# Rapid repair kinetics of pyrimidine(6-4)pyrimidone photoproducts in human cells are due to excision rather than conformational change

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## ABSTRACT

**UV-induced pyrimidine(6-4)pyrimidone photoproducts in DNA of mammalian cells are apparently repaired much more rapidly than cyclobutane dimers. Since only immunological assays for (6-4) photoproducts have been sensitive enough for repair measurements, it was possible that these apparently rapid repair kinetics reflected a change in physical conformation of antibody-binding sites, resulting in epitope loss rather than excision. To discriminate between these possibilities, we developed a procedure to photochemically convert (6-4) photoproducts to single-strand breaks in UV-irradiated DNA with a background low enough to permit repair measurements. Analysis of a specific DNA sequence indicated that photoinduced alkali-labile sites (PALS) were induced with the same site-specificity as (6-4) photoproducts. Normal human and xeroderma pigmentosum (XP) variant cells rapidly excised (6-4) photoproducts measured as PALS, but little repair was seen in cells from XP complementation group A. These repair kinetics corresponded to those determined in the same samples by radioimmunoassay of (6-4) photoproducts. Thus we conclude that the rapid repair of (6-4) photoproducts observed in UV-irradiated human cells is not the result of a conformational change resulting in epitope loss, but reflects excision of this photoproduct from DNA.**

## INTRODUCTION

Sunlight-induced human skin cancers are likely caused by DNA damage produced by the UV portion of the solar spectrum. However, the heterogeneous nature of UV damage to DNA has limited the understanding of initial molecular events in sunlight carcinogenesis. Studies of the biological effects of the cyclobutane pyrimidine dimer in DNA of human cells have been facilitated by the relative abundance of this photoproduct and by its specific recognition and cleavage *in vitro* by UV endonucleases such as the *den*V gene product of bacteriophage T4. These factors have allowed analysis of cyclobutane dimer induction and repair in human cells irradiated with low UV fluences, demonstrating that cells derived from patients suffering from the cancer-prone inherited disorder xeroderma pigmentosum (XP) are deficient in excising cyclobutane dimers. The pyrimidine cyclobutane dimer is thus considered an important determinant of the lethal, mutagenic, and carcinogenic effects of UV light [1].

Recent evidence has suggested that another type of UV-induced DNA damage, the pyrimidine(6-4)pyrimidone photoproduct, or (6-4) photoproduct, may play a significant role in mutagenesis and in inducing human skin cancer [2,3]. Various techniques have been applied to quantify (6-4) photoproduct induction and repair in cells cultured from normal individuals or those with skin cancer predisposition syndromes [4,5], but only immunological assays have possessed the sensitivity required for DNA repair measurements in UV-irradiated mammalian cells [6–9]. The removal of antibody-binding sites associated with (6-4) photoproducts is very efficient, with 80% lost in the first 3 hours after UV irradiation [10]. These kinetics of apparent (6-4) photoproduct repair are an order of magnitude greater than observed for repair of cyclobutane dimers measured as the removal of UV endonuclease-sensitive sites (ESS) [10, 11]. UV hypersensitive human XP and several mutant hamster cell lines have been shown by radioimmunoassay (RIA) to be deficient in removal of both (6-4) photoproducts and cyclobutane dimers [7,12,13]. Although extremely sensitive, the RIA has two inherent limitations: 1) as a competitive binding assay, it measures relative rather than absolute numbers of binding sites in DNA, and 2) structural modification of the binding site (*i.e.*, the (6-4) photoproduct) may render it unrecognizable by antibody, resulting in a false inference of (6-4) photoproduct excision and removal. Thus, although a large body of data has been collected using RIA to measure (6-4) photoproduct repair in a variety of cell types [14], interpretation of these data must take into account the caveat that these measurements may represent modification of the (6-4) photoproduct, resulting in loss of the epitope recognized by RIA, rather than actual excision.

To address these limitations, we have developed in the current investigation a photochemical method for determining (6-4)

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photoproducts in DNA of mammalian cells irradiated at low UV fluences. In the studies presented here, we have used this method to analyze the excision repair of (6-4) photoproducts in human cells. Since an earlier study suggested that 'photolysis' of the (6-4) photoproduct by wavelengths of light near its absorbance maximum (315–320 nm) produced a secondary photoproduct rather than photo- or photoenzymatic reversal [15] , the photochemical properties of this secondary photoproduct were of interest. High-performance liquid chromatography (HPLC) of UV-irradiated pyrimidine dinucleotides was used to isolate a photoproduct resulting from the secondary UVB irradiation of the Thy(6-4)Thy photoproduct [16], and its structure was identified as a Dewar pyrimidinone produced by photoisomerization [17]. A rabbit antiserum raised against DNA containing this photoproduct [18] revealed that the sites recognized by this antibody were labile to mild alkaline hydrolysis (0.1 M NaOH at 23 °C) [19]. With this knowledge, we anticipated the feasibility of converting (6-4) photoproducts to alkali-labile sites that could be manifested as single-strand breaks (SSB) in DNA under mild conditions of alkaline hydrolysis. Like UV-induced ESS, these breaks could then be measured by conventional techniques such as agarose gel electrophoresis of supercoiled molecules or sedimentation of high molecular weight DNA extracted from mammalian cells on alkaline sucrose gradients. Photoinduced alkali-labile sites (PALS) were indeed induced in DNA with the same site specificity as (6-4) photoproducts and were rapidly excised in XP variant and repair proficient human cells, but not in XP complementation group A cells. The kinetics of PALS removal in these human cell lines corresponded to those determined for (6-4) photoproduct repair by RIA of the same samples.

## MATERIALS AND METHODS

### Cell lines and culture conditions

XP group A (XP12RO) and variant (XP4BE) cell lines were purchased from the American Type Culture Collection (Rockville, MD), and a skin fibroblast cell line used as the normal control (AG1518A) was purchased from the Institute for Medical Research (Camden, NJ). All cell lines were maintained as monolayer cultures at 37 °C in a humidified atmosphere of 5% $CO_2$/95% air in a-modified Eagle's minimal essential medium (Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, penicillin (50 μg/ml) and streptomycin (50 units/ml).

### DNA isolation

Human cells were prelabeled for 2–3 days in medium containing 0.1 μCi/ml [$^{14}$C]thymidine (61 mCi/mmol) (Amersham, Arlington Heights, IL) or 0.3 μCi/ml [$^{3}$H]thymidine (25 Ci/mmol) (Amersham). Subconfluent cultures containing $^{14}$C-labeled DNA were UV irradiated and either harvested immediately or incubated for 6 h. Each cell lysate from irradiated samples was combined with cell lysate from unirradiated, [$^{3}$H]thymidine labeled cells prior to further DNA purification. Samples were incubated overnight at 37 °C in a solution containing 10 mM Tris-HCl (pH 7.8),1 mM EDTA, 0.5% sodium dodecyl sulfate, 0.2 mg/ml proteinase K (Boehringer Mannheim, Indianopolis, IN), extracted with 0.1 M Tris-HCl (pH 8)-saturated phenol followed by chloroform:isoamyl alcohol (24:1), and then precipitated from 70% ethanol after addition of 0.1 volume 3.3 M sodium acetate. DNA precipitates were collected by centrifugation at 4 °C in a swinging bucket rotor at 15,900×$g$ for 30 min and then washed by centrifugation with cold 70% ethanol. For determinations of (6-4) photoproduct repair, about 60–100 μg DNA was resuspended in 1 mM Tris-HCl (pH 7.8), 0.1 mM EDTA.

Plasmids pUC19 and p1N2D3D4H were purified by a standard cleared-lysate method [20]. Plasmid DNA was diluted in $H_2O$ prior to UV irradiation.

### UV irradiation and photoisomerization

Light sources used for irradiating cells and DNA samples were provided either by two GE 15T8 germicidal lamps emitting predominantly 254 nm light (General Electric, Cleveland, OH), or by four Westinghouse FS20 fluorescent sunlamps (Westinghouse Electric, Somerset, NJ) filtered through Mylar 500D (Du Pont, Wilmington, DE) (cutoff 320 nm and below). Emission spectra for the UVB light source with Mylar filters have been published [21]. The Mylar-filtered sunlamp was used in preference to other UV sources, such as 313-nm monochromatic light, since its emission spectrum overlaps the (6-4) photoproduct photoisomerization action spectrum without significantly overlapping action spectra for photoproducts induced by shorter wavelengths [18]. Previous RIA data indicated that 36 kJ/m$^2$ (equivalent to 200 kJ/m$^2$ measured at the peak wavelength of 320 nm) from the Mylar-filtered sunlamp (measured at 365 nm) removed greater than 90% of the binding sites associated with (6-4) photoproducts while inducing negligible levels of cyclobutane dimers [18]; this fluence was produced by 2–3 h exposures of DNA at 10–15 cm from sunlamps.

### Alkaline hydrolysis

Plasmid DNA was incubated in 45 μl 50 mM Tris, 0.4 M NaOH (pH 13.5) at a concentration of 45 μg/ml for 18–20 h at 23 °C in the dark. Immediately prior to gel electrophoresis, samples were neutralized with HCl. The alkali concentration required to manifest PALS with minimum nonspecific breakage was determined and found to lie between 0.2 and 0.6 M NaOH, with an optimum at 0.4 M NaOH (pH 13.5). Human cell DNA was treated with the same concentration of NaOH but was incubated for a shorter time (2–3 h) prior to layering, without neutralization, on 0.1 M NaOH alkaline sucrose gradients prepared as described by Clarkson and Hewitt [22].

### UV endonuclease digestion

UV-irradiated form I (supercoiled) pUC19 and purified human cell DNA were incubated with excess UV endonuclease from *Micrococcus luteus* (Applied Genetics, Freeport NY) in 50 mM Tris-HCl (pH 8.0), 10 mM NaCl, 1 mM EDTA for 2 h at 37 °C prior to gel electrophoresis or alkaline sucrose gradient sedimentation.

### Agarose gel electrophoresis

After treatment, form I and form II DNA conformers were separated by electrophoresis overnight at 20–30 V in 0.8% agarose gels in 40 mM Tris (pH 7.8), 20 mM sodium acetate, 10 mM EDTA (TAE) electrophoresis buffer containing 0.5 μg/ml ethidium bromide [20]. The corresponding form I$^{alk}$ and form II$^{alk}$ bands in DNA samples treated with alkali and then neutralized were resolved by electrophoresis for 4–5 h at 100 V in TAE agarose gels. After electrophoresis, the gels were photographed using Polaroid type 55 film and negatives were scanned at 700 nm using a Zeineh Soft Laser scanning densitometer (Biomed Instruments, Fullerton, CA). Peaks

representing form I and form $I^{alk}$ were integrated and the percentage of unnicked molecules remaining after UV irradiation and cleavage with UV endonuclease or treatment with 0.4 M NaOH was determined.

To confirm identities of the various gel bands, double- and single-stranded DNA conformations were distinguished by green and red fluorescence, respectively, after staining with acridine orange. After gel electrophoresis in the presence of ethidium bromide, untreated and alkali-treated form I and II DNAs were destained overnight in dilute phosphate buffer; the gel was restained with acridine orange (30 μg/ml) for 30 min and then destained for several hours in dilute phosphate buffer prior to reexamination of fluorescence following the procedure of McMaster and Carmichael [23].

### Sequence-specific determination of (6-4) photoproducts

To measure photoproducts at individual nucleotide base pairs, plasmid p1N2D3D4H [24] containing a c-K-*ras* minigene was digested with restriction enzymes at unique *Cla*I and *Sal*I sites and 3′ end-labeled at the *Cla*I site using the Klenow fragment of DNA polymerase I and [$^{32}$P]TTP [25]. The 2 kilobase pair (kb), singly end-labeled fragment was purified and UV irradiated as described above. To detect the presence of cyclobutane pyrimidine dimers at individual base pairs, end-labeled DNA was resuspended in 15 μl of reaction buffer containing 10 mM Tris-HCl (pH 8), 10 mM EDTA, 100 mM NaCl, 1 mg/ml bovine serum albumin, and 1 μl of T4 *den*V endonuclease (T4 *den*V endonuclease was a gift of Dr. A. Ganesan; 1 μl incised $2\times10^{12}$ cyclobutane dimers/min at 37 °C). The DNA was incubated for 15 min at 37 °C, phenol extracted, and ethanol precipitated.

To detect pyrimidine(6-4)pyrimidone photoproducts, end-labeled DNA samples were incubated with 0.1 M piperidine at 90 °C for 20 min and then lyophilized. To detect PALS, samples irradiated with UVB light were incubated or not further incubated in alkali reaction conditions as described above; unirradiated DNA was used in control experiments. Samples (100 μl volumes) also contained 10 mM EDTA and 1 μg salmon sperm DNA. In some experiments, samples were irradiated with 100–300 kJ/m$^2$ 313-nm light from a monochromator (J-Y Optical Systems, Methuchen, NJ). Alkali-treated samples were neutralized and precipitated. Samples were electrophoresed on 8% acrylamide sequencing gels with Maxam-Gilbert G+A and C+T sequencing reactions as markers.

### Analysis of repair on alkaline sucrose gradients

Alkaline sucrose gradients were prepared as described [22]. Samples (0.2 ml each) were carefully layered on gradients which were then centrifuged in a Beckman SW27 swinging bucket rotor at 17,000 rpm using an integrator to determine the $w^2$ t of $1.72\times10^9$ (approx. 14.5 h). Fractions of 1.2 ml were collected from the top by siphoning through a Buchler Densiflow apparatus (Haake Buchler Instruments, Saddle Brook, NJ). Radioactivity was measured after the addition of 10 ml Scintiverse II (Fisher Scientific, Pittsburgh, PA), containing 0.06% acetic acid, using a $^3$H/$^{14}$C dual-label program on the LKB Model 1209 Rackbeta scintillation counter (LKB Diagnostics, Gaithersburg, MD). The weighted mean of each profile was calculated and weight-averaged molecular weights were determined by reference to the sedimentation properties of bacteriophage lambda DNA using the formula $D_1/D_2 = (M_1/M_2)^{0.4}$, where $M_1$ and $D_1$ are the molecular weights and weighted mean fractions of lambda DNA and $M_2$ and $D_2$ represent these values for sample DNA [26]. Induction of SSB were calculated according to the methods described by Brash [27].

### Radioimmunoassay

Preparation of the antiserum and details of the assay have been published previously [11].

## RESULTS

### Analysis of Photoinduced Alkali-Labile Sites in UV-Irradiated Plasmid DNA

We anticipated that the alkali treatment would also alter the conformation of supercoiled plasmid DNA molecules and thus their electrophoretic mobility after neutralization. Therefore, prior to analysis of UV-irradiated plasmid DNA samples by photoisomerization and alkali treatment, we measured SSB induction by *M. luteus* UV endonuclease with pUC19 DNA containing cyclobutane pyrimidine dimers and then examined the effect of alkaline denaturation. As expected, UV-irradiated form I pUC19 DNA (2686 bp) was converted to open circular form II DNA in a dose-dependent manner by cleavage with the cyclobutane dimer-specific UV endonuclease from *M. luteus* (Fig. 1A, lanes 1–8). The loss of form I DNA with increasing UV fluence was linear on a semilogarithmic plot (Fig. 1B, open triangles). The $D_{37}$ value calculated from this curve indicated that, on average, one ESS was induced per plasmid molecule after irradiation with 23 J/m$^2$ 254-nm light. From the size of the plasmid and the $D_{37}$ value, the induction rate was calculated to be 1 ESS/2686 bp$\times$660 Da $\cdot$ bp$^{-1}$/23 J $\cdot$ m$^{-2}$ = 2.45 ESS/10$^8$ Da/J $\cdot$ m$^{-2}$. We obtained similar values using larger plasmids (data not shown), and these results agreed as well with frequencies of cyclobutane dimer induction previously determined for plasmid pSV2catSVgpt [28].

Alkali treatment and neutralization of form I pUC19 DNA produced a new band, form $I^{alk}$, migrating with slightly increased electrophoretic mobility (Fig. 1A, *cf* lanes 1, 9). Similar treatment of UV endonuclease-nicked plasmid DNA resulted in a form $II^{alk}$ band migrating slightly faster than form $I^{alk}$ DNA (Fig. 1A, lanes 9, 10), although with noticeably increased electrophoretic mobility compared with its untreated counterpart (lane 8). To unambiguously identify the gel bands corresponding to alkali-treated forms I and II plasmid DNA, UV-irradiated form I pUC19 DNA was digested with UV endonuclease and the resulting form II band was separated by gel electrophoresis and purified by electroelution. Isolated form I and II DNA samples were then treated overnight with 0.4 M NaOH (pH 13.5), neutralized with equimolar amounts of HCl, and electrophoresed alongside untreated samples in a 0.8% TAE agarose gel. Ethidium bromide was removed by washing, and the gel was restained with acridine orange. Untreated form I DNA (electrophoresis pattern as in lane 1, Fig. 1A) fluoresced green, and untreated form II DNA (as in lane 8) was red with green highlights; alkali-treated forms $I^{alk}$ (lane 9) and $II^{alk}$ (lane 10) both fluoresced red (data are not shown). These results unambiguously identified DNA bands and indicated that: 1) form I DNA was primarily double-stranded as stained in these gels; 2) the nicked form II DNA had a significant single-stranded component in these conditions of electrophoresis and acridine staining that determined substantial red fluorescence; and 3) both DNA conformers treated with alkali were primarily single-stranded even after neutralization, and exhibited altered electrophoretic mobilities.

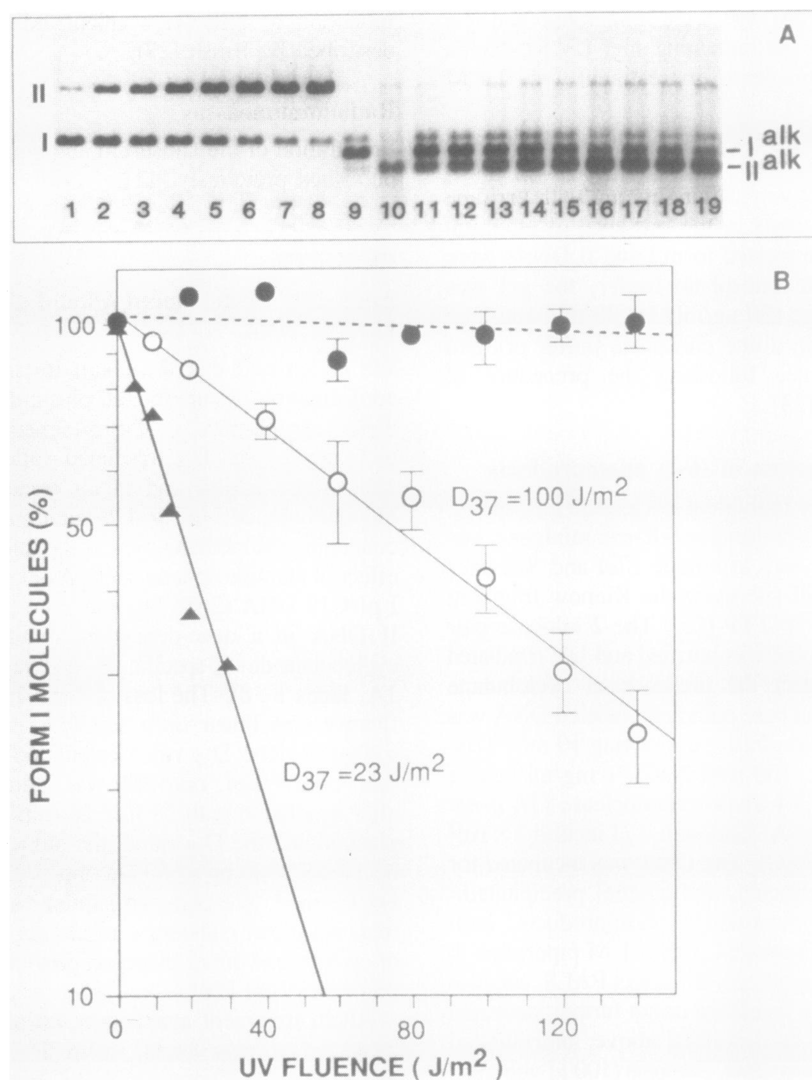


**Fig. 1.** Induction of ESS and PALS in pUC19 DNA by 254-nm light irradiation.
*A*, Lanes 1–8, results of TAE agarose gel electrophoresis of pUC19 DNA that was 254-nm light-irradiated with increasing UV fluences and treated with *M. luteus* UV endonuclease as described in the text. Lane 9, supercoiled pUC19 DNA that was treated overnight at 23 °C with 0.4 M NaOH and neutralized immediately prior to electrophoresis. Lane 10, nicked pUC19 DNA that was treated overnight at 23 °C with 0.4 M NaOH and neutralized immediately prior to electrophoresis. Lanes 11–19, pUC19 DNA that was 254-nm light-irradiated, reirradiated with 36 kJ/m$^2$ Mylar-filtered sunlamp UVB, treated overnight at 23 °C with 0.4 M NaOH and neutralized immediately prior to electrophoresis. *B*, Loss of form I pUC19 DNA (determined by soft laser scanning as described in the text) as a function of initial 254-nm light irradiation: DNA that was irradiated with 254-nm light and then treated overnight at 23 °C with 0.4 M NaOH, and neutralized immediately prior to electrophoresis (●——● ); 254-nm light-irradiated DNA that was reirradiated with 36 kJ/m$^2$ sunlamp UVB light, treated overnight at 23 °C with 0.4 M NaOH and neutralized immediately prior to electrophoresis (data from lanes 11–19 in Fig. 1A, ○——○); 254-nm light-irradiated DNA that was treated with *M. luteus* UV endonuclease as described in the text and electrophoresed without any subsequent treatment (data from lanes 1–6 in Fig. 1A, ▲——▲).

As for the form I band, loss of the form $I^{alk}$ band could be quantified by densitometry of gel photographs and thus the number of *M. luteus* UV-induced ESS in UV-irradiated pUC19 DNA could be measured after alkali treatment, neutralization, and gel electrophoresis. The ESS dose-response curve derived from measurements of the loss of the form $I^{alk}$ band after incubation with *M. luteus* UV endonuclease, followed by alkali and neutralization treatments of UV-irradiated pUC19 DNA, closely resembled that for UV-irradiated form I DNA treated with UV endonuclease but not subsequently treated with alkali (data not shown).

From data represented in Fig. 1B, it is evident that negligible numbers of sites sensitive to prolonged incubation at pH 13.5 were induced in pUC19 DNA by low fluences of 254-nm light alone (*i.e.*, without UV endonuclease treatment) over a range of UV fluences up to 140 J/m$^2$. However, when these same samples were also irradiated with high fluences of sunlamp UV prior to alkali treatment and neutralization, the amount of form $I^{alk}$ DNA migrating in gels decreased exponentially with UV fluence (Fig. 1A, lanes 11–19; Fig. 1B, closed circles). The sunlamp treatment thus produced alkali-labile sites from photoproducts induced by low fluences of UVC light. The $D_{37}$ value determined from a dose-response curve, based on analysis of a large number (*i.e.*, $n = 72$) of individual samples, yielded an induction rate of 0.56 PALS/$10^8$ Da/J · m$^{-2}$, a rate that was 23% the frequency of cyclobutane dimers induced by 254-nm light irradiation of pUC19 DNA.

In Fig. 2 the loss of form $I^{alk}$ DNA irradiated with 100 J/m$^2$ 254 nm UV light is plotted against increasing fluence of FS20 sunlamp radiation filtered through Mylar 500D. The production

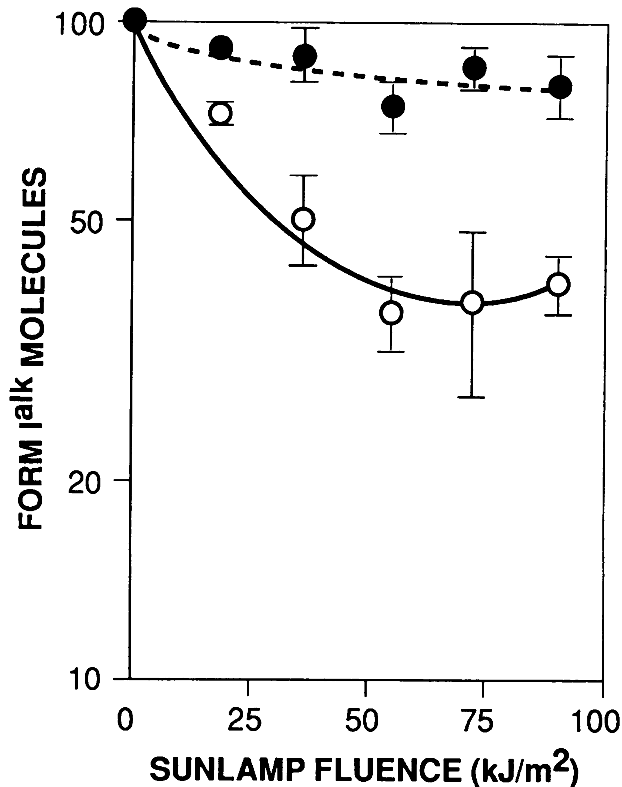


**Fig. 2.** Production of PALS in 254-nm light-irradiated pUC19 DNA by subsequent irradiation with sunlamp UVB light. Plasmid pUC19 DNA irradiated with 100 J/m$^2$ 254-nm light (○——○), or unirradiated (●——●), was next irradiated with increasing fluences of Mylar-filtered sunlamp UVB light, incubated overnight at 23 °C with 0.4 M NaOH, and neutralized immediately prior to electrophoresis on TAE agarose gels. Loss of form I DNA was determined by soft laser scanning of photographic negatives of gels as described in the text.

of alkali-labile sites by sunlamp UV was dose-dependent up to 40 kJ/m$^2$ 365-nm UV light; beyond this dose, few additional sites were produced. In the absence of 254-nm UV damage, this dose of photoisomerizing light produced few alkali-labile sites in pUC19 DNA (Fig. 2, closed circles). Thus, the PALS assay revealed SSB in 254-nm light-irradiated DNA in a manner consistent with their originating by photoisomerization of (6-4) photoproducts, with a low background level of nonspecific DNA breakage.

## Analysis of Photoinduced Alkali-Labile Sites in a Specific DNA Sequence

To identify the sites of induction of PALS, cleavages produced by this treatment were analyzed at the level of DNA sequence in the c-K-*ras* minigene sequence contained in plasmid pIN2D3D4H. First, 254 nm UV-irradiated DNA was incubated at various conditions of alkali and temperature (Fig. 3A). As previously reported (5), a 0.1 M NaOH treatment at 90 °C revealed (6-4) photoproducts (Fig. 3A, lane 2), whereas room temperature treatment did not break the DNA (Fig. 3A, lane 3). Increasing the NaOH concentration to 1 M at room temperature, however, revealed a small fraction of (6-4) photoproducts (Fig. 3A, lane 4), indicating that the alkali lability of (6-4) photoproducts depends on both alkali concentration and temperature. In contrast, when DNA irradiated with 254-nm light was reirradiated with 313-nm UVB light in order to photoisomerize (6-4) photoproducts to Dewar isomers, room temperature alkali treatment caused extensive DNA cleavages specifically at sites of (6-4) photoproducts (Fig. 3A, lane 5), even in excess of that generated by treatment with 0.1 M NaOH at 90 °C. In these conditions, even very rare T< >T and C< >T (6-4) photoproducts were apparent.

The specificity of the photoisomerization/alkali treatment was next investigated using filtered sunlamp UVB light and 0.4 M NaOH (Fig. 3B). In the absence of alkali treatment, neither 254-nm UV light, sunlamp UVB irradiation, nor 254-nm plus sunlamp irradiation of DNA led to specific DNA cleavage (lanes 1–3). (The gel is overexposed to emphasize faint bands; the nonspecific breakage in each lane results from roundtrip interstate shipping of end-labeled DNA.) Even in the presence of alkali, neither 254-nm nor sunlamp UVB irradiation generated cleavage at (6-4) photoproducts (Fig. 3B, lanes 8 and 9). However, when photoisomerizing sunlamp UVB light was administered after 254-nm irradiation, room temperature alkali treatment induced cleavage bands (Fig. 3B, lane 10) at the same sites and with the same relative intensities as with the 90 °C piperidine treatment (Fig. 3B, lane 7), although at only approximately one-third the frequency. The close correlation of cleavage sites in this sequence suggests that the photo-labile lesion is equivalent to the hot alkali-labile lesion previously identified as a (6-4) photoproduct on sequencing gels [5].

When photoisomerization was followed by alkali treatment at 90 °C rather than at room temperature (Fig. 3B, lane 11), the intensities of DNA cleavage bands increased on the order of 20-fold. These intensities exceeded those generated by hot piperidine treatment (lane 7) and also revealed T< >T and C< >T (6-4) photoproducts. The intensity of (6-4) photoproduct bands identified by this method was comparable to the intensity of cyclobutane pyrimidine dimer bands determined as ESS (Fig. 3B, lane 6).

## Repair of Photoinduced Alkali-Labile Sites in Human Cells

Representative alkaline sucrose gradient profiles showing the induction and repair of PALS in DNA of normal and UV-sensitive human cells are shown in Fig. 4. The number of SSB per $10^8$ Da is shown in Table 1. The first panel in each row of Fig. 4 shows the reduction in molecular weight of 254-nm light-irradiated DNA treated with 0.4 M NaOH for 2 h without sunlamp UVB irradiation (*i.e.*, no photoisomerization). It can be seen that 30 J/m$^2$ of 254-nm light induced about 0.7 breaks/$10^8$ Da in DNA. This level of background DNA breakage required both 254-nm light and 0.4 M alkali and did not increase after overnight incubation in alkali or decrease significantly after the 6 h repair incubation (data not shown).

In Fig. 4, the center panel of each row shows the production of alkali-labile sites in irradiated DNA as a result of photoisomerization and subsequent alkali treatment. An average of 1.9 breaks/$10^8$ Da was observed after 30 J/m$^2$ 254-nm light. Since alkali treatment alone produced 0.7 breaks/$10^8$ Da, the PALS induction rate was 0.04 PALS/$10^8$ Da/J · m$^{-2}$. The induction of these lesions increased in a dose-dependent manner for doses of up to at least 60 J/m$^2$ UVC light (not shown). Prolonged incubation of photoisomerized samples in alkali increased the number of nonspecific breaks but did not increase the difference in DNA size between irradiated and unirradiated samples. In the last panels, the effect of a 6 h recovery period is shown. In normal human cells (AG1518A), about 75% of alkali-labile lesions induced after 30 J/m$^2$ were removed by 6 h after 254-nm light irradiation. Since, as pointed out above,

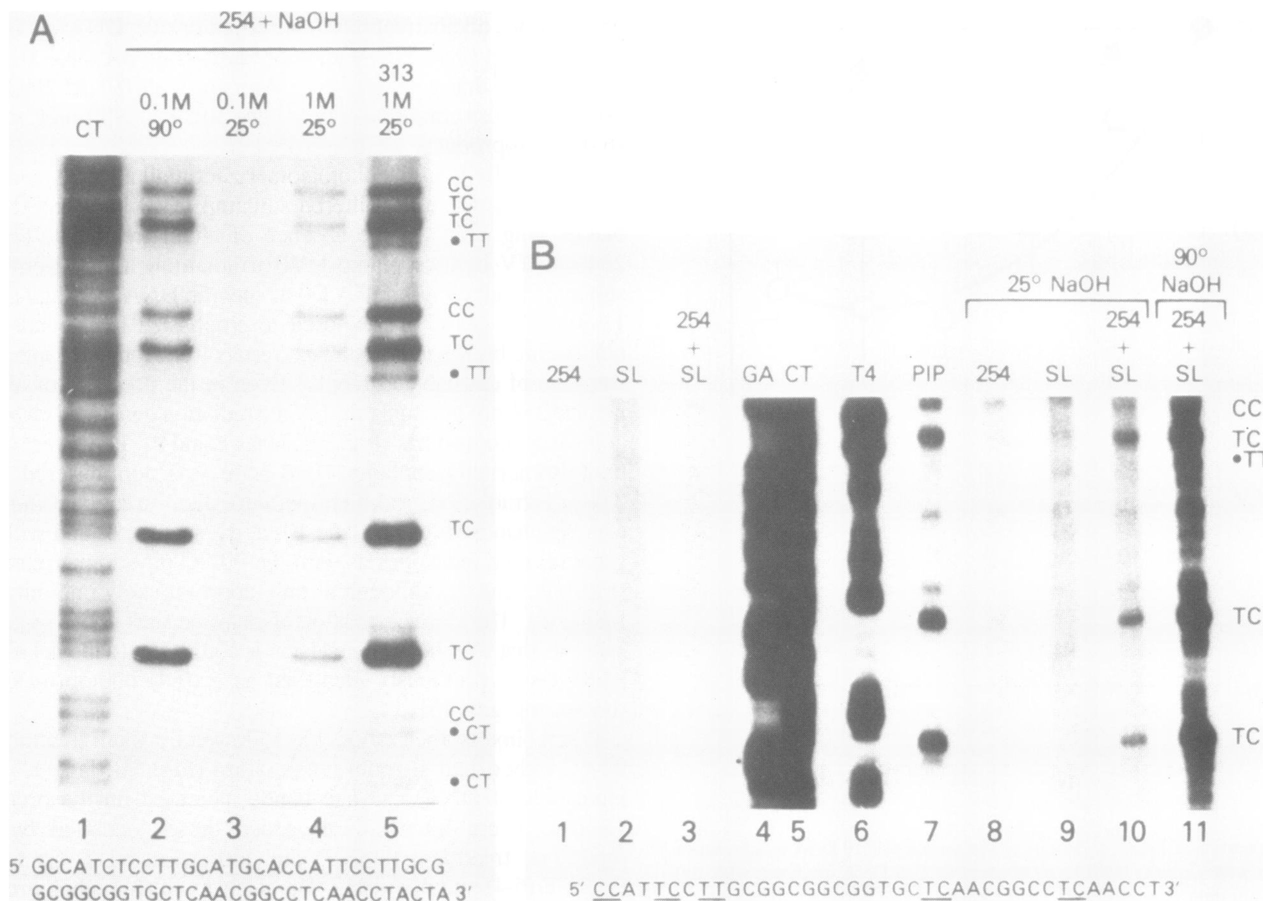


**Fig. 3.** Site specificity of PALS induction in DNA in various conditions of alkali and photoisomerizing light treatments. *A*, Lane 1, C + T Maxam-Gilbert DNA sequencing lane. Lanes 2–5, DNA irradiated with 1000 J/m$^2$ 254 nm light and then treated with NaOH: Lane 2, DNA treated with 0.1 M NaOH at 90 °C for 20 min and then neutralized and precipitated. Lane 3, DNA treated with 0.1 M NaOH at 23 °C for 3 h. Lane 4, DNA treated with 1.0 M NaOH at 23 °C for 3 h. Lane 5, DNA reirradiated with 300 kJ/m$^2$ monochromatic 313-nm light, treated with 1.0 M NaOH at 23 °C for 3 h, neutralized, and precipitated. *B*, Lane 1, 254-nm irradiation only (500 J/m$^2$). Lane 2, sunlamp only (40 kJ/m$^2$ at 365-nm). Lane 3, 254-nm plus sunlamp. Lanes 4 and 5, G + A and C + T Maxam-Gilbert DNA sequencing lanes. Lane 6, treatment with T4 *den*V cyclobutane dimer-specific endonuclease (loaded with twice the number of cpm as other lanes). Lane 7, treatment with 1 M piperidine at 90 °C for 20 min, to reveal (6–4) photoproducts. Lanes 8–10, treated with 0.4 M NaOH at 23 °C for 3 h: Lane 8, 254-nm irradiation only. Lane 9, sunlamp only. Lane 10, 254-nm plus sunlamp. Lane 11, same as lane 10 except alkali treatment was 90 °C for 20 min. The DNA sequences are shown below each panel; the 5′ end corresponds to the top of the gel. Dipyrimidine sites of particular interest, including sites of T< >T and C< >T (6–4) photoproducts, are indicated to the right of each gel.

**Table I:** Repair of PALS in Human Cell Lines Determined on Alkaline Sucrose Gradients

| Cell Lines | Repair Time (h) | Sunlamp Photolysis | $M_r(\times 10^{-7})$ Da (S.E.M.) 0 J/m$^2$ | 30 J/m$^2$ | Breaks/$10^8$Da | Percentage Remaining (6-4) Photoproducts |
|---|---|---|---|---|---|---|
| AG1518A | 0 | – | 1.73 (0) | 1.62 (0) | 0.39 | |
| | 0 | + | 1.38 (0.09) | 1.10 (0.08) | 1.84 | |
| | 6 | + | 1.37 (0.09) | 1.24 (0.08) | 0.77 | 20 |
| XP12RO | 0 | – | 1.74 (0.03) | 1.53 (0.02) | 0.79 | |
| | 0 | + | 1.33 (0.06) | 1.07 (0.02) | 1.82 | |
| | 6 | + | 1.41 (0.03) | 1.15 (0.02) | 1.58 | 90 |
| XP4BE | 0 | – | 1.79 (0.05) | 1.55 (0.03) | 0.87 | |
| | 0 | + | 1.40 (0.02) | 1.08 (0.01) | 2.12 | |
| | 6 | + | 1.47 (0.06) | 1.30 (0.04) | 0.89 | 10 |

breaks produced in the absence of photoisomerization persisted beyond 6 h, this observed repair was due primarily to the removal of PALS. XP variant cells (XP4BE) also removed nearly all of the PALS by 6 h, but XP group A cells (XP12RO) were PALS repair-deficient.

Numerical results obtained for the induction and repair of cyclobutane pyrimidine dimers, measured as ESS, are shown in Table 2. The average induction rate in human cells was 1.43 ESS/$10^8$ Da/J · m$^{-2}$, approximately half the rate determined in pUC19 DNA irradiated *in vitro*. Substantial repair (60%–65%)

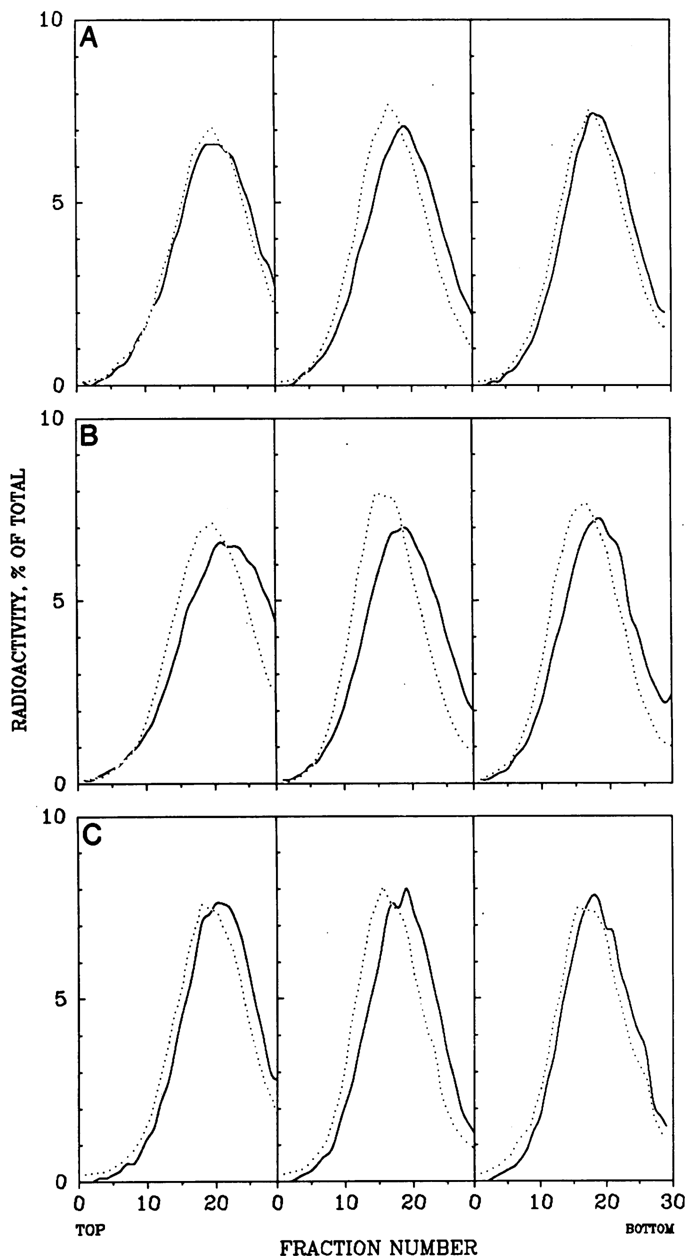


**Fig. 4.** Induction and repair of PALS in 254-nm light-irradiated human cells determined by alkaline sucrose gradient centrifugation analysis. Representative alkaline sucrose gradient profiles of DNA analyzed after isolation from human cell lines that were irradiated with 30 J/m$^2$ 254-nm light and variously treated before layering on gradients are shown. Left panels, cells were lysed immediately after 254-nm light irradiation, and DNA was isolated and alkali treated as described in the text and layered on gradients. Center panels, cells were lysed immediately after 254-nm light irradiation, DNA was isolated, treated with sunlamp UVB light, alkali-treated, and layered on gradients. Right panels, cells were incubated 6 h before being lysed, DNA was isolated, treated with sunlamp UVB light, alkali-treated and layered on gradients. *A*, AG1518A cells derived from an individual with normal UV sensitivity. *B*, UV-sensitive XP12RO cells derived from an individual belonging to XP complementation group A. *C*, UV-sensitive XP4BE cells derived from an individual of the XP variant class. Dotted lines represent [$^3$H]-labeled DNA counts from unirradiated cells included as an internal standard as described in the text; solid lines represent [$^{14}$C]-labeled DNA counts from irradiated samples.

of these sites was evident in normal and XP variant cells after 24 h, whereas no removal was observed in the XP group A cell line.

**Radioimmunoassay of (6-4) Photoproducts in DNA**

Repair of (6-4) photoproducts in normal AG1518A and UV-sensitive XP12RO and XP4BE cell lines was determined by RIA in the same samples for which repair was determined as PALS removal. These data (Table 1, last column) show that by 6 h, there was almost complete removal of antibody-binding sites to (6-4) photoproducts in AG1518A and XP4BE cell lines, while there was little loss of antibody-binding sites in the XP12RO (XP group A) cell line. These results confirm data previously reported for (6-4) photoproduct repair as determined by RIA in these cell lines irradiated at lower UV fluences [12].

## DISCUSSION

Sites of PALS induction in a c-K-*ras* DNA sequence corresponded to sites labile to hot piperidine, a technique used extensively to express (6-4) photoproducts as breaks on DNA sequencing gels. Sites of PALS occurred primarily at T< >C dipyrimidines and less often at C< >C sequences, consistent with the sequence specificity of (6-4) photoproduct induction [5]. The kinetics for producing alkali-labile sites by sunlamp UVB irradiation of 254 nm light-irradiated pUC19 DNA closely resembled those for both loss of (6-4) photoproducts and the production of (6-4) photoisomerization products determined by RIAs specific for each of these lesions [18,19].

In pUC19 DNA, (6-4) photoproducts assayed as PALS were induced at 23% the frequency of cyclobutane dimers, a rate significantly higher than that previously reported [29,30]. Incubation of the UVB-photoisomerized, 254 nm light-irradiated c-K-*ras* sequence with higher alkali concentrations or with hot alkali revealed a frequency of (6-4) photoproducts severalfold higher than previously shown with hot piperidine and revealed rare T< >T and C< >T (6-4) photoproducts. This result evidently arises from the continuum of alkali-lability of the Dewar photoisomer (Fig. 3), combined with its much greater lability compared to the (6-4) photoproduct. Since the relative frequencies at different sites were unchanged, previous conclusions concerning the role of (6-4) photoproducts in the site specificity of mutagenesis (2) are not altered. The quantitative role of (6-4) photoproducts in lethality and mutagenesis, however, may be greater than previously believed.

Shielding by cellular components or differences in the biochemical environment proximal to the DNA are known to reduce the effectiveness of UV irradiation in inducing cyclobutane dimers by about 50% [5]. In the current study, the rate of cyclobutane dimer induction in human cell DNA was roughly half that in pUC19 DNA. Each rate was very similar to published data using the corresponding technique [22,28]. Since the action spectrum for inducing (6-4) photoproducts is the same as for cyclobutane dimers in the UVC region (31), cellular shielding would be expected to have a similar effect on the yield of both photoproducts. However, the rate of (6-4) photoproduct induction expressed as PALS in DNA of human cells irradiated *in vivo* was less than 10% that observed in pUC19 DNA irradiated *in vitro*. In contrast, RIA data indicated that the number of antibody-binding sites associated with (6-4) photoproducts was comparable in plasmid DNA and isolated mammalian DNA irradiated *in vitro*, and also comparable to the number of (6-4) photoproducts induced in DNA isolated from mammalian cells irradiated *in vivo* (data not shown). Therefore, considering the continuum of PALS expression observed in the c-K-*ras* sequence, it is probable that not all Dewar photoisomers were converted to SSB in DNA

**Table II:** Repair of ESS in Human Cell Lines Determined on Alkaline Sucrose Gradients

| Cell Line | Repair Time (h) | $M_r(\times 10^{-7})$ Da 0 J/m² | 5 J/m² | Breaks/10⁸ Da |
|---|---|---|---|---|
| AG1518A | 0 | 1.69 | 0.76 | 7.24 |
| | 24 | 1.94 | 1.23 | 2.98 |
| XP12RO | 0 | 1.44 | 0.75 | 6.39 |
| | 24 | 1.78 | 0.77 | 7.37 |
| XP4BE | 0 | 2.06 | 0.79 | 7.80 |
| | 24 | 2.05 | 1.30 | 2.81 |

isolated from 254-nm light-irradiated human cells that was subsequently UVB irradiated and treated in alkaline conditions sufficiently mild to minimize nonspecific breakage; mild alkali treatment to maintain integrity of high molecular weight DNA was necessary, however, to allow the analysis of repair on alkaline sucrose gradients. Since the induction rate of the (6-4) photoproduct is known to be pH dependent [4], it is possible that the proximal biochemical environment in the cell may also be a factor in inducing (6-4) photoproducts in DNA of cells irradiated *in vivo*.

Repair of (6-4) photoproducts has been investigated by RIA in numerous cultured mammalian cell lines, and there is evidence that defective removal of this lesion may account for a significant proportion of the UV cytotoxicity observed in certain UV-sensitive mammalian cells [32,33]; in addition, the kinetics of (6-4) photoproduct repair in mammalian cells have been observed to be much more rapid than for cyclobutane dimer removal [10,11], and to correlate with kinetics of early repair events such as repair replication and recovery of DNA synthesis after UV irradiation (reviewed in [14]). Recently, two studies using human cell extracts to examine excision of UV-induced photoproducts *in vitro* have shown that (6-4) photoproducts are much better substrates than cyclobutane dimers *in vitro* [34,35], and probably account for the majority of repair synthesis [35]. Rapid kinetics of (6-4) photoproduct removal (as opposed to modification) *in vivo* would thus be consistent with evidence provided by both cellular and biochemical studies. To determine whether the results of experiments measuring (6-4) photoproduct repair by RIA and by expression of PALS with mild alkali treatment were consistent, we investigated (6-4) photoproduct repair in human cells using both techniques. In 254-nm light-irradiated, repair proficient AG1518A human cells, about 75% of PALS were removed by 6 h after irradiation (Table 1). These repair kinetics were in substantial agreement with those determined by RIA of the same samples, indicating that 80−90% of the antibody-binding sites associated with (6-4) photoproducts were lost in 6 h. In contrast, few (6-4) photoproducts, determined either as PALS or antibody-binding sites, were repaired in XP complementation group A (XP12RO) cells.

Repair of PALS in XP variant (XP4BE) cells, which exhibit intermediate UV sensitivity and have been shown to be proficient for (6-4) photoproduct repair by RIA [12], was also examined. XP variant cells are derived from patients manifesting clinical symptoms characteristic of classical XP cases, are defective in their ability to replicate damaged DNA, and yet appear to repair cyclobutane dimers at normal rates. Using a treatment resembling that described here, Francis and Regan [36] reported that XP variant cells (XP4BE) were deficient in removing a UV-induced photosensitive lesion from DNA, raising the possibility that the (6-4) photoproduct is cytotoxic in XP variant cells and might contribute to the clinical consequences of UV light exposure in these individuals. However, our RIA analyses showed these same XP variant cells to be as proficient as normal cells in removing antibody-binding sites associated with (6-4) photoproducts [12]; this result is confirmed in the present study, which shows that by 6 h, 254-nm light-irradiated XP4BE cells had removed nearly all (6-4) photoproducts determined as PALS (Table 1).

The photochemistry of the lesion detected by Francis and Reagan is consistent with its identity as a (6-4) photoproduct, yet it was expressed as SSB in alkaline sucrose gradients about sixfold less frequently than PALS described here. This discrepancy may be due to differences in the methods of determination. In their procedure, intact 254-nm light irradiated cells were reirradiated with 313-nm monochromatic light and then directly lysed on alkaline sucrose gradients. Since subsequent reirradiation of cell lysates with near UV light did not increase the yield of alkali-labile sites in DNA, it is possible that alkali treatment of whole cells may have reduced site-specific cleavages at Dewar photoisomers, thus reducing the yield of SSB on alkaline sucrose gradients. Such an effect is consistent with our observation that expression of PALS in sequencing gels lies on a continuum of alkali lability. This reasoning leads to the predictions that 1) cellular components and the microenvironment in DNA may influence the expression of the Dewar photoisomer as SSB in DNA, both in conditions of analysis, as Francis and Regan reported [36], and we have described here, and also perhaps *in vivo*, and 2) the actual induction of (6-4) photoproducts in DNA of mammalian cells irradiated with 254-nm light may have been previously underestimated to a significant degree.

In summary, the biochemical method developed here for detecting the induction and repair of (6-4) photoproducts in DNA has allowed the kinetics of (6-4) photoproduct repair in normal and UV-sensitive human cells to be measured on alkaline sucrose gradients as SSB derived from alkali-labile sites after UVB photoisomerization. The kinetics of repair of PALS were the same as those determined for loss of antibody-binding sites in RIA of the same DNA samples and in RIAs of DNA isolated from numerous mammalian cell lines, tissues and organisms after 254-nm light irradiation [14]. Our results are also consistent with those of other biochemical studies using human cell extracts which showed that (6-4) photoproducts are substantially better substrates for excision *in vitro* than cyclobutane pyrimidine dimers [32,33]. This correlation confirms the specificity of a RIA that has been used extensively to measure the repair of (6-4) photoproducts in diverse biological systems and indicates that loss of antibody-binding reflects excision rather than modification of this lesion to result in loss of epitope. These data also indicate that XP variant cells are not defective in excision of (6-4) photoproducts.

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