Published online 10 February 2009 *Nucleic Acids Research, 2009, Vol. 37, No. 6* ***1951–1961***
doi:10.1093/nar/gkp070

Reactive oxygen species generated by thiopurine/UVA cause irreparable transcription-blocking DNA lesions

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Received December 12, 2008; Revised and Accepted January 21, 2009

ABSTRACT

Long-term treatment with the anticancer and immunosuppressant thiopurines, azathioprine or 6-mercaptopurine, is associated with acute skin sensitivity to ultraviolet A (UVA) radiation and a high risk of skin cancer. 6-thioguanine (6-TG) that accumulates in the DNA of thiopurine-treated patients interacts with UVA to generate reactive oxygen species. These cause lethal and mutagenic DNA damage. Here we show that the UVA/DNA 6-TG interaction rapidly, and essentially irreversibly, inhibits transcription in cultured human cells and provokes polyubiquitylation of the major subunit of RNA polymerase II (RNAPII). *In vitro*, 6-TG photoproducts, including the previously characterized guanine-6-sulfonate, in the transcribed DNA strand, are potent blocks to RNAPII transcription whereas 6-TG is only slightly inhibitory. *In vivo*, guanine-6-sulfonate is removed poorly from DNA and persists to a similar extent in the DNA of nucleotide excision repair-proficient and defective cells. Furthermore, transcription coupled repair-deficient Cockayne syndrome cells are not hypersensitive to UVA/6-TG, indicating that potentially lethal photoproducts are not selectively excised from transcribed DNA. Since persistent transcription-blocking DNA lesions are associated with acute skin responses to sunlight and the development of skin cancer, our findings have implications for skin cancer in patients undergoing thiopurine therapy.

INTRODUCTION

The thiopurines, 6-thioguanine (6-TG), azathioprine (aza) and 6-mercaptopurine are immunosuppressants and anti-inflammatory agents that are also used in the treatment of cancer. They are prodrugs and their metabolism culminates in the formation of 6-thioguanine nucleotides and the incorporation of 6-TG into nucleic acids (1,2). The long-term use of aza results in detectable DNA 6-TG in patients' lymphocytes and skin cells (3,4) and continuous immunosuppression in organ transplant patients is associated with an incidence of skin cancer that is up to 200-fold higher than that of non-immunosuppressed individuals. Sunlight exposure is a cofactor in this increased skin cancer risk (5). UVA (wavelengths 320–400 nm) comprises more than 95% of the ultraviolet (UV) radiation in incident sunlight. Despite its abundance, UVA is normally considered to be less harmful than the shorter wavelength UVB because it is absorbed only weakly by DNA. UVA can, however, cause DNA damage via photosensitized reactions following absorbance by non-DNA cellular chromophores (6). Unlike the canonical DNA bases, thiopurines do absorb UVA and 6-TG has an absorbance maximum at 340 nm. Incorporation of 6-TG therefore introduces a strong UVA photosensitizer into DNA. This suggests a possible mechanism by which sunlight and aza might interact to promote the development of skin cancer. The UVA energy absorbed by DNA 6-TG generates reactive oxygen species (ROS), with singlet oxygen $^1O_2$ as a major product (7). ROS cause DNA damage, and one target for oxidation is DNA 6-TG itself. One oxidized form of the thiobase, guanine-6-sulfonate (G^{SO3}), is a strong block to replication in primer extension assays *in vitro* but can be bypassed by error-prone DNA polymerases (4,7).

Proteins also react with $^1O_2$; histidine and aromatic aminoacids are particularly susceptible to oxidation (8). The proteins involved in DNA metabolism are likely to be vulnerable to attack by any $^1O_2$ generated within DNA itself. This susceptibility is illustrated by the covalent photochemical cross-linking between subunits of replisome-associated PCNA (9). It seems likely that this intersubunit crosslinking of an important DNA replication and repair factor is an example of a more general oxidation of DNA-associated proteins.

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Consistent with damage to both DNA and its associated proteins, the combination of DNA 6-TG and UVA is detrimental to living cells. 6-TG and UVA combine synergistically to cause cell death and mutation, to inhibit replication, and to provoke a p53-dependent DNA damage response (4,7). We previously noted that the response of the transcriptionally regulated p21 gene is attenuated at higher levels of DNA damage and suggested that this might reflect a general inhibition of transcription.

Transcript elongation by RNA polymerase II (RNAPII) is stalled by DNA lesions such as UVC-induced pyrimidine dimers (10). Although some DNA damage may be bypassed by RNAPII with or without the help of transcription elongation factors (11,12), no specific lesion-bypass RNA polymerases have been identified. Transcription-blocking DNA damage is selectively removed from transcribed DNA by transcription coupled nucleotide excision repair (TCR) (13). In addition to most of the nucleotide excision repair (NER) factors, TCR requires the CSA and CSB proteins, and CSA- or CSB-deficient cells are defective in TCR and are sensitive to agents that introduce transcription-blocking DNA lesions. If TCR fails, an irreversibly arrested RNAPII at a DNA lesion site is removed from the DNA via polyubiquitylation and degradation, most likely as a strategy of last resort to restart transcription (14).

Structurally, RNAPII resembles an incompletely closed clamp which encircles transcribed DNA (15). During transcription, DNA enters RNAPII via the jaws of the clamp and progresses through a large central cleft to the active site of the enzyme. The cleft and parts of the jaws are formed by the two largest RNAPII subunits Rpb1 and Rpb2 which contact the DNA template and RNA transcript. Because of this close contact, the Rpb1 and Rpb2 subunits might be particularly susceptible to oxidation by ROS generated in DNA.

Here we show that combined UVA and 6-TG treatment induces a rapid, profound, and apparently irreversible block to transcription in human cells. This inhibition is dependent on the presence of 6-TG in DNA rather than RNA. Transcription arrest by photochemically activated 6-TG does not reflect photochemical crosslinking of RNAPII subunits. Instead inhibition is accompanied by polyubiquitylation of RNAPII, suggesting the presence of persistent DNA lesions that block transcript elongation. The ability of a single template DNA G^{SO3} to block RNAPII transcription *in vitro* is consistent with this possibility. Quantification of photoproducts in cellular DNA indicates that DNA G^{SO3} is highly persistent and a poor substrate for excision by NER. Finally, TCR-defective CSA and CSB cells are no more sensitive to killing by 6-TG/UVA than their TCR-competent counterparts, confirming that potentially lethal transcription-arresting DNA lesions persist even when TCR is functional. We conclude that photochemical activation of DNA 6-TG produces lesions which block RNAPII transcription, induce RNAPII polyubiquitylation and degradation, and are not effectively excised from transcribed DNA.

MATERIALS AND METHODS

Cell culture

The human mismatch-repair defective CCRF-CEM leukaemia cell line and the lymphoblastoid cell lines GM005, AG7075 and GM2345 were cultured in RPMI. The SV40-transformed human fibroblast cell lines MRC5VA, CS1AN and CS3B were grown in DMEM. All media were supplemented with 10% fetal calf serum.

Incorporation of [^{3}H]-uridine and [^{3}H]-thymidine into nucleic acids

Cells were grown in medium containing 6-TG for 24 h, washed and resuspended in PBS before irradiation with 10 kJ/m^2 UVA. After irradiation, cells were returned to normal medium and pulsed with [5′ ^{3}H]-uridine (1 μCi/ml; 999 GBq/mmol) or [5′ ^{3}H]-thymidine (1 μCi/ml; 511 GBq/mmol) for 15 min at 37°C at appropriate time points. Trichloroacetic acid insoluble radioactivity in 2×10^6 cells was determined by scintillation counting. All experiments were performed in duplicate. To measure the effect of DNA 6-TG on RNA synthesis, cells were preincubated for 2 h in normal medium containing 1 mM hydroxyurea (HU) before treatment with 6-TG and UVA as described above. One millimolar HU was also present throughout the radiolabelling period.

Chromatography

To determine the extent of 6-TG incorporation into nucleic acids, cells were treated with 0.8 μM (CCRF-CEM, GM005, AG7075) or 0.6 μM (MRC5VA, CS3B, CS1AN) 6-TG for 48 h to achieve similar incorporation levels of 6-TG into DNA among the different cell lines. They were then washed with PBS and DNA and RNA were extracted using the Wizard Genomic DNA purification kit (Promega, Madison, WI) or Trizol (Invitrogen, Carlsbad, CA), respectively, according to the manufacturers' instructions. Nucleic acids (40 μg) were digested to nucleosides with 10 units of nuclease P1 (Sigma, St. Louis, MO) for 1 h at 50°C followed by two units of alkaline phosphatase (1 h at 37°C). Nucleosides were separated by HPLC as described (4) and 6-TG 2′-deoxy- and ribonucleosides quantified by their absorbance at 342 nm. 6-TG substitution was expressed as a percentage of total 2′-deoxyguanosine or guanosine in the same samples.

Excision of photoproducts

Cells were grown for 48 h in medium containing 1 μM 6-TG. After washing and resuspension in PBS, they were irradiated with 5 kJ/m^2 UVA and returned to fresh medium. Immediately after irradiation and at times up to 48 h later, DNA was extracted from aliquots of 10^6 cells using the Wizard Genomic purification kit (Promega) and digested to nucleosides. Following separation by HPLC (4), 6-TG 2′-deoxyriboside was quantified by its A_{342} and dG^{SO3} by its fluorescence (Excitation 324 nm, Emission 408 nm) using an external calibration curve of authentic standards. In a control experiment, we measured excision of UVC-induced 6-4

Pyrimidine:Pyrimidone photoproducts (6-4PPs), which are repaired by NER. For this purpose, cells were irradiated with 30 J/m^2 UVC and DNA extracted at indicated time points. ELISA was performed as described (16). Briefly, quadruplicate samples of DNA (400 ng) were placed in 96-well plates precoated with protamine sulphate. 6-4PPs were detected using anti-6-4PP (MBL, 1:1000 dilution) primary antibody, a biotinylated secondary antibody (1:2000 dilution, Zymed, San Francisco) and strepatavidin-coupled peroxidase (1:10 000 dilution, Zymed).

In vitro transcription

Oligonucleotide sequences were as follows: Transcribed strand G: 5′-CCCTTTCCTACCTACATACACCACAC ACCACACCCA**G**CCCAACCCCTTTCCCCTTCCCCT TTTCCCTTACCCCTCTCCATACCACACCACCTTA CCTACCACCCACCTTCCCTTACCCTTCCAX-3′ (X = biotin). Transcribed strand TG is the same as G, except that it contains a single 6-TG instead of the G (shown above in bold) at position 37. Nontranscribed strand: 5′-TGGAAGGGTAAGGGAAGGTGGGTGGT AGGTAAGGTGGTGTGGTATGGAGAGGGGTAA GGGAAAAGGGGAAGGGGAAAGGGGTTGGGCT GGGTGTGGTGTGTGGTGTATGTAGGTAGGAAA GGG-3′. RNAprimer: 5′-AUGGAGAGG-3′. Purified *Saccharomyces cerevisiae* RNAPII was a kind gift of Jesper Svejstrup.

The RNA primer was radiolabelled using T4 polynucleotide kinase (New England Biolabs, Ipswich, MA). Prior to ternary complex formation, transcribed strands G and TG were irradiated with indicated doses of UVA or treated with MMPP (5 μM final concentration) for 20 min at RT before ethanol precipitation and resuspension in water. Ternary complexes were assembled from oligonucleotides and isolated with Streptavidin MagneSphere magnetic particles (Promega) as described (17). Elongation was carried out for 10 min at 30°C in 1× transcription buffer (20 mM Tris, pH 7.9, 40 mM KCl, 2 mM $MgCl_2$, 1 mM β-mercaptoethanol), 1 U/μl RNAsin and 50 μM each nucleotide. Stop buffer (20 mM EDTA, 800 μg/ml Proteinase K, 0.5% SDS) was added immediately afterwards and the mixture incubated for 30 min at 37°C. Magnetic beads were then removed, and the RNA products were purified by phenol/chloroform extraction and ethanol precipitation before being resuspended in 95% formamide and run on a 12% polyacrylamide gel.

During assembly of the complex, the RNA primer places RNAPII 34 bases from G/TG. Transcription elongation on undamaged DNA in the absence of CTP results in arrest at the same site as upon oxidation of 6-TG, giving rise to an RNA product of 43 bases.

Immunoblotting

Proteins were separated on 3–8% Tris–acetate or 4–20% Tris–glycine gels in Tris–acetate/SDS or Tris–Glycine/SDS running buffer (Invitrogen). Total 20 μg protein was loaded per lane. Following transfer, membranes were probed with antibodies against Rpb2 (1:1000 dilution, abcam, Cambridge, UK), Rpb3 (1:1000 dilution, abcam), Rpb1(1:10 000 dilution, kindly provided by Anindya Roy), p53Ser15 (1:1000 dilution, Cell Signalling), p53 (1:1000 dilution, DAKO, Glostrup, Denmark) and actin (1:5000 dilution, abcam). Antigen–antibody complexes were detected by ECL western blotting detection reagent (GE Healthcare, Little Chalfont, UK).

Immunoprecipitation

A total of 250 μg extract proteins were preincubated with 25 μl Rpb1 antibody for 1 h at 4°C before 75 μl of protein G plus agarose beads (Santa Cruz, Santa Cruz, CA) were added and the mixture incubated overnight at 4°C with continuous inversion. The next day, beads were recovered, washed, and resuspended in PBS before boiling for 15 min. After removal of the beads by centrifugation, immunoprecipitated material was loaded onto a 3–8% polyacrylamide gel. After transfer, membranes were probed with an antibody against ubiquitin (1:1000 dilution, MBL, Woburn, MA).

Cell survival

Cells were treated with 6-TG for 48 h, washed and resuspended in PBS before irradiation with 10 kJ/m^2 (fibroblasts) or 5 kJ/m^2 (lymphoblastoid cells) UVA. Fibroblasts were then seeded in normal medium into 96-well plates (1000 cells/well) and the fraction of viable cells was assayed 10 days later using the MTT assay. Lymphoblastoid cells were replated at a density of 1×10^5 cells/ml in normal medium and survival was assayed five days later by trypan blue exclusion. To measure survival after exposure to UVC, cells were irradiated with 10 J/m^2 and processed as described above. All experiments were performed in triplicate and repeated at least twice.

RESULTS

Inhibition of transcription *in vivo*

We have previously shown that the interaction between DNA 6-TG and UVA causes inhibition of replication, and provokes the p53 DNA damage response. In that study, we noted that at higher levels of photochemical damage, induction of the transcriptionally activated p21 protein was attenuated and suggested that this might reflect global inhibition of transcription by DNA 6-TG photoproducts (7). To investigate this point further, we compared p53 phosphorylation after 6-TG/UVA and after exposure to 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB), a known inhibitor of transcription. CCRF-CEM cells were cultured in the presence of 0.8 μM 6-TG for 24 h to allow substitution of DNA by the thiobase, then irradiated with 10 kJ/m^2 UVA. Phosphorylation of p53 Ser15 was determined by western blotting. Figure 1A shows that combined 6-TG/UVA induced p53 phosphorylation. This was detectable 1 h after radiation and by 4 h, phosphorylation had increased substantially in parallel with stabilization of the p53 protein. The level of Ser15 phosphorylation was

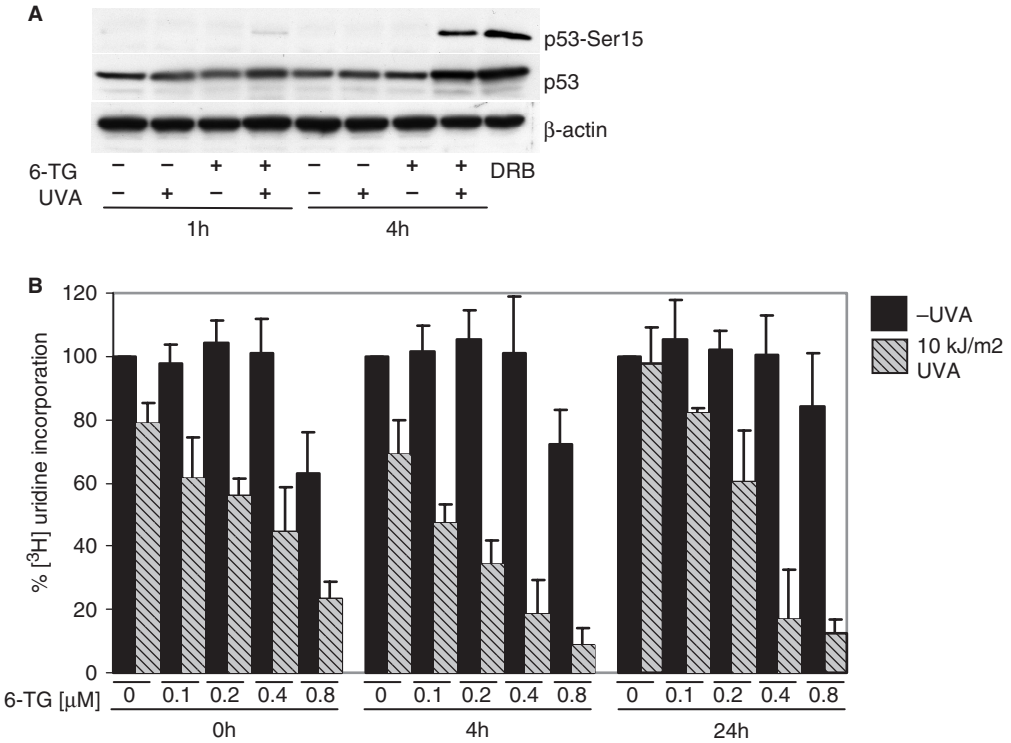

Figure 1. Transcription in cells containing DNA 6-TG following UVA irradiation. (**A**) Activation of p53. Extracts were prepared at the times indicated from cells grown for 24 h in 0.8 μM 6-TG and irradiated with 10 kJ/m^2 UVA. Phosphorylation of p53Ser15 was analysed by western blotting. Control cells were treated for 18 h with 50 μM DRB. (**B**) RNA synthesis. CCRF-CEM cells were grown in the presence or absence of 6-TG for 24 h before irradiation with 10 kJ/m^2 UVA as indicated. They were then returned to normal medium. Incorporation of [^{3}H-uridine] into nascent RNA was measured for 15 min at the indicated times. Values are expressed as percentage of the incorporation by non-6-TG-treated, non-irradiated cells. Each measurement was performed in duplicate and the mean values of three independent experiments are shown.

comparable to that following overnight treatment with the transcription inhibitor DRB. Neither 6-TG nor UVA alone induced detectable p53 phosphorylation.

Measurements of [^{3}H]-uridine incorporation into nascent RNA in CCRF-CEM cells containing DNA 6-TG indicated that RNA synthesis was inhibited immediately after UVA exposure (Figure 1B). Four hours later, incorporation was reduced still further. After 24 h, recovery of transcription was minimal and was apparent only in cells treated with the lowest 6-TG doses. In non-6-TG-treated cells, 10 kJ/m^2 UVA induced a modest decrease of RNA synthesis (20–30%) four hours after irradiation. This inhibition was reversible and after 24 h, incorporation was comparable to that of unirradiated cells. Growth in 6-TG alone did not significantly affect RNA synthesis except at the highest concentration (0.8 μM) which resulted in an inhibition of around 40%. [^{3}H]-uridine incorporation was inhibited to a similar extent by 6-TG/UVA in unrelated HCT116 colorectal cells (data not shown). Taken together, these results indicate that the products of UVA-mediated photochemical activation of DNA 6-TG significantly inhibit transcription.

6-TG is incorporated into DNA and RNA and interference with transcription might be mediated by 6-TG photoproducts in either transcribed DNA or the RNA product. We examined these two possibilities. First, the extent of 6-TG incorporation into DNA and RNA of CCRF-CEM cells was measured directly. DNA and RNA extracted from cells grown in 0.8 μM 6-TG for 24 h, was digested to nucleosides which were separated by HPLC. 2′-deoxyribo- and ribo-6-TG were detected and quantified by their absorbance at 342 nm. DNA was ~20-fold more heavily substituted with 6-TG than RNA. 0.06% of RNA guanine was substituted by 6-TG whereas 6-TG replaced 1.1% of guanine in DNA from the same cells (Figure 2A).

To determine whether inhibition of transcription was due to DNA or RNA 6-TG, CCRF-CEM cells were allowed to incorporate 6-TG into RNA, but not DNA by growth in the presence of the replication inhibitor HU. Pre-treatment of CCRF-CEM cells with 1 mM HU for 2 h reduced the level of DNA replication by >95%. Cells were then grown for a further 24 h in HU plus 6-TG (0.8 μM) and then irradiated with 10 kJ/m^2 UVA. RNA and DNA synthesis was analysed one hour later. Figure 2B shows that exclusion of 6-TG from DNA by HU treatment effectively abolished the inhibition of transcription by combined 6-TG and UVA. After UVA radiation, [^{3}H]-uridine incorporation in the 6-TG + HU treated cells (bar 8, left panel) was >6-fold higher than in cells not treated with HU (bar 6, left panel) and was

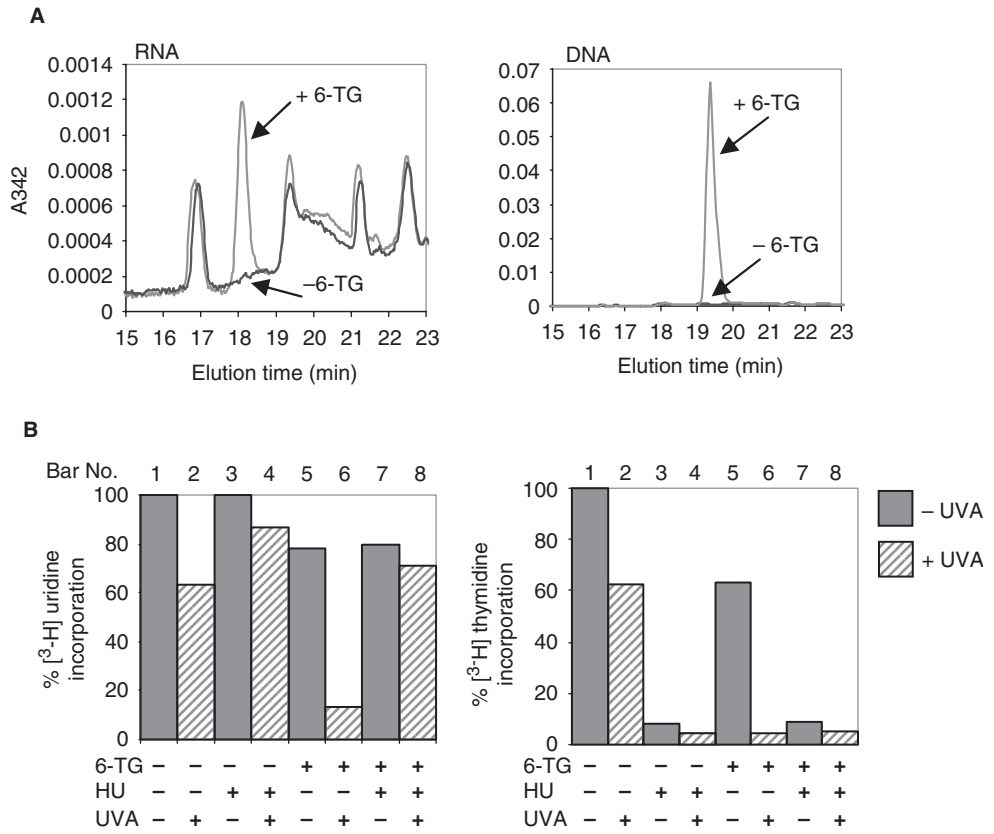

Figure 2. The effects of RNA and DNA 6-TG. (**A**) 6-TG substitution. CCRF-CEM cells were treated with 0.8 μM 6-TG for 24 h. DNA and RNA were extracted and digested to nucleosides and analysed by HPLC. 6-TG nucleosides were detected by A_{342}. (**B**) 6-TG in DNA versus RNA as inhibitors of transcription. CCRF-CEM cells treated as indicated for 2 h with 1 mM HU were grown further in the presence or absence of 0.8 μM 6-TG and/or 1 mM HU for 24 h. Following UVA irradiation (10 kJ/m^2) and an additional 1 h incubation in the presence or absence of HU, cells were labelled with 1 μCi/ml [^{3}H-uridine] or 1 μCi/ml [^{3}H-thymidine] for 15 min and incorporation of radioactivity into TCA-insoluble material was determined by scintillation counting.

similar to the incorporation by cells treated with either 6-TG or UVA alone (bars 2 and 5, left panel). These differences were also apparent four hours after irradiation (results not shown). The low level of transcription inhibition caused by either UVA or 6-TG alone was largely unaffected by HU-treatment (compare bars 2 and 4 or bars 5 and 7, left panel). HU treatment and 6-TG/UVA reduced [^{3}H]-thymidine incorporation to similar extents (Figure 2B, right panel) confirming previous observations that replication is profoundly inhibited by photoactivation of 6-TG. These findings indicate that UVA-mediated inhibition of transcription in 6-TG-treated cells is largely due to DNA 6-TG. 6-TG incorporation into RNA is significantly lower than into DNA and this makes no measurable contribution to the photochemical inhibition of transcription.

Modification of RNA Pol II

Since UVA activation of DNA 6-TG can cause protein modification (9) and arrest at UVC-induced DNA lesions triggers polyubiquitylation and degradation of the Rpb1 subunit of RNAPII (17,18), we examined the effect of UVA/6-TG on the transcription machinery itself. Extracts of 6-TG-treated and UVA irradiated CCRF-CEM cells were analysed by immunoblotting (Figure 3A). Between 1 and 4 h after irradiation, increasing levels of a slowly migrating form of Rpb1were noted in extracts of cells treated with both 6-TG and UVA. This form was not observed after UVA or 6-TG treatment alone. The behaviour of the modified Rpb1 was consistent with polyubiquitylation and this was confirmed by immunoprecipitation followed by western blotting with a ubiquitin-specific antibody (Figure 3B). In addition to the ubiquitylation of Rpb1, 6-TG/UVA induced a rapid modification of Rpb2, whereas Rpb3 was stable and remained apparently unaltered over 4 h.

We did not observe discrete slowly migrating forms of Rpb1 or Rpb2 that would be consistent with covalent crosslinking of the RNAPII subunits. This suggests that the effects of 6-TG/UVA treatment on RNAPII reflect cellular responses to the presence of DNA lesions rather than direct modification of the proteins.

Inhibition of transcription by 6-TG oxidation products *in vitro*

UVA irradiation of 6-TG generates reactive oxygen species that can oxidize 6-TG to guanine sulfonate (G^{SO3}). Since this photoproduct is a powerful block to DNA

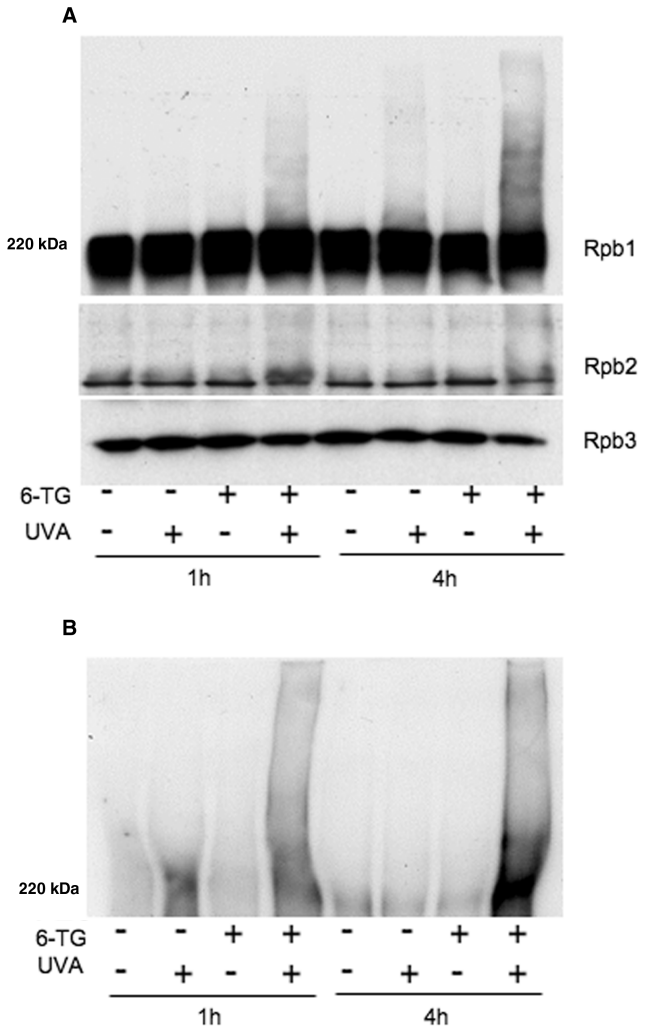

Figure 3. Modification of RNA polymerase II subunits following UVA irradiation of cells containing DNA 6-TG. CCRF-CEM cells were grown in 0.8 μM 6-TG for 24 h before 10 kJ/m^2 UVA irradiation. Cell extracts were prepared at the indicated times after irradiation. (**A**) The three largest subunits of RNAPII analyzed by western blotting. (**B**) Rpb1 immunoprecipitated from extracts of treated cells was analysed by western blotting with an anti-ubiquitin antibody.

replication, we examined its effect on transcription. To do this, we used an established transcription system (19) with highly purified yeast RNAPII to address whether a single DNA G^{SO3} is sufficient to stall elongating transcription complexes. The active site of Rpb1 is 100% conserved from yeast to humans and both human and yeast RNAPIIs share a very similar subunit structure. Indeed, many human subunits can substitute for their yeast counterparts in a functional RNAPII (20). The *in vitro* transcription assay comprises RNAPII, a radiolabelled RNA primer, a nontranscribed 124-mer oligodeoxyribonucleotide and a transcribed 124-mer strand containing either a G or a 6-TG at position 87 (Figure 4A). To examine the effects of G^{SO3}, the single 6-TG in the transcribed strand was selectively oxidized by treating the oligonucleotide with the mild oxidizing agent MMPP or with UVA. HPLC analysis of MMPP-treated oligonucleotides confirmed stoichiometric conversion of 6-TG to G^{SO3} (data not shown). Elongation-competent RNAPII/DNA/RNA ternary transcription complexes were assembled by annealing appropriate oligonucleotides and adding purified RNAPII. Elongation was started by the addition of nucleoside triphosphates. In the assembled complex, the 9-mer RNA primer places RNAPII 34 bases upstream of position 87. The sequence of the transcribed strand ensures that transcript elongation on undamaged DNA in the absence of CTP arrests at position 87 giving rise to an RNA product of 43 bases (Figure 4B, lane 2). In the presence of all four NTPs, the transcription run-off product is 80 nucleotides long (Figure 4B, lane 1). A single 6-TG at position 87 in the transcribed strand caused some reduction in the yield of run-off transcripts (Figure 4B, compare lanes 1 and 3). In contrast, G^{SO3} in the same position completely blocked transcript elongation. UVA irradiation of the 6-TG containing template induced a similar profound inhibition of transcription, even at the lowest dose of 20 kJ/m^2. In each case, selective oxidation to G^{SO3} by MMPP or conversion to photoproducts by UVA, transcript termination occurred one base before the modified 6-TG. This is consistent with the inability of DNA G^{SO3} to form stable base pairs. As expected, MMPP treatment or UVA irradiation of the transcribed strand containing guanine had no detectable effect on transcription and levels of run-off products.

DNA 6-TG oxidation products and nucleotide excision repair

G^{SO3} significantly reduces the stability of oligonucleotide duplexes. It is unable to form stable base pairs with canonical DNA bases (7) and is likely to cause significant distortion of duplex DNA. Since it is also a strong block to transcription and replication, we examined whether these UVA-induced photoproducts might be subject to repair by NER. To do this, we compared the persistence of DNA G^{SO3} in excision repair-proficient GM005 and GM2345 XP group A lymphoblasts. Following 48-h treatment with 1 μM 6-TG, the levels of DNA substitution by the thiobase were similar (1% of DNA G) as determined by HPLC analysis of digested DNA. UVA (5 kJ/m^2) induced $\sim 2 \times 10^5$ DNA G^{SO3} lesions per cell. In DNA extracted at times up to 48 h after radiation, the amount of G^{SO3} remained effectively unchanged (Figure 5). Lesion persistence was independent of NER status and neither GM005 nor GM2345 cells excised G^{SO3} to a measurable extent. As a control, we examined by ELISA the introduction and persistence of UVC-induced 6–4 photoproducts. As expected, these were efficiently excised in an NER-dependent fashion. Approximately 90% were excised by 2 h post irradiation in the NER proficient GM005 cells whereas the levels remained unchanged in the XPA GM2345 cells (Figure 5). We conclude that the G^{SO3} photoproduct of DNA 6-TG is a poor substrate for NER.

To address the possible involvement of transcription-coupled nucleotide excision repair (TCR) in excising potentially lethal 6-TG photoproducts from transcribed DNA, we examined the effects of 6-TG/UVA in TCR-defective Cockayne's Syndrome cells. We first investigated

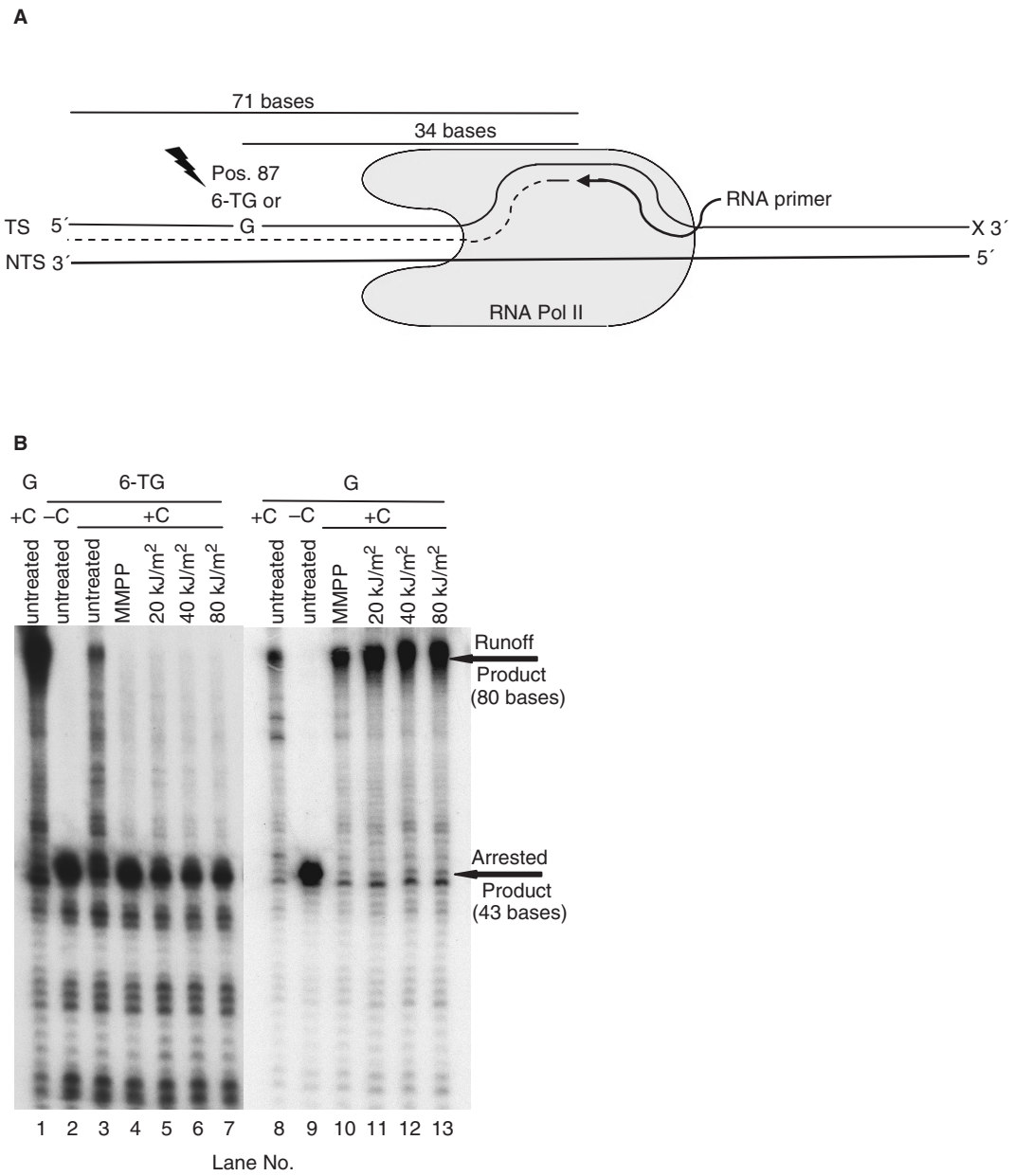

Figure 4. Inhibition of RNAPII transcription *in vitro* by oxidised template 6-TG. (**A**) *In vitro* transcription set-up. The transcription system comprises a radiolabelled 9-mer RNA primer and two 124-mer DNA oligonucleotides. The 124-mer transcribed strands contain either a single G or 6-TG at position 87. (**B**) Oxidation products of 6-TG block *in vitro* transcription. Transcribed strand oligonucleotides were treated with MMPP or UVA prior to formation of the ternary complexes. These were supplemented with GTP, ATP and UTP and transcription was initiated by purified *S. cerevisiae* RNAPII in the presence or absence of CTP. Transcription products were analysed by gel electrophoresis.

Rpb1 modification. Figure 6A shows that 6-TG/UVA provoked the Rpb1 modification associated with blocked transcription independently of a functional TCR machinery. Western blotting indicated that 6-TG/UVA induced comparable Rpb1 polyubiquitylation in CSB-defective CS1AN, CSA-defective CS3B, and repair-proficient MRC5VA fibroblasts. Thus, CSA and CSB cells mount an appropriate response to the presence of transcription-blocking photoproducts and ubiquitylate the Rpb1 subunit of RNAPII to ensure clearance of blocked transcription.

Cell viability assays revealed no significant differences in survival between the 6-TG/UVA treated TCR-deficient CS1AN and CS3B cells and the repair-proficient MRC5VA controls (Figure 6B, left panel). The extent of DNA substitution by 6-TG was similar in each of the cell lines. In MRC5VA and CS1AN, 6-TG replaced 0.3% of DNA G after treatment with 0.8 μM 6-TG. The corresponding value for CS3B was 0.2%. The similar resistance to 6-TG/UVA of the CSA (AG7075) and TCR-proficient (GM005) lymphoblastoid cell lines provided further evidence that TCR does not influence sensitivity to

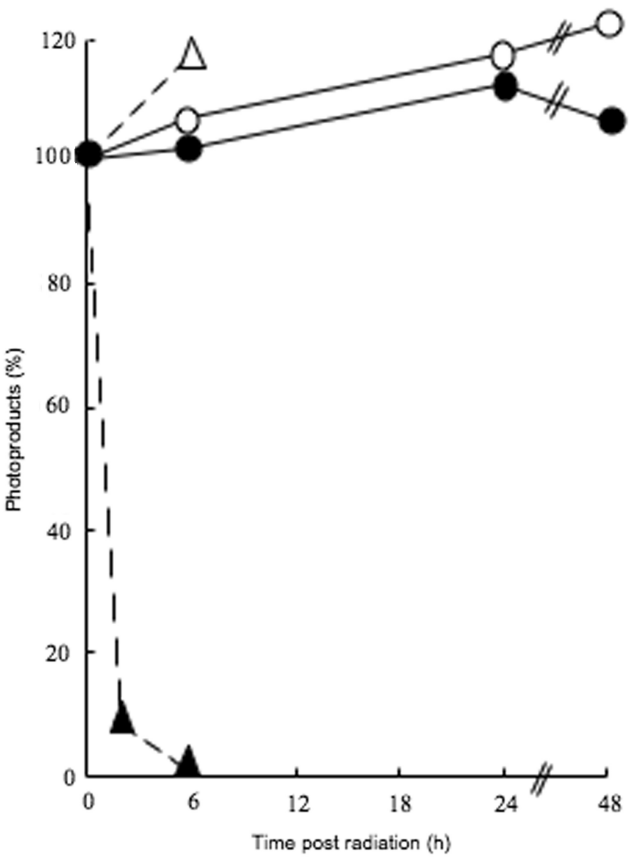

Figure 5. Excision of 6-TG/UVA photoproducts. GM005 (filled circle) and GM2345 (open circle) cells were grown for 48 h in 1 μM 6-TG and irradiated with 5 kJ/m^2 UVA. DNA extracted from 10^6 cells at the times shown was digested to 2′-deoxynucleosides which were separated by HPLC. 6-TGdR was quantified by A_{342} and dG^{SO3} by fluorescence. The initial dG^{SO3}:6-TGdR ratio immediately after irradiation was set to 100%. In a separate experiment, the same cells were irradiated with 30 J/m^2 UVC. DNA extracted at the times shown was analysed for 6–4 photoproducts by ELISA. GM005 (filled triangle) and GM2345 (open triangle).

photochemical 6-TG damage (Figure 6B, right panel). As a control, the hypersensitivity of the CS cell lines to UVC radiation was confirmed (Figure 6C).

We conclude that UVA-induced DNA 6-TG photoproducts provoke appropriate modification of RNAPII in both TCR competent and deficient cells. The similar sensitivity to 6-TG/UVA of CSA, CSB and wild-type cells contrasts to the hypersensitivity of the CS cells to UVC radiation and indicates that potentially cytotoxic DNA 6-TG photolesions are not removed by TCR.

DISCUSSION

We previously showed that treatment of cells with 6-TG and low doses of UVA causes oxidative DNA damage and that DNA 6-TG is a major target of oxidation. The same treatment also induces oxidation of DNA guanine to 8-oxoguanine (8-oxoG) (21). Whilst cells are protected against the effects of DNA 8-oxoG by several efficient DNA repair pathways, we show here that 6-TG photoproducts are not excised from DNA. They induce a powerful, and largely irreversible, arrest of replication and transcription which triggers robust induction of the p53 DNA damage response (4,7).

Conversion to thioguanine nucleotides and incorporation of 6-TG into DNA is a significant contributor to the clinical effects of thiopurines. 6-TG can also be incorporated into RNA (22) although there appear to be fewer documented measurements of the extent of RNA substitution. In the cultured cells we used, RNA substitution by 6-TG is around 20-fold lower than that of DNA- and UVA-induced transcription inhibition in 6-TG treated cells is a consequence of photochemical DNA, rather than RNA, damage. Consistent with these findings, a single G^{SO3} in the transcribed DNA strand also arrests transcript elongation by RNAPII in an *in vitro* assay. This easily identifiable product of oxidation of DNA 6-TG which is unable to form stable base pairs with canonical DNA bases (7), is also a strong block to DNA replication *in vitro* and it seems likely that an inability to direct an incoming NTP contributes to its effect on transcript elongation.

UVA introduces lesions in DNA containing 6-TG via the formation of ROS. In particular, singlet oxygen ($^1O_2$) is generated in a Type II photochemical reaction. $^1O_2$ is particularly hazardous because it damages not only DNA, but also proteins (8). DNA transactions such as replication, repair, and transcription all involve proteins or protein complexes that interact intimately with DNA and might be particularly vulnerable to oxidation by $^1O_2$ generated in DNA. The covalent oxidative crosslinking of PCNA subunits provides an example of this susceptibility (9). Unlike PCNA, free RNA PolII does not form a closed toroidal structure. Nevertheless, the actively transcribing enzyme adopts a similar clamping strategy *via* intimate and highly stable contacts with its DNA substrate and RNA product. 6-TG/UVA did not cause similar covalent crosslinking among RNAPII subunits. Instead, it provoked a rapid and extensive polyubiquitylation of Rpb1 and an as yet uncharacterized modification of the Rpb2 subunit, consistent with the presence of irreversible transcription-arresting photochemical DNA lesions (14). In yeast, ubiquitylation of Rpb1 by the Def1 ubiquitylation factor is regarded as a strategy of last resort to clear irreversibly stalled RNAPII complexes by targeting them for degradation by the proteosome. In yeast, the Rpb1 subunit undergoes polyubiquitylation whereas Rpb2 apparently remains unmodified. In the human cells used in this study, we consistently observed modification of the Rpb2, but not the Rpb3 subunit. Whether this difference represents a slightly different strategy by which human cells deal with dangerous blocked transcription complexes is currently unclear. The observation provides additional evidence for the presence of obstructive DNA 6-TG photolesions in transcribed DNA of UVA irradiated cells.

Replication and transcription blocking DNA lesions are frequently substrates for NER, a versatile DNA repair system that removes a variety of bulky DNA adducts (23). DNA G^{SO3} was not actively removed and persisted to the same extent in NER-proficient and

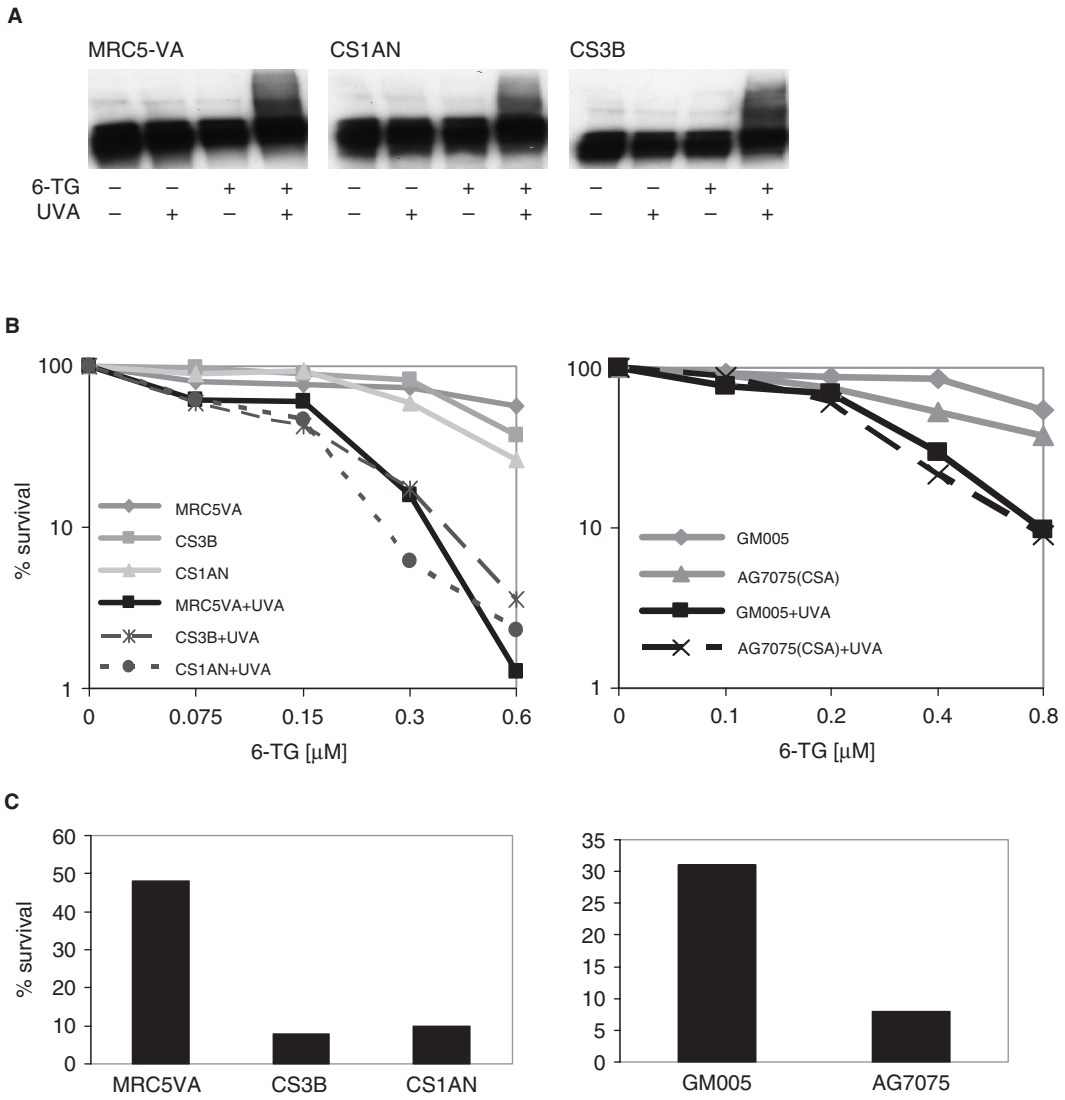

Figure 6. Sensitivity of Cockayne syndrome cells to 6-TG/UVA. (**A**) Rpb1 polyubiquitylation. CSA- and CSB-deficient and proficient SV40-transformed fibroblasts were grown in the presence of 0.6 μM 6-TG for 48 h and irradiated with 10 kJ/m2 UVA. Four hours after irradiation, cell extracts were prepared and Rpb1 was analysed by western blotting. (**B**) Survival after treatment with 6-TG/UVA. CSA- and CSB-deficient and proficient SV40-transformed fibroblasts and EBV-transformed lymphoblastoid cells were grown in the presence of indicated doses of 6-TG for 48 h before irradiation with 10 kJ/m2 (fibroblasts, left panel) or 5 kJ/m2 UVA (lymphoblastoid cells, right panel). Survival of fibroblasts was determined 10 days after irradiation by MTT assay. Survival of lymphoblastoid cells was assessed by trypan blue exclusion 5 days after UVA treatment. (**C**) Survival after UVC irradiation. Cells (not treated with 6-TG) were irradiated with 10 J/m2 UVC. Survival was assessed as in (B).

defective cells. Defects in either of the two NER subpathways, global or transcription coupled (TCR) repair, are associated with UV sensitivity. The complete absence of NER—for example in xeroderma pigmentosum group A—confers extreme UVB sensitivity and susceptibility to sunlight-induced skin cancer. The selective TCR defects in Cockayne's syndrome (CS) Group A or B patients cause increased sunlight sensitivity which reflects the persistence of transcription-blocking UVB DNA photoproducts. TCR-proficient and CS cells responded to 6-TG/UVA by ubiquitylating the Rpb1 subunit of RNApolII, confirming the efficient detection of transcription-blocking DNA damage. The similar sensitivity of wild-type and CSB cells to 6-TG/UVA indicates that these transcription-inhibiting photoproducts are not subject to TCR. The XPA and CSB phenotypes are recapitulated in mouse models. Significantly, XPA and CSB mice are particularly susceptible to the induction of erythema/oedema by UVB (24,25)—an observation that seems to connect the induction and persistence of transcription-blocking DNA lesions to these physiological end points. $Csb^{-/-}$ mice are also susceptible to UVB-induced skin cancer. We have previously reported that the skin of patients taking azathioprine contains measurable DNA 6-TG (4) and is hypersensitive to erythema induction by UVA but not UVB (26). These observations suggest that the skin cells of these patients are particularly susceptible to the formation of transcription-blocking

6-TG/UVA photolesions. We note in this regard, that erythema in the form of sunburn, is a significant risk factor for the development of skin malignancies (27). The link between sunlight and skin cancer is well established and mutations in critical tumor suppressor genes generally bear the hallmark of UVB exposure (28–30). The composition of incident UV light and the deeper penetration of the longer UVA wavelengths means that vulnerable 6-TG-containing skin cells are exposed to up to 10 times more UVA than UVB. Investigation of the effects of UVA and DNA 6-TG in physiologically relevant cell types such as keratinocytes may now be warranted.

In summary, 6-TG/UVA induced damage to cellular DNA rapidly, and largely irreversibly, inhibits transcription. DNA 6-TG photoproducts, including the previously characterized G^{SO3}, are potent blocks to transcript elongation by RNAPII *in vitro*. In agreement with this inhibitory effect on RNAPII, photoactivation of DNA 6-TG *in vivo* provokes the polyubiquitylation of RNAPII subunits that indicates persistent transcription blockage by DNA lesions. This RNAPII modification occurs in both NER competent and TCR-defective cells. The normal sensitivity of CSA and CSB cells to killing by 6-TG/UVA suggests that lethal 6-TG photoproducts have structural features that allow them to escape recognition and processing by excision repair. Persistent transcription-blocking DNA lesions induce erythema and are associated with the development of skin cancer. Our findings suggest that UVA may be a carcinogenic hazard for patients taking thiopurines.

ACKNOWLEDGEMENTS

We are grateful to Jesper Svejstrup for discussions and Stefan Sigurdsson for assistance with the *in vitro* transcription assay. The Rpb1 antibody was a kind gift of Anindya Roy.

FUNDING

This work was supported by Cancer Research UK. Funding for open access charge: Cancer Research UK.

Conflict of interest statement. None declared.

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