasts

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We investigated the contribution of the global and the transcription-coupled nucleotide excision repair pathway to the removal of structurally different DNA lesions. The repair kinetics of UV-induced cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) were determined in an active and inactive gene in normal human fibroblasts and in xeroderma pigmentosum group C (XP-C) fibroblasts. Previously we have shown that in normal human cells exposed to a UV dose of 10 J/m^2 repair of CPDs takes place via two pathways: global repair and transcription-coupled repair, the latter being responsible for accelerated repair of CPDs in the transcribed strand of active genes. So far, no clear evidence for transcription-coupled repair of 6-4PPs has been presented. Here we demonstrate that 6-4PPs really form a target for transcription-coupled repair. In XP-C cells, exposed to 30 J/m^2 and only capable of performing transcription-coupled repair, CPDs as well as 6-4PPs are removed selectively and with similar kinetics from the transcribed strand of the adenosine deaminase (ADA) gene. The non-transcribed strand of the ADA gene and the inactive 754 gene are hardly repaired. In contrast to XP-C cells, normal cells exposed to 30 J/m^2 lack strand-specific repair of both 6-4PPs and CPDs, suggesting that transcription-coupled repair is overruled by global repair, probably due to severe inhibition of transcription at this high UV dose. The much more rapid repair of 6-4PPs compared with CPDs in normal cells may be related to higher affinity of the global repair system for the former lesion. In XP-C cells the similarity of the rate of repair of both 6-4PPs and CPDs in the transcribed strand at 30 J/m^2 indicates that transcription-coupled repair of photolesions takes place in a sequential way. Our results strongly suggest that the significance of transcription-coupled repair for removal of lesions depends on the type of lesion and on the dose employed.

Key words: cyclobutane pyrimidine dimer/pyrimidine (6-4) pyrimidone photoproduct/transcription-coupled repair/UV/xeroderma pigmentosum

Introduction

There is now convincing evidence that DNA repair processes are not homogeneously distributed over the genome. For a variety of DNA lesions it has been shown that nucleotide excision repair takes place preferentially in transcriptionally active DNA. In mammalian cells cyclobutane pyrimidine dimers (CPDs) induced by UV irradiation and certain types of DNA adducts induced by chemical agents such as benzo(a)pyrene diol epoxide, aflatoxin B1 and psoralen are removed faster and more efficiently from active genes than from inactive genes or regions of non-coding DNA (Bohr *et al.*, 1985; Mellon *et al.*, 1986; Thomas *et al.*, 1989; Venema *et al.*, 1990; Islas *et al.*, 1991; Leadon and Lawrence, 1991; Chen *et al.*, 1992; Ruven *et al.*, 1993). On the other hand, no such preference was demonstrated for lesions induced by 4-nitroquinoline-1-oxide or *N*-acetoxyaminofluorene, assumed to be processed by the same repair pathway as UV damage (Tang *et al.*, 1989; Snyderwine and Bohr, 1992). Thus it is not clear at present which parameters actually determine whether a lesion will be repaired preferentially or not.

The phenomenon of preferential repair has been studied most thoroughly for UV-induced DNA damage. In human cells, UV-induced CPDs have been shown to be preferentially removed from active rather than inactive genes (Mellon *et al.*, 1987; Venema *et al.*, 1991). Moreover, in many cases it has been shown that within active genes, repair of CPDs is faster in the transcribed than in the non-transcribed strand. However, the non-transcribed strand was still repaired more efficiently than DNA in inactive genes. Convincing evidence for preferential repair of potentially active genes, even in the absence of transcription, was obtained with a human cell line in which transcription of the housekeeping gene coding for adenosine deaminase (ADA) was abolished by a promoter deletion. The efficiency of repair of the template strand decreased to the level of the non-transcribed strand but was still higher than that of inactive genes (Venema *et al.*, 1992). Obviously, two pathways are involved in the complex mechanism of preferential repair of active genes. We proposed that one pathway concerns the targeting or concentration of global repair activity towards (potentially) active DNA (Venema *et al.*, 1992). A second pathway involves a close coupling of repair to RNA polymerase II-driven transcription (Leadon and Lawrence, 1991; Christians and Hanawalt, 1992). This transcription-coupled repair pathway specifically acts on the transcribed strand

and appears to be superimposed on the preferential repair of active genes.

In this study we addressed the question of whether transcription-coupled repair acts on structurally different lesions with equal efficiency and in a sequential way. We investigated the kinetics of repair of UV-induced CPDs and pyrimidine (6-4) pyrimidone photoproducts (6-4PPs) in active genes in normal and xeroderma pigmentosum group C (XP-C) fibroblasts. In XP-C cells only the transcription-coupled repair pathway is active. These cells selectively remove CPDs from the transcribed strand of active genes with kinetics similar to those observed in normal cells (Venema *et al.*, 1991) and therefore are very suitable for establishing the kinetics of removal of 6-4PPs by the transcription-coupled repair pathway.

6-4PPs are induced at a lower frequency than CPDs and are removed much faster from the genome overall (Mitchell, 1988), but may play a key role in UV-induced cytotoxicity and mutagenesis (McGregor *et al.*, 1991; Zdzienicka *et al.*, 1992; Gibbs *et al.*, 1993). In spite of its biological importance, detailed information on intragenomic heterogeneity of induction and repair of 6-4PPs is limited, mainly due to technical limitations in measuring 6-4PP frequencies in specific sequences. Heterogeneity in induction of 6-4PPs does exist at the nucleosome level as 6-4PPs are predominantly induced in linker DNA (Mitchell *et al.*, 1990). In spite of this heterogeneity no clear differences in induction of 6-4PPs were found between transcribed and non-transcribed sequences in hamster and *Drosophila* cells (Thomas *et al.*, 1989; De Cock *et al.*, 1992). Although in mammalian cells repair of 6-4PPs may be faster in an active than an inactive gene (Thomas *et al.*, 1989), no convincing evidence has been presented for strand-specific repair of 6-4PPs.

To investigate the role of transcription-coupled repair and global repair in removal of 6-4PPs from active and inactive sequences we adapted the method developed by Thomas *et al.* (1989) using the *Escherichia coli* excinuclease complex UvrABC. This complex is capable of incising the DNA at a variety of lesions including CPDs and 6-4PPs (Van Houten, 1990). To determine 6-4PPs specifically, CPDs were first removed from the DNA by *in vitro* photoreactivation. We measured induction and repair of 6-4PPs in the transcribed and non-transcribed strand of the active ADA gene and in the inactive 754 gene in primary fibroblasts from two normal individuals and two XP-C patients. We found that in normal human cells 6-4PPs are repaired very rapidly both in active and in inactive genes with a clear preference for active genes. No difference in repair kinetics of 6-4PPs between the transcribed strand and non-transcribed strand of the ADA gene was observed. Moreover, repair of 6-4PPs was much faster than that of CPDs measured in the same experiments. In XP-C cells, however, 6-4PPs were repaired with the same kinetics as CPDs, and selectively in the transcribed strand of the active ADA gene. We propose that in XP-C cells both CPDs and 6-4PPs are repaired in a sequential way via the same transcription-coupled pathway. However, in normal human cells transcription-coupled repair of 6-4PPs proceeds slowly compared with the global repair, at least at higher doses, and thus does not contribute significantly to repair of 6-4PPs in active genes.

Results

To measure the frequency of UV-induced 6-4PPs in specific DNA fragments, highly purified *E.coli* UvrABC proteins (Visse *et al.*, 1992) were used to incise at sites of DNA photolesions. Since the UvrABC excinuclease complex will recognize both CPDs and 6-4PPs, it is necessary to remove CPDs prior to incubation with the UvrABC enzyme complex. CPDs were completely removed from purified DNA samples by photoreactivation employing *Anacystis nidulans* photolyase and photoreactivating light. The remaining lesions are thought to be predominantly 6-4PPs. The absence of significant amounts of other types of DNA lesions, such as thymine hydrates and glycols in DNA obtained from UV-irradiated cells, was demonstrated by the insensitivity of the DNA to digestion with the enzyme *E.coli* endonuclease III (data not shown).

After photoreactivation of CPDs in the DNA and checking this reaction for completeness with T4 endonuclease V, the *E.coli* UvrABC excinuclease complex was used to introduce single-stranded breaks in the DNA at the sites of remaining lesions. The DNA samples were subjected to electrophoresis in alkaline agarose gels, Southern blotted and hybridized with radiolabeled specific probes. In this assay repair is seen as the reappearance of full-size restriction fragments in lanes containing UvrABC-digested DNA (Figures 3A and 4A). Bands on autoradiograms were quantified by densitometry and lesion frequencies per fragment were calculated from the ratio of full-size restriction bands in lanes containing treated and non-treated DNA using the Poisson expression. Repair of 6-4PPs was measured in the active ADA gene in a 5′ located 19.9 kb *Bcl*I fragment (ADA template strand is transcribed) and in a 3′ located 18.5 kb *Eco*RI fragment (both strands are transcribed, Lattier *et al.*, 1989; Figure 1). Repair in the inactive X-chromosomal 754 gene was measured in a 14 kb *Eco*RI fragment. Since CPDs are induced at a much higher frequency than 6-4PPs it is not feasible to measure CPDs and 6-4PPs in the same restriction fragments at the UV dose of 30 J/m^2 used in repair experiments. Therefore, in order to have one to two CPDs per fragment which is required for accurate measurement of repair, CPD frequencies at the dose of 30 J/m^2 were measured in a smaller fragment, in this case a 7.2 kb *Eco*RI fragment located in the middle of the ADA gene (Figure 1).

Incision efficiency of the UvrABC enzyme complex

Before performing repair studies, several pilot studies with the UvrABC complex were performed. First the efficiency of the enzyme reaction was checked by comparing single and repeated incubations of DNA with UvrABC. An autoradiogram of such an experiment is shown in Figure 2. Obviously a single incubation of the DNA with UvrABC is sufficient for cutting all sensitive lesions in the DNA, since the second incubation did not result in the appearance of more breaks.

Because the UvrABC complex showed low but variable aspecific activity on DNA obtained from non-irradiated cells, a DNA sample isolated from unirradiated cells in the same experiment was also incubated with UvrABC. The nicking frequency measured in this sample was used

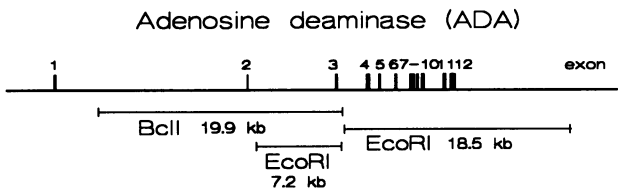

Fig. 1. Genomic map of the human adenosine deaminase gene showing exons and relevant restriction fragments.

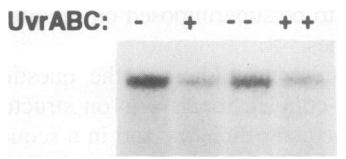

Fig. 2. Autoradiogram showing that the UvrABC enzyme complex cuts all sensitive sites in one single incubation. DNA from cells irradiated with 30 J/m^2 UV was purified, restricted with *Eco*RI, photoreactivated and, out of four samples with equal amounts of DNA, two were treated (+) and two were mock treated (−) with UvrABC. The samples were purified as described in Materials and methods and two samples (one treated, one mock treated) were treated or mock treated (− −, + +) in a second incubation. Subsequently the four samples were subjected to agarose gel electrophoresis, blotted and hybridized with the ADA Be probe.

to correct the lesion frequencies measured in the irradiated samples. The average 'aspecific' nicking frequency was 0.13/10 kb. To check whether the aspecific nicking by the enzyme complex was influenced by the lesion frequency in the DNA, 0.5 μg unirradiated DNA from the *Drosophila melanogaster* Kc cell line was added to the DNA samples used for UvrABC incubations. The nicking frequency in this unirradiated DNA, measured in a 15.9 kb *Xho*I fragment containing the *GART* gene, was the same whether added to human DNA obtained immediately after irradiation or to human DNA obtained at various times after UV irradiation. This clearly indicates that the extent of aspecific nicking is not influenced by the presence of DNA lesions in the reaction mixture.

Induction of 6-4PP in specific sequences

To determine the relative frequencies of CPDs and 6-4PPs in active and inactive genes, cells were irradiated with various UV doses. Lesion frequencies in specific sequences were determined as described in Materials and methods. A linear increase in 6-4PP induction was observed up to 40 J/m^2 (not shown) and from these data an average induction frequency of 0.015 6-4PP/10 kb/J/m^2 was determined (corrected for aspecific cutting), which is ~30% of the CPD frequency. 6-4PP induction levels did not show large variations between the different fragments analyzed, i.e. both fragments of the active ADA gene and the inactive 754 gene. However, a slight difference in induction was observed between the transcribed strand and the non-transcribed strand of the ADA gene: in the non-transcribed strand an ~1.2-fold higher induction of photolesions (CPDs as well as 6-4PPs) compared with the transcribed strand was measured, which correlates with the 1.2-fold higher number of possible dimer sites (two adjacent pyrimidines) in the non-transcribed strand.

In order to obtain the desirable induction of approximately one 6-4PP per fragment of 15–20 kb, repair studies were performed at a dose of 30 J/m^2.

Repair of 6-4PP and CPD in normal human fibroblasts

Confluent primary human fibroblasts (VH16 and VH25) derived from two normal individuals were irradiated with a UV dose of 30 J/m^2. Repair of UV-induced CPDs and 6-4PPs was measured as described above. Autoradiograms of a representative experiment are shown in Figure 3A. Since no significant differences in repair kinetics between the two cell lines were observed, the combined data are presented in Figure 3B and C. Repair of 6-4PP measured in the 5′ located 19.9 kb *Bcl*I fragment and the 3′ located 18.5 kb *Eco*RI fragment was very fast: 50% of the lesions were removed within 2 h. After 8 h the repair was complete. No differences in the rate of repair could be detected between the transcribed strand and the non-transcribed strand of the *Bcl*I fragment. However, as is shown in Figure 3B, the initial rate of repair of the inactive 754 gene appeared to be lower than that of the ADA gene (17% repair after 2 h).

In Figure 3C values are plotted for CPD repair obtained in the same experiments as those for 6-4PP repair, i.e. at a dose of 30 J/m^2. Additionally, previously published CPD repair data obtained at 10 J/m^2 are plotted in Figure 3C (Venema *et al.*, 1991). There was a large difference in the rate of repair between CPDs and 6-4PPs, the latter being repaired ~5-fold faster. Repair of CPDs at this dose, measured in a small restriction fragment (Figure 1), was much slower than at the dose of 10 J/m^2, which was used in previous experiments to elucidate the kinetics of CPDs repair in transcribed and non-transcribed sequences (Venema *et al.*, 1991). At 30 J/m^2 only 64% of the CPDs were removed after 24 h, compared with 100% at 10 J/m^2. Moreover, at the higher dose no significant differences in repair kinetics were found between the transcribed strand and the non-transcribed strand of the ADA gene, whereas after 10 J/m^2 repair of the transcribed strand was much faster than that of the non-transcribed.

Repair of 6-4PPs and CPDs in XP-C fibroblasts

Repair of 6-4PPs was measured in primary fibroblasts (XP1TE and XP21RO) derived from two XP-C patients. In a previous study it was shown that these cells remove CPDs only from the transcribed strand of active genes (measured at a UV dose of 10 J/m^2; Venema *et al.*, 1991). The 6-4PP repair experiments were performed as described for normal cells, using a UV dose of 30 J/m^2. Autoradiograms of a typical experiment with XP21RO cells are shown in Figure 4A. No clear differences in rate or extent of repair were detected between the two XP-C cell lines and therefore the combined data are presented in Figure 4B and C. Compared with normal human fibroblasts, XP-C cells removed 6-4PPs very slowly from the active ADA gene: 33% and 62% were removed from the 3′ located *Eco*RI fragment (measured in both strands using a double-stranded probe) in XP-C cells after 24 and 36 h respectively, compared with 100% within 8 h in normal cells (Figure 3B). The transcribed strand of the 5′ located *Bcl*I fragment of the ADA gene was repaired with similar slow kinetics (81% after 36 h), whilst only few 6-4PPs were removed from the non-transcribed strand (24% after 36 h). Comparing Figure 4B with C it becomes clear that

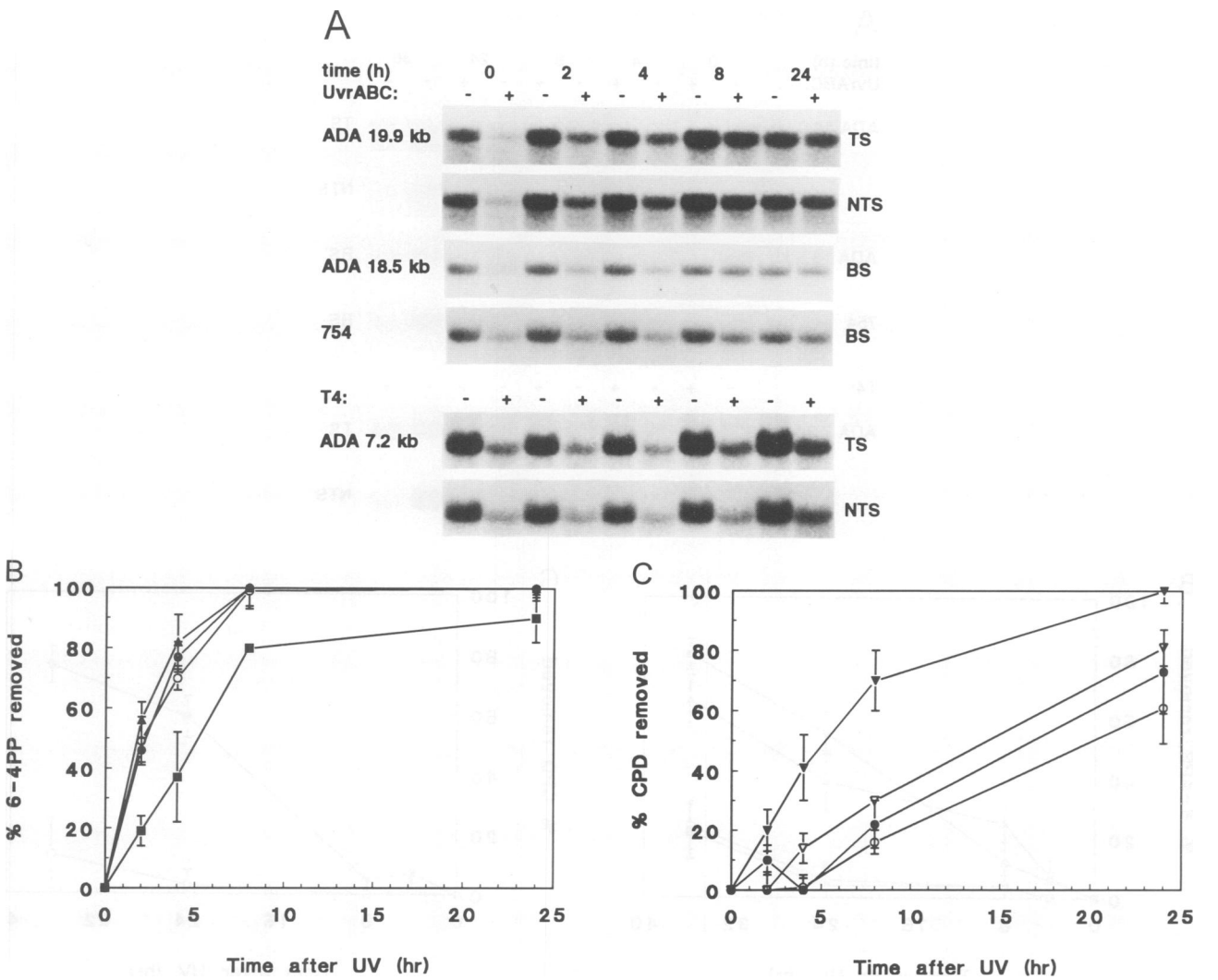

Fig. 3. Autoradiograms (A) and graphs (B and C) showing removal of 6-4PP and CPD from the active ADA gene and the inactive 754 gene in normal human fibroblasts irradiated with 30 J/m^2 UV. Removal of 6-4PP was measured in *Eco*RI restriction fragments of the ADA gene (18.5 kb) and the 754 gene using probes recognizing both strands and in an ADA *Bcl*I fragment using probes recognizing either the transcribed or the non-transcribed strand. CPD removal was measured in a 7.2 kb ADA *Eco*RI fragment, also using strand-specific probes. (**A**) Autoradiograms representing the induction and repair of 6-4PP (UvrABC) and CPD (T4) in the active ADA gene and the inactive 754 gene. (**B**) Repair of 6-4PP: (▲) ADA *Eco*RI 18.5 kb BS, (●) ADA *Bcl*I TS, (○) ADA *Bcl*I NTS, (■) 754 BS. (**C**) Repair of CPD: 30 J/m^2: (●) ADA *Eco*RI 7.2 kb TS, (○) ADA *Eco*RI 7.2 kb NTS; 10 J/m^2: (▼) ADA *Bcl*I 19.9 kb TS, (▽) ADA *Bcl*I 19.9 kb NTS. CPD repair data obtained at 10 J/m^2 were previously published (Venema *et al.*, 1991). Bars represent standard errors of the mean (SEM). BS = both strands; TS = transcribed strand; NTS = non-transcribed strand.

in XP-C cells CPDs as well as 6-4PPs are preferentially removed from the transcribed strand of the active ADA gene, with almost the same kinetics, whereas the non-transcribed sequences show only slight repair for both lesions after long incubation times. Compared with our previously published data on CPD repair in XP-C cells exposed to a UV dose of 10 J/m^2 (Venema *et al.*, 1991), repair after the higher dose of 30 J/m^2 was strongly retarded. At 10 J/m^2 repair of CPDs in the *Eco*RI fragment of the ADA gene was 80% after 8 h and complete within 24 h, whereas in the current study even after 36 h 25% of the CPDs were still not repaired.

Discussion

The relative induction of 6-4PPs versus CPDs by UV-C irradiation is somewhat controversial (reviewed by Mitchell, 1988). In early studies very low relative frequencies of 6-4PPs were observed in acid hydrolysates of UV irradiated *E.coli* DNA (6-4PP amounting to 5–7% of all photoproducts). Hot alkali treatment, however, resulted in an estimation of 6-4PP induction of 15–35% of CPDs, i.e. 13–25% of all photoproducts. Immunoprecipitation of radiolabeled DNA fragments with use of specific antibodies against CPDs or 6-4PPs led to the conclusion that 6-4PPs are induced at half the level of CPDs, i.e. 20–35% of all photoproducts (Mitchell, 1988). Thomas *et al.* (1989) and de Cock *et al.* (1992), using UvrABC excinuclease to detect non-CPD lesions in photoreactivated DNA, estimated the amount of 6-4PPs to be 30–40% of all photolesions in transcribed and non-transcribed sequences in hamster and *Drosophila* cells respectively.

We adapted the method described by Thomas *et al.* in order to determine the non-CPD lesion frequency in DNA photoreactivated *in vitro*, presumed to be predominantly 6-4PPs. Indeed incubation of photoreactivated DNA from

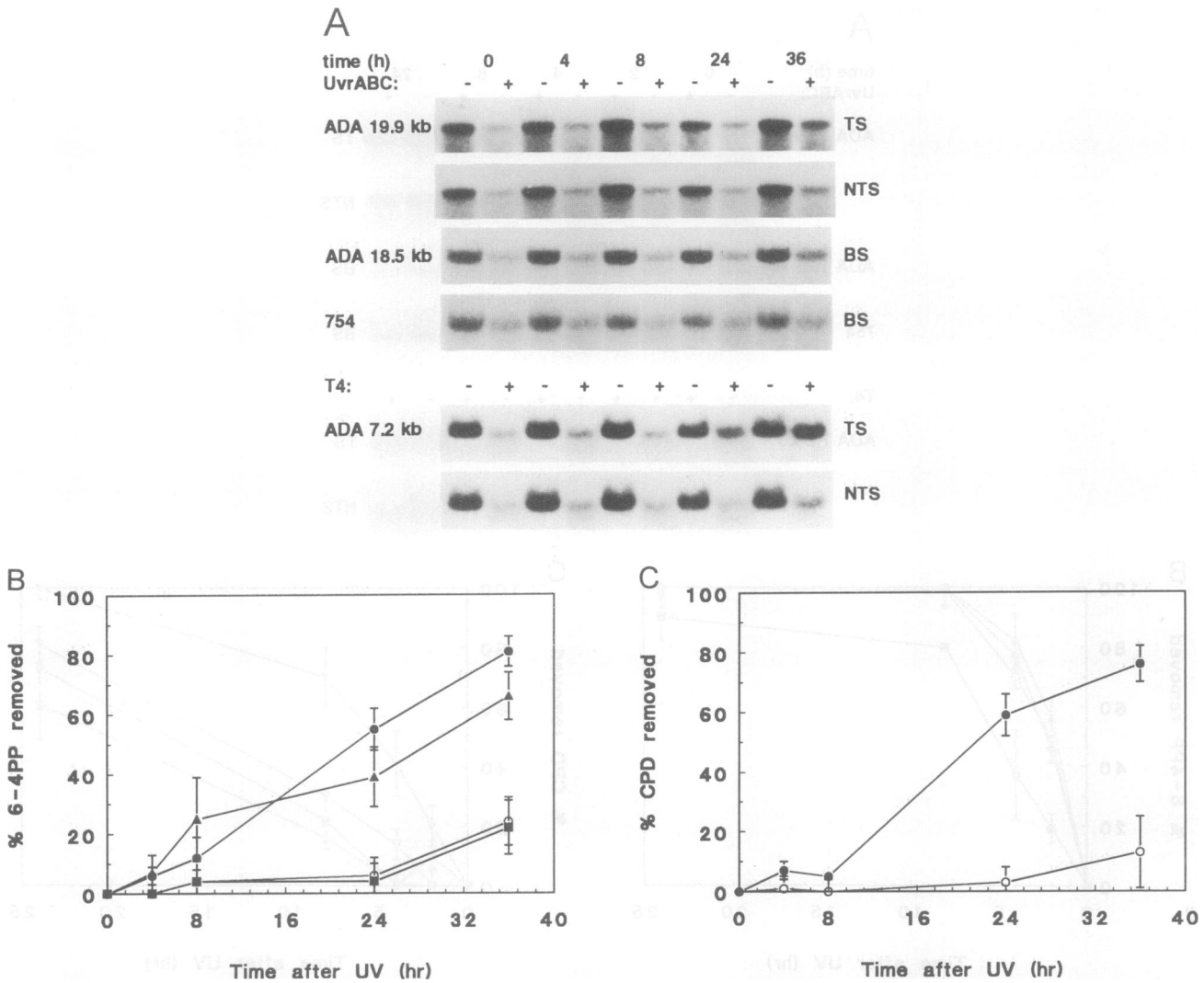

Fig. 4. Autoradiograms (A) and graphs (B and C) showing removal of 6-4PP and CPD from the active ADA gene and the inactive 754 gene in primary xeroderma pigmentosum group C fibroblasts, in fragments and using probes as described in the legend of Fig. 3. (**A**) Autoradiograms representing the induction and repair of 6-4PP (UvrABC) and CPD (T4) in the active ADA gene and the inactive 754 gene. (**B**) Repair of 6-4PP: (▲) ADA *Eco*RI 18.5 kb BS, (●) ADA *Bcl*I TS, (○) ADA *Bcl*I NTS, (■) 754 BS. (**C**): Repair of CPD: (●) ADA *Eco*RI 7.2 kb TS, (○) ADA *Eco*RI 7.2 kb NTS. Bars represent standard errors of the mean (SEM). BS = both strands; TS = transcribed strand; NTS = non-transcribed strand.

irradiated cells with endonuclease III revealed that the DNA did not contain detectable amounts of damaged bases such as thymine hydrates and glycols. This observation is consistent with the finding that the frequency of endonuclease III-sensitive sites is ~100-fold lower than the frequency of CPDs following UV-C irradiation (Epe *et al.*, 1992). We conclude that remaining UvrABC-sensitive sites consisted predominantly of 6-4PPs. The highly purified UvrA, B and C proteins employed in our study make scissions at all sites of sensitive lesions present in the DNA in one single incubation as shown in Figure 3, which enabled us to make a reliable estimation of 6-4PP induction and repair. In agreement with the data summarized by Mitchell (1988) we observed an induction frequency of 0.015 6-4PP/10 kb/J/m^2 (i.e. 30% of the amount of CPDs) in all fragments analyzed. A slight difference in induction of 6-4PPs as well as CPDs between transcribed strand and non-transcribed strand of the ADA gene emerged in our study. Nevertheless our data demonstrate that, as with CPDs, the frequency of 6-4PPs induction in active and inactive genes is very similar and that induction of both types of photolesions is not influenced by possible differences in chromatin configuration when measured at the gene level.

A relatively high UV dose of 30 J/m^2 had to be employed in order to induce one to two 6-4PPs per DNA fragment of 14–20 kb. This lesion frequency is required for reliable measurement of induction and repair of DNA lesions in specific sequences. In normal human fibroblasts, repair of 6-4PPs appeared to be very fast: within 8 h all 6-4PPs were removed from the active ADA gene. No strand-specificity was found, the non-transcribed strand being repaired as rapidly and efficiently as the transcribed strand. The rate of repair of 6-4PPs in the inactive 754 gene, however, was considerably slower than in the active ADA gene.

Several factors might contribute to the difference in repair kinetics between the active and the inactive gene. Since at higher UV doses the non-transcribed strand of the ADA gene is repaired as rapidly as the transcribed

strand, transcription itself as a factor of importance can be excluded. However, transcriptional activity is usually accompanied by hyperacetylation of nucleosome core histones, known to stimulate repair (Ramanathan and Smerdon, 1989), and by a reduced level of methylation of DNA (Adams, 1990). In CHO cells, reduction of the level of methylation mediated by 5-aza-cytidine treatment was accompanied by increased UV-induced excision repair measured in the genome overall and in specific genes (Ho *et al.*, 1989). DNA of inactive X-chromosomal loci is known to be heavily methylated (Adams, 1990) which may lead to less efficient repair in these regions of the genome. Another factor that could play a role in the preferential repair of 6-4PPs in active genes is the positioning of active genes proximal to the nuclear matrix. Recently the transcription factor TFIIH was shown to include proteins playing a role in the global repair pathway (Schaeffer *et al.*, 1993; Drapkin *et al.*, 1994). Given the fact that transcription complexes have been shown to be located at the nuclear matrix (Jackson *et al.*, 1993), it becomes likely that compartmentalization of these complexes facilitates both transcription and repair. As a consequence, genes that are located proximal to the nuclear matrix have a prevalence to be repaired.

CPD repair in normal human fibroblasts exposed to 30 J/m^2 appeared to occur with only slight strand-specificity and to be much less efficient than repair of 6-4PPs. The slightly slower repair rate of the non-transcribed compared with the transcribed strand might be related to the somewhat higher induction of CPDs (1.2-fold) in the non-transcribed strand. In previous experiments, using a dose of 10 J/m^2, CPDs were shown to be removed preferentially from the transcribed strand of active genes. This accelerated repair of the transcribed strand appeared to be dependent on transcription (transcription-coupled repair). An obvious explanation for the lack of strand-specific repair at high UV doses is that in normal cells transcription-coupled repair of CPDs (and also of 6-4PPs), although active, is not detectable because of the much higher efficiency of the global repair system at high doses, and the inhibition of transcription. Nevertheless it is clear that CPDs are recognized much less efficiently by the global repair machinery than 6-4PPs.

Repair of 6-4PPs in XP-C fibroblasts exposed to 30 J/m^2 showed quite a different pattern from that of normal cells. The transcribed strand of the ADA gene was selectively repaired, but repair was very slow compared with normal cells. The non-transcribed strand of the ADA gene as well as the inactive 754 gene showed only slight repair of 6-4PPs 36 h after UV irradiation. In a previous study we showed that CPD repair in XP-C cells is restricted to the transcribed strand of active genes. In the current study CPD repair measured in the same experiments as 6-4PP repair was slow but selective for the transcribed strand of the ADA gene, the non-transcribed strand being only slightly repaired after long post-UV incubation times. Taken together these results suggest that in XP-C cells 6-4PPs and CPDs are repaired via the same pathway, which is coupled to active transcription (Carreau and Hunting, 1992). The fact that the kinetics of repair of 6-4PPs and the much more abundant CPDs are similar, strongly suggests that 6-4PPs and CPDs are repaired in a sequential way and that no lesions are bypassed by the transcription-coupled repair complex.

The combined data from normal and XP-C cells indicate that repair of active genes is governed by two repair pathways: transcription-coupled repair and global repair. The relative efficiency of DNA damage processing in active genes by the two repair pathways may depend on the type and frequency of lesions induced. For transcription-coupled repair it was proposed that RNA polymerase stalled at a DNA lesion acts as a damage antenna for repair enzymes (Van Houten, 1990). This has already been shown to be the case for *E.coli* in which a transcription–repair coupling factor is attracted by the stalled RNA polymerase complex. Whether a stalled polymerase plus transcript actually dissociates from the template or continues to transcribe after repair of the lesion is not yet clear. In *E.coli* the transcription coupled repair factor appears to remove the polymerase plus nascent RNA from the template and thus allows repair (Selby and Sancar, 1993). However, a recent study by Donahue *et al.* suggests that in an *in vitro* transcription system in which RNA polymerase II, eukaryotic transcription initiation factors and elongation factor TFIIS were employed, neither the RNA polymerase nor the nascent RNA strand dissociates from the DNA template at the site of CPDs (Donahue *et al.*, 1994). It remains to be resolved whether eukaryotic transcription, blocked at DNA lesions, is resumed after repair of the lesion without release of the nascent transcript, or whether repair requires the dissociation of the RNA polymerase and the nascent transcript from the template. Our data obtained with XP-C suggest that transcription-coupled repair is a processive repair pathway, in a sense that the different photolesions are sequentially removed from the transcribed strand, in the 3′ to 5′ direction, by the concerted action of transcription and repair. This implies that the more lesions are introduced into the template, the more the progression of the transcription–repair complex will be retarded. After its action as a damage antenna, the RNA polymerase is removed and transcription has to restart at the promoter site before it will encounter the next lesion (Selby and Sancar, 1993). At the dose of 30 J/m^2 approximately eight lesions (six CPDs, two 6-4PPs) have to be removed to make the ADA template (30 kb) damage-free. Moreover, the inhibition of initiation of RNA synthesis may be more severe at high UV doses, resulting in a strong impairment of transcription-coupled repair. The prolonged inhibition of transcription initiation and elongation at high UV doses enables the global repair system to compete with transcription-coupled repair in the case of CPDs, accounting for the loss of strand-specificity. This is reflected in the more rapid repair of CPDs in the ADA gene in normal cells compared with XP-C cells. A crucial point is that one has to assume that the efficiency of the global repair system is less affected by the high UV dose than transcription-coupled repair. Unfortunately, specific data on saturation kinetics of transcription-coupled repair are currently not available. Taken together it is obvious that repair of 6-4PPs in normal cells is dominated by the global repair pathway as indicated by the large difference between processing of 6-4PPs in XP-C (exclusively transcription-coupled repair) and normal cells (transcription-coupled repair and global repair), and that repair of 6-4PPs by

transcription-coupled repair is simply too slow to compete effectively with the global repair pathway.

Whether repair of photolesions in active genes in normal cells is dominated by transcription-coupled repair apparently depends on the type of lesion and on the UV dose employed. Generally speaking the lower the UV dose, the larger the contribution of transcription-coupled repair to repair of active genes. Consequently we cannot exclude that in cells exposed to a low UV dose (e.g. 2–12 J/m^2, used for mutation induction experiments; Vrieling *et al.*, 1991), 6-4PP repair may be dominated by transcription-coupled repair. Unfortunately, no proper method has been developed yet for measuring repair of 6-4PPs at the level of the gene at such low doses. However, mutation studies in hamster cells suggested that a UV dose effect on transcription-coupled repair of DNA photolesions might exist. At a low UV dose (2 J/m^2) a strong bias for mutation induction in the HPRT gene towards the non-transcribed strand was observed, consistent with the preferential repair of UV-induced lesions in the transcribed strand. However, at a higher UV dose (12 J/m^2) at which CPDs were still preferentially repaired, the strand bias was much less pronounced (Vrieling *et al.*, 1991). A possible explanation for this phenomenon is that at 12 J/m^2 transcription-coupled repair of 6-4PPs is less efficient than at 2 J/m^2 and is overruled by the global repair pathway. Anyway, one could conclude that transcription-coupled repair of DNA lesions in the absence of global repair is not sufficient to protect cells from high mutation rates. The lack of repair of 6-4PPs in the non-transcribed strand of active genes in XP-C might explain the enhanced UV-induced mutagenicity of these cells compared with normal human cells (Tatsumi *et al.*, 1987).

Materials and methods

Cell cultures

Monolayer cultures of normal human (VH16 and VH25) and xeroderma pigmentosum group C (XP1TE and XP21RO) primary fibroblasts were grown in 94 mm Petri dishes in Ham's F10 medium (without hypoxanthine and thymidine) supplemented with 15% fetal calf serum and antibiotics in a 2.5% CO_2 atmosphere.

UV irradiation and DNA isolation

UV irradiation and isolation of DNA were performed essentially as described previously (Venema *et al.*, 1991). Briefly, confluent cells were washed with PBS, irradiated with a Philips TUV lamp (predominantly 254 nm) at a dose rate of 0.2 W/m^2, incubated for various periods of time in medium and lysed. DNA was isolated and purified by phenol and chloroform extractions, precipitated with ethanol, resuspended and treated with RNase. The purified DNA was incubated with the appropriate restriction enzymes, again purified by phenol and chloroform extractions and ethanol precipitated. It was not necessary to separate replicated DNA from parental DNA by CsCl gradient centrifugation because replication activity in confluent cells is negligible (Venema *et al.*, 1992).

Measurement of CPD

CPD frequencies in restriction fragments of genes were measured as described previously (Venema *et al.*, 1991). Briefly, equal amounts of DNA were either treated or mock treated with the CPD-specific enzyme T4 endonuclease V. The samples were subjected to electrophoresis in an alkaline 0.6% agarose gel and transferred to a nylon membrane (Hybond N+, Amersham) which was hybridized with a radiolabeled gene-specific probe. Autoradiograms were made and full-size fragments were quantified by videodensitometric scanning. CPD frequencies were calculated by comparing the density of the band in the treated sample with that in the mock treated sample using the Poisson expression.

Measurement of endonuclease III sensitive sites

In order to check the DNA for the presence of thymine hydrates and glycols, equal amounts of DNA were treated or mock treated with *E.coli* endonuclease III (kindly provided by Dr L.Roza, TNO, Rijswijk, The Netherlands) in a buffer containing 100 mM NaCl, 10 mM Tris pH 7.5, 10 mM EDTA and incubated for 20 min at 37°C. After incubation the samples were loaded on an alkaline 0.6% agarose gel which was processed as described above.

Measurement of 6-4PP

For gene-specific measurement of 6-4PP frequencies the method described by Thomas *et al.* (1989) was used with a number of modifications. In this assay single-stranded DNA breaks are introduced at the sites of lesions by the *E.coli* UvrABC excinuclease complex (Visse *et al.*, 1992). This complex will cut the DNA at any lesion that causes a helical distortion: in the case of short wave UV irradiation, predominantly CPDs and 6-4PPs. Prior to incubation of the DNA with the UvrABC complex, CPDs were removed from the DNA by *in vitro* photoreactivation. The DNA was mixed with photolyase derived from *Anacystis nidulans* (kindly provided by Dr A.Eeker, Erasmus University, Rotterdam, The Netherlands; Eker *et al.*, 1990) in a buffer containing 10 mM KH_2PO_4 (pH 7.4), 100 mM NaCl, 5 mM β-mercaptoethanol and 100 μg/ml BSA, and exposed to 425 nm light for 1 h at room temperature. Subsequently the samples were incubated with proteinase K and SDS for 30 min at 37°C, the DNA was purified by phenol and chloroform extraction, and precipitated with ethanol. Photoreactivation was checked for completeness by treatment of DNA samples with T4 endonuclease V and subsequent Southern analysis. Equal amounts of DNA were either treated or mock treated with UvrABC (2 pmol of each subunit per μg DNA). Prior to incubation with the DNA the subunits UvrA and UvrB were pre-incubated together in the presence of 75 mM KCl, 10 mM $MgCl_2$, 50 mM Tris pH 7.5 and 2 mM ATP for 10 min at 37°C to form the pre-incision complex. UvrC was added to the pre-incubation mix, the mixture of the three subunits was added to the DNA and the samples were incubated for 1 h at 37°C. After incubation 10 mM EDTA and 0.1% SDS were added and the DNA was purified by phenol and chloroform extraction, precipitated with ethanol and dissolved in TE. The samples were electrophoresed in an alkaline 0.6% agarose gel. After Southern blotting and hybridization with a gene-specific probe, autoradiograms were made and 6-4PP frequencies were calculated as described for CPD.

Preparation of ^{32}P-labeled double- or single-stranded probes

From the human ADA cDNA clone pLL the *Pst*I fragments Ba (exons 1–5), Bo (exons 6–11) and Be (exon 12) were subcloned in pUC19 (Berkvens *et al.*, 1987). A plasmid containing a 2.0 kb *Hin*dIII fragment of 754 cloned into pAT153 was obtained from Dr B.Bakker (Leiden University, The Netherlands). A pEMBL 18+ plasmid, containing a genomic *Xba*I partial restriction fragment that includes the *Drosophila Gart* gene, was used for hybridizations with *Drosophila* DNA.

The double-stranded probes were labeled with [α-^{32}P]dATP by random primer extension (Feinberg and Vogelstein, 1983). For the preparation of strand-specific probes, linear PCR was performed on double-stranded templates containing the ADA Ba or Bo fragment, using one specific primer, 2.5 mM dCTP, dGTP and dTTP and 800 μCi/ml [α-^{32}P]dATP (3000 Ci/mmol) (Ruven *et al.*, 1994). The strand-specificity of the probe was checked by including dot blots with control single-stranded DNA in the hybridizations.

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