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The Sensitivity of Cockayne's Syndrome Cells to DNA-Damaging Agents Is Not due to Defective Transcription-Coupled Repair of Active Genes

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Two of the hallmarks of Cockayne's syndrome (CS) are the hypersensitivity of cells to UV light and the lack of recovery of the ability to synthesize RNA following exposure of cells to UV light, in spite of the normal repair capacity at the overall genome level. The prolonged repressed RNA synthesis has been attributed to a defect in transcription-coupled repair, resulting in slow removal of DNA lesions from the transcribed strand of active genes. This model predicts that the sensitivity of CS cells to another DNA-damaging agent, i.e., the UV-mimetic agent *N*-acetoxy-2-acetylaminofluorene (NA-AAF), should also be associated with a lack of resumption of RNA synthesis and defective transcription-coupled repair of NA-AAF-induced DNA adducts. We tested this by measuring the rate of excision of DNA adducts in the adenosine deaminase gene of primary normal human fibroblasts and two CS (complementation group A and B) fibroblast strains. High-performance liquid chromatography analysis of DNA adducts revealed that *N*-(deoxyguanosin-8-yl)-2-aminofluorene (dG-C8-AF) was the main adduct induced by NA-AAF in both normal and CS cells. No differences were found between normal and CS cells with respect to induction of this lesion either at the level of the genome overall or at the gene level. Moreover, repair of dG-C8-AF in the active adenosine deaminase gene occurred at similar rates and without strand specificity in normal and CS cells, indicating that transcription-coupled repair does not contribute significantly to repair of dG-C8-AF in active genes. Yet CS cells are threefold more sensitive to NA-AAF than are normal cells and are unable to recover the ability to synthesize RNA. Our data rule out defective transcription-coupled repair as the cause of the increased sensitivity of CS cells to DNA-damaging agents and suggest that the cellular sensitivity and the prolonged repressed RNA synthesis are primarily due to a transcription defect. We hypothesize that upon treatment of cells with either UV or NA-AAF, the basal transcription factor TFIIH becomes involved in nucleotide excision repair and that the CS gene products are involved in the conversion of TFIIH back to the transcription function. In this view, the CS proteins act as repair-transcription uncoupling factors. If the uncoupling process is defective, RNA synthesis will stay repressed, causing cellular sensitivity. Since transcription is essential for transcription-coupled repair, the CS defect will affect those lesions whose repair is predominantly transcription coupled, i.e., UV-induced cyclobutane pyrimidine dimers.

The nucleotide excision repair (NER) process, which recognizes a wide variety of DNA lesions, can be subdivided into two different pathways, i.e., the transcription-coupled repair pathway and the global genome repair pathway. Removal of DNA lesions by the transcription-coupled repair pathway is confined to the transcribed strand of transcriptionally active genes and is dependent on transcription. The global genome repair pathway acts on DNA lesions across the entire genome, including transcriptionally active genes. From the repair phenotypes of repair-proficient and -deficient mammalian cell lines, it is obvious that a large number of factors are common to both pathways but that in addition, genetic factors which are specific for one of the two exist. This is visualized most strikingly in cell lines belonging to xeroderma pigmentosum (XP) complementation group C, which were found to be deficient in the global genome repair pathway but fully capable of performing transcription-coupled repair (21, 43). On the contrary, cells belonging to Cockayne's syndrome (CS) complementation group A or B exhibit a deficiency in transcription-coupled repair but possess a normal global genome repair pathways (40, 45). Insights into the mechanisms of NER pathways are gained from the cloning of repair genes and characterization of encoded proteins. An alternative approach to unraveling the mechanisms of the NER pathways is to study the repair of structurally different lesions in repair-proficient and repair-deficient cell lines, the latter only capable of removing DNA lesions by one of the two subpathways. The final goal is to establish a relationship between the structure of a DNA lesion and the efficiency of its recognition and removal of either pathway.

A major part of the research so far has focused on the repair of the two major UV-induced DNA photolesions, i.e., cyclobutane pyrimidine dimers (CPD) and 6-4 photoproducts (6-4 PP). In normal human cells exposed to 10 J of UV light per m^2, CPD are repaired faster in transcriptionally active genes than in inactive genes or the genome overall (24, 29, 44). This

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preferential repair of CPD in active genes is, at least partly, caused by the fact that the transcription-coupled repair pathway leads to an accelerated removal of CPD from the transcribed strand of active genes in comparison with the removal rate for the nontranscribed strand or the genome overall (30, 46). The situation for 6-4 PP is different, because in normal human cells strand-specific repair of 6-4 PP in active genes is masked because of a very efficient removal of 6-4 PP by the global genome repair pathway (41).

To get more insights into the mechanisms of repair of bulky DNA lesions by the NER subpathways, we started to investigate the kinetics of repair of bulky adducts, structurally different from UV-induced photolesions. As the DNA-damaging agent we chose *N*-acetoxy-2-acetylaminofluorene (NA-AAF), which is generally considered a UV-mimetic agent: cell lines which are sensitive to UV also show sensitivity to NA-AAF (14, 26), and lesions induced by NA-AAF are processed by NER (3, 15, 32). Moreover, Brown et al. (8) found that the repair of the NA-AAF-induced lesions was inhibited when UV photolesions were subsequently induced. However, some contradictions to this point have been reported as well. Ahmed and Setlow (1, 2) found additive repair of both lesions under conditions where repair was saturated, indicating two different repair pathways. Interestingly, Tang et al. (36) studied the repair of NA-AAF-induced DNA adducts in CHO cells in transcribed and nonexpressed sequences and found similar rates and extends of repair in the two sequences. This is in contrast to the selective removal of CPD from the transcribed strand of active genes in hamster cells. NA-AAF reacts preferentially with guanine and induces two major types of lesions: the deacetylated *N*-(deoxyguanosine-8-yl)-2-aminofluorene (dG-C8-AF) and the acetylated *N*-(deoxyguanosin-8-yl)-2-acetylaminofluorene (dG-C8-AAF). The ratio of induction of the two lesions is dependent on tissue, species, or even strain, because of the different enzymatic activities involved in deacetylation. The two types of lesions have different effects on the DNA conformation and therefore may be processed differently by the two NER subpathways. dG-C8-AAF lesions are in syn conformation, causing a local denaturation of about 5 bp, referred to as the insertion-denaturation model (16, 17). In contrast, dG-C8-AF lesions are preferentially in the anti conformation, causing no or only a slight helix distortion or denaturation (12, 25). In human cells, NA-AAF treatment leads to the induction of dG-C8-AF but not dG-C8-AAF (37).

The role of NER in repair of dG-C8-AF adducts was convincingly demonstrated by the enhanced sensitivity and lack of repair of lesions in NER-deficient XP group A fibroblasts (3). Interestingly, CS cells, defective in transcription-coupled repair, were also sensitive to the cytotoxic effects of NA-AAF (48), suggesting that NA-AAF-induced lesions in active genes are processed by the transcription-coupled repair pathway. To test this hypothesis and to find a clue to the NA-AAF sensitivity of CS cells, we determined the repair of NA-AAF-induced DNA adducts in primary normal human fibroblasts and two CS cell lines, CS1AN (complementation group B) and CS3BE (complementation group A). Both CS cell lines lack the ability to perform transcription-coupled repair of UV-induced CPD (41, 45) and X-ray-induced DNA damage (23). In this paper, we show that the major lesion induced by NA-AAF in human fibroblasts is dG-C8-AF and that CS cells remove this lesion from active genes with the same kinetics and to the same extent as normal cells do. In addition, in both normal and CS cells dG-C8-AF lesions are repaired with similar kinetics and efficiencies in the transcribed and nontranscribed strands, suggesting that transcription-coupled repair does not significantly contribute to the removal of dG-C8-AF from the transcribed strand of active genes. In spite of this, CS cells are sensitive to the cell-killing effects of NA-AAF and are not capable of resuming RNA synthesis after inhibition by NA-AAF, whereas normal cells do recover the ability to synthesize RNA. Our results suggest that the sensitivity of CS cells to DNA-damaging agents is primarily due to a transcription defect. We hypothesize that upon treatment of cells with either NA-AAF or UV, the basal transcription factor TFIIH becomes involved in NER and that the CS gene products are subsequently involved in the conversion of TFIIH from a repair function back to a transcription function. As a consequence, the defect in the ability to resume RNA synthesis in CS cells would not be due to a defect in transcription-coupled repair but is caused by a defect in transcription initiation following exposure to DNA-damaging agents.

MATERIALS AND METHODS

Cell lines and culture conditions. Primary fibroblasts from a healthy individual, cell line VH25D, and primary CS fibroblasts, CS1AN (complementation group B) and CS3BE (complementation group A), were cultured in Ham's F10 medium (without hypoxanthine and thymidine) supplemented with 15% fetal calf serum and antibiotics at 37°C and 2.5% CO_2. In experiments aimed to determine the initial frequency and the repair 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) and 1.6 μM thymidine. For repair replication and for RNA synthesis experiments, exponentially growing cells were prelabelled with $^{32}P_i$ (0.3 μCi/ml) and [^{14}C]uridine (0.03 μCi/ml, 60 mCi/mmol), respectively. After the labelling period, the medium was replaced by label-free medium and cells were allowed to grow to confluence.

Survival studies. Cell survival following treatment with NA-AAF was determined by measuring the colony-forming ability of the treated cells relative to that of the untreated control. A total of 500 to 1,000 cells were seeded in 94-mm-diameter petri dishes, allowed to attach for 16 h, and incubated with 0, 5, 10, 15, and 20 μM NA-AAF for 30 min at 37°C in complete medium. After incubation, the cells were washed twice with phosphate-buffered saline (PBS) and fresh medium was added to the cells. After 7 days, the medium was replaced by fresh medium, and 10 to 14 days after plating, the cells were rinsed twice with 0.9% NaCl and colonies were stained with methylene blue.

HPLC analysis. Confluent cells were incubated with 5 μM [^{3}H]NA-AAF (2 mCi/ml, 649 mCi/mmol) for 30 min at 37°C and 5% CO_2 and immediately lysed. The DNA was purified and RNase A treated, and the specific activity (disintegrations per minute per microgram) of single-stranded DNA was determined by scintillation counting in Plasmasol. For high-performance liquid chromatography (HPLC) analysis, 7,000 dpm of DNA (20 to 30 μg) was freeze-dried overnight and subsequently hydrolyzed in 1 ml of trifluoroacetic acid (Uvasol quality; Merck). The hydrolyzed DNA was resolved in 150 μl of eluent buffer, containing 70% 50 mM ammonium formate, pH 5.6, and 30% methanol, and a 15-μl aliquot was used to determine the specific activity of the hydrolysate. The remainder of the DNA was separated on a Nucleosil HPLC column by using a gradient of 30% methanol–70% ammonium formate to 60% methanol–40% ammonium formate. As a control, dG-C8-AAF and dG-C8-AF markers were also run on the Nucleosil HPLC column. From the distribution pattern of radioactivity, the relative induction of dG-C8-AF and dG-C8-AAF and other minor adducts was determined.

Measurement of RNA synthesis. [^{14}C]uridine-prelabelled confluent cells were incubated with 5 or 15 μM NA-AAF for 30 min at 37°C. After the cells had been washed twice with PBS, fresh medium was added to the cells and the cells were pulsed-labelled with [^{3}H]uridine (10 μCi/ml) for 30 min at 37°C at different time points after NA-AAF treatment. The cells were washed three times with PBS and lysed in 150 mM NaCl–10 mM Tris HCl (pH 8.0)–1 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–100 μg of proteinase K per ml at 37°C overnight. RNA was trichloroacetic acid precipitated (final concentration, 10%), kept for 2 h at 4°C, and collected on GF/C filters by filtration. The ^{14}C and ^{3}H contents of the filters were measured by liquid scintillation counting.

DNA repair replication and unscheduled DNA synthesis (UDS) measurement. DNA repair replication was measured by the radioisotope and density labelling technique described by Van Zeeland et al. (42). Confluent ^{32}P-prelabelled cells were treated with 30, 45, and 90 μM NA-AAF for 30 min at 37°C and 5% CO_2. After the cells had been washed twice with PBS, fresh medium containing 10 μM 5-bromodeoxyuridine (BUdR)–1 μM fluorodeoxyuridine (FUdR) and [^{3}H]thymidine (5 μCi/ml, 82 Ci/mmol) was added and the cells were kept at 37°C and 2.5% CO_2 for 24 h. The cells were lysed in 150 mM NaCl–10 mM Tris HCl (pH 8.0)–1 mM EDTA–0.5% Sarkosyl–100 μg of proteinase K per ml at 37°C overnight. The DNA was sheared and subjected to neutral cesium chloride density gradient centrifugation to separate parental DNA from replicated DNA. The gradients were fractionated, and the four topmost fractions of the parental peak were pooled. Subsequently, alkaline cesium chloride density gradient centrifu-

gation was performed to deplete parental DNA from remnants of replicated DNA due to incomplete separation in the neutral gradient. The gradients were fractionated and trichloroacetic acid precipitated, and the ^{32}P and ^{3}H contents of the fractions were measured. The specific activity of the ^{32}P-labelled DNA (^{32}P counts per minute per microgram) was determined from cell cultures which were lysed at time zero. Repair replication was expressed as ^{3}H counts per minute per micrograms of DNA.

UDS was performed as described by Vermeulen et al. (47). Briefly, fibroblasts grown on coverslips were washed with PBS and either treated with 16 μM NA-AAF for 30 min in total medium or exposed to UV (16 J/m^2). The cells were incubated with [^{3}H]thymidine for 30 min either during the treatment with NA-AAF or directly following treatment. Subsequently the cells were washed three times with PBS, fixed, and processed for autoradiography, and the number of grains above the nuclei was determined.

DNA probes. Double-stranded DNA probes were radioactively labelled with [^{32}P]dATP by random primer extension (13). The *Pst*I fragments BA (exons 1 to 4 and part of exon 5) and BE (exon 12) of the human adenosine deaminase (ADA) cDNA clone pLL (4) were amplified by PCR. Strand-specific single-stranded probes were radioactively labelled with [^{32}P]dATP by a linear PCR, using a single primer recognizing one strand specifically (33). Strand specificity of the probes was checked on dot blots by hybridization to single-stranded M13 vectors containing the probes in either of the two orientations. A plasmid containing a 2.0-kb *Hin*dIII fragment of the 754 gene cloned into pAT153 was obtained from B. Bakker (Leiden University, Leiden, The Netherlands) (20).

Determination of NA-AAF-induced DNA adducts in specific genomic 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, 15, and 20 μM NA-AAF, in complete medium, for 30 min at 37°C. After the cells had been washed 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. After washing of the cells twice with PBS, they were either lysed immediately or incubated for 2, 4, 8, 24, 32, or 48 h in complete medium supplemented with 10 μM BUdR–1 μM FUdR.

DNA was isolated as described previously (44). Briefly, cells were lysed in 150 mM NaCl–10 mM Tris HCl (pH 8.0)–1 mM EDTA–0.5% SDS–100 μg of proteinase K per ml at 37°C overnight. The DNA was purified by phenol and chloroform extractions, precipitated with ethanol, resuspended in 10 mM Tris HCl (pH 8.0)–1 mM EDTA (TE), and digested overnight with either *Eco*RI at 37°C or *Bcl*I at 50 °C. To separate the parental DNA from the replicated DNA, neutral cesium chloride gradient centrifugation was performed. Fractions containing the parental DNA were pooled, dialyzed against 1× and 0.05× TE, and dried by vacuum centrifugation. Finally, the samples were resolved in water and the frequency of NA-AAF-induced DNA adducts was determined by in vitro incision at the sites of adducts employing the UvrABC excinuclease complex of *Escherichia coli* as described previously (41). Equal amounts of DNA (5 μg) were either treated with the UvrABC excinuclease complex or mock-treated. The incubation mixture, containing 50 mM Tris (pH 8.0), 10 mM $MgCl_2$, 75 mM KCl, and 2 mM ATP, as well as the UvrA and UvrB subunits, was preincubated for 5 min at 37°C, after which the UvrC protein was added. The final concentration of each of the three Uvr proteins was 15 pmol per reaction. The DNA samples were incubated for 1 h at 37°C. Because the UvrABC complex exhibited a low, but variable, nicking activity on DNA from cells not exposed to NA-AAF, a DNA sample isolated from untreated cells was also incubated with UvrABC. After the incubation, the reaction was stopped by adding 10 mM EDTA and 0.5% SDS. Prior to alkaline agarose gel electrophoresis, the DNA samples were phenol and chloroform extracted, ethanol precipitated, and resuspended in 15 μl of TE. Electrophoresis of the DNA samples was performed in 0.8% alkaline agarose gels and was followed by Southern blotting and hybridization. Autoradiograms were made, and the intensities of full-size fragments were quantified by using a videodensitometer (model 60; Bio-Rad). The number of UvrABC-sensitive sites per fragment was calculated from the relative band intensities of full-size restriction fragments in the lanes containing the treated and mock-treated samples, assuming a Poisson distribution of lesions (5).

RESULTS

Cytotoxic effects of NA-AAF treatment. Survival of the cells after exposure to NA-AAF was determined by measurement of their colony-forming ability. Figure 1 shows that the two CS cell lines, CS3BE and CS1AN, were more sensitive to NA-AAF than was the normal human cell line VH25D. This enhanced sensitivity of CS cells is in accordance with the two- to threefold-enhanced sensitivity of CS cell lines to cytotoxic effects of NA-AAF previously described by Wade and Chu (48).

Formation of DNA adducts. The enhanced sensitivity of CS cells to NA-AAF may be related to differences from normal fibroblasts in frequencies and patterns of induction of various types of DNA adducts. Since NA-AAF is capable of inducing

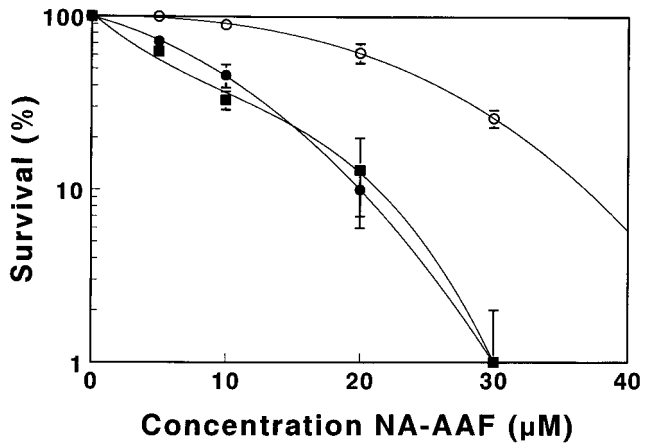

FIG. 1. Cell killing effects of NA-AAF on VH25D primary normal human fibroblasts (○), CS1AN CS cells (complementation group B) (●), and CS3BE CS cells (complementation group A) (■). The error bars represent standard errors of the mean.

two major types of lesions, i.e., dG-C8-AF and dG-C8-AAF, in addition to some minor lesions, we employed HPLC analysis of DNA treated with [^{3}H]NA-AAF to determine the induction of DNA adducts in normal and CS cells (39). Figure 2 shows that the distribution patterns of radioactivity in the chromatograms were very similar for CS1AN and VH25D and that the deacetylated dG-C8-AF adduct was the major type of lesion induced. In fact, there was no significant amount of radioactivity present at the position of dG-C8-AAF, indicating that the vast majority of adducts consist of dG-C8-AF. Similar observations were

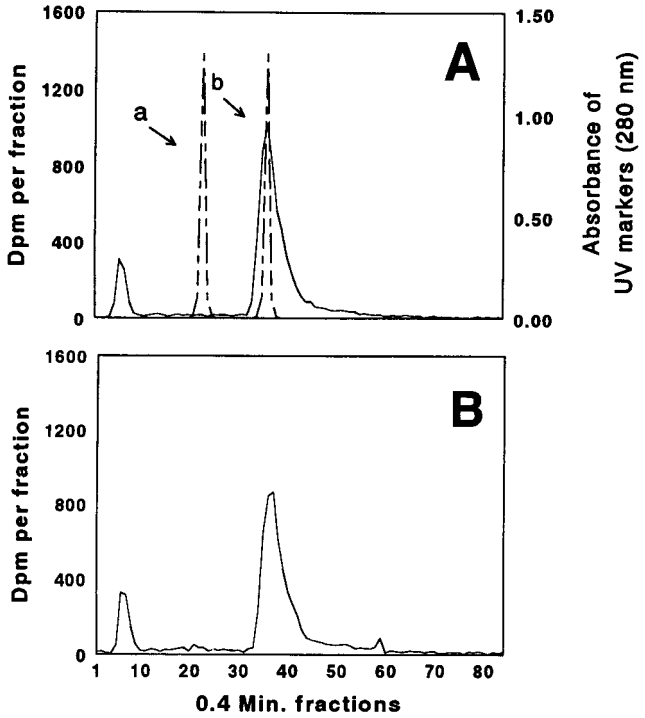

FIG. 2. HPLC profile of trifluoroacetic acid-hydrolyzed DNA obtained from cells treated with 5 μM ^{3}H-labelled NA-AAF. The dotted lines of peaks a and b represent, respectively, the positions of the standards dG-C8-AAF and dG-C8-AF. (A) Normal cell line VH25D; (B) CS cell line CS1AN.

made for CS3BE cells (data not shown). From a single experiment, we determined frequencies of 0.13, 0.10, and 0.15 dG-C8-AF-induced lesions per μM per 10 kb in normal, CS3BE, and CS1AN cells, respectively. These findings indicate that there are no major differences between normal and CS cells regarding the relative frequency and the types of NA-AAF-induced DNA adducts.

Effects of NA-AAF treatment on RNA synthesis. One of hallmarks of CS is the lack of resumption of RNA synthesis after UV inhibition, a phenomenon which has been attributed to impaired transcription-coupled repair in CS (40). We addressed the question of the extent to which RNA synthesis was inhibited by NA-AAF treatment. Figure 3A shows that RNA synthesis inhibition is dose dependent in both normal and CS1AN cells and that the levels of inhibition are similar. However, CS and normal cells differ in their capabilities to resume NA-AAF-inhibited RNA synthesis: Fig. 3B shows that normal cells are able to resume RNA synthesis, after treatment with 15 μM NA-AAF, at up to 80% of the level in untreated cells by 8 h posttreatment, whereas the two CS cell lines lack the ability to resume RNA synthesis. Also, treatment with 5 μM NA-AAF (the concentration used to study gene-specific repair) leads to a lack of recovery of the ability to synthesize RNA after inhibition in CS cells whereas normal cells efficiently resume the inhibited RNA synthesis (Fig. 3C).

Repair replication and UDS measurement. The repair of NA-AAF-induced DNA adducts in the genome overall in normal human and CS cell lines was assessed by measurement of repair replication. ^{32}P-prelabelled cells were treated with different doses of NA-AAF (30, 45, and 90 μM) and incubated for 24 h in the presence of [^{3}H]thymidine, BUdR, and FUdR. The specific activity of the ^{32}P-prelabelled DNA was determined for each cell line, to allow comparison of the levels of repair replication in the three cell lines. The results (Fig. 4) indicate that the extent of repair synthesis following NA-AAF treatment does not substantially differ between the normal human and the CS cell lines.

To assess possible differences in repair activity between normal and CS cells during treatment or during the initial period following treatment, cells were treated with NA-AAF, concomitantly labelled with [^{3}H]thymidine or labelled for 30 min immediately after the NA-AAF treatment, and processed for measurement of UDS. Table 1 shows that there were no clear differences in UDS among the tested cell lines, although the CS3BE cells exhibited somewhat higher UDS values. The level of UV-induced UDS was higher than that of NA-AAF-induced UDS, when the comparison was based on lesion frequency; about three times more lesions are induced by 16 μM NA-AAF treatment than by 16-J/m^2 UV irradiation.

Induction and repair of NA-AAF adducts in active and inactive genes. The induction of dG-C8-AF adducts in restriction fragments of genes was measured as a function of the dose, by using UvrABC excinuclease to introduce single-stranded-DNA breaks in the DNA near the sites of lesions. The DNA samples were then subjected to alkaline agarose gel electrophoresis, Southern blotted, and hybridized with radiolabelled probes recognizing specific restriction fragments. In this assay, the presence of DNA lesions is seen as the reduction of the intensities of full-size restriction fragments in lanes containing UvrABC-digested DNA in comparison with results for undigested DNA. Intensities of bands in autoradiograms were quantified, and lesion frequencies were calculated from the ratio of the band intensities of full-size fragments in lanes containing UvrABC-treated DNA to those of full-size fragments in lanes containing nontreated DNA by employing the Poisson distribution (5).

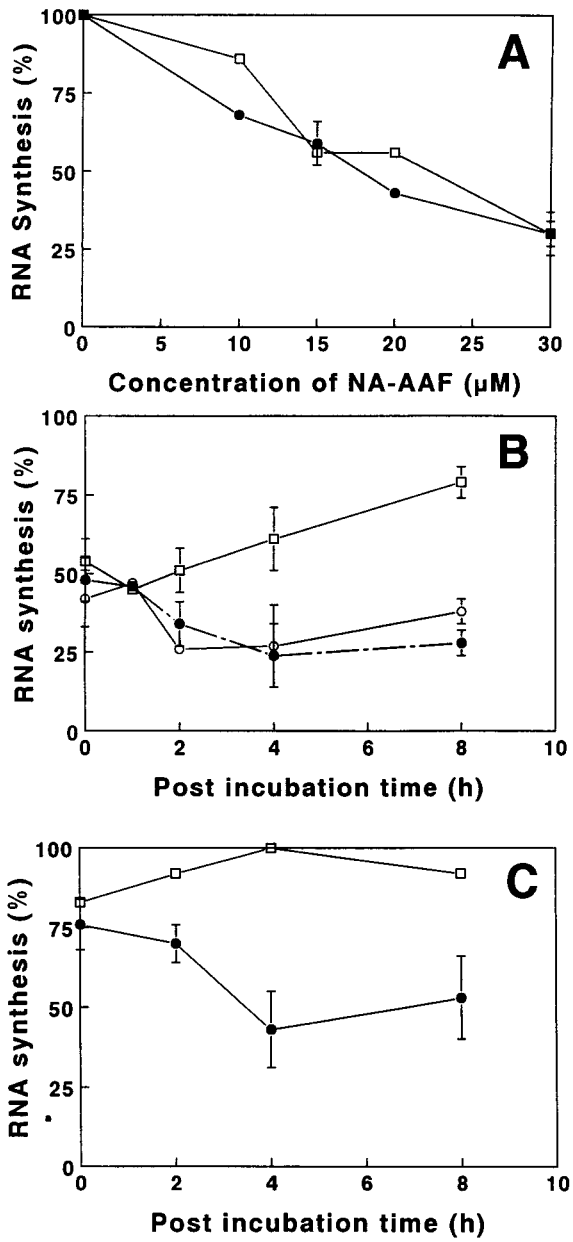

FIG. 3. (A) Inhibition of RNA synthesis after treatment with different doses of NA-AAF. □, VH25D; ●, CS1AN. (B) Resumption of RNA synthesis after treatment with 15 μM NA-AAF. □, VH25D; ○, CS1AN; ●, CS3BE. (C) Resumption of RNA synthesis after treatment with 5 μM NA-AAF. □, VH25D; ●, CS (average for CS1AN and CS3BE). In all three panels, RNA synthesis after treatment with NA-AAF is measured by 30 min of pulse labelling and expressed relative to levels in corresponding untreated cells. The error bars represent standard errors of the mean.

The capability of the UvrABC excinuclease to incise dG-C8-AF-containing DNA has been described previously (36). To check the efficiency of cutting dG-C8-AF-containing DNA by UvrABC excinuclease under our experimental conditions, we performed single and repeated incubations with the enzyme complex as previously described for determination of 6-4 PP (41). These experiments revealed that a single incubation of DNA with UvrABC is sufficient for complete cutting of all sensitive lesions in the DNA, as repeated incubation did not further increase the number of breaks.

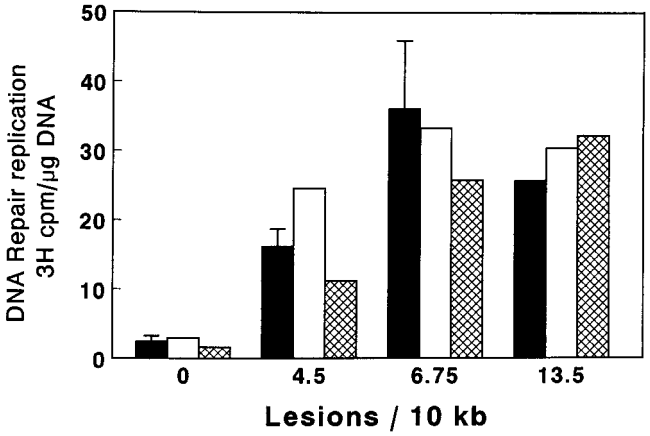

FIG. 4. Repair replication in normal human fibroblasts and CS fibroblasts after treatment with 30 (4.5 lesions per 10 kb), 45 (6.75 lesions per 10 kb), and 90 (13.5 lesions per 10 kb) μM NA-AAF and after incubation for 24 h. Closed bars, VH25D; open bars, CS1AN; cross-hatched bars, CS3BE. The error bars represent standard errors of the mean.

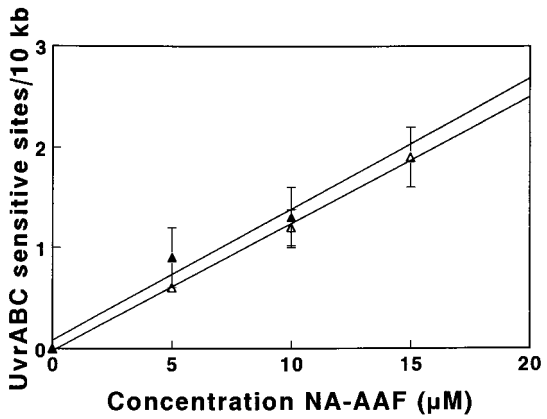

FIG. 5. Induction of UvrABC-sensitive sites in active and inactive genes after treatment with different doses of NA-AAF. Induction was measured in the 18.5-kb *Eco*RI fragment of the active ADA gene (open symbols) and the 14-kb fragment of the inactive 754 gene (closed symbols). The data represent the average lesion frequencies measured in the three cell lines used in this study. The error bars represent standard errors of the mean.

To determine the induction of dG-C8-AF in active and inactive genes as a function of dose, normal and CS cells were treated with various concentrations of NA-AAF and lesion frequencies were measured in a 3′-end-located 18.5-kb *Eco*RI fragment of the ADA gene and a 14-kb *Eco*RI fragment of the X-chromosomal inactive 754 gene by employing the same filter. A linear increase in the number of NA-AAF-induced lesions was observed up to 15 μM (Fig. 5). Moreover, the induction frequencies were very similar for the active ADA gene and the inactive 754 gene, with an average induction frequency of 0.15 adducts per μM per 10 kb. This frequency is very similar to the frequency in the genome overall as measured by HPLC analysis. There was no significant difference between the different cell lines in the induction of lesions. The optimal initial frequency of adducts in repair experiments is one adduct per restriction fragment. This adduct frequency is reached by using a dose of 5 μM NA-AAF, which results in about 0.75 adducts per 10 kb and equals the frequency of CPD per fragment induced by 10 J of UV-C light per m^2. A concentration of 5 μM results in a 40% reduction of clonal survival in CS cells, whereas the clonal survival in normal cells is unaffected.

The kinetics of repair of NA-AAF-induced lesions in transcriptionally active and inactive genes in cells treated with 5 μM NA-AAF were measured by using the active ADA gene and the inactive 754 gene. Repair in the ADA gene was measured in the 3′-end-located 18.5-kb *Eco*RI fragment (both strands are transcribed) (22). Figure 6 shows that normal human fibroblasts exhibited rather inefficient repair of the ADA gene, leading to approximately 50% repair after 24 h. However, the removal of dG-C8-AF adducts clearly proceeds in a biphasic manner. During the first 8 h after treatment, about 40% of the dG-C8-AF adducts are removed, after which repair of an additional 10% of dG-C8-AF adducts proceeds at a much slower rate (Fig. 6). In spite of the rather slow repair of dG-C8-AF, the active ADA gene was preferentially repaired relative to the inactive 754 gene, which exhibited only 25% removal of dG-C8-AF after 24 h. Like normal cells, CS3BE cells showed preferential repair of dG-C8-AF in the active ADA gene. The differences in repair rates for the ADA and 754 genes between normal and CS cells shown in Fig. 6 are similar to the differences found among different normal human primary fibroblasts for repair of UV-induced CPD (44); these differences are most likely due to experimental variation and strain differences.

In order to detect possible differences in kinetics of removal of dG-C8-AF adducts between the transcribed and nontranscribed strands of the ADA gene, measurements of repair in the 5′-end-located 19.9-kb *Bcl*I fragment (only the ADA template strand is transcribed [22]) were performed with strand-specific probes. Figure 7 represents autoradiograms of experiments to determine repair measurements in the ADA *Bcl*I

TABLE 1. Number of ^{3}H-labelled grains per nucleus after treatment of normal human and CS cells with UV or NA-AAF

Cell line	^{3}H-labelled grains/nucleus[a]			
	UV, 16 J/m^2		NA-AAF, 16 μM	
	0–30 min	30–60 min	0–30 min	30–60 min
Normal	19.1 ± 1.6	31.3 ± 1.7	14.2 ± 1.0	11.2 ± 0.8
CS1AN	30.6 ± 1.1	30.8 ± 1.5	12.4 ± 0.8	10.5 ± 0.8
CS3BE	35.9 ± 1.9	40.1 ± 1.7	20.4 ± 1.1	14.6 ± 1.0

[a] Data are presented as mean values ± standard errors of the mean.

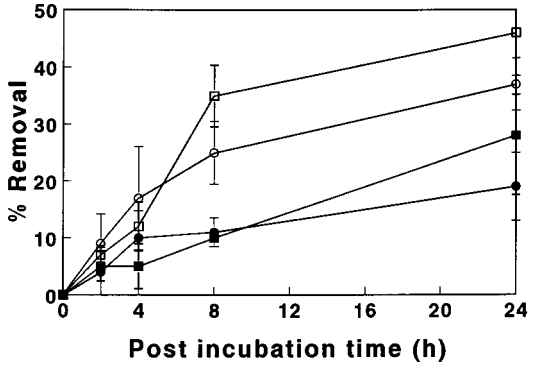

FIG. 6. Removal of dG-C8-AF from the 18.5-kb *Eco*RI fragment of the ADA gene and the 14-kb fragment of the 754 gene in primary normal human fibroblasts VH25D and in CS fibroblasts CS3BE. Lesion frequencies are measured by the UvrABC assay. VH25D: □, ADA; ■, 754. CS3BE: ○, ADA; ●, 754. The error bars represent standard errors of the mean.

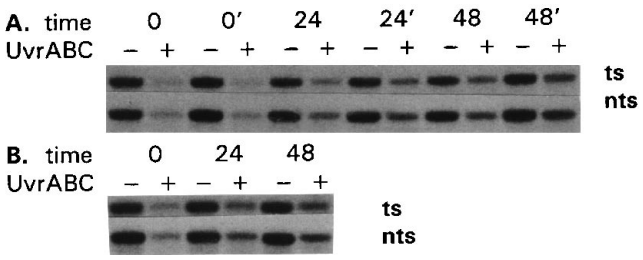

FIG. 7. Autoradiograms showing removal of dG-C8-AF from the 19.9-kb *Bcl*I ADA fragment of normal cells, VH25D (A), and CS cells, CS1AN (B). The repair in the ADA gene was analyzed with strand-specific probes recognizing the transcribed strand (ts) and the nontranscribed strand (nts). Panel A represents the autoradiograms for two independent experiments.

fragment in normal and CS cells done by using strand-specific probes. In normal cells, no difference in the rate of repair between the two strands could be detected (Fig. 7 and 8A), and repair occurred with the same kinetics as seen for the *Eco*RI fragment of the ADA gene, as analyzed with the double-stranded probes (Fig. 6). Also, both CS cell lines exhibited a lack of strand specificity for repair of dG-C8-AF adducts in the *Bcl*I fragment of the ADA gene (Fig. 7 and 8B) and displayed repair kinetics very similar to those of normal cells.

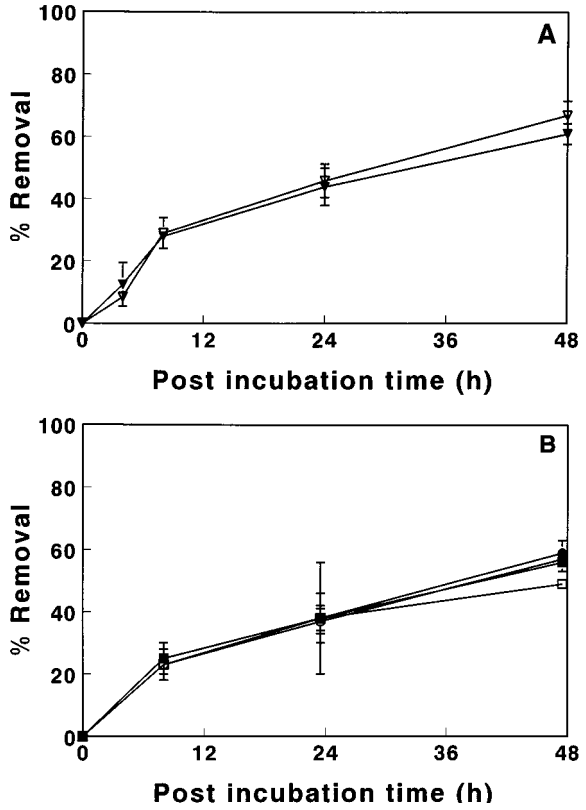

FIG. 8. Strand-specific analysis of the removal of dG-C8-AF from the 19.9-kb *Bcl*I fragment of the ADA gene. (A) Removal of dG-C8-AF from the transcribed strand (open symbols) and the nontranscribed strand (closed symbols) in VH25D fibroblasts. (B) Removal of dG-C8-AF from the transcribed strand (open symbols) and the nontranscribed strand (closed symbols) in CS1AN (○ and ●) and CS3BE (□ and ■) fibroblasts. The 8-h time point represents data for CS3BE only. The error bars represent standard errors of the mean.

DISCUSSION

Primary CS fibroblasts belonging to complementation groups A and B appeared to be two- to threefold more sensitive to the cytotoxic effects of NA-AAF than normal cells, as previously described by Wade and Chu (48). Exposure of DNA to NA-AAF is known to induce two major DNA adducts, i.e., dG-C8-AAF and dG-C8-AF. Here we show that AAF adducts are apparently not formed in the DNA of NA-AAF-treated human fibroblasts, in agreement with the results of a previous study of NA-AAF-induced DNA adducts in human cells (37). The lack of dG-C8-AAF formation is most likely due to rapid deacetylation of acetylated NA-AAF derivates, an activity common to most of the mammalian systems since treatment of cultured cells mainly results in deacetylated adducts (18). Nevertheless, we cannot rule out the possibility that the frequency of formation of dG-C8-AAF adducts might be too low (less than 1% of the level of dG-C8-AAF adducts) to be detected with our HPLC assay. No major differences were detected between normal and CS fibroblasts with respect to the types and frequencies of NA-AAF-induced DNA adducts, demonstrating that the enhanced sensitivity of CS cells to NA-AAF cannot be attributed to a higher level of DNA adduct formation or to different types of DNA lesions.

At the overall genome level, UDS and repair replication measurements did not reveal any indication for defective repair in CS cells. The finding that UDS after NA-AAF treatment was significantly less than it was following UV radiation (when the comparison was made at equal adduct levels) demonstrates that at least during the initial time period after treatment, the repair of dG-C8-AF adducts is rather inefficient. We have employed UvrABC excinuclease to investigate the induction and removal of NA-AAF-induced adducts in active and inactive sequences. Tang et al. (37) showed that UvrABC excinuclease incises at sites of all dG-C8-AF adducts in DNA fragments. In agreement with their results, we found that the lesion frequency in total DNA as determined by HPLC analysis is very similar to the frequency of occurrence of DNA lesions in restriction fragments of active and inactive genes as quantified by the UvrABC assay. Both in the active ADA gene and in the inactive 754 gene, repair appeared to be rather inefficient: less than 50% repair of lesions in 24 h. Moreover, the adduct removal in the inactive gene occurred more slowly and was less efficient than in the active gene. This slow repair of dG-C8-AF adducts in the inactive gene is in line with previous studies showing that other bulky lesions, i.e., UV-induced photolesions 6-4 PP and CPD (40, 45) and benzo(*a*)pyrene-induced lesions (9), are repaired considerably more slowly in X-chromosomal loci than in active genes. For the active ADA gene, the repair of dG-C8-AF adducts is clearly biphasic: 40% of the total adducts are repaired during the first 8 h, and then there is additional removal, but at a slower rate. A biphasic repair pattern has also been observed at the overall genome level (31) and in the dihydrofolate reductase domain (36) in Chinese hamster cells. The differential rate of removal of dG-C8-AF adducts may be related to different DNA lesion conformations. Conformational heterogeneity of dG-C8-AF adducts has been basically related to two different conformations. The two models proposed are an insertion-denaturation model in which the aminofluorene moiety (in syn conformation) stacks between adjacent bases, causing local distortions of the DNA helix, and an outside binding model in which the aminofluorene moiety (in anti conformation) resides outside the DNA helix, thereby causing little distortion. It is conceivable that lesions in syn conformation may form a better substrate

for nucleotide excision repair enzymes than do lesions in anti conformation.

One of the hallmarks of CS cells is the lack of recovery of the ability to synthesize RNA after inhibition by exposure to DNA-damaging agents, first demonstrated by Mayne and Lehmann for UV light (27). This impairment of RNA synthesis might actually be the cause of the enhanced cytotoxic effects of DNA-damaging agents in CS cells. Exposure to NA-AAF resulted in a dose-dependent inhibition of RNA synthesis in CS and normal cells. The resumption of RNA synthesis after repression by NA-AAF treatment resembled the resumption of RNA synthesis after UV exposure: normal cells are able to recover the ability to synthesize RNA after inhibition by NA-AAF, whereas CS cells fail to resume RNA synthesis. On the basis of adduct frequencies, the inhibition of RNA synthesis was rather inefficient: approximately 50% inhibition is achieved at an adduct frequency of 2 dG-C8-AF adducts per 10 kb, suggesting that multiple DNA adducts per transcription unit are required to inhibit RNA synthesis. For comparison, with UV-C light 50% inhibition is reached at 0.5 photolesions per 10 kb (unpublished results). Since the majority of [^{3}H]uridine incorporation during a short pulse is due to RNA polymerase II-driven transcription, we conclude that dG-C8-AF adducts form only weak blocks for RNA polymerase II. This conclusion is supported by two recent studies. McGregor et al. observed that only one of six dG-C8-AF adducts in plasmid DNA was capable of blocking T7 RNA polymerase-driven transcription in vitro (28). Using a plasmid template with a site-specific dG-C8-AF, Donahue et al. reported a weak arrest of RNA polymerase II-driven in vitro transcription (11).

In the active ADA gene no strand specificity was found, the nontranscribed strand being repaired as rapidly as the transcribed strand. A similar result has been reported for the removal of dG-C8-AF from the hypoxanthine phosphoribosyltransferase gene in human fibroblasts (28). These data suggest that the repair of dG-C8-AF adducts is dominated by the global genome repair pathway with no or only a minor contribution of transcription-coupled repair. This raises the question of whether dG-C8-AF adducts are actually targets for transcription-coupled repair. Recently we demonstrated that another bulky lesion, 6-4 PP, is not removed in a strand-specific manner in normal human fibroblasts but that this type of lesion is processed by transcription-coupled repair in XP group C cells lacking a global genome repair pathway (41). Similar observations have been made for dG-C8-AF-adducts in XP group C cells, indicating that dG-C8-AF adducts induced at 5 μM can be repaired by the transcription-coupled repair pathway (unpublished data), even though the inhibition of RNA synthesis (stalled transcripts are presumed to be the trigger for transcription-coupled repair) at the dose of 5 μM is relatively weak (about 25% inhibition).

On the basis of defective repair of CPD in ribosomal genes in CS cells, Christians and Hanawalt (10) concluded that the defect in CS cells is not limited to a deficiency in a transcription-repair coupling factor. Our data also indicate that increased sensitivity of the CS cells to the killing effect of NA-AAF is not due to a primary defect in transcription-coupled repair as was hypothesized from the lack of strand-specific repair of UV-induced CPD in CS (40). As with exposure to UV light, CS cells exhibited an apparently normal level of repair in the genome overall following NA-AAF exposure. However, a major difference emerges when levels of processing of CPD and dG-C8-AF adducts in CS and normal fibroblasts are compared. CS cells are deficient in the accelerated repair of CPD in the transcribed strand, exhibiting equal rates of repair of the transcribed and nontranscribed strands of the ADA gene (39). The lack of RNA synthesis resumption after UV damage in CS cells has been attributed to this failure to perform transcription-coupled repair, resulting in a slow removal of transcription-blocking photolesions from the transcribed strand. The absence of a strand bias, as well as similar rates and efficiencies of repair of dG-C8-AF adducts in the ADA gene in normal and CS cells, rules out defective preferential repair of active genes as the cause of increased sensitivity of CS cells to NA-AAF and the lack of RNA synthesis resumption. An alternative approach to explain the cellular response of CS cells to DNA-damaging agents is to assume that the CS gene products are involved in the regulation of transcription following DNA damage. Recent studies have shown that the XP group B (XP-B) and XP-D genes encode polypeptides that are subunits of the RNA polymerase II basal transcription factor TFIIH essential for RNA polymerase II transcription initiation, as well as essential components in nucleotide excision repair. Bootsma and Hoeijmakers (6) and Svejstrup et al. (35) have proposed a model in which the transcription initiation complex and the repair complex share a common factor, i.e., core TFIIH. In the absence of DNA damage, core TFIIH associates with RNA polymerase II and essential transcription factors at a promoter, whereas in the presence of DNA damage the core TFIIH might be recruited for association with additional NER proteins.

To explain our results, we propose that the CS proteins are essential factors allowing the core complex to switch from a repair function back to transcription: in this view, the CS proteins act as repair-transcription uncoupling factors. Mutations in the CS genes will then inevitably lead to a lack of transcription initiation. As a consequence, the mutations in the CS genes will directly affect those lesions whose repair in active genes is predominantly transcription coupled, i.e., UV-induced CPD. We note here that our results do not exclude the possibility of a role of CS gene products in transcription-coupled repair such as that described for the *E. coli* transcription-coupling repair factor (34). Recently, the CS group A and B genes have been cloned and the encoded proteins have been purified (19, 38). Interestingly, the CS group A protein interacts with the p44 protein, a subunit of TFIIH, and with the CS group B protein. It is conceivable that mutations that affect the interaction of CS proteins with TFIIH may result in a CS phenotype. Besides mutations in the CS proteins themselves, mutations in subunits of TFIIH may result in the clinical phenotype of CS patients. In this respect, specific mutations in XP-B and XP-D genes may exert their effects by interfering with CS proteins, thereby impairing the putative conversion of the TFIIH core complex from a repair function to a transcription function. This hypothesis is supported by the observed repair phenotype of XP-D cells from patients without the CS phenotype and XP-D cells from patients with a CS phenotype (XP-D/CS). Fibroblasts of an XP-D/CS patient exhibited an enhanced sensitivity to UV irradiation in comparison with sensitivity of XP-D fibroblasts, despite a level of 30 to 40% repair in the genome overall in comparison with rates for normal cells (7). The observation that neither XP-D (XP3NE) nor XP-D/CS (XP8BR and XPCS2) fibroblasts exhibit significant removal of CPD from their active genes (unpublished results) favors the hypothesis of a transcription defect in XP-D/CS cells instead of a repair defect underlying the enhanced UV sensitivity relative to that of XP-D cells.

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