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Formation and Repair of Psoralen-DNA Adducts and Pyrimidine Dimers in Human DNA and Chromatin

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DNA damage and repair in human cells exposed to ultraviolet light (254 nm) or to psoralen derivatives plus 360 nm light were compared by means of a variety of analytic techniques. The two kinds of damage show considerable structural similarity; both involve cyclobutyl bonds to 5,6 positions of pyrimidines as major products and have various minor products. In purified DNA, pyrimidine dimers, but not psoralen adducts, cause structural distortions that are substances for digestion with single-strand-specific nucleases. Whereas pyrimidine dimers are randomly produced in chromatin, psoralen adducts, are concentrated approximately 2- to 4-fold in linker regions of chromatin at doses that are not highly lethal. Chromatin shows considerable mobility; assignment of DNA to linker or core regions is not permanent, and psoralen adducts initially concentrated in linker regions become randomized after 10 hr. Pyrimidine dimers and psoralen adducts are excised by normal cells but not by repair-deficient xeroderma pigmentosum cells. This repair process requires DNA polymerase alpha, but its rate in ultraviolet-damaged cells is twice that in psoralen-damaged cells. Conversion of monoadducts to DNA–DNA crosslinks reduces the rate of repair because of the increased complexity of the damaged site.

Introduction

Psoralens are a family of compounds of plant and synthetic origin that have had extensive clinical applications in dermatology (*1–4*) and many experimental applications as probes for DNA, RNA, and chromatin structure (*5–10*). They form specific covalent adducts with DNA pyrimidines after illumination with 360 nm light (*11–14*). Some psoralens, such as trimethylpsoralen and 8-methoxypsoralen, can form both monoadducts and DNA–DNA crosslinks, whereas others, such as angelicin and methylisopsoralen, can form only monoadducts (*12,15–17*). A major monoadduct consists of a psoralen molecule intercalated into DNA and covalently attached to the 5,6 positions of thymine, forming a cyclobutyl linkage (*11*). This structure is similar to the cyclobutyl pyrimidine dimer formed by irradiation of DNA with short-wave ultraviolet (UV) light. We have therefore used psoralen monoadducts and UV-induced pyrimidine dimers as two closely similar, naturally occurring lesions in DNA to highlight the common general features underlying DNA damage and repair in human cells. In this report we emphasize new results obtained using mainly 5-methylisopsoralen, which forms only monoadducts, and compare then with current knowledge about pyrimidine dimers from UV light.

Materials and Methods

Human fibroblasts of various kinds were obtained from skin biopsies or from the Human Genetic Mutant Cell Bank (Institute for Medical Research, Camden, NJ). These included various normal fibroblasts, normal SV40-transformed cells (GM637), xeroderma pigmentosum (XP) fibroblasts of various complementation groups, and an SV40-transformed XP cell line of complementation group A (XP12RO). Cells were grown in Eagle's minimal essential medium with 10% fetal calf serum and antibiotics in 5% CO_2 at 37°C. For most experiments, cells were first grown in [^{14}C]thymidine ([^{14}C]dThd, 0.01 μCi/mL; specific activity, 55 mCi/mole) to ensure that the DNA was uniformly ^{14}C-labeled. The medium was replaced with fresh unlabeled medium 1 to 2 days before cells were used for experiments.

Cultures were irradiated with UV light (254 nm, 1.3 J/m^2/sec, calibrated by a YSI radiometer model 52) or

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exposed to unlabeled 5-methylisopsoralen (5-MIP) at various concentrations in phosphate-buffered saline, and irradiated on ice with 360 nm light (total dose, 4.5×10^4 J/m^2 in 30 min.). When labeled psoralens were used, cultures were trypsinized, resuspended in phosphate-buffered saline containing 2 to 20 μCi of various generally labeled psoralen derivatives (4,5,6-trimethylpsoralen, 17.6 Ci/mole; 8-methoxypsoralen, 2.2 Ci/mole; 5-MIP, 6 Ci/mole; HRI Associates, Inc., Emeryville, CA) and exposed to 360 nm light for 10 to 30 min (22.5 J/m^2/sec). For some samples, the DNA was isolated, digested to nucleosides by a combination of nucleases (*18*) and analyzed by high-performance liquid chromatography. The toxicity of 5-MIP plus 360 nm light was determined by the method of Kantor et al. (*19*) for UV light. Under our conditions, the survival after exposure to 10 μCi/mL 5-MIP plus 30 min of 360 nm light was 60% for normal cells and 10% for XP12RO cells. This dose therefore represents one at which there is considerable cell survival. From the specific activity of DNA isolated from cells exposed to 5-MIP, we estimate that exposure to 2 and 20 μCi/mL plus 30 min of 360 nm light produced adduct frequencies of 5 and 50 per 10^7 nucleotides, respectively.

To determine the distribution of labeled psoralen adducts in nuclease sensitive regions of chromatin, cell suspensions were washed, and nuclei were isolated and then digested for various times with staphylococcal nuclease, as previously described (*20*). The fractions of [^{3}H]psoralen adducts and [^{14}C]dThd label solubilized were calculated as previously described (*20*).

To study the effect of cell growth on the distribution of labeled psoralen adducts, some cultures were seeded into normal medium and allowed to grow for up to 24 hr. For some experiments, the medium was supplemented with inhibitors: aphidicolin (3×10^{-6} M), cycloheximide (3.5×10^{-5} M), 3-aminobenzamide (10^{-3} M), actinomycin D (4×10^{-7} M), or α-amanatin (10^{-6} M). Cultures were harvested, and nuclei were isolated and digested with staphylococcal nuclease as described.

Repair was assayed by determining both the loss of [^{3}H]psoralen adducts from purified DNA as a function of time in culture, and the rate at which single-strand breaks accumulated in cells when excision repair was inhibited by cytosine arabinoside or aphidicolin. The loss of adducts was determined from the specific activity of DNA isolated from cells harvested at various times after exposure to [^{3}H]5-MIP. To inhibit repair, cultures exposed to unlabeled 5-MIP or UV light were grown for various times with hydroxyurea (2 mM), cytosine arabinoside (20 μM), or aphidicolin (20 μM). Cells were rinsed with SSC (0.15 M sodium chloride, 0.015 M sodium citrate), scraped into 0.5 to 1.0 mL of SSC, irradiated with 10 Gy of X-rays (300 kVp Maxitron), and layered onto a 0.5-mL alkaline lysis solution (0.1 M NaOH, 0.02 M Na_2EDTA) on top of 30-mL preformed 5 to 20% alkaline sucrose gradients. Sedimentation was started immediately and continued for 4 hr at 25,000 rpm, after which fractions were collected and processed for determination of ^{3}H and ^{14}C radioactivity, as described previously (*21*). Number- and weight-average molecular weights and the number of breaks accumulated per control DNA molecule (approximately 2×10^8 daltons) were calculated. In these calculations, the radioactivity was summed from the minimum on the high molecular weight sides of the profiles to background on the low molecular weight side.

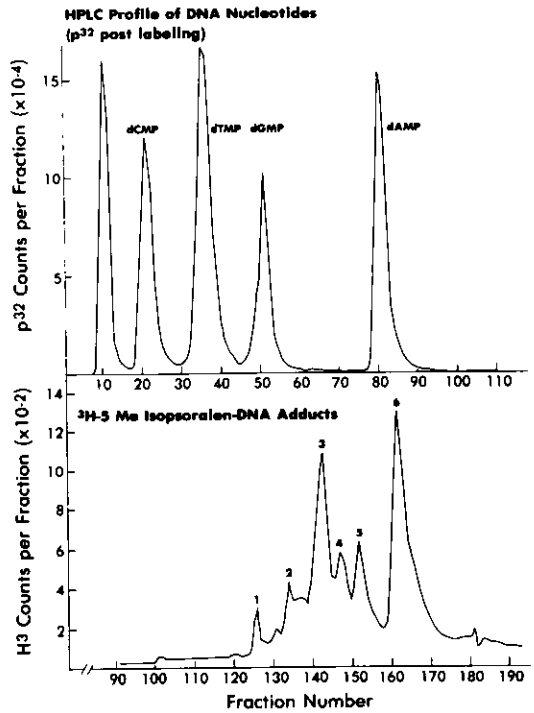

FIGURE 1. High-pressure liquid chromatographic profiles of DNA mononucleotides and [^{3}H]5-MIP adducts. Mononucleotides were eluted in the routine manner, which causes long retention of bulky aromatic psoralen adducts. After release of the nucleotides, the psoralen adducts were eluted with a low-salt buffer.

To determine the sensitivity of various adducts or pyrimidine dimers to single-strand-specific nuclease, samples of ^{3}H- and ^{14}C-labeled DNA and [^{3}H]5-MIP-bound DNA (containing approximately 0.5 adducts in 10^5 daltons, as determined from the specific activity) were precipitated with EtOH and redissolved in Sl buffer (0.3 M NaCl, 0.03 M NaAc, 3 mM $ZnCl_2$, pH 4.5). One ^{3}H-labeled DNA sample was irradiated with 39 J/m^2 UV light (254 nm), which produced 0.86 ± 0.10% thymines as dimers (approximately 0.8 dimers in 10^5 daltons). Some samples of labeled DNA were heat-denatured in a boiling water bath and then quenched in ice. Aliquots (100 μL) of the various labeled DNAs were incubated at 37°C for 30 min with Sl nuclease (1 to 1000 units/ml) (P-L Biochemicals, Milwaukee, WI). After nuclease digestion, the UV-irradiated DNA was precipitated with 5-trichloroacetic acid (5%), digested with formic acid, and analyzed for pyrimidine dimers by using one-dimensional thin-layer chromatography (*22*). All other samples were precipitated with 0.1 N HCl, and the percentage of radioactivity remaining precipitable was determined as previously described (*23*). These measurements provided data on the percentage of sin-

gle- and double-stranded DNA, 5-MIP adduct-labeled DNA, and pyrimidine dimers in DNA that were resistant to digestion by single-strand-specific nucleases.

Results

DNA Damage and Sl Nuclease Sensitivity

UV light and 5-MIP plus 360 nm light both produce a variety of major and minor products in DNA. The UV-induced products have been well characterized and include cyclobutane and azetine pyrimidine dimers and a small frequency of other lesions. Azetine dimers appear to have much greater mutagenicity and antigenicity than cyclobutane dimers, despite their numerical minority (*24–26*).

The DNA from cells exposed to 5-MIP plus 360 nm light shows up to six separate peaks in analysis by high-pressure liquid chromatography (Fig. 1). These peaks are in the process of being identified, and their relative biological importance have yet to be determined.

There is a difference in structural abnormalities generated in DNA by UV light and by 5-MIP adducts that can be detected by Sl nuclease digestion. Several previous studies have demonstrated that various forms of DNA damage create structural distortion in DNA large enough to be recognized by single-strand-specific nucleases (*27–30*). Over a wide range of concentrations of nuclease, complete digestion of single-stranded DNA was achieved with little attack of double-stranded DNA (Fig. 2). Pyrimidine dimers in double-stranded DNA were selectively removed at Sl concentrations of 100 to

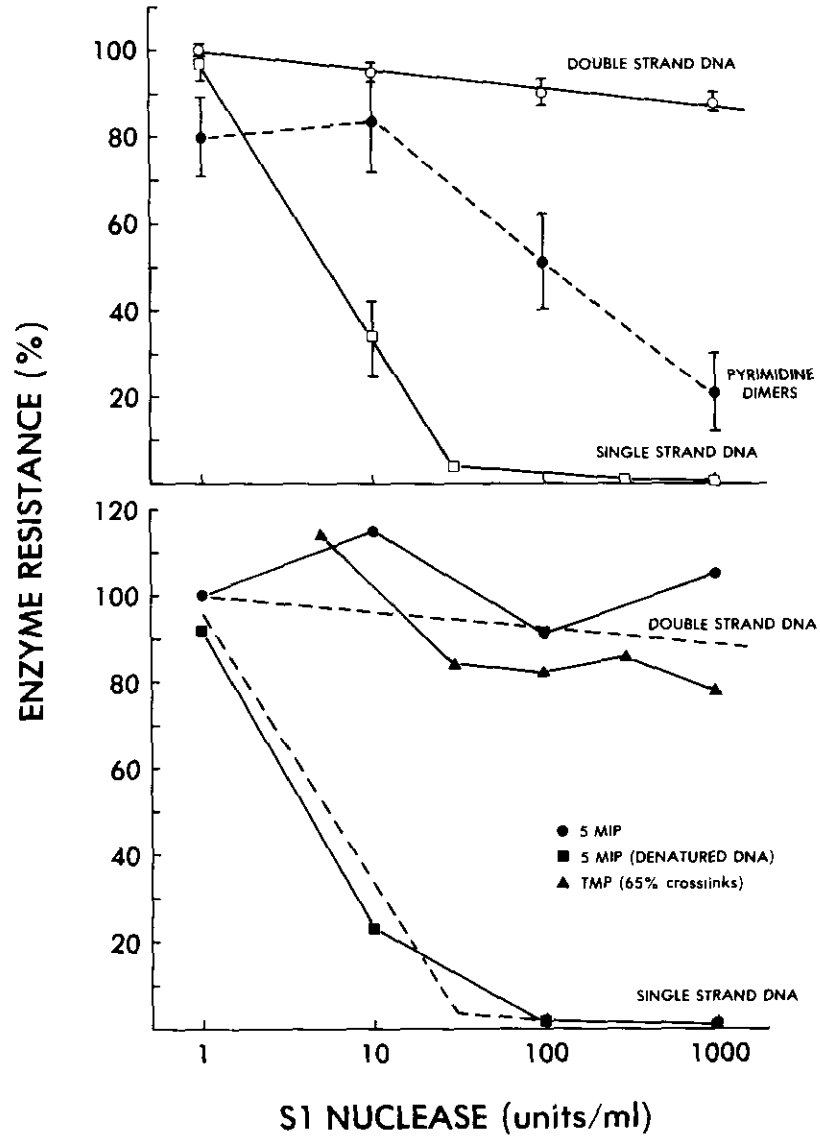

FIGURE 2. Acid-soluble nucleotides released from purified DNA incubated with various concentrations of Sl nuclease (37°C, 30 min): (top) (○) ^{14}C-labeled double-stranded DNA; (□) ^{14}C-labeled single-stranded DNA; (●) cyclobutane pyrimidine dimers; (bottom) (●) [^{3}H]5-MIP-labeled DNA; (■) [^{3}H]5-MIP-labeled DNA denatured by boiling for 10 min and quenching in ice; (▲) [^{3}H]trimethylpsoralen (crosslink frequency determined by heat denaturation followed by Sl nuclease digestion).

1000 units/mL, leaving 20% of the initial dimers and approximately 90% of double-stranded DNA, but no single-stranded DNA. The 5-MIP adducts and those from other crosslinking psoralens in double-stranded DNA were resistant to even the maximum concentration of S1 nuclease. This was not due to these adducts being refractory to nuclease attack, because they were fully digested to acid-soluble products after heat denaturation of the DNA to which they were bound. Pyrimidine dimers therefore created sufficient distortions in DNA to be substrates for S1 nuclease digestion, whereas psoralen adducts in DNA did not.

Digestion of Nuclei Containing 5-Methylisopsoralen

Cells that had been labeled with [^{14}C]dThd and exposed to [^{3}H]5-MIP plus 360 nm light were harvested, and nuclei were isolated and incubated with staphylococcal nuclease to determine the proportions of labeled DNA that were in nuclease-sensitive and -resistant regions (Fig. 3). This nuclease rapidly digests the linker regions between nucleosomes, producing DNA fragments approximately 140 base pairs long.

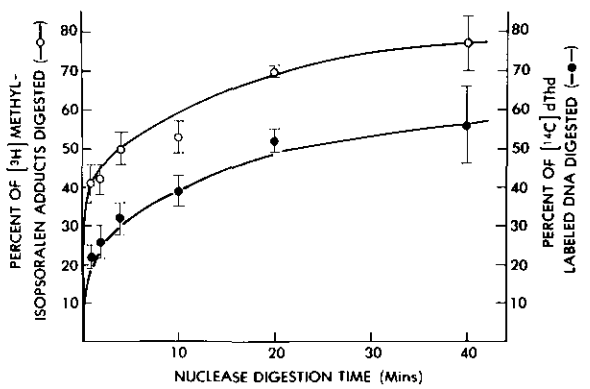

FIGURE 3. Percentage of [^{3}H]5-MIP adducts (○) and ^{14}C-labeled DNA (●) rendered soluble by micrococcal nuclease as a function of digestion time using nuclei prepared from XP12RO cells.

The digestion of ^{14}C and ^{3}H label showed two phases: an initial rapid release of acid-soluble nucleotides that was complete within 5 min, followed by a slower release at longer times. The ^{14}C curve from 10 to 40 min was extrapolated through a zero time value of approximately 30%, corresponding to the percentage of DNA in linker regions of chromatin (60 base pairs per 200). The initial release therefore represented rapid breakdown of the linker DNA, whereas the slower release represented complete destruction of core DNA. A larger percentage of [^{3}H]5-MIP adducts was released by nuclease action, indicating that these adducts are more concentrated in linker than core DNA. (Fig. 3). Similar digestion kinetics have been observed for psoralens that can form crosslinks (trimethylpsoralen, 8-methoxypsoralen) and those that form exclusively monoadducts (angelicin, 5-MIP) (*31*). These nuclease digestion data can be analyzed according to a simplified algebraic model that permits calculation of the relative proportion of [^{3}H]psoralen adducts in linker compared to core DNA (*31*). These calculations indicated that the relative proportion of [^{3}H]psoralen adducts per nucleotide was between 2 and 4 for 5-MIP and angelicin (Fig. 4). These values are much less than the previously reported value of approximately 10 for trimethylpsoralen (*5*), but this previous result was obtained at saturating levels of adduct formation. A slight trend in which increasing proportions of linker adducts are found at higher doses can be seen in the present data (Fig. 4).

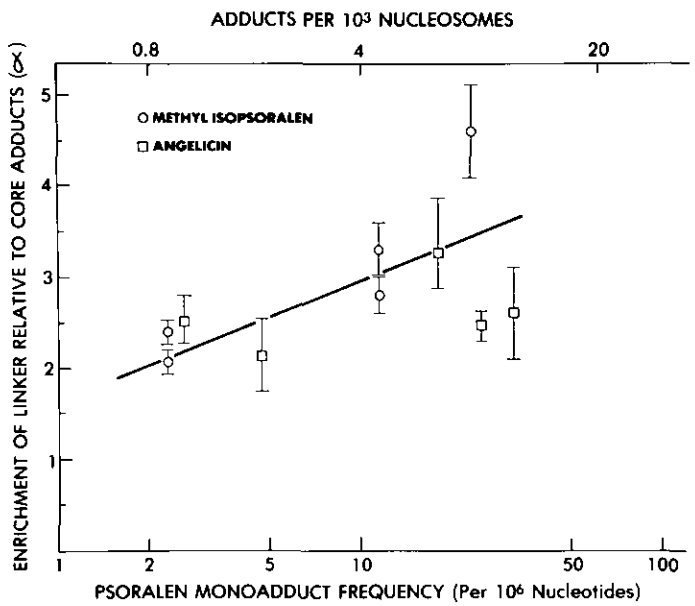

FIGURE 4. Enrichment of linker relative to core [^{3}H]5-MIP-labeled DNA and [^{3}H]angelicin-DNA adducts as a function of the total adduct frequency in DNA. Enrichment is defined as the ratio of adducts per nucleotide in linker DNA divided by the adducts per nucleotide in core DNA and can be calculated from data such as that shown in Fig. 3 using the formulae discussed previously (*31*). Reproduced by permission of Elsevier Science Publishers.

Nucleosome Movement

When cells were allowed to grow in fresh medium after exposure to 5-MIP, the proportion of [^{3}H]psoralen adducts in linker DNA declined steadily in both excision-proficient (GM637) and excision-defective (XP12RO) cells (Fig. 5). By 12 hr the initial 2.5-fold concentration of [^{3}H]5-MIP adducts in linker sites declined to a value slightly above 1.0, representing nearly complete randomization (Fig. 5). This decline was similar in excision-proficient and -defective cells and is therefore unrelated to excision of damaged sites. Instead, the steady decline reflects progressive randomization of bound adducts, which could be a consequence of a number of biochemical events. When cells were grown in inhibitors of DNA replication, RNA transcription or protein synthesis, the progressive randomization of psoralen adducts was not affected by any of these inhibitors, implying that it is independent of most of the major physiologic processes (*31*).

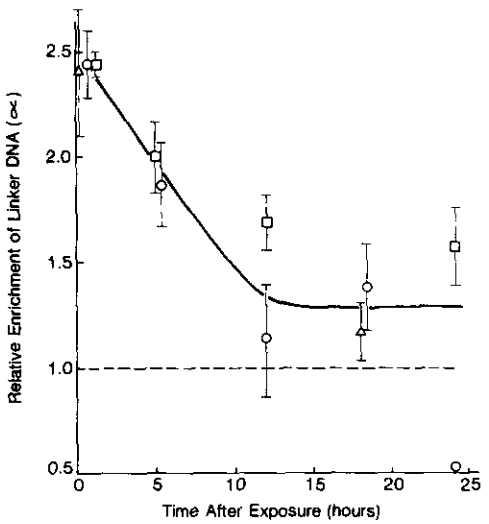

FIGURE 5. Decline of the relative enrichment of linker sites for [^{3}H]5-MIP adducts as a function of time of growth: (□) normal GM637 cells; (○) repair-deficient XP12RO cells. Reproduced by permission of Elsevier Science Publishers.

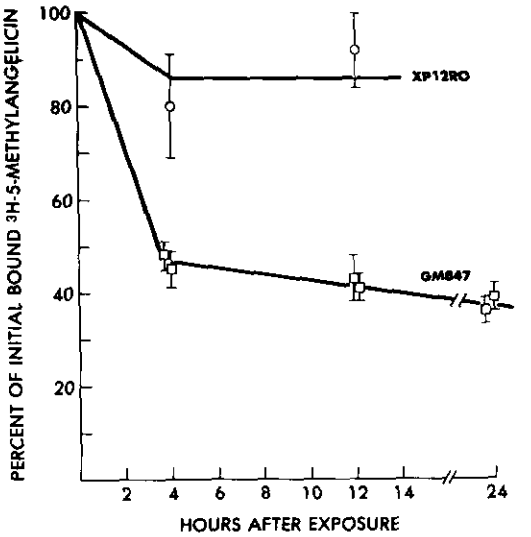

FIGURE 6. Excision of [^{3}H]5-MIP adducts from DNA as a function of time of growth after exposure: (□) normal GM847 cells; (○) repair-deficient XP12RO cells. Reproduced by permission of Williams & Wilkins.

Excision Repair of Psoralen Monoadducts

When normal (GM847) and XP12RO cells were exposed to [^{3}H]5-MIP plus 360 nm light, and then harvested after various periods of growth, normal cells showed a rapid loss of ^{3}H-labeled adducts (Fig. 6). XP12RO cells, however, appeared unable to excise these adducts, which is consistent with the well-established absence of pyrimidine dimer excision in this cell type.

Precise comparison of rates of repair of 5-MIP adducts and UV-induced pyrimidine dimers was made by blocking repair with cytosine arabinoside and determining the number of single-strand breaks in the DNA as a function of time after exposure. Breaks accumulated steadily up to 7 hr after exposure (Fig. 7). Exposure to 360 nm light (4.5×10^4 J/m^2) immediately before irradiation with UV light did not alter the number of single-strand breaks observed. This indicates that exposure to 360 nm light does not interfere with repair of UV damage over short times. At all time periods, fewer breaks were observed in 5-MIP-damaged cells than in UV-damaged cells, indicating a consistently lower rate of repair throughout the time interval. This is consistent with previous observations of lower levels of repair replication at saturating doses of 5-MIP plus 360 nm light in comparison to UV light (*32*).

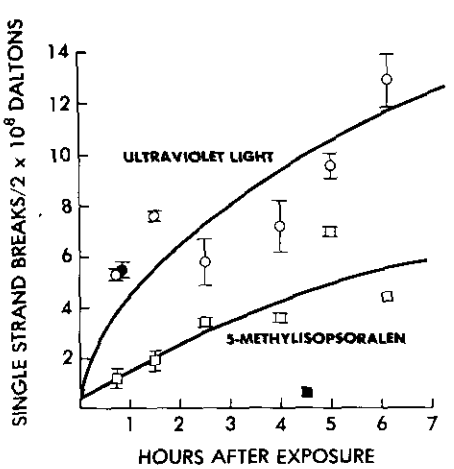

FIGURE 7. Accumulation of single-strand breaks in normal human fibroblasts exposed to UV light (13 J/m^2) or 5-MIP (10 μM) plus 360 nm light and grown for up to 6 hr in cytosine arabinoside (20 μ) and hydroxyurea (2 mM): (○) exposed to UV light; (●) exposed to 360 nm light before UV light; (□) exposed to 5-MIP plus 360 nm light; (■) exposed to 360 nm light alone. Mean and standard errors are shown.

Repair Replication in Relation to Monoadducts and Crosslinks

The psoralens provide a unique opportunity to study repair as a function of the character of DNA lesions. This is especially relevant to the use of unscheduled synthesis (or repair replication) as indicators of the amount of DNA damage. When cells are exposed to a potentially crosslinking psoralen (e.g., 8-methoxypsoralen, 8-MOP) they will attempt repair of both monoadducts and crosslinks simultaneously. If noncovalently bound psoralen is washed out of cells after an initial exposure, the proportion of crosslinks to monoadducts can be increased by a second irradiation with 360 nm light, with no net increase in the absolute frequency of lesions. This experiment was carried out in cells exposed to 8-MOP (125 μM) that were then allowed to repair after the second exposure. Analysis of repair replication after single exposures of near-UV light showed maximum repair at approximately 15,000 J/m^2 (Fig. 8). At higher near-UV doses, there was a sudden decrease in the amount of repair replication in 8-MOP-treated cells. For a split-dose protocol, cells were first irradiated in the presence of 8-MOP and then rinsed and irradiated again in phosphate-buffered saline alone to enhance crosslinking without changing the absolute number of lesions in DNA. A second irradiation with 30,000

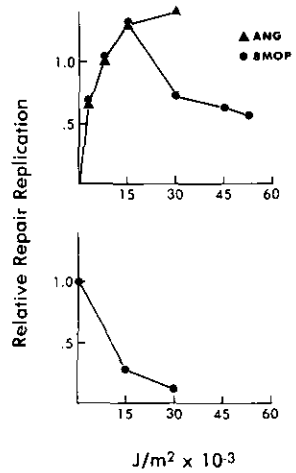

FIGURE 8. *(Top)* Repair replication in normal GM637 cells exposed to 8-MOP (125 μM) (●) or angelicin (125 μM) (▲) and exposed to increasing doses of 360 nm light. *(Bottom)* Repair replication in GM637 cells exposed to 8-MOP (125 μM) plus 360 nm (7,500 J/m²), washed, and irradiated again in phosphate-buffered saline with increasing doses of 360 nm light. Repair synthesis after a single exposure to a psoralen plus 7,500 J/m² 360 nm light was normalized to a value of 1.0 in both panels.

J/m² showed a decline in repair replication to 10% of that with exposure to 7,500 J/m² alone (Fig. 8). These observations indicate that conversion of monoadducts to crosslinks causes a reduction in repair replication.

Discussion

The damage to DNA caused by UV light is similar in many ways to that produced by psoralens plus 360 nm light. Both sets of damaged sites involve pyrimidines, and a major lesion in both sets involve the 5,6 double bond. UV light makes cyclobutane pyrimidine dimers using these bonds; psoralens are covalently added to pyrimidines by these bonds (*11*). In addition, numerous other products are also made in DNA that have different relative importance with respect to cell lethality, mutagenicity, and carcinogenicity. With UV light, the major product, the cyclobutane dimer, is of minor significance for mutagenesis, and a quantitatively minor product, the azetidine dimer, is the main mutagenic lesion (*25*). The relative biological importance of the various psoralen adducts to DNA has yet to be determined.

Despite the similarities in the DNA damage produced by UV and psoralens, our studies have highlighted a number of important differences both in the nature of the damage and in its repair. Pyrimidine dimers appeared to generate sufficient distortions in DNA to act as substrates for Sl nuclease (Fig. 2), as previously reported by a number of investigators (*27–29*). This may be due in part to the tendency for pyrimidine dimers to be formed in clusters rather than truly randomly (*33*). Both monoadducts and crosslinks from various psoralen derivatives were resistant to Sl nuclease (Fig. 2). These adducts therefore do not create distortions in the DNA large enough to be recognized by single-strand-specific nucleases. This difference between pyrimidine dimers and 5-MIP adducts is reminiscent of the study by Yamasaki et al. (*30*) in which the C-8 adduct in DNA from N-acetoxyacetylaminofluorene was sensitive to Sl digestion but the N^2 adduct was not. These different structural distortions may play a role in modifying the relative affinity of specific repair enzymes for pyrimidine dimers, psoralen adducts, and other carcinogen-DNA adducts.

Psoralens capable of forming monoadducts and crosslinks produced a higher concentration of adducts in linker compared to core regions of DNA (Figs. 4 and 5). The degree of enrichment was far less than that reported previously (*5*) because the earlier work was performed at saturating frequencies of bound psoralen adducts. Low doses that permitted high levels of cell survival (10–60%) produced only a low concentration of adducts in linker sites, but the trend to greater enrichment at higher exposure concentrations was clearly evident (Fig. 4). Assumptions about the location of most of the bound adducts must therefore be made with careful attention to the particular frequency of bound adducts produced in a given experimental situation. These results contrast with those previously observed with UV light, in which pyrimidine dimers were found randomly in different nucleosomal regions (*34*). Other carcinogens also have various relative affinities for linker and core DNA. Aflatoxin adducts concentrate at an approximately 5-fold higher frequency in linker regions that in core regions of DNA *in vivo* (*35*). Benzo(a)pyrenediol adducts concentrate in linker regions at frequencies approximately 3- to 4-fold (*36–38*) and up to 10-fold (*39*) higher. Bromomethylbenz(a)anthracene, in contrast, forms more adducts in core DNA than in linker DNA (*40*).

When cells were allowed to grow for various periods of time, the proportion of psoralen adducts that were initially in linker regions declined steadily, reflecting progressive randomization of the adducts (Fig. 5). This randomization did not appear to be caused by any of the major metabolic processes associated with DNA (e.g., replication, repair, transcription, protein synthesis). It occurred to the same extent in repair-competent and -deficient cells (*41,42*) and therefore cannot be due to preferential excision of adducts from the nuclease-sensitive regions (Fig. 5). Instead, these results imply that excision of [^{3}H]5-MIP adducts must occur at similar rates from linker and core DNA in normal cells. Otherwise, the decrease in ^{3}H-labeled adducts from linker DNA would have been more rapid in normal cells than in excision-defective cells, because excision of over half the adducts occurred in normal cells over the time course of the randomization (Fig. 6). The excision of pyrimidine dimers similarly occurs at the same rate from different regions of chromatin (*34*), as does excision of 7-bromomethylbenz(a)anthracene adducts (*40*).

Observations of a slow movement of chromatin are in contrast to the much more rapid conformational changes associated with DNA repair (*31,43,45–48*) and DNA replication (*49,50*). Typically, the half-time for changes in sensitivity of repaired sites to staphylococcal nuclease is of the order of 5 to 10 min, in contrast to several

hours for the movement detected with psoralen adducts.

In general, the repair of damage from UV light and from 5-MIP plus 360 nm light have a close resemblance. Repair shows similar dose-response relationships, a similar major role for DNA polymerase alpha revealed by inhibition of repair by aphidicolin and cytosine arabinoside, and similar relative degrees of excision-repair deficiency found in xeroderma pigmentosum groups A, C, and D (*51,52*). A reasonable assumption, therefore, is that the same repair system acts on both UV- and 5-MIP-induced damage. Some subtle differences do exist, especially the lower maximum amounts and rates of repair seen at high doses of 5-MIP compared to UV light (Fig. 7) (*32,52*).

Differences in distribution of dimers and 5-MIP adducts in chromatin are not likely to cause the different extents of repair. Pyrimidine dimers (*34*) and psoralen adducts (*31*) (Fig. 5) have been shown to be excised randomly, independent of their nucleosomal location. Instead, these suggest that the same repair system operates on pyrimidine dimers at about twice the efficiency with which it operates on 5-MIP adducts. The underlying mechanisms could involve differences in the efficiency of recognition, binding constants, or rates of endonucleolytic cleavage. Our results with single-strand-specific nucleases bear on these possibilities (Fig. 2). Pyrimidine dimers create sufficient distortion in DNA to be recognized by S1 nuclease, whereas psoralen adducts do not. Pyrimidine dimers may therefore generate a large signal for recognition by repair enzymes or associated single-strand-specific binding protein.

DNA–DNA crosslinks involving psoralen molecules, however, appear to generate a different response compared to the same frequency of monoadducts (Fig. 8). Crosslinks appear to be repaired much less efficiently than monoadducts, and much less repair replication is observed over similar labeling periods. The amount of label incorporated by repair replication (or unscheduled synthesis) in this situation is much more strongly dependent on the molecular structure of the DNA adducts than on their mere number.

These studies highlight a series of comparative observations on the localization in DNA and chromatin and the repair of a number of lesions of human importance. Extension of the observations with psoralen adducts to situations involving human exposure during psoralen-UVA therapy is planned for the near future.

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