Nucleic Acids Research, 1996, Vol. 24, No. 23 **4653–4659**

The sensitivity of human fibroblasts to *N*-acetoxy-2-acetylaminofluorene is determined by the extent of transcription-coupled repair, and/or their capability to counteract RNA synthesis inhibition

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Received September 5, 1996; Revised and Accepted October 22, 1996

ABSTRACT

Nucleotide excision repair (NER) mechanism is the major pathway responsible for the removal of a large variety of bulky lesions from the genome. Two different NER subpathways have been identified, i.e. the transcription-coupled and the global genome repair pathways. For DNA-damage induced by ultraviolet light both transcription-coupled repair and global genome repair are essential to confer resistance to cytotoxic effects. To gain further insight into the contribution of NER subpathways in the repair of bulky lesions and in their prevention of biological effects we measured the rate of repair of dG-C8-AF in active and inactive genes in normal human cells, XP-C cells (only transcription-coupled repair) and XP-A cells (completely NER-deficient) exposed to NA-AAF. XP-C cells are only slightly more sensitive to NA-AAF than normal cells and, like normal cells, they are able to recover RNA synthesis repressed by the treatment. In contrast, XP-A cells are sensitive to NA-AAF and unable to recover from RNA synthesis inhibition. Repair of dG-C8-AF in the active ADA gene proceeds in a biphasic way and without strand specificity, with a subclass of lesions quickly repaired during the first 8 h after treatment. Repair in the inactive 754 gene occurs more slowly than in the ADA gene. In XP-C cells, repair of dG-C8-AF in the ADA gene is confined to the transcribed strand and occurs at about half the rate of repair seen in normal cells. Repair in the inactive 754 gene in XP-C cells is virtually absent. Consistent with these results we found that repair replication in XP-C is drastically reduced when compared with normal cells and abolished by α-amanitin indicating that the repair in XP-C cells is mediated by transcription-coupled repair only. Our data suggest that dG-C8-AF is a target for transcription-coupled repair and that this repair pathway is the main pathway or recovery of RNA synthesis inhibition conferring resistance to cytotoxic effects of NA-AAF. In spite of this, repair of dG-C8-AF in active genes in normal cells by transcription-coupled repair and global genome repair is not additive, but dominated by global genome repair. This indicates that the subset of lesions which are capable of stalling RNA polymerase II, and are, therefore, a substrate for TCR, are also the lesions which are very efficiently recognized by the global genome repair system.

INTRODUCTION

Nucleotide excision repair (NER) is a major pathway by which a large variety of bulky DNA lesions are removed from the genome. Two different NER subpathways have been identified, i.e. the global genome repair pathway and the transcription-coupled repair pathway. Both pathways have been well characterized for their role in the repair of ultraviolet (UV) light-induced cyclobutane pyrimidine dimers (CPDs) in mammalian cells. In human cells the global genome repair pathway processes CPDs with different efficiencies across the genome: (potentially) active genes are repaired at a much faster rate than non-expressed regions of the genome (1). In addition, CPDs are excised by the transcription-coupled repair pathway more rapidly from the transcribed strand of active genes than from the non-transcribed strand (2,3). This accelerated repair of CPDs in the transcribed strand of active genes is dependent on transcription (4). It has been proposed that stalled RNA polymerase may act as a signal to target the NER apparatus to the site of a lesion. From the repair phenotype of UV-sensitive human cells it is obvious that specific genetic factors are involved in the NER subpathways. Xeroderma pigmentosum group C (XP-C) fibroblasts appear to be fully capable of performing transcription-coupled repair, but are defective in global genome repair, resulting in the lack of CPD repair in non-transcribed DNA including the non-transcribed strand of active genes (3,5). In contrast with CPDs, pyrimidine 6-4 pyrimidone photoproducts

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(6-4 PP) are not preferentially removed from the transcribed strand of active genes in normal cells. This lack of strand-specific repair of 6-4 PP in transcriptionally active genes has been shown to be the result of a very efficient removal of these lesions by the global genome repair pathway overruling the removal of 6-4 PP by transcription-coupled repair (6).

The molecular basis for the observed differences in repair of 6-4 PP and CPD by the global repair pathway most likely lies in local alterations of DNA conformation induced by the photolesions. Further information on the efficiency of processing of other bulky DNA lesions by the global genome and by the transcription-coupled repair pathways will provide insights in the relationship between the structure of a DNA lesion, its transcription inhibiting potency, and the efficiency to be processed by both NER subpathways (with respect to repair of lesions in active genes). In this study we focussed on the role of the global genome and transcription-coupled repair pathways in the repair of *N*-acetoxy-2-acetylaminofluorene (NA-AAF)-induced DNA adducts in active and inactive genes.

NA-AAF causes lesions in DNA which are substrate to NER (7–10). Although treatment of cells with NA-AAF may principally lead to the formation of two major lesions, i.e. the deacetylated *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) and the acetylated *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF), the main lesion induced in human fibroblasts appears to be the deacetylated dG-C8-AF. Recent studies with normal human fibroblasts (11,12) and NA-AAF-sensitive Cockayne syndrome cells (CS) (12) revealed that transcription-coupled repair does not contribute significantly to the repair of dG-C8-AF in active genes. These observations, as well as the reported marginal blockage of transcription by dG-C8-AF in *in vitro* transcription assays (11,13), raise the question whether dG-C8-AF lesions are actually targets for transcription-coupled repair. To assess the role of transcription-coupled repair in the repair of the dG-C8-AF lesion we compared the recovery of NA-AAF inhibited RNA synthesis, the repair kinetics of dG-C8-AF in active genes and the cytotoxic effects in normal cells, XP-A fibroblasts (completely deficient in NER) and XP-C fibroblasts (only capable of performing transcription-coupled repair). In this study we demonstrate that dG-C8-AF lesions are a target for both the global genome repair pathway and the transcription-coupled repair pathway. Both pathways remove dG-C8-AF from active genes with biphasic kinetics, but the efficiency of repair by global genome repair is ~2-fold higher than by transcription-coupled repair. The biological relevance of transcription-coupled repair of dG-C8-AF is illustrated by the recovery of RNA synthesis and the mild cytotoxic effects of NA-AAF in XP-C cells when compared with XP cells belonging to complementation group A and compared with CS cells. Our data indicate that repair of dG-C8-AF in active genes by the two NER subpathways is not simply additive, and suggest that those lesions which are removed by the transcription-coupled repair process are also an efficient substrate for the global genome repair.

MATERIALS AND METHODS

Cell lines and culture conditions

Primary fibroblasts from a normal individual, VH25D, and primary xeroderma pigmentosum fibroblasts, XP21RO (complementation group C) and XP25RO (complementation group A) were cultured at 37°C in 2.5% CO_2 atmosphere using Ham's F10 medium (without hypoxanthine and thymidine) supplemented with 15% Fetal Bovine Serum and antibiotics. In experiments aimed to determine the initial frequency and the removal of adducts in defined genomic sequences, exponentially growing cells were prelabelled for 2 days with [^{3}H]thymidine (0.06 µCi/ml, 82 Ci/mmol). For measurements of repair replication or RNA synthesis, exponentially growing cells were prelabelled with [^{32}P]orthophosphate (0.3 µCi/ml) or [^{14}C]uridine (0.03 µCi/ml, 60 mCi/mmol), respectively.

Cell survival studies

Cell survival following treatment with NA-AAF was determined by measuring the colony forming ability of the treated cells relative to the untreated control. 500–1000 cells were seeded in 94 mm Petri dishes, allowed to attach for 16 h, and incubated with 0, 5, 10, 15, 20 or 30 µM NA-AAF for 30 min at 37°C in complete medium. After incubation fresh medium was added to the cells and 10–14 days after plating colonies were stained with methylene blue.

HPLC analysis of NA-AAF-induced DNA adducts

Confluent cells were incubated with 5 µM of [^{3}H]NA-AAF (649 mCi/mmol) for 30 min at 37°C, and immediately lysed in 150 mM NaCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 0.5% SDS and 100 µg/ml proteinase K at 37°C overnight. The DNA was purified by phenol extraction, RNase A-treated and the specific activity (d.p.m./µg) of DNA was determined by scintillation counting in Plasmasol. HPLC analysis was performed as described previously (12).

Measurement of RNA synthesis, DNA repair replication and unscheduled DNA synthesis

RNA synthesis measurements, DNA repair replication and unscheduled DNA synthesis (UDS) were performed as described by van Oosterwijk *et al.* (12).

α-Amanitine studies

Cell cultures were incubated for 5 h with α-amanitine (1 µg/ml) prior to treatment with 90 µM NA-AAF. After NA-AAF treatment, cells were washed twice with PBS and medium containing α-amanitine was added to the cells. To allow repair, cells were incubated for 24 h in this medium supplemented with [^{3}H]thymidine, 5 µCi/ml (82 Ci/mmol) and BUdR/FUdR (10 µM/1 µM). To determine the amount of incorporated radioactivity the same protocol as previously described for repair replication measurements was followed (12). As a control, cells were treated with only α-amanitine or NA-AAF, or not treated at all.

DNA probes

Double-stranded DNA probes were radioactively labelled with [^{32}P]dATP by random primer extension (14). Strand-specific single-stranded probes were radioactively labelled with [^{32}P]dATP by a linear polymerase chain reaction, using a single primer recognizing specifically one strand (15).

Determination of NA-AAF-induced DNA adducts in defined sequences

In order to measure DNA adduct frequencies in defined genomic sequences as a function of dose, [^{3}H]thymidine prelabelled confluent cells were incubated with 0, 5, 10 or 15 μM NA-AAF in complete medium for 30 min at 37°C. After washing the cells twice with PBS, they were lysed. In repair experiments ^{3}H-prelabelled confluent cells were incubated with 5 μM NA-AAF in complete medium for 30 min at 37°C, and either lysed immediately or incubated for up to 48 h in complete medium supplemented with BUdR/FUdR (10 μM/1 μM).

DNA was isolated and purified by phenol–chloroform extractions and digested with restriction enzymes (1,12). The frequency of NA-AAF-induced DNA-adducts per restriction fragment was determined by incision at the sites of adducts employing the UvrABC excinuclease complex of *Escherichia coli* as described previously (6,12).

Determination of BUdR-labelled repair patches in active and inactive genes

The distribution of BUdR-labelled repair sites in restriction fragments of genes was studied by an immunochemical method previously described (16). The method is based on the separation of restriction fragments containing BUdR-labelled repair patches from non-BUdR containing DNA using specific antibodies. Aliquots of 5 μM NA-AAF treated cells were incubated for different post-incubation periods (0, 2, 4, 8 and 24 h) in medium containing BUdR/FUdR (10 μM/1 μM). DNA was isolated, restricted, and caesium chloride gradients were performed twice to separate parental DNA from replicated DNA. The parental DNA was denaturated and subsequently incubated with a monoclonal antibody raised against BUdR in DNA, a biotinylated Goat-anti-Mouse antibody (GαMbio) and polymeric magnetic particles coated with streptavidin (Dynabeads M-280, Dynal). The bound DNA (repaired, BUdR containing) was separated from the unbound DNA (unrepaired) using a magnetic particle concentrator (MPC-E, Dynal), and both fractions were applied to Hybond N$^+$ using a slotblot apparatus (Schleicher and Schuell). The blots were hybridized with gene-specific ^{32}P-labelled probes and the amount of ^{32}P in the bound and unbound fractions was determined using a Betascope 603 blot analyser (Betagen Corp.).

RESULTS

Cytotoxic effects of NA-AAF treatment

To determine the cytotoxic effects of NA-AAF on cells after a 30 min exposure, we measured the colony forming ability relative to untreated cells. Figure 1 shows that the xeroderma pigmentosum cell line XP21RO (complementation group C) is only slightly more sensitive to NA-AAF treatment than the normal human VH25D cell line, in contrast with XP25RO cells (complementation group A). The survival of XP21RO cells following NA-AAF treatment is also better when compared with the Cockayne syndrome cell line CS3BE (complementation group A).

Formation of DNA adducts

HPLC analysis of DNA obtained from confluent cells treated with ^{3}H-labelled NA-AAF revealed that the distribution patterns of radioactivity in the chromatograms were very similar for VH25D and XP21RO, and that the major lesion induced in both cell lines after NA-AAF treatment is the deacetylated dG-C8-AF lesion with a frequency of 0.13 and 0.20 NA-AAF lesions/μM/10 kb for normal and XP21RO cells respectively.

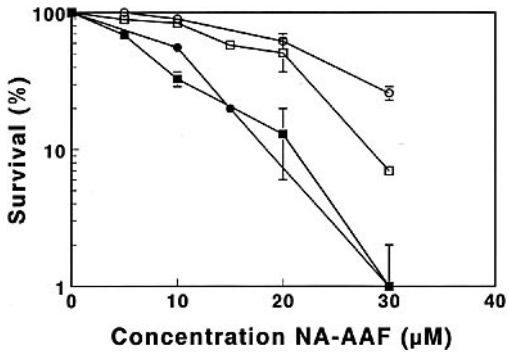

Figure 1. Cytotoxic effects of NA-AAF on primary normal human fibroblasts VH25D (○), xeroderma pigmentosum fibroblasts XP21RO (complementation group C) (□), XP25RO (complementation group A) (●) and Cockayne's syndrome fibroblasts CS3BE (complementation group A) (■). The CS3BE data are taken from (12). The error bars represent standard error of the mean (SEM).

Effects of NA-AAF treatment on RNA synthesis

Since a stalled transcription complex is considered to be an essential intermediate in transcription-coupled repair, we investigated the inhibition of RNA synthesis by NA-AAF treatment. Figure 2A shows that RNA synthesis measured during a 30 min pulse immediately after treatment with NA-AAF is inhibited in a dose-dependent way and the extent of inhibition is the same in normal and XP-C cells. Next we determined the capabilities of these two cell lines as well as XP-A cells to recover from NA-AAF-inhibited RNA synthesis. Figure 2B shows that both normal and XP-C cells, after treatment with 15 μM NA-AAF, are capable of recovering the inhibited RNA synthesis with the same kinetics. This in contrast with XP-A cells, which are not able to recover RNA synthesis after NA-AAF treatment of 15 μM.

Repair replication and UDS measurement

To assess the genome overall repair of NA-AAF induced DNA adducts in normal and XP-C cells, we measured the level of DNA repair replication. ^{32}P-prelabelled cells were treated with 30, 45 or 90 μM NA-AAF and were allowed to carry out repair for 24 h in the presence of [^{3}H]thymidine, BUdR and FUdR. The results, shown in Figure 3, indicate that the extent of repair synthesis measured over a period of 24 h following NA-AAF treatment is substantially lower in XP-C cells when compared with normal cells.

Additionally, the level of UDS was determined in the two cell types. Cells were labelled with [^{3}H]thymidine during the initial 30 min following NA-AAF exposure, and processed for measurement of UDS. The number of ^{3}H-labelled grains per nuclei was significantly lower in the case of the XP-C cells compared with normal cells, 2.2 and 11.2 grains per nuclei respectively. Thus both methods, DNA repair replication and UDS, indicate a reduced level (10–20%) of genome overall repair in XP-C cells, when compared with normal human cells. This is true for short (2 h) as well as long (24 h) periods of repair.

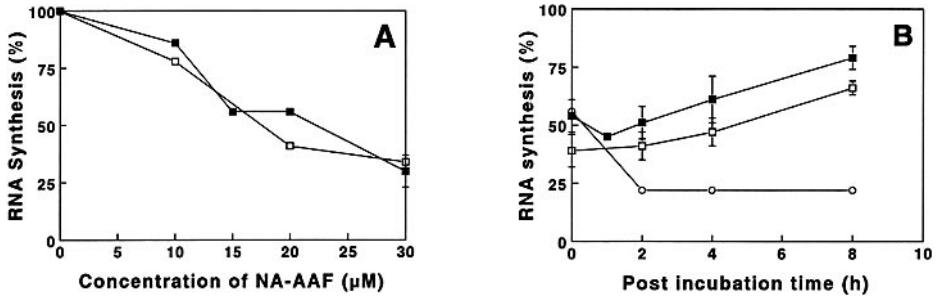

Figure 2. (A) Inhibition of RNA synthesis after treatment with different doses of NA-AAF. VH25D (■); XP21RO (□). (**B**) RNA synthesis relative to untreated cells at various post incubation times after treatment with 15μM NA-AAF. VH25D (■); XP21RO (□); XP25RO (○). In both panels RNA synthesis after treatment with NA-AAF is measured by 30 min pulse labelling with [^{3}H]uridine and expressed relative to corresponding untreated cells. The error bars represent SEM.

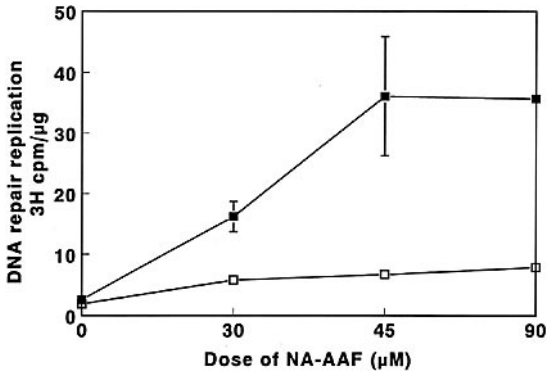

Figure 3. Repair replication in normal human fibroblasts (■) and xeroderma pigmentosum fibroblasts (complementation group C) (□) during 24 h following treatment with 30, 45 and 90 μM NA-AAF.

To address the effect of RNA polymerase II driven transcription on repair of NA-AAF-induced lesions, we investigated the effect of α-amanitine, an inhibitor of RNA polymerase II, on the level of DNA repair synthesis. Previous experiments by Carreau and Hunting (17) indicated that treatment of UV-irradiated XP-C cells with α-amanitine resulted in a drastic reduction of repair replication, whereas no such effect was seen in normal human cells. Table 1 shows that the incorporation of [^{3}H]thymidine in XP-C cells after NA-AAF treatment is substantially reduced (66%) in the presence of α-amanitine. This in contrast with the normal cells which did not show a decrease in repair replication in the presence of α-amanitine. We note here that the amount of repair replication in normal cells actually increased in the presence of α-amanitin; possibly due to the effects of α-amanitin on nucleotide pools. Surprisingly, untreated cells also showed an increase in incorporation in the presence of the inhibitor, although untreated cells are not expected to incorporate the ^{3}H label in parental DNA. The reason of this incorporation is not clear.

Induction and removal of NA-AAF adducts in active and inactive genes

To examine the frequency of NA-AAF adducts in restriction fragments of genes, we employed the UvrABC excinuclease to introduce a single-stranded DNA break in the DNA at each site of a lesion. After treatment with UvrABC excinuclease the DNA samples were subjected to alkaline agarose gel electrophoresis, Southern blotting and hybridization with radiolabelled specific probes. The presence of DNA lesions is seen as a reduction in intensity of bands of full size restriction fragments in lanes containing UvrABC-digested DNA compared with the intensity of the same bands in lanes with undigested DNA. The ratio of band intensities was used to quantify lesion frequencies in restriction fragments employing the Poisson distribution (18).

Normal and XP-C cells were treated with various concentrations of NA-AAF to determine the frequency of induction of NA-AAF adducts in active and inactive genes. The lesion frequencies were measured in a 3′ located 18.5 kb *Eco*RI fragment of the active ADA gene and a 14 kb *Eco*RI fragment of the X-chromosomal inactive 754 gene employing the same filter. Moreover, the induction frequencies were very similar for both genes and for both cell lines with an average induction frequency of 0.15 adducts/μM/10 kb, which is very similar to the average frequency in the genome overall as measured by HPLC analysis (0.17 adducts/μM/10 kb). Since the methodology for the quantification of adducts in defined sequences is most sensitive in the range of one adduct per restriction fragment, a dose of 5μM was chosen, inducing ~0.75 adducts/10 kb, for the measurement of gene-specific repair.

To determine the kinetics of repair of NA-AAF induced lesions in transcriptionally active and inactive genes, we measured the frequencies of DNA lesions in restriction fragments of the active adenosine deaminase (ADA) gene and the inactive 754 gene at different time intervals after 5 μM NA-AAF treatment. In the ADA gene, repair was measured in the 3′ located *Eco*RI fragment, in which both strands contain transcription units (19). Figure 4A shows that in normal cells the removal of dG-C8-AF adducts from the *Eco*RI fragment of the ADA gene clearly proceeds in a biphasic manner. During the first 8 h after NA-AAF treatment, ~40% of the dG-C8-AF adducts is removed, followed by a slower rate of repair in the subsequent 16 h. It is clear that the repair of dG-C8-AF adducts in the transcribed ADA gene of normal human fibroblasts VH25D is significantly faster than in the inactive 754 gene. In the XP21RO fibroblasts the rate of repair is slower: ~30% of the dG-C8-AF adducts in the *Eco*RI fragment of the ADA gene is removed in 24 h, whereas the removal of dG-C8-AF adducts from the inactive 754 gene is virtually absent.

Table 1. The effect of α-amanitine treatment (1 μg/ml) on the incorporation of [^{3}H]thymidine in ^{14}C-prelabelled cells from primary normal human fibroblasts (VH25D) and xeroderma pigmentosum fibroblasts (XP21RO) after treatment with 90 μM NA-AAF (αA/NA-AAF)

Cell line	Ratio ^{3}H/^{14}C Untreated	αA	αA/NA-AAF	NA-AAF	% Inhibition
VH25D	0.45	1.50	12.40	6.70	0
XP21RO	0.23	0.38	0.56	0.76	66

Data are average of two experiments.

An alternative method employed to measure gene-specific repair is based on the incorporation of BUdR in repair patches during the repair of DNA lesions and extraction of DNA fragments containing BUdR-labelled repair patches using an antibody specific for BUdR containing DNA. The percent of a specific restriction fragment bound by the antibody is then analyzed on slot blots which are hybridized by gene-specific probes. The increase of bound restriction fragment is a measure for the removal of damage from that fragment by DNA repair. The kinetics of repair as determined by the analysis of BUdR-labelled DNA closely resembled the kinetics found by employing UvrABC excinuclease (Fig. 4B).

To detect possible differences in kinetics of removal of dG-C8-AF adducts from the transcribed and the non-transcribed strand of the ADA gene, repair measurements were performed in the ADA *Bcl*I fragment employing strand-specific probes. In contrast with the *Eco*RI fragment of the ADA gene, the *Bcl*I fragment contains a transcription unit only on the ADA template strand (19). Figure 5 shows an example of an autoradiogram obtained after hybridization of filters containing *Bcl*I-restricted DNA after hybridization with strand-specific probes. In VH25D cells no difference in the rate of repair could be detected between the two strands in normal cells (Fig. 6). Furthermore, the kinetics of removal of dG-C8-AF observed in the *Bcl*I fragment was the same as in the *Eco*RI fragment of the ADA gene measured with the double strand probes (Fig. 4). As mentioned above, the latter fragment covers a part of the ADA gene in which both strands are transcribed. However, the XP-C cells clearly exhibited strand specificity for repair of dG-C8-AF adducts in the ADA *Bcl*I fragment (Fig. 6), revealing the existence of transcription coupled repair of dG-C8-AF adducts.

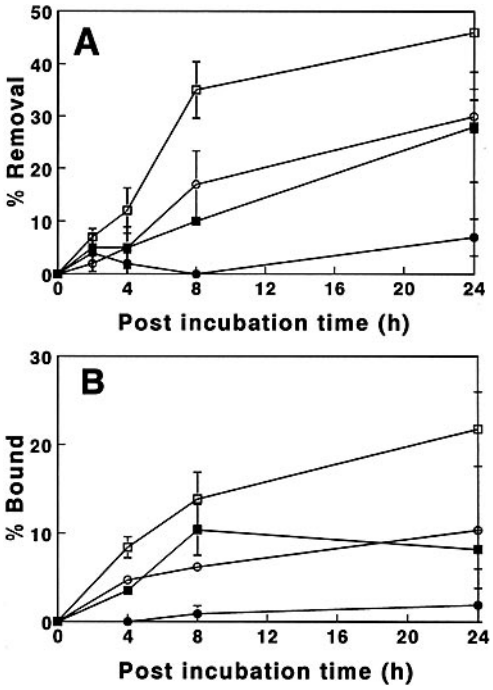

Figure 4. (A) Removal of dG-C8-AF from the 18.5 kb *Eco*RI fragment of the ADA gene and the 14 kb *Eco*RI fragment of the 754 gene in primary normal human fibroblasts VH25D [ADA (□); 754 (■)] and in xeroderma pigmentosum fibroblasts XP21RO [ADA (○); 754 (●)]. Lesion frequencies are measured by the UvrABC assay. (**B**) Percent of DNA bound by antibodies recognizing BUdR in DNA. The DNA was subsequently analysed on slotblots using probes recognizing the 18.5 kb *Eco*RI fragment of the ADA gene and the 14 kb *Eco*RI fragment of the 754 gene in primary normal human fibroblasts VH25D [ADA (□); 754 (■)] and in repair-deficient fibroblasts XP21RO [ADA (○); 754 (●)]. The error bars represent SEM.

DISCUSSION

Primary XP-C fibroblasts appear to be somewhat more sensitive to the cytotoxic effects of NA-AAF than normal cells but less sensitive when compared with XP-A fibroblasts or CS-A and CS-B fibroblasts (12). This differential sensitivity of the various human fibroblast strains cannot be attributed to variations in frequencies or types of DNA lesions induced by NA-AAF exposure. The results of the current study and a previous investigation by Amacher and Lieberman (20) demonstrate that in primary normal human fibroblasts, as well as in XP and CS cells, NA-AAF induces dG-C8-AF as the major lesion.

The relatively mild cytotoxic effect of NA-AAF in XP-C cells is in contrast with the low level of genome overall repair of NA-AAF induced DNA lesions in these cells when compared with normal human cells. This reduced repair of NA-AAF adducts in XP-C cells resembles the poor genome overall repair of UV-induced photolesions in these cells (1,5). Analogous to repair of UV-damage, the residual repair of NA-AAF induced DNA damage in XP-C cells is confined to transcriptionally active genes, because treatment of XP-C cells with α-amanitin, an inhibitor of RNA polymerase II, severely inhibited NA-AAF induced repair replication, whereas the inhibitor had no inhibitory effect on repair replication in normal cells. A similar inhibitory effect of α-amanitine on repair synthesis in UV-irradiated confluent XP-C cells has been reported by Carreau and Hunting (17). Thus our data indicate that removal of NA-AAF induced DNA lesions in XP-C cells depends on transcription and that dG-C8-AF lesions are actually targets for transcription-coupled repair.

It has been proposed that transcription-coupled repair is driven by stalled transcripts which act as signals to target repair enzymes to the sites of the DNA lesions (21,22). Indeed, exposure to NA-AAF resulted in a dose-dependent inhibition of RNA synthesis in XP-C and normal cells, however the inhibition of RNA synthesis is rather inefficient: ~50% inhibition is achieved

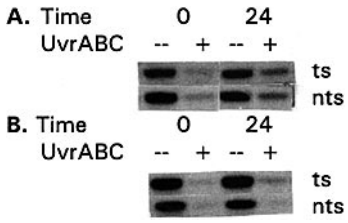

Figure 5. Autoradiograms showing removal of dG-C8-AF from the 19.9 kb *Bcl*I ADA fragment in primary normal human cells, VH25D (**A**) and xeroderma pigmentosum cells, XP21RO (**B**). The ADA fragments are analysed with strand-specific probes recognizing the transcribed (ts) or the non-transcribed strand (nts).

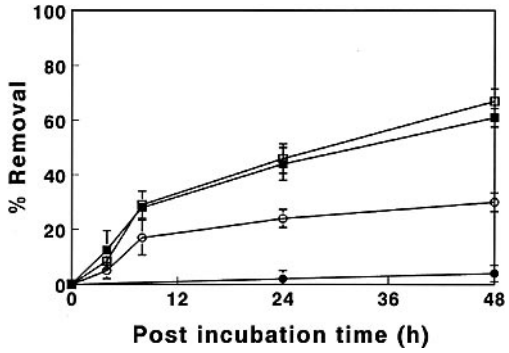

Figure 6. Strand-specific analysis of the removal of dG-C8-AF from the 19.9 kb *Bcl*I fragment of the ADA gene in primary normal human fibroblasts VH25D [transcribed strand (□); non-transcribed strand (■)] and xeroderma pigmentosum fibroblasts XP21RO [transcribed strand (○); non-transcribed strand (●)]. The error bars represent SEM.

at an adduct frequency of two adducts/10 kb suggesting that for efficient inhibition of RNA synthesis multiple DNA adducts per transcription unit are required. Thus we conclude that dG-C8-AF adducts form only weak blocks for RNA polymerase II driven transcription. This conclusion has also been drawn from *in vitro* transcription assays (11,13). Both normal human and XP-C cells are able to recover NA-AAF inhibited RNA synthesis within 8 h, but XP-A cells lack this recovery. In this respect, NA-AAF exposure mimics the response of XP fibroblasts to UV-irradiation, as XP-C cells are capable of recovering UV-inhibited RNA synthesis in contrast with XP-A cells (23). It is tempting to say that the recovery of XP-C cells from NA-AAF-repressed RNA synthesis is due to their capacity to perform transcription-coupled repair, and that this accounts for the relative mild sensitivity of XP-C cells to NA-AAF.

To show directly the role of transcription-coupled repair in removal of dG-C8-AF adducts, we investigated the induction and removal of NA-AAF-induced adducts in transcribed sequences. Tang *et al*. (24) demonstrated that purified UvrABC excinuclease can incise dG-C8-AF adducts in linear plasmid DNA fragment with high efficiency. Our results indicate that dG-C8-AF adducts in large genomic fragments are also efficiently recognized by UvrABC excinuclease. HPLC analysis of ^{3}H-labelled dG-C8-AF in total DNA revealed adduct frequencies very similar to those quantified in restriction fragments of active and inactive genes by the UvrABC assay. Repair of dG-C8-AF in the *Eco*RI fragment of the active ADA gene in normal cells appeared to be rather slow (50% removed after 24 h), but clearly exceeded the repair of this adduct in the inactive 754 gene (30% removed after 24 h). In XP-C cells repair of dG-C8-AF occurred at a significantly slower rate than in normal human cells exhibiting only 30% removal from the active ADA gene after 24 h. The repair of dG-C8-AF in the inactive 754 gene in the XP-C cells was almost absent demonstrating selective repair of this lesion in active genes in these cells. The differences revealed by the UvrABC assay between active and inactive genes on one hand and between normal and XP-C cells on the other hand were confirmed by gene-specific analysis of BUdR-labelled repair patches in both human cell strains.

In normal cells no strand-specific repair of dG-C8-AF lesions was observed in the *Bcl*I fragment of the ADA gene, the transcribed strand being repaired as rapidly as the non-transcribed strand. However, in XP-C cells the transcribed strand of the ADA gene was repaired more rapidly and efficiently than the non-transcribed strand. In fact, repair of dG-C8-AF in the non-transcribed strand of the ADA gene was barely detectable. The rate of dG-C8-AF repair in the transcribed strand of the ADA gene in XP-C was about half of the rate observed in normal cells, and closely resembled the removal of dG-C8-AF from the 3′ located *Eco*RI fragment of the ADA gene, measured in both strands of the gene. A similar observation was made for repair of UV-induced CPD and 6-4 PP in the *Eco*RI and the *Bcl*I ADA restriction fragments in XP-C cells (3,6), which has been explained by the transcriptional organisation of the ADA gene. Both strands of the *Eco*RI fragment contain transcription units and therefore it is expected that the repair of dG-C8-AF in the *Eco*RI fragment measured by a DNA probe recognizing both strands is similar to the repair of the transcribed strand determined in the *Bcl*I fragment of the ADA gene. Thus, taken together, all data suggest that dG-C8-AF lesions are target for the transcription-coupled repair pathway, but that the efficiency of repair of dG-C8-AF by transcription-coupled repair is rather low compared with UV-induced CPD (25) or benzo[*a*]pyrene adducts (26). The same holds for the efficiency of dG-C8-AF removal by the global genome repair.

In UV-irradiated XP-C cells, cytotoxic effects are much more pronounced in exponentially growing cells than in confluent cells (27). The mild sensitivity of non-dividing XP-C cells to UV-light has been related to the capability of XP-C cells of performing transcription-coupled repair and to restore RNA synthesis after damage induction. In growing cells unrepaired lesions residing in the non-transcribed part of the genome will interfere with replication leading to severe lethal effects of UV-light in dividing XP-C cells. Our results suggest that in the case of NA-AAF exposure, transcription-coupled repair plays an important role in counteracting the lethal effects, but importantly not only in confluent cells but also in exponentially growing cells. Thus, the relative contribution of both NER pathways to counteract the lethal effects of genotoxic agents strongly depends on the type of lesions induced. In the case of UV-induced lesions, transcription-coupled repair and global genome repair are both required to alleviate their cytotoxic effects in dividing cells: efficient transcription-coupled repair in concert with the capability of recovering UV-inhibited transcription or efficient global genome repair alone (XP-C and CS respectively) are insufficient to confer resistance to lethal effects of UV-light. This situation is different for the dG-C8-AF lesion. The ability to recover NA-AAF-inhibited RNA synthesis is the major factor that determines cellular

survival after NA-AAF treatment as deduced from the mild sensitivity of XP-C cells. Most likely, global genome repair has a minor effect on cytotoxicity due to the weak replication blocking potency of dG-C8-AF lesions. However, the question remains whether the restoration of inhibited RNA synthesis or the capability to perform TCR (which depends on RNA synthesis) explains the mild sensitivity of the XP-C cells for NA-AAF.

In normal human cells removal of dG-C8-AF by the global genome repair is rather inefficient and its kinetics are clearly biphasic. The latter may be related to different DNA-lesion conformations. Conformational heterogeneity of AF-adducts has been basically related to two different conformations: i.e. an insertion–denaturation model has been proposed in which the AF-moiety (in *syn* conformation) stacks between adjacent bases causing local distortions of the DNA helix, and an outside binding model with the AF moiety (in *anti* conformation) outside the helix causing little distortion (28–30). It is conceivable that lesions in *syn* conformation form a better substrate for nucleotide excision repair enzymes than lesions in the *anti* conformation. Lesions in *syn* conformation may also form efficient blockage to transcription and be processed by transcription-coupled repair. Thus the same subclass of lesion may be an efficient substrate for both global genome and transcription-coupled repair. This is consistent with the notion that the biphasic kinetics of dG-C8-AF removal by transcription-coupled repair in XP-C resemble the biphasic removal of dG-C8-AF from the ADA gene in normal human cells. In principal, the repair of dG-C8-AF in the ADA gene in normal human fibroblasts could be mediated by both the global genome and the transcription-coupled repair pathways. Yet it is clear that transcription-coupled repair does not contribute significantly to removal of dG-C8-AF lesions in the ADA gene in normal cells. This is remarkable since processing of dG-C8-AF by transcription-coupled repair (i.e. in XP-C cells) amounts to 50% of the repair of dG-C8-AF in active genes in normal cells. This suggests that repair of dG-C8-AF in active genes by transcription-coupled repair and global repair is not additive, but that repair in normal cells is dominated by the global genome repair pathway. A possible explanation is that a subset of lesions which are capable of blocking transcription are also the lesions which are very efficiently recognized by the global genome repair pathway. We assume that the repair of this subclass of lesions by the global genome repair pathway will be even more efficient than by the transcription-coupled repair pathway; a situation similar to repair of UV-induced 6-4 PP (6). As a consequence, normal cells will remove these transcription blocking lesions by the global genome repair pathway before transcription-coupled repair can act on them. It is a challenge to verify the above mentioned hypothesis and to identify the structural parameters which influence recognition of dG-C8-AF adducts by the NER pathways. One way to proceed is to apply techniques that allow the study of repair at the nucleotide level such as ligation-mediated PCR (31).

ACKNOWLEDGEMENTS

The UvrABC proteins used in this study were obtained from Dr P. van de Putte, Department of Molecular Genetics, Leiden Institute of Chemical Research, Leiden University, The Netherlands. The UDS measurements were performed by Dr K. Jaspers, MGC–Department of Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands. We are grateful to Drs S. Bol and J. H. N. Meerman for help in performing the HPLC analysis. This study was supported by a grant of the Medical Genetics Centre South-West Netherlands and the European Commission (contract EV5V-CT94-0397).

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