The residual repair capacity of xeroderma pigmentosum complementation group C fibroblasts is highly specific for transcriptionally active DNA

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ABSTRACT

We have measured removal of pyrimidine dimers in defined DNA sequences in confluent and actively growing normal human and xeroderma pigmentosum complementation group C (XP-C) fibroblasts exposed to 10 J/m² UV-irradiation. In normal fibroblasts 45% and 90% of the dimers are removed from the transcriptionally active adenosine deaminase (ADA) gene within 4 and 24 hours after irradiation respectively. Equal repair efficiencies are found in fragments located entirely within the transcription unit or partly in the 3' flanking region of the ADA gene. The rate and extent of dimer removal from the dihydrofolate reductase (DHFR) gene is very similar to that of the ADA gene. Repair of the transcriptionally inactive 754 locus is less efficient: 18% and 52% of the dimers are removed within 4 and 24 hours respectively. In spite of the limited overall repair capacity, confluent XP-C fibroblasts efficiently remove dimers from the ADA and DHFR genes: about 90% and 50% within 24 hours respectively. The 3' end of the ADA gene is repaired as efficiently as in normal human fibroblasts, but less efficient repair occurs in DNA fragments located in the DHFR gene and at the 5' end of the ADA gene. Repair of the inactive 754 locus does not exceed the very slow rate of dimer removal from the genome overall. Confluent and actively growing XP-C cells show similar efficiencies of repair of the ADA, DHFR and 754 genes. Our findings suggest the existence of two independently operating pathways directed towards repair of pyrimidine dimers in either active or inactive chromatin. XP-C cells have lost the capacity to repair inactive chromatin, but are still able to repair active chromatin.

INTRODUCTION

Xeroderma pigmentosum (XP) is a rare genetic disorder with clinical and cellular hypersensitivity to ultraviolet (UV) light.

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is as inefficient as from the genome overall. Although these results seem inconsistent, it has been suggested that not all active regions are repaired equally well and that there may be a subset of domains that are repairable in non-dividing XP-C cells (16).

In an attempt to further characterize the repaired domains in XP-C cells we measured repair of pyrimidine dimers induced by 254 nm UV in the transcriptionally active adenosine deaminase (ADA) and dihydrofolate reductase (DHFR) genes and the transcriptionally inactive 754 locus using the procedures described by Bohr et al. (17). The 754 locus is located on the X-chromosome near the Duchenne’s muscular dystrophy locus (DMD) and is not transcribed in primary human fibroblasts (18). Both in non-dividing normal human and XP-C cells repair of the ADA gene as well as the DHFR gene was found to be very efficient. Normal cells also performed efficient repair of pyrimidine dimers from the 754 locus, but at a slower rate than found for the active ADA and DHFR genes. In XP-C cells pyrimidine dimers were very poorly removed from the 754 locus.

**MATERIALS AND METHODS**

**Cell culture and prelabeling of the cells.** Diploid normal human (VH16, VH25) and xeroderma pigmentosum (XP1TE, XP21RO, XP6RO) fibroblasts were cultured in Ham’s F10 medium supplemented with 15% fetal calf serum and antibiotics in a 2.5% CO₂ atmosphere. Routinely cell populations were grown to confluence and then split 1 : 3. To prelabel the cells 0.1 µCi/ml 3H-thymidine (100 mCi/mmol) or 0.01 µCi/ml 14C-thymidine (50 mCi/mmol) was added to the medium and the cells were cultured for 3 days. The medium was then replaced by fresh label-free medium and cell growth was continued to confluence. In experiments aimed to study repair in stationary cells, confluent prelabeled cell populations were trypsinized and inoculated in normal medium at a 2.5 fold lower density than the confluent cells. The cells were then cultured for 10 days with replacement of medium every 3 days to reach stationary phase. In experiments aimed to study repair in exponentially growing cells, confluent prelabeled cells were trypsinized and inoculated in normal medium at a 3.5 fold lower density. Repair experiments were started 40 hours after splitting the cells.

**UV-irradiation and post-UV incubation of the cells.** Prior to irradiation the medium was removed and stored at 37°C. The cells were rinsed with prewarmed phosphate-buffered saline and irradiated with a Philips T.U.V. lamp (predominantly 254 nm) at a dose rate of 0.2 W/m². Cells were either lysed immediately or incubated for various periods of time in the original medium supplemented with 10 µM bromodeoxyuridine (BrdU) and 1 µM fluorodeoxyuridine (FdU). BrdU/FdU was added to allow separation of parental DNA and replicated (hybrid density) DNA, since the presence of replicated dimer free DNA would lead to an overestimation of DNA repair.

**Isolation and purification of DNA.** Lysis of the cells was achieved by addition of lysis buffer (10 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 0.5% SDS and 100 µg/ml proteinase K) and subsequent incubation for 16 hr at 37°C. DNA was further purified by phenol and chloroform extraction. After alcohol precipitation and centrifugation pellets were dissolved in TE buffer (10 mM Tris (pH 8.0), 1 mM EDTA) and digested with 50 µg/ml RNase A for 1 hr at 37°C. After precipitation and resuspension in TE the DNA was incubated for 16 hr with the appropriate restriction enzyme at an enzyme concentration of 2.5 U/µg DNA using digestion conditions recommended by the manufacturer. All digestions were checked for completion on agarose gels. The DNA samples were adjusted to a final concentration of 10 mM EDTA and centrifuged to equilibrium in CsCl density gradients. The gradients were fractionated and aliquots were counted in liquid scintillation counter to determine parental and hybrid density DNA. Fractions containing parental density DNA were pooled and dialyzed against TE. The DNA concentration was determined by UV absorption at 260 nm. The DNA was concentrated with butanol and precipitated with ethanol in the presence of 150 mM NaCl. The DNA was collected by centrifugation and dissolved in TE at a final concentration of 1 µg/µl.

**T4 endonuclease V digestion.** 15 µg of DNA was incubated with T4 endonuclease V (19) for 15 min at 37°C in a reaction buffer containing 10 mM Tris (pH 8.0), 80 mM NaCl and 10 mM EDTA. An equivalent amount of DNA not receiving enzyme was incubated with enzyme buffer. Incubation was terminated by adding alkaline loading buffer to each sample to final concentrations of 50 mM NaOH, 1 mM EDTA, 2.5% Ficoll and 0.025% bromocresol purple. Routinely UV-irradiated plasmid DNA (four T4 endonuclease V sensitive sites per kb) or genomic DNA (15 J/m²) was incubated with T4 endonuclease V in various concentration ratios of DNA to enzyme to check for complete digestion of pyrimidine dimers under the conditions used.

**Gel electrophoresis, Southern transfer and hybridizations.** Electrophoresis of DNA samples was performed in 0.6% alkaline agarose gels for 16 hr at 20 V in a buffer containing 30 mM NaOH and 1 mM EDTA. Based on radioactivity equivalent amounts of DNA treated either with or without enzyme were loaded on the gel. After electrophoresis the DNA was transferred to a support membrane in alkaline/high salt transfer solution. Filters were hybridized for 40 hr at 42°C in 2.5 ml of a solution containing 40% formamide, 5× SSPE, 5× Denhardt, 1% SDS and 100 µg/ml denatured salmon sperm DNA. DNA probes were labeled with α-32P-dATP by random primer extension (20). After hybridization filters were washed at 65°C with a final wash step at 1× SSPE, 1% SDS. Filters were exposed to either Fuji or Kodak XAR-5 x-ray films at −80°C using an intensifying screen. Band intensities were quantitated using a Biorad Video Densitometer Model 620. The average number of ESS per fragment was calculated from the densities of full length fragments using the Poisson expression as described by Bohr et al. (17).

**Measurement of DNA synthesis.** To monitor the amount of DNA synthesis, subcultured 14C-prelabeled cells were pulse labeled with 5 µCi/ml 3H-thymidine (80 Ci/m mole) for 60 min at various times after subculturing. Cells were lysed and DNA was purified by phenol extraction. The relative amount of DNA synthesis was determined from the 3H/14C ratio after liquid scintillation counting.

**Determination of T4 endonuclease sensitive sites in total cellular DNA.** The number of T4 endonuclease sensitive sites in total DNA was determined as described by Van Zeeland et al. (21). Briefly, confluent 14C-prelabeled cells were permeabilized and exposed to 2 M NaCl before T4 endonuclease V treatment. The
number of endonuclease sensitive sites in DNA was assayed by sedimentation in alkaline sucrose gradients upon which the cells were lysed directly.

DNA probes. From the human ADA cDNA clone pLL the PstI fragments BA (exons 1–5 partly), BO (exons 5 partly-11) and BE (exon 12) were subcloned in pUC19 (22). A plasmid containing a 2.0 kb HindIII fragment of 754 cloned into pAT153 was obtained from B. Bakker (University of Leiden, The Netherlands). A plasmid designated pBH31R1.8 (23) containing a 1.8 kb genomic EcoRI fragment from the human DHFR gene was a gift of V. Bohr (National Institute of Health, U.S.A.).

RESULTS

1. Induction of pyrimidine dimers in the ADA gene and in total cellular DNA. The frequency of pyrimidine dimers in DNA was assayed using the enzyme T4 endonuclease V, which specifically incises DNA at pyrimidine dimers (endonuclease sensitive sites). The frequency of endonuclease sensitive sites (ESS) was determined in an 18.5 kb EcoRI fragment of the ADA gene (Fig. 1), according to procedures described by Bohr et al. (17). DNA samples from UV-irradiated cells were treated with either T4 endonuclease V or with enzyme buffer, electrophoresed in alkaline agarose gels and transferred to a support membrane. After hybridization and autoradiography the pyrimidine dimer frequency was determined from the relative densities of the full length fragments in the treated and non-treated DNA samples using the Poisson distribution. The frequency of ESS in total cellular DNA was determined by centrifugation of DNA in alkaline sucrose gradients (20). Table 1 shows that the UV dose response for the induction of pyrimidine dimers in the ADA gene and total DNA was very similar. These results rule out the possibility that differences in repair efficiency of the ADA gene and of the genome overall might be caused by differences in dimer content. During the course of the experiments dimer frequencies in all loci analyzed were found to be similar to the frequency in the genome overall. These results are consistent with previous reports (17,24) that dimer induction is uniform throughout the genome. Control experiments indicated that T4 endonuclease V treatment had no effect on DNA of unirradiated cells.

2. Removal of pyrimidine dimers from defined DNA sequences in confluent human fibroblasts. Repair analysis was performed in confluent fibroblasts which were exposed to 10 J/m² and incubated for up to 24 hours after UV-irradiation in the presence of bromodeoxyuridine. Even after 24 hr virtually no hybrid density DNA was present in CsCl density gradients, confirming the stationary state of the cells. Repair of the transcriptionally active ADA gene was analyzed in two restriction fragments generated by either BclI or EcoRI (Fig. 1). The 19.9 kb BclI fragment is entirely located within the ADA gene and contains exons 2 and 3. The 18.5 kb EcoRI fragment is located at the 3' end of the gene, containing 9 kb of flanking sequences. Filters containing EcoRI restricted DNA can also be used to measure repair in a 14 kb fragment of the transcriptionally inactive 754 locus (18) enabling us to accurately measure relative repair rates of both loci.

Figure 1. Molecular organization of the human ADA and DHFR genes. Filled boxes represent exons of the genes. EcoRI, BclI and HindIII restriction sites are indicated. The solid line indicates the genomic DHFR probe. The size in kb is also indicated. The ADA and DHFR maps are derived from ref. 40 and 41, respectively.

Table 1. Frequency of pyrimidine dimers per 10 kb of DNA

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1 18.5 kb EcoRI fragment of the ADA gene
2 Measured in alkaline sucrose gradients (see ref. 21)
dimers were removed from the ADA BclI fragment within 24 hr after UV-irradiation. Within 4 hr after UV-irradiation, repair in this fragment was twofold faster than in the genome overall. Removal of pyrimidine dimers from the ADA EcoRI fragment was as fast and as efficient as from the BclI fragment. The initial rate of removal of pyrimidine dimers from the 754 locus was comparable to that of the genome overall. After 24 hr, however, repair of the 754 locus appeared to be less than that of the genome overall.

The results obtained with confluent XP-C cells are also shown in Fig. 2 and Table 2. The residual repair capacity of the XP-C cells used in this study is approximately 15% of normal repair levels, as measured by repair replication (data not shown). In spite of the limited overall repair capacity, XP-C cells efficiently removed pyrimidine dimers from the ADA gene (Fig. 2 and Table 2). In two different XP-C cell lines, approximately 70% and 90% of the pyrimidine dimers were removed from the BclI and the EcoRI fragments of the ADA gene within 24 hr after irradiation. In contrast, repair of the 754 locus was as inefficient as repair of the genome overall: less than 10% of the pyrimidine dimers were removed within 24 hr after treatment. It should be noted that the initial pyrimidine dimer frequency in XP-C cells correlates well with the frequency in normal human VH16 cells (0.75 ESS/10 J/m²/10 kb).

To find out whether efficient repair in XP-C cells is a general feature of transcriptionally active DNA, we measured removal of pyrimidine dimers from the DHFR housekeeping gene. Repair was examined in a 22 kb HindIII fragment largely located within the DHFR transcription unit (Fig. 1). Over 80% of the pyrimidine dimers were removed from this fragment in normal cells incubated for 24 hr after treatment, whereas less efficient repair (50%) occurred in XP-C cells (Fig. 2 and Table 2). Our data in Table 2 also show that the repair efficiencies in the various DNA fragments in three different XP-C cell lines with comparable levels of repair replication were very similar.

3. Removal of pyrimidine dimers in exponentially growing cells.

To determine the most appropriate time to analyze repair in actively growing cells, experiments were performed in which 14C-thymidine prelabeled cells were pulse labeled for 1 hr with 3H-thymidine at various times after subculturing of the confluent cells. DNA synthesis was monitored by isolating DNA from these cells and calculating the relative amount of 3H-thymidine incorporation at each time point. For both VH16 and XP1TE cells DNA synthesis was found to be maximal between 30 and 50 hr after subculturing (data not shown). Therefore, all experiments with exponentially growing cells were performed during this time period. After UV-irradiation approximately 15% and less than 5% of the parental DNA was replicated within 24 hr in VH16 and XP1TE cells respectively, as indicated by the amount of hybrid density DNA in CsCl gradients (data not shown). Fig. 3 shows autoradiograms of repair analyses in the ADA EcoRI fragment and the DHFR HindIII fragment. Normal cells were able to perform fast and efficient repair of the ADA gene (see also Table 3) with about 80% of the pyrimidine dimers being removed within 24 hr after irradiation. The 754 locus was repaired to a lesser extent. XP-C cells showed efficient repair of the ADA gene similar to that observed in normal cells. After 24 hr 90% of the pyrimidine dimers were removed. However, pyrimidine dimer removal from the 754 locus was very low in XP1TE cells, similar to the limited repair of the genome overall. Similar to confluent cells, exponentially growing XP-C cells performed less efficient repair of the DHFR gene than normal cells, although still much more efficient than the repair of pyrimidine dimers from the genome overall.

**DISCUSSION**

A number of different biochemical approaches have revealed that the residual excision repair in XP-C cells is clustered in domains comprising approximately 10% of the genome (8,24). In this

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the ADA gene and that the region of preferential repair extends beyond the 3' end of the ADA gene. The latter may be related to the possibility that the 'repair domain' is larger than the actual transcribed unit itself in a way similar to the observation that DNase I sensitivity associated with active genes is not limited to the coding sequences of genes but extends both to 5' and 3' regions to define an 'active domain' (27,28,29). Furthermore, the recently reported existence of a transcription unit on the opposite strand beyond the 3' end of the ADA gene (30) could add to the observation of efficient repair of the ADA EcoRI fragment.

Based on the observed preferential localization of UV-induced repair synthesis at the nuclear matrix in confluent XP-C cells we have suggested that XP-C cells are proficient in repair of active genes, but have a defect in repair of inactive regions (11). The results shown in this study fully support this hypothesis. 24 hr after UV-irradiation repair of the 3' end of the ADA gene in XP-C fibroblasts is as efficient as in normal human fibroblasts. The transcriptionally inactive 754 locus, however, is very poorly repaired and its repair does not exceed the inefficient removal of pyrimidine dimers from the genome overall. In contrast to data published by Bohr et al. (15) we also note substantial repair of pyrimidine dimers from the DHFR gene during a 24 hr period, although this repair occurs to a lesser extent than in normal cells. In comparison to normal human cells the various DNA fragments derived from active genes are repaired with different efficiencies in the three XP-C cell lines employed in this study. Repair of the EcoRI fragment of the ADA gene mimics most closely the repair in normal cells, both with respect to rate and extent of repair. Removal of pyrimidine dimers from the ADA BglII fragment and the DHFR gene tends to occur more slowly and to a lesser extent than in normal cells. A possible explanation for these differences may be that efficient repair of active genes in XP-C cells is restricted to transcribed strands only as has been described for Chinese hamster cells (31). Recently, RNA polymerase II and RNA polymerase III antisense transcripts have been reported at the 3' and 5' end of the ADA gene respectively (30). Whereas the role of RNA polymerase III transcription in DNA repair remains to be defined, antisense transcription at the 3' end of the ADA gene could account for a repair efficiency of the ADA EcoRI fragment in XP-C cells very similar to normal cells. The maximum level of repair in the DHFR gene in XP-C cells is consistent with a maximum level of 50 to 60% repair of the DHFR gene in hamster cells (31) and further supports the abovementioned hypothesis.

Impairment or lack of recovery of transcription after inhibition by UV has been implicated in UV-induced cytotoxicity (32), since it is known that pyrimidine dimers efficiently terminate transcription (33) and inactivate gene expression (34). Therefore, efficient removal of pyrimidine dimers from all or a subset of active genes in XP-C cells would restore UV-inhibited RNA synthesis (4) and could explain the relatively high resistance of non-dividing XP-C cells to UV-light. With respect to kinetics of dimer removal XP-C cells resemble Chinese hamster cells, which efficiently remove pyrimidine dimers from active genes, but inefficiently from non-expressed DNA or the genome overall (17; Venema, unpublished results). Stationary XP-C cells are only slightly more sensitive to the lethal effects of UV than hamster cells. This relatively small difference in UV-sensitivity is remarkable if the repair capacity for another major UV-induced lesion, the (6-4)photoproduct, is considered. XP-C cells very poorly remove (6-4)photoproducts from the genome overall, whereas Chinese hamster cells are able to perform fast and study we have attempted to further characterize the repairable regions of the XP-C genome by measuring the removal of pyrimidine dimers from restriction fragments of the transcriptionally active ADA and DHFR genes and the inactive 754 locus. The ADA gene is a housekeeping gene which is constitutively expressed in all human tissues including skin (26). By Northern analysis transcription of the X-chromosomal 754 locus has been detected in adenovirus transformed retina cells, but not in HeLa cells or primary skin fibroblasts (18). Repair analysis in both genes was performed using the same Southern blot. In this way we could make an accurate comparison of relative repair rates by excluding variations due to unequal loading of different gels. We have shown that in confluent fibroblasts from two different normal human cell lines exposed to 10 J/m² UV pyrimidine dimers are removed faster and to a greater extent from the expressed ADA gene than from the transcriptionally inactive 754 locus. The rate of dimer removal from the 754 locus is similar to that of the genome overall, although after 24 hr the extent of repair of the 754 locus appears to be somewhat lower. Mellon et al. (24) showed preferential repair of pyrimidine dimers from the human DHFR gene. Our results provide a second human gene subject to preferential repair, indicating that preferential repair may be a general feature of active genes in human cells. Measurement of repair efficiencies in two adjacent ADA fragments revealed that the rate of repair is fairly constant within the ADA gene and that the region of preferential repair extends beyond the 3' end of the ADA gene. The latter may be related to the possibility that the 'repair domain' is larger than the actual transcribed unit itself in a way similar to the observation that DNase I sensitivity associated with active genes is not limited to the coding sequences of genes but extends both to 5' and 3' regions to define an 'active domain' (27,28,29). Furthermore, the recently reported existence of a transcription unit on the opposite strand beyond the 3' end of the ADA gene (30) could add to the observation of efficient repair of the ADA EcoRI fragment.

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**Figure 3.** Repair of pyrimidine dimers in exponentially growing human fibroblasts UV-irradiated with 10 J/m² and incubated for 8 and 24 hr. DNA was isolated and processed as described in the legend to Fig. 2. Southern blots were hybridized with ADA and DHFR probes. A. Normal human fibroblasts (VH16); B. XP-C fibroblasts (XP1TE).

**TABLE 3.** Percent pyrimidine dimer removal in exponentially growing normal human (VH16) and XP-C (XP1TE) fibroblasts. Numbers in parentheses represent SEM.

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<th>Cell line</th>
<th>Repair time (hr)</th>
<th>ADA EcoRI</th>
<th>754 EcoRI</th>
<th>DHFR EcoRI</th>
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<td>XP1TE</td>
<td>8</td>
<td>57 (3)</td>
<td>1 (1)</td>
<td>45</td>
<td></td>
</tr>
<tr>
<td></td>
<td>24</td>
<td>94 (5)</td>
<td>12 (7)</td>
<td>54</td>
<td></td>
</tr>
<tr>
<td>No. of experiments:</td>
<td>2</td>
<td>2</td>
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efficient repair of these lesions (35). The cytotoxic effects of 
(6-4)-photoproducts have been demonstrated by comparing the 
kinetcs of removal of pyrimidine dimers and (6-4)-photoproducts 
in a UV-sensitive XP-A cell line and its UV-resistant revertant 
(36). Our results suggest that in stationary cells the effects of 
(6-4)-photoproducts on cytotoxicity are limited and that survival 
of UV-irradiated stationary cells mainly depends on efficient 
removal of pyrimidine dimers (and possibly also 
(6-4)-photoproducts) from active genes.

We have shown that proliferating and stationary XP-C cells are 
equally efficient in removal of pyrimidine dimers from the ADA 
and DHFR genes. Preferential repair of active genes in 
proliferating XP-C cells is in line with previously reported 
preferential repair of nuclear matrix associated DNA in these 
cells (9). Our results are inconsistent with a random distribution 
of repaired sites in proliferating XP-C cells as was observed by 
Cleaver (37). The strong UV-sensitivity of actively growing XP-C 
cells (4,24) may be caused by the severe cytotoxic effects of 
(6-4)-photoproducts in these cells. Efficient repair of active genes, 
however, offers an explanation for the recovery of UV-inhibited 
RNA- and DNA-synthesis with kinetics similar to those in XP 
variant cells (4). In XP cell lines belonging to complementation 
groups A, D and G such a recovery was not observed. Similar 
efficiencies of repair of active genes in non-dividing and 
exponentially growing cells, as observed in both normal and XP-
C cells, may be a general feature of mammalian cells. Confluent 
and actively growing mouse 3T3 fibroblasts showed similar 
relative efficiencies of repair of proto-oncogenes (38). 
Furthermore, the rate of repair of the DHFR gene in a 
proliferating human cell line (26) is similar to that of the ADA 
gene in confluent fibroblasts described in this study.

In conclusion, our data show that repair pathways operating in 
active and inactive chromatin are at least partially independent. 
XP-C cells have lost the ability to repair inactive chromatin. It 
is interesting to note that the reverse situation may exist in cells 
from patients with another UV-sensitive disorder, Cockayne's 
syndrome. These cells have a normal overall repair capacity, but 
appear to be unable to perform efficient repair of active genes 
(39).

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