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Repair by human cell extracts of single (6-4) and cyclobutane thymine-thymine photoproducts in DNA

DAVID E. SZYMKOWSKI*, CHRISTOPHER W. LAWRENCE†, AND RICHARD D. WOOD*‡

*Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Herts EN6 3LD, United Kingdom; and †University of Rochester Medical Center, Department of Biophysics, 601 Elmwood Avenue, Rochester, NY 14642

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ABSTRACT **One *cis-syn* cyclobutane thymine dimer or one (6-4) thymine-thymine photoproduct was built into an identical sequence of a closed-circular M13 duplex DNA, and nucleotide excision repair synthesis carried out by human cell extracts in the area containing each lesion was determined. Extracts from normal cells repaired the (6-4) photoproduct with a patch size of ≈20–30 nucleotides, but repair was at least 10-fold lower at the cyclobutane dimer. The (6-4) lesion was repaired with comparable efficiency to a single acetylaminofluorene-guanine adduct in a similar location. Extract from nucleotide excision repair-deficient xeroderma pigmentosum group A cells could not remove any of these adducts but could complete repair of the lesions after incision with *Escherichia coli* UvrABC proteins. This direct comparison of repair of two UV photoproducts, in an *in vitro* system where chromatin assembly and transcription are absent, suggests that the more rapid repair of the (6-4) lesion observed in the mammalian cell genome overall is due in part to a significant difference in the ability of the repair complex to locate and incise these lesions in DNA.**

When DNA is irradiated with ultraviolet light (UV), the two major types of damage produced are the *cis-syn* cyclobutane pyrimidine dimer and pyrimidine(6-4)-pyrimidinone lesions (1, 2), formed in about a 3:1 ratio (3). The toxic and mutagenic effects of these photoproducts have been much studied, but the contribution of each to the biological effects of UV is not easily determined (4–7). Both lesions are removed by nucleotide excision repair of DNA, and neither *uvr* mutants of *Escherichia coli* (8, 9) nor human cells representing the skin cancer-prone condition xeroderma pigmentosum (XP) group A can excise either type of photoproduct (10, 11). However, mammalian cells appear to process these two lesions differently. For example, cyclobutane dimers are removed from overall nuclear DNA ≈5-fold more slowly than (6-4) photoproducts (3, 7, 11, 12). In addition, revertants of XP-A cells have been isolated that have regained the ability to repair (6-4) photoproducts but not cyclobutane dimers in the bulk of the genome (13).

What accounts for the difference in removal of these photoproducts from genomic DNA? One possibility is that the nucleotide excision repair system may recognize or incise each with significantly different efficiencies. Alternatively, there may be two partially redundant repair systems, only one of which efficiently removes (6-4) photoproducts (14, 15). (6-4) lesions might also be preferentially repaired if they are more accessible in chromatin to the repair complex, a possibility supported by evidence that (6-4) photoproducts (unlike cyclobutane dimers) form with higher yield in linker regions of nucleosomes than in the core (16, 17).

There are significant limitations to the techniques that have been used to examine the repair of UV-induced lesions. In many analyses, enzymatic photoreactivation is used to remove cyclobutane dimers and the remaining biological effects are attributed to (6-4) photoproducts. This is an oversimplification because it assumes that photoreactivation is 100% efficient at dimer reversal and because UV light induces other photoproducts (1). Another complication is that photoreactivating illumination may convert a fraction of (6-4) photoproducts to the Dewar isomer (3, 18), which can be more toxic than the (6-4) lesion (6). Photoreactivation may also increase the rate of (6-4) lesion repair in cells by reducing the overall damage load (19). Direct comparisons of the removal of (6-4) photoproducts and cyclobutane dimers from irradiated DNA are complex because the relative induction of each lesion varies considerably from site to site depending on the DNA sequence context, and these different sequences can in turn be repaired with different efficiencies (20–22). Further, assays for the (6-4) lesion often assume that every (6-4) photoproduct is labile at high pH and temperature, but this assumption is incorrect (23). Immunological assays are valuable, but they do not measure completion of repair or quantify the absolute amounts of photoproducts removed (10, 23).

To eliminate these difficulties we have used a direct approach. Circular DNA duplexes were constructed with one cyclobutane thymine dimer or one (6-4) photoproduct in an identical sequence, and the repair synthesis stimulated by each lesion was measured in a cell-free assay, using extracts from repair-proficient and repair-deficient human cell lines.

MATERIALS AND METHODS

DNA Containing Single Defined Lesions. The oligonucleotide 5′-GCAAGTTGGAG-3′ was irradiated at 254 nm to form the (6-4) thymine-thymine photoproduct or irradiated with fluorescent sunlamps in the presence of acetophenone to obtain a *cis-syn* cyclobutane dimer (5). The damaged oligonucleotides were isolated as described (6), with modifications to improve on purity (C.W.L., unpublished). The oligonucleotide with a (6-4) photoproduct was determined to be >95% pure, and that with a cyclobutane dimer, >99.5% pure. Minor contaminants consisted primarily of truncated (unligatable) products. The oligonucleotide 5′-CCAACCACACCGAATTCACCACC-3′ was treated with *N*-acetoxyacetylaminofluorene (*N*-acetoxy-AAF) (by R. Fuchs, Institut de Biologie Moléculaire et Cellulaire, Strasbourg, France) to

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Abbreviations: AAF, acetylaminofluorene; XP, xeroderma pigmentosum; XPAC, XP group A-complementing protein; M13mp18TT, the M13 construct with a priming site for oligonucleotides containing thymine-thymine photoproducts; M13mp18TT-U, -64, and -CPD, constructs synthesized with an undamaged oligonucleotide or with oligonucleotides containing a single (6-4) photoproduct or *cis-syn* cyclobutane thymine dimer, respectively; M13mp18G, the M13 construct with a priming site for *N*-acetoxy-AAF-modified oligonucleotide; M13mp18G-U and -AAF, constructs synthesized with oligonucleotides either undamaged or containing a single AAF adduct, respectively.

‡To whom reprint requests should be addressed.

form an AAF adduct at the C8 position of the guanine, and the modified oligonucleotide was purified to homogeneity as described (24).

The vector M13mp18TT was constructed by replacing the 33-bp *Eco*RI–*Sal* I fragment of M13mp18 with an insert formed by annealing the oligonucleotides 5′-AATTCCCTG-GCTCCAACTTGCCTGG-3′ and 5′-TCGACCAG-GCAAGTTGGAGCCAGGG-3′. M13mp18G was constructed by replacing the same fragment with an insert formed by annealing the oligonucleotides 5′-AATTGCCTG-GTTGGTGGTGAATTCGGTGTGGTGGTTCCTGG-3′ and 5′-TCGACCAGGAACCACCACACCGAATTCACCAC-CAACCAGGC-3′. Closed circular DNA containing defined lesions was produced by priming 75 μg of (+) strand M13mp18 derivative (200 ng/μl) with a 3-fold molar excess of damaged or undamaged oligonucleotide and incubating with T4 DNA polymerase holoenzyme, single-stranded DNA binding protein (Stratech, Luton, U.K.), and T4 DNA ligase (25, 26). Closed circular product was separated from the small amount (<5%) of unligated DNA by ethidium bromide/CsCl equilibrium centrifugation. The AAF adduct in M13mp18G was located in a unique *Eco*RI site, and digestion with *Eco*RI before purification on density gradients removed any molecules not containing a properly located AAF adduct.

Circular DNA synthesized using the oligonucleotide containing a (6-4) photoproduct was ligated inefficiently to form closed duplex, though the priming and DNA synthesis steps were as efficient as with undamaged control oligonucleotide. The poor ligation was apparently due to melting of the 5′ end of the damaged oligonucleotide from the template and subsequent extension by the DNA polymerase, creating a 5′ single-stranded "tail." To synthesize duplex DNA containing a (6-4) photoproduct, a 3-fold molar excess of the damaged 11-mer was annealed to single-stranded M13mp18TT, then one-third as much oligonucleotide of sequence 5′-GCCTGCAGGTCGACCAG-3′ was annealed immediately 5′ to the (6-4)-containing 11-mer, and the two oligonucleotides were ligated prior to addition of polymerase. Tests using a 5′ ^{32}P-labeled (6-4) oligonucleotide confirmed efficient ligation of the oligonucleotides prior to DNA synthesis and incorporation of the damaged oligonucleotide into closed circular product. The 3-fold excess of damaged oligonucleotide ensured that no closed circular product was formed containing only upstream oligonucleotide.

Plasmid DNA. Plasmid pBluescript KS$^+$ (pBS, 3.0 kb, Stratagene) was irradiated with UV (254 nm) light (450 J/m^2), treated with *E. coli* Nth protein to incise DNA containing stable pyrimidine hydrates (≈0.6 per molecule), and purified on gradients to remove nicked circular DNA (27). As a control, plasmid pHM14 (3.7 kb) was treated identically but not irradiated.

***In Vitro* Repair Synthesis.** Reaction mixtures (50 μl) contained 200 μg of cell extract protein (28), DNA, and buffer including α-^{32}P-labeled deoxynucleotides (26). Repair assays with irradiated plasmids contained 250 ng each of UV-irradiated pBS and undamaged pHM14. Repair assays of single-lesion M13 substrates contained 100 ng of DNA. Reaction mixtures were incubated at 30°C for 3 hr and DNA was isolated as described (27). When indicated, samples were treated with *E. coli* UvrABC enzyme (29) before adding cell extract protein. For globally irradiated plasmids, DNA was linearized with *Bam*HI and separated on a 0.8% agarose gel that included standards to measure DNA recovery (≈70%). The yield of each plasmid was determined by scanning densitometry of a photographic negative. Liquid scintillation spectroscopy of bands from the dried gel was used to quantify DNA synthesis. For single-lesion M13 substrates, DNA was digested with *Bst*NI, and the nine resulting fragments were resolved on a 12% polyacrylamide gel. Fixed and dried gels were exposed to flashed x-ray film. Autoradiographs (exposed without intensifying screens) were quantified by densitometry and band intensities were compared to those on equivalent autoradiographs of gels containing a dilution series of a ^{32}P-labeled *Msp* I digest of pBR322. Bands on the latter gels were quantified by liquid scintillation spectroscopy and used as standards to determine absolute synthesis.

RESULTS

Oligonucleotides containing either a *cis-syn* cyclobutane thymine dimer, a (6-4) thymine-thymine photoproduct, or no lesion were purified and incorporated into closed circular M13 duplex DNA (Fig. 1). Separation in CsCl density gradients was used to eliminate unligated circles. The presence of a single *cis-syn* cyclobutane thymine dimer in M13mp18TT-CPD was verified by digestion with an excess of T4 endonuclease V. This completely nicked M13mp18TT-CPD, with no effect on circles containing a (6-4) lesion or on undamaged DNA (Fig. 2*A*). To further confirm that photoproducts were correctly and uniquely located, DNA polymerase was used to synthesize DNA from a primer beginning at a position 42 nt 3′ to the damaged site; each photoproduct blocked synthesis at the nucleotide immediately preceding the TT sequence (Fig. 2*B*). As a comparable substrate that is known to stimulate appreciable DNA repair synthesis in this *in vitro* system, an M13 duplex containing a single AAF lesion on guanine was also synthesized (Fig. 1), and the location of the adduct was verified by primer extension and resistance of the duplex to linearization with *Eco*RI (not shown).

To measure DNA synthesis at each lesion, the three single-lesion duplexes containing either a (6-4) photoproduct (M13mp18TT-64), a *cis-syn* cyclobutane thymine dimer (M13mp18TT-CPD), or an AAF adduct on guanine (M13mp18G-AAF), with appropriate control duplexes for each (M13mp18TT-U and M13mp18G-U), were incubated with human cell extracts under conditions supporting repair synthesis in damaged DNA. Results with human lymphoblastoid GM1953 and HeLa cell extracts are shown in Fig. 3; similar results were obtained with extract from the human fibroblast cell line 1BR.3N. Repair synthesis was readily apparent in the 15-bp *Bst*NI restriction fragment containing the (6-4) photoproduct. In contrast, the single *cis-syn* cyclobutane thymine dimer did not stimulate measurable DNA synthesis in this fragment. Quantification shows that the (6-4) TT photoproduct is repaired with an efficiency at least 10-fold greater than the cyclobutane TT dimer at an identical

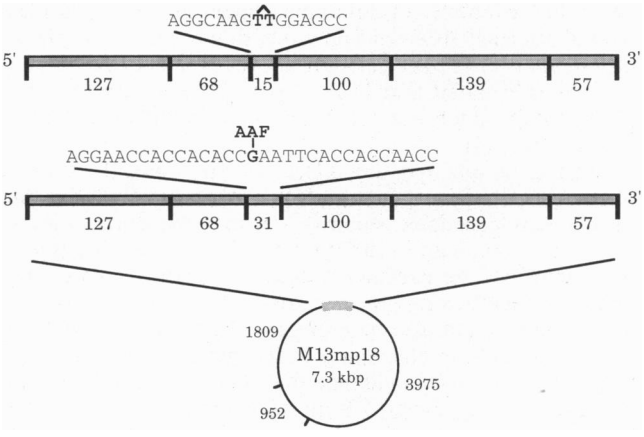

FIG. 1. Substrates containing defined DNA lesions. The (−) strand contains either a single *cis-syn* cyclobutane thymine dimer or a (6-4) photoproduct (top line, in M13mp18TT) or a guanine-AAF adduct (second line, in M13mp18G). The sequences for the damage-containing regions are shown, as well as fragment sizes (bp) after *Bst*NI digestion.

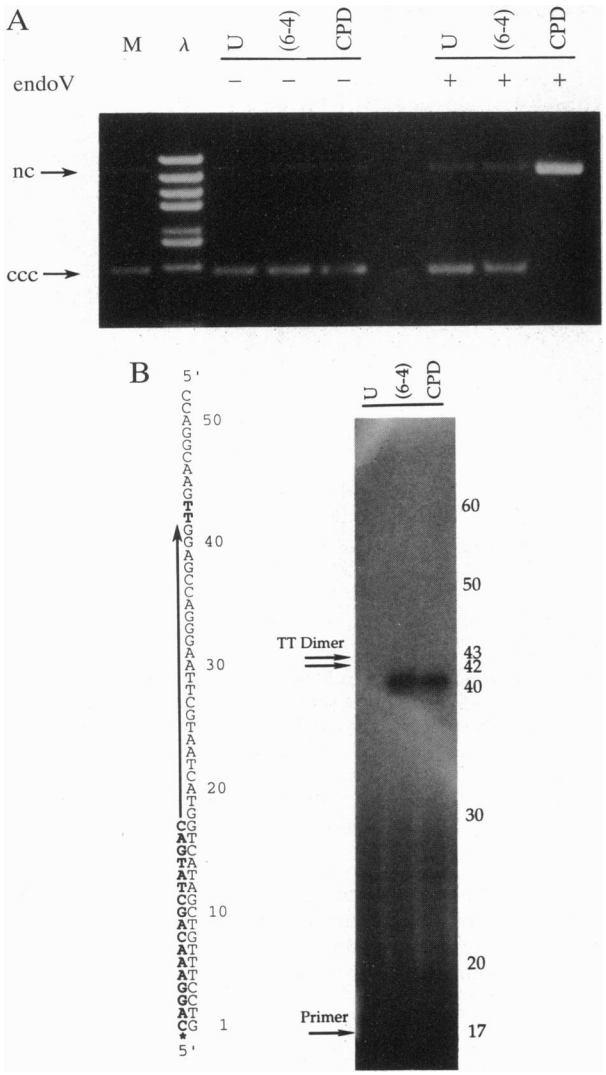

FIG. 2. Confirmation of presence of dithymine lesions. (*A*) Constructs containing a (6-4) photoproduct, cyclobutane thymine dimer (CPD), or undamaged control (U) were treated with T4 endonuclease V (endoV) at 37°C (30) for 1 hr (+) or mock-treated (−). Samples were incubated 10 min at 65°C before separating nicked circular (nc) and covalently closed circular (ccc) forms by ethidium bromide/agarose gel electrophoresis. M, ccc form M13mp18TT; λ, *Bst*EII digest of λ DNA. (*B*) Substrates were denatured, and the oligonucleotide 5′-CAGGAAACAGCTATGAC-3′ (bold) was annealed 3′ to the damage and then extended with *E. coli* DNA polymerase I (Klenow fragment). Products were separated on a denaturing gel and compared to a DNA sequencing ladder for size reference (position 1 is nt 6204 of M13mp18 DNA). Both lesions blocked elongation immediately before the thymine dinucleotide (25–26 nt from the 3′ end of the primer) and longer products at the top of the gel were barely detectable. Primer extension on undamaged control DNA gave products >200 nt long.

site (Fig. 3*C*). The relative difference may be larger, but the detection of low amounts of repair is limited by the damage-independent background synthesis that occurs throughout the entire M13 molecule and is present in proportional amounts in each *Bst*NI fragment. This synthesis is initiated at either preexisting nicks or those formed by nonspecific nucleases present in cell extracts (25, 26) and is primarily confined to the three largest fragments, which comprise 93% of the M13 DNA.

The level of repair of the (6-4) lesion was measured in two ways. First, repair reactions containing a mixture of UV-irradiated pBS and undamaged pHM14 plasmids were done simultaneously under identical conditions (the results in Fig. 4 for global UV damage correspond to those shown in Fig. 5 using single-lesion substrates). Quantification of the data in Fig. 4 shows incorporation of ≈15 dNMP per kbp of pBS, irradiated with UV light (450 J/m^2) to give ≈3 cyclobutane dimers and ≈1 (6-4) photoproduct per kbp (31). If repair efficiency at each lesion were identical, then the dimer would contribute 75% of repair synthesis, and the (6-4) lesion, 25%. However, Fig. 3 indicates that, conservatively, 90% of synthesis occurs at (6-4) lesions and 10% at cyclobutane dimers. Therefore, in the UV-irradiated plasmid, 90%[1 (6-4) lesion/kbp]/{90%[1 (6-4) lesion/kbp] + 10%(3 CPD lesions/kbp)} = ≈75% of synthesis is due to the (6-4) lesion, with only ≈25% induced by the cyclobutane dimer. This result is consistent with our earlier data showing that photoreactivation reduced repair synthesis *in vitro* by 20–30% (31). Assuming that the repair patch for a (6-4) photoproduct is ≈28 nt long (see below) then the efficiency of repair for the (6-4) photoproduct in globally irradiated DNA was [75%(15 nt/kbp)][(1 lesion/kbp)]$^{-1}$[28 nt/patch]$^{-1}$ = ≈40%. This number may be inexact for various reasons (such as inaccurate values for [^{32}P]dNTP specific activities), but the data indicate substantial removal of (6-4) photoproducts from irradiated plasmid. Irradiating plasmid with 450 J/m^2 induces an amount of damage that is above the linear range of the assay (31); a higher proportion of photoproducts might be removed at a lower fluence. Incubating beyond 3 hr gives little increase in the overall level of repair synthesis (32).

The second method to measure repair at each lesion was to quantify synthesis in the 15-bp fragment containing the single (6-4) photoproduct in M13mp18TT-64, by comparison with calibration curves derived from identically exposed autoradiographs of standards. This direct estimate showed that ≈4% of the (6-4) photoproduct in M13mp18TT-64 was removed by extract in the experiment in Fig. 3*A* and ≈0.4% of the (6-4) photoproduct in Fig. 3*B*. Repair in the single lesion substrate appears to be considerably less efficient than in globally irradiated plasmid. The single photoproduct repair rate may be reduced relative to that in irradiated plasmid because the density of damage is much lower in M13mp18TT-64 (1 lesion/7.3-kbp molecule) than in irradiated pBS (12 lesions/3.0-kbp molecule). This low local concentration of damage in single-lesion substrates might be below the K_m of the repair complex.

The distribution of DNA synthesis in the damaged and flanking fragments allows an independent estimate of the size of the repair patch associated with removal of the (6-4) photoproduct, assuming the size is discrete and constant. Fig. 3*C* indicates that ≈60% of above-background synthesis induced by the (6-4) photoproduct occurred in the 15-bp region containing the lesion at nt 8–9 compared to ≈30% in the immediate 68-bp 5′ region and ≈10% in the 100-bp 3′ region. If repair synthesis occurred at every position in the 15-nt region, then the number of dNMPs incorporated in the 68-bp region is (30%/60%)15 = ≈7.5, and in the 100-bp region (10%/60%)15 = ≈2.5. This gives an estimated patch size of roughly 25 nt, consistent with *in vivo* data (33) and close to various *in vitro* measurements (25, 26, 34–36).

Extract from an XP-A cell line could not perform detectable repair of the two photoproducts or the AAF-guanine adduct (Fig. 5). This confirms that DNA synthesis by normal cell extracts at the site of the lesions is due to nucleotide excision repair. However, adding *E. coli* UvrABC proteins to substrates, to introduce incisions flanking each lesion prior to addition of human cell extract, circumvented the XP defect and permitted DNA repair synthesis at the sites of the lesions (Fig. 5). Treatment of DNA with UvrABC also increased label incorporation (≈2.5-fold) by HeLa extract specifically in the region containing the (6-4) lesion and stimulated repair at the cyclobutane dimer and AAF adduct (Fig. 5). This

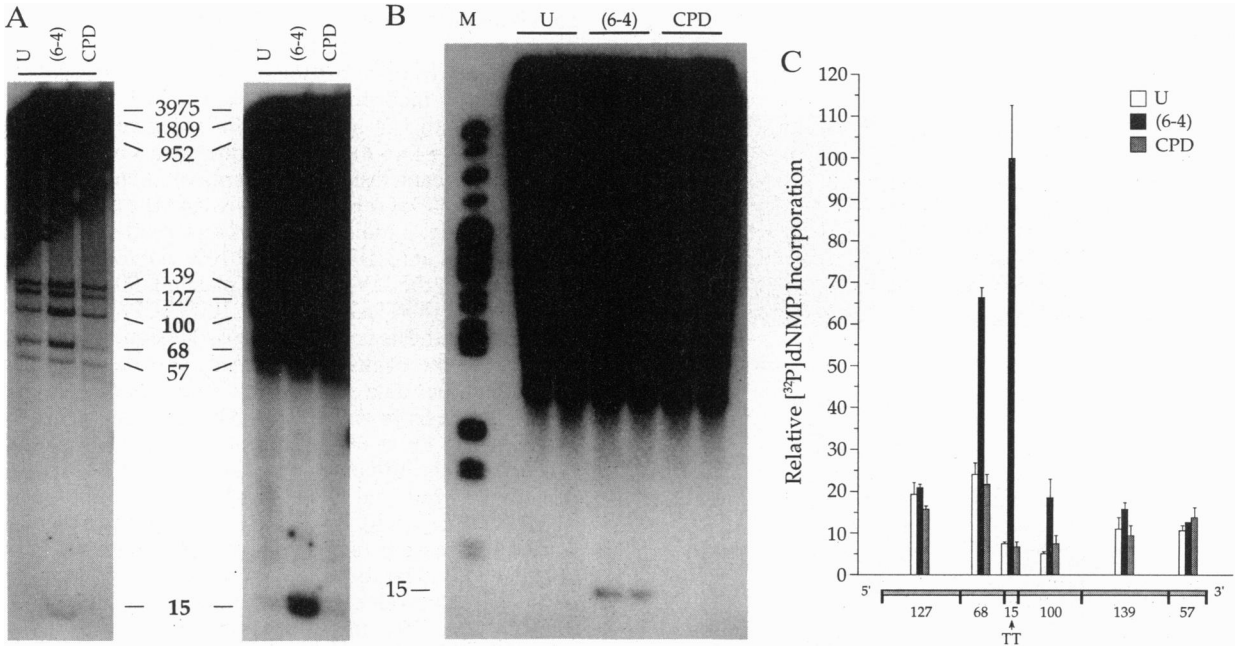

FIG. 3. Repair of single UV photoproducts in normal cell extracts. (*A*) Extract from normal human lymphoblastoid GM1953 cells. A less intense exposure (*Left*) without intensifying screens indicates how relative DNA synthesis in each of the six fragments surrounding the damage (Fig. 1) was quantified. An overexposure with screens (*Right*) shows synthesis in the 15-bp fragment containing the (6-4) photoproduct relative to the cyclobutane dimer-containing and control fragments. (*B*) Extract from HeLa cells, showing duplicate reactions containing control, (6-4) photoproduct, or cyclobutane dimer-containing M13mp18TT. M is a radiolabeled *Msp* I digest of pBR322 (a size marker). (*C*) Quantification of DNA synthesis in the experiment shown in *B* (using a less intense exposure of the autoradiograph, without screens) for the six fragments surrounding the lesion. The normalized incorporation in each region takes into account the size and base composition of each *Bst*NI fragment. Averages of duplicate reactions are shown, with bars spanning each point.

stimulation by UvrABC suggests that damage-recognition or incision are rate-limiting steps in repair even with HeLa cell extract. The more pronounced ability of UvrABC to stimulate synthesis at the AAF adduct may arise because the lesion appears to be a better substrate for UvrABC than either UV photoproduct (22, 37). Taking into account base composition, the repair synthesis caused by an AAF adduct (in the absence of UvrABC) was ≈1–2.5 times that caused by a (6-4) lesion.

DISCUSSION

The simplest explanation for the observed ≥10-fold difference in repair of the two types of UV lesions is that the nucleotide excision repair system active in human cell extracts recognizes or incises the (6-4) photoproduct much better than the cyclobutane dimer, perhaps because the (6-4) bond causes more distortion or unwinding of the DNA helix (38, 39). UvrABC pretreatment stimulates DNA synthesis by cell extracts in the order AAF > (6-4) > CPD, which also

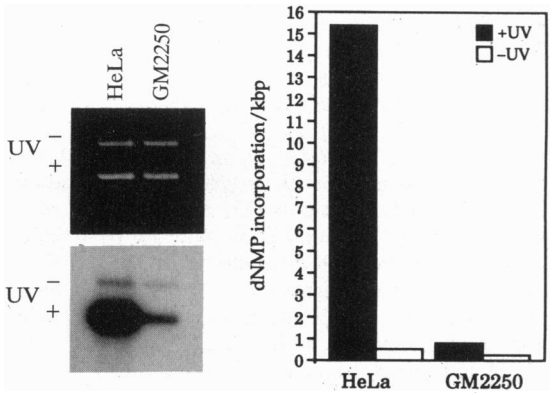

FIG. 4. Repair of plasmid DNA containing global UV damage. Equal amounts of irradiated plasmid pBS (UV +) and unirradiated plasmid pHM14 (UV −) were incubated in a repair assay with HeLa or GM2250 cell extracts. (*Left*) Ethidium bromide-stained gel and autoradiograph. (*Right*) Quantification of DNA synthesis.

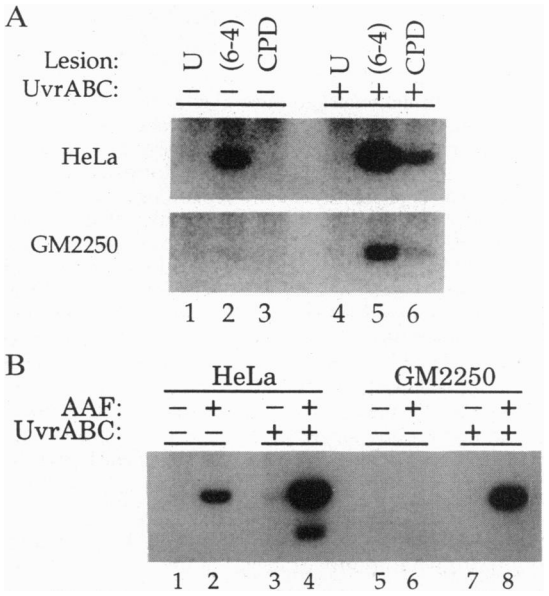

FIG. 5. Stimulation of DNA synthesis at single lesions by UvrABC. Substrates were incubated with *E. coli* UvrABC prior to addition of cell extract (only the damage-containing *Bst*NI fragment is shown). (*A*) Extracts from HeLa cells or XP-A cells (GM2250, patient XP12BE), with (+) or without (−) UvrABC in reactions with M13mp18TT-U, -64, and -CPD. (*B*) HeLa or XP-A extract, with (+) or without (−) UvrABC in reactions with M13mp18G-AAF and -U. The additional band in lane 4 may represent a small population of incomplete, unligated repair patches that migrate faster than the 31-bp *Bst*NI double-stranded fragment.

describes the relative UvrABC incision efficiency at each lesion.

Huang *et al.* (36) did not detect repair synthesis in DNA substrates containing a single cyclobutane thymine dimer, and our data agree with this result. They could, however, detect repair when four dimers were clustered within 800 bp on a 4300-bp plasmid. Incisions in this substrate were located at the ≈22nd phosphodiester bond 5′ and ≈6th bond 3′ to the cyclobutane dimers, corresponding to an asymmetric patch of ≈27–29 nt. The distribution of DNA repair synthesis we report for the (6-4) photoproduct, and for an AAF lesion (26), suggests that these are repaired with an asymmetric patch of similar size and that this mode of incision by the human nucleotide excision repair system is a general one. The presence of closely spaced multiple lesions appears to stimulate repair of cyclobutane dimers *in vitro* (36), but the basis for this effect is unclear. The human nucleotide excision repair complex is not highly processive *in vitro* (35), so this would not account for synergistic repair of dimers. However, a high density of lesions might be expected to help maintain a greater local concentration of repair proteins near the DNA, increasing the probability of assembling a complete, multiprotein repair complex.

The XPAC protein (XP group A-complementing protein) is a key component of the nucleotide excision repair mechanism. XPAC binds to UV-irradiated DNA (28), with a greater affinity for (6-4) photoproducts than for cyclobutane dimers (C. J. Jones and R.D.W., unpublished). At least one other cellular polypeptide also has an affinity for UV-irradiated DNA containing (6-4) photoproducts (40). These proteins may contribute to the difference in repair efficiency of the two UV lesions.

The more efficient repair of (6-4) photoproducts compared to cyclobutane dimers emphasizes that cells have other methods of dealing with the more abundant dimer. For example, cyclobutane pyrimidine dimers appear to be better tolerated in cellular genomes than (6-4) photoproducts, and cells can complete DNA replication with a large number of dimers still present (30, 41). In addition, cyclobutane dimers are preferentially removed from transcribed genes (42). In the present analysis many complications of *in vivo* studies have been eliminated by comparing UV photoproducts in isolation under identical conditions. The results suggest that the difference in the repair of the two types of dipyrimidine photoproducts in mammalian cells is due in part to a fundamental difference in the ability of the repair complex to process each lesion.

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