Phototriggered Formation and Repair of DNA Containing a Site-Specific Single Strand Break of the Type Produced by Ionizing Radiation or AP Lyase Activity†

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ABSTRACT: DNA strand breaks are produced by a variety of agents and processes such as ionizing radiation, xenobiotics, oxidative metabolism, and enzymatic processing of DNA base damage. One of the major types of strand breaks produced by these processes is a single nucleotide gap terminating in 5′- and 3′-phosphates. Previously, we had developed a method for sequence-specifically producing such phosphate-terminated strand breaks in an oligodeoxynucleotide by way of two photochemically activated (caged) building blocks placed in tandem. We now report the design and synthesis of a single caged building block consisting of 1,3-(2-nitrophenyl)-1,3-propanediol, for producing phosphate-terminated strand breaks, and its use producing such a break at a specific site in a double-stranded circular DNA vector. To produce the site-specific break in a duplex vector, a primer containing the caged single strand break was extended opposite the single strand form of a circular DNA vector followed by enzymatic ligation and purification. The single strand break could then be formed in quantitative yield by irradiation of the vector with 365 nm light. In contrast to a previous study, it was found that the strand break can be repaired by *Escherichia coli* DNA polymerase I and *E. coli* DNA ligase alone, though less efficiently than in the presence of the 3′-phosphate processing enzyme *E. coli* endonuclease IV. Repair in the absence of endonuclease IV could be attributed to hydrolysis of the 3′-phosphate in the presence of dNTP and to a lesser extent to exonucleolytic removal of the 3′-phosphate-bearing terminal nucleotide by way of the 3′ → 5′ exonuclease activity of polymerase I. This work demonstrates that specialized 3′-end processing enzymes such as endonuclease IV or exonuclease III are not absolutely required for repair of phosphate-terminated gaps. In addition to preparing single strand breaks, the caged building block described should also be useful for preparing double strand breaks and multiply damaged sites that might otherwise be difficult to prepare by other methods due to their lability.

Ionizing radiation induces a wide array of DNA lesions, including various types of base damage, apurinic/apyrimidinic (AP)[1] sites and single and double strand breaks (*1*). Among these, DNA single strand breaks are the most prevalent (*2*, *3*) and have recently been shown to be mutagenic (*4*−*6*). How these lesions are processed by repair, replication, and transcription enzymes is not well understood, in large part because the large diversity of damage produced by ionizing radiation and its limited sequence specificity (*7*) make structure−activity correlations hard to establish.

Single strand breaks formed by ionizing radiation generally consist of a one-nucleotide gap containing a 5′-phosphate and either a 3′-phosphate or a 3′-phosphoglycolate terminus (*8*) that result from hydroxyl radical abstraction of the deoxyribose hydrogens (*9*). Single nucleotide gaps terminating in both 5′- and 3′-phosphates are also formed during base excision repair by DNA glycosylases with associated AP lyase activity (*10*). One straightforward method for preparing such substrates has been to anneal two sets of synthetic oligodeoxynucleotides to a complementary single strand DNA, one bearing the 5′-phosphate and the other bearing the 3′-phosphate (*11*). For repair and mutagenesis studies in vivo, it would be preferable to incorporate a single strand break site-specifically into a phagemid or shuttle vector. A procedure for incorporating a single nucleotide gap terminating in a 3′-phosphoglycolate and a 5′-phosphate by way of synthetically prepared oligodeoxynucleotide precursors has been reported, but it is rather laborious and inefficient (*12*). A more simple and general method to introduce a single strand break site-specifically into a circular duplex vector would be to prime second strand synthesis opposite the single strand form of a circular vector with an oligodeoxynucleotide containing the single strand break precursor. This method has been successfully used to incorporate various types of DNA damage site-specifically into circular duplex vectors and facilitates purification of the desired substrate (*13*−*16*). One readily available precursor to a phosphate-terminated

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[1] Abbreviations: AP, apurinic/apyrimidinic; ddNTP, dideoxynucleotide triphosphate; dNTP, deoxynucleotide triphosphate; DTT, dithiothreitol; endonuclease IV, *Escherichia coli* endonuclease IV; exo⁻, 3′ → 5′ exonuclease deficient; KF, Klenow fragment of *E. coli* DNA polymerase I; PNK, T4 polynucleotide kinase; polymerase I, *E. coli* DNA polymerase I.

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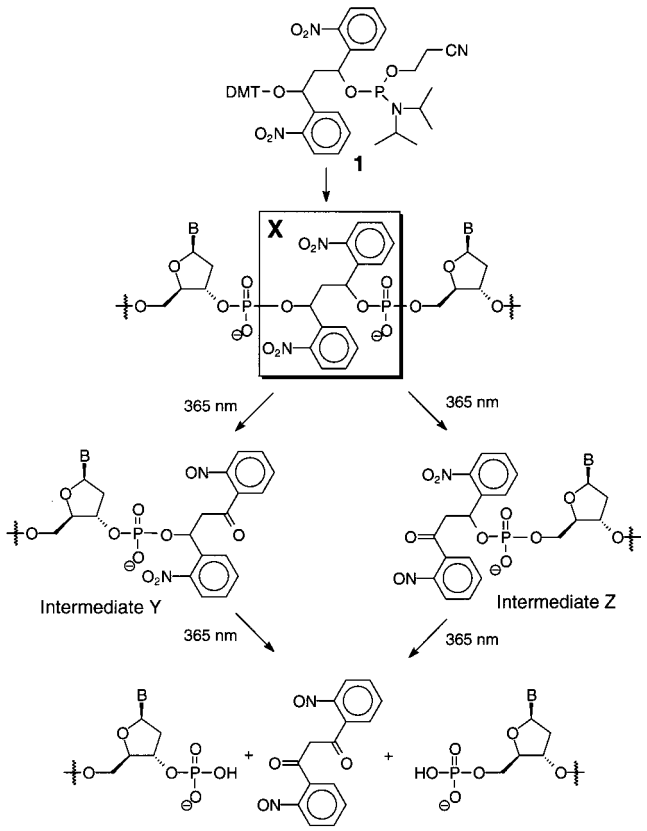

FIGURE 1: Design of a single building block for the phototriggered formation of DNA strand breaks with 5′- and 3′-phosphate termini. Irradiation can first lead to either intermediate Y or intermediate Z, which upon further irradiation can both lead to the phosphate-terminated break.

single strand break is deoxyuridine, which can be converted to a break by uracil glycosylase and an AP endonuclease (*1*). Though such a procedure may be generally useful for the preparation of single strand breaks for in vitro studies, it may not be useful for future in vivo studies where it would be desirable to trigger single strand break formation at a particular step in a transfection protocol. An enzymatic method may also not work for preparing substrates bearing closely spaced or opposed single strand breaks because of interference from one abasic site or break on the enzymatic processing of the other (*11*). In addition, the use of enzymes might necessitate their removal under conditions that may cause premature double strand breaks to arise. A phototriggered precursor to a single strand break bearing phosphate termini would circumvent such problems.

Herein, we report the design (Figure 1) and synthesis (Figure 2) of a single building block that can be used to site-specifically and sequence-independently introduce a phosphate-terminated break into an oligodeoxynucleotide in quantitative yield upon irradiation with 365 nm light. We show how this building block can be used to introduce such a single strand break site-specifically and sequence-independently into a circular duplex vector. In the process of characterizing the single strand break, we discovered that it can be repaired by *Escherichia coli* polymerase I and DNA ligase alone and does not require another enzyme, such as *E. coli* endonuclease IV or exonuclease III, to remove the 3′-phosphate as previously thought (*17*). We also show that both *E. coli* polymerase I and the Klenow fragment (KF) but not exonuclease-deficient KF are capable of extending

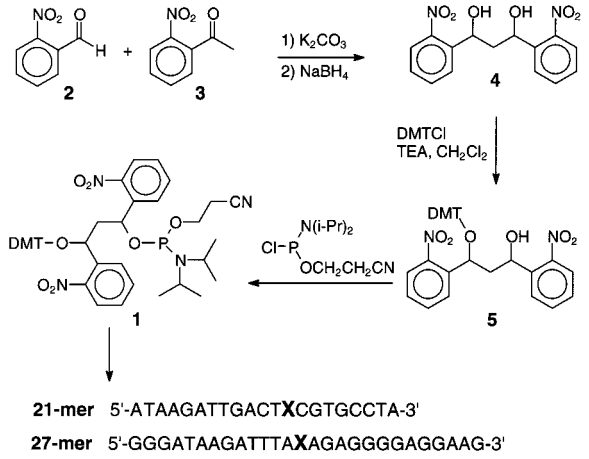

FIGURE 2: Synthesis of the building block.

the 3′-phosphate-terminated primers by first removing the 3′-phosphate or the 3′-phosphorylated nucleotide via their 3′ → 5′ exonuclease activity.

MATERIALS AND METHODS

Enzymes, Reagents, and Instrumentation. T4 polynucleotide kinase, *E. coli* DNA polymerase I, Klenow fragment (KF), exo$^-$ KF, Taq DNA ligase, and *E. coli* DNA ligase were purchased from New England Biolabs. One unit of the pol I and KF polymerases used herein corresponds to approximately 0.74 pmol. The Stoffel fragment of Taq DNA polymerase was obtained from PE Biosystems. *E. coli* endonuclease IV was from Epicentre Technologies. One unit of this enzyme corresponds to about 0.16 pmol. [γ-^{32}P]ATP (6000 Ci/mmol) and [α-^{32}P]dATP (6000 Ci/mmol) were purchased from Amersham Pharmacia Biotech. The double strand plasmid pIES(+)/DraI was provided by Dr. Ingrid E. Simon. Oligodeoxynucleotides were synthesized on an Applied Biosystems 380B DNA synthesizer. NMR (^{1}H, ^{13}C, ^{31}P) spectra were obtained on a Varian XL-300 spectrometer referenced to TMS (^{1}H NMR, δ 0.00), $CDCl_3$ (^{13}C NMR, δ 77.0), or 85% phosphoric acid (^{31}P NMR, δ 0.00) peaks. IR spectra were recorded either neat or as a film on potassium bromide plates on a Mattson Instruments Polaris FT-IR spectrometer. High-resolution gel electrophoresis was carried out on 15% denaturing polyacrylamide gels (mono/bis, 19:1) and quantified using a Molecular Dynamics PhosphorImager and ImageQuant software version 3.3 or 1% agarose gels (1 μg/mL ethidium bromide) for the vector DNA.

*1-O-(4,4′-Dimethoxytrityl)-1,3-(2-nitrophenyl)-1,3-propanediol (**5**).* To a solution of **4** (*18*) (235 mg, 0.74 mmol) in CH_2Cl_2 (8 mL) were added Et_3N (0.52 mL, 3.7 mmol) and dimethoxytrityl chloride (276 mg, 0.81 mmol) at 0 °C. The mixture was stirred for 1 h at 0 °C and 2 h at room temperature, diluted with CH_2Cl_2 (30 mL), and washed with saturated $NaHCO_3$ solution (10 mL). The organic phase was dried over Na_2SO_4(s), filtered, and concentrated under reduced pressure. The residue was flash chromatographed on silica gel (EtOAc/hexane, 3:7) to give 335 mg (73%) of pale yellow foam: ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.97−7.91 (m, 1H), 7.82−7.80 (m, 2H), 7.62−7.57 (m, 1H), 7.50−7.41 (m, 3H), 7.36−7.14 (m, 10H), 6.74−6.61 (m, 4H), 5.75−5.45 (m, 2H), 4.46 (s, 1H, OH), 3.75−3.72 (m, 6H), 2.45−2.04 (m, 2H); ^{13}C NMR (75 MHz, $CDCl_3$) δ 158.7, 158.6, 158.4, 158.3, 146.9, 146.1, 145.2, 144.4, 139.8, 139.5,

138.2, 135.8, 135.5, 134.9, 134.8, 133.5, 133.4, 132.7, 130.7, 130.3, 130.2, 130.1, 129.8, 128.2, 128.1, 128.0, 127.9, 127.8, 127.7, 127.4, 127.3, 127.2, 126.7, 124.4, 124.2, 124.1, 123.7, 113.2, 113.1, 113.0, 112.9, 89.3, 87.9, 71.3, 68.8, 66.4, 65.8, 55.1, 55.0, 48.5, 43.7; IR (film) ν_{max} 3350 (br, OH), 1541, 1510, 1458, 1443, 1283, 1242, 1191 cm^{-1}; HRMS (FAB) calcd for $C_{36}H_{32}N_2O_8Li$ ($M + Li^+$) 627.2319, found 627.2311.

*Caged Strand Break Building Block **1***. To a solution of **5** (112 mg, 0.18 mmol) in CH_2Cl_2 (3 mL) was added Et_3N (0.50 mL, 3.6 mmol). The mixture was stirred for 5 min before addition of 2-cyanoethyl diisopropylchlorophosphoramidite (80 μL, 0.36 mmol). The reaction mixture was stirred at room temperature for 15 min, and saturated $NaHCO_3$ solution (5 mL) was added. After separation of the layers, the aqueous phase was further extracted with CH_2Cl_2 (10 mL × 2). The combined organic extracts were dried over Na_2SO_4(s), filtered, and concentrated. The residue was flash chromatographed on silica gel (Et_3N pretreated) and eluted with EtOAc/hexane (1:3) to afford 134 mg (91%) of pale yellow foam: ^{31}P NMR (121.5 MHz, acetone-d_6) δ 152.43, 150.04, 149.43, 148.57; ^1H NMR (300 MHz, acetone-d_6) δ 7.90−7.12 (m, 17H), 6.74−6.61 (m, 4H), 5.86−4.97 (m, 2H), 3.74−3.72 (m, 6H), 3.92−3.31 (m, 4H), 3.00−2.28 (m, 4H), 1.22−1.04 (m, 9H), 0.82−0.80 (m, 3H); IR (film) ν_{max} 1541, 1510, 1459, 1443, 1401, 1381, 1304, 1242, 1191 cm^{-1}; HRMS (FAB) calcd for $C_{45}H_{50}N_4O_9P$ ($M + H^+$) 821.3315, found 821.3300.

Oligodeoxynucleotide Synthesis. Oligodeoxynucleotides were synthesized and deprotected under standard automated β-cyanoethyl phosphoramidite DNA synthesis conditions with the exception that the coupling time was extended to 30 min for the caged single strand break building block, which was coupled with an efficiency of about 80%.

Oligodeoxynucleotide Photocleavage Experiments. The oligodeoxynucleotide 21-mer (10 pmol) was either 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase or 3′-end-labeled by primer extension opposite d(CTTAGGCACGAGTCAATCTTATAC) with [α-^{32}P]dATP and KF according to standard methods (*19*). The 5′-end-labeled 21-mer and 3′-end-labeled 22-mer were purified by PAGE, desalted by ethanol precipitation, and dissolved in 20 mM Tris-HCl (pH 7.0) buffer. The 5′-end-labeled 21-mer solution (50 μL, 40 nM) or 3′-end-labeled 22-mer solution (50 μL, 40 nM) was irradiated in a 1.5 mL Eppendorf tube cap with 365 nm light from a hand-held UV lamp (Ultra-Violet Products, UVGL-25 720 $\mu W/cm^2$ at 7.6 cm) at a distance of 1 cm for 32 min at 0 °C. Aliquots of 5 μL were taken at 0, 1, 2, 4, 8, 16, and 32 min of irradiation and electrophoresed on a denaturing 15% polyacrylamide gel. For the 5′-end-labeled 21-mer, another 5 μL aliquot was taken after 32 min of irradiation and treated with T4 polynucleotide kinase by adding 1 μL of the supplied 10× kinase buffer, 3.8 μL of doubly distilled H_2O, and 0.2 μL of T4 polynucleotide kinase and incubating for 30 min at 37 °C.

Construction of the Photocleavable Double Strand DNA Plasmid. The single strand form of pIES(+)/DraI was obtained by superinfection of pIES(+)/DraI-infected *E. coli* XL1 blue cells with VCSM13 helper phage (Stratagene). Phage particles were purified by banding through a CsCl gradient. After dialysis, the single strand pIES(+)/DraI was isolated by phenol extraction and ethanol precipitation. The caged single strand break-containing oligodeoxynucleotide 27-mer was 5′-phosphorylated with T4 polynucleotide kinase and purified by PAGE, and 100 pmol was annealed to 20 pmol of the single strand pIES(+)/DraI. The primed pIES(+)/DraI was extended with the Stoffel fragment of Taq DNA polymerase (100 units) and ligated with Taq DNA ligase (400 units) in a total reaction of 0.5 mL containing 20 mM Tris-HCl (pH 7.6), 25 mM potassium acetate, 10 mM magnesium acetate, 10 mM DTT, 1 mM NAD^+, 0.1% Triton X-100, and 200 μM each dNTP for 30 min at 65 °C. The fully ligated photocleavable building block containing pIES(+)/DraI molecules was separated from unligated and unreplicated molecules by 1% agarose gel. The desired gel slices were excised, and the photocleavable double strand DNA plasmid was recovered by the QIAquick gel extraction kit (QIAGEN).

Plasmid Photocleavage and Repair Assays. The photocleavable plasmid solution (40 μL, 40 nM) was irradiated as described for the oligonucleotides. Aliquots of 5 μL were taken at 0, 1, 2, 4, 8, 16, and 32 min of irradiation and analyzed on a 1% agarose gel (1 μg/mL ethidium bromide). For repair assays, the photocleavable plasmid solution (40 μL, 40 nM) was irradiated with 365 nm light for 30 min at 0 °C to convert the strand break precursor to a single strand break. Aliquots of this solution (5 μL) were used in each 10 μL reaction mixture containing 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 26 μM NAD^+, 25 μg/mL BSA, and 200 μM each dNTP. Endonuclease IV (1 unit), polymerase I (1 unit), and *E. coli* DNA ligase (5 units) were used as indicated. The reactions were incubated for 30 min at 37 °C and electrophoresed on a 1% agarose gel (1 μg/mL ethidium bromide). The gels were photographed through a Kodak 23A filter, and the image was scanned and analyzed by Scion Image software. A correction factor of 0.7 was applied to the intensity of the form IV DNA to account for differences in affinity of form II and form IV DNA for ethidium bromide. The factor was derived from analysis of an equimolar mixture of form III DNA and form IV DNA that contained some form II DNA, making the assumption that form II DNA and form III DNA bind ethidium bromide equally well.

Enzymatic Assays of the Single Strand Break-Containing 27-mer DNA Duplex. The 27-mer was 5′-end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, purified by PAGE, desalted by ethanol precipitation, and dissolved in doubly distilled H_2O. The 5′-end-labeled 27-mer was annealed to its complementary strand (molar ratio 1:1.1). The duplex DNA was irradiated with 365 nm light for 30 min at 0 °C to convert the photocleavable precursor to a strand break. The 27-mer duplex substrate (final concentration of 20 nM) was mixed on ice with the enzymes in 5 μL of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 26 μM NAD^+, 25 μg/mL BSA, and 200 μM dATP or ddATP (as indicated). Endonuclease IV (0.4 unit), polymerase I (1 unit), KF (1 unit), or exo$^-$ KF (1 unit) were used as indicated. The reactions were incubated for 15 min at 22 °C except for the endonuclease IV reaction which was 2 min at 22 °C and stopped by the addition of 5 μL of formamide loading buffer (95% formamide, 20 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were heated at 90 °C for 10 min prior to being loaded on a 15% denaturing polyacrylamide gel. For a time−course study the single strand break-containing 27-mer DNA duplex (20 nM) was mixed

on ice with polymerase I (0.25 unit) or KF (5 units) in 25 μL of 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM DTT, 26 μM NAD^+, 25 μg/mL BSA, and 200 μM dATP, in the presence or absence of endonuclease IV (1 unit). The reactions were incubated for 16 min at 22 °C. Aliquots of 5 μL were taken at 1, 2, 4, 8, and 16 min, and the reaction was stopped by the addition of 5 μL of formamide loading buffer (95% formamide, 20 mM EDTA, 0.025% bromophenol blue, 0.025% xylene cyanol). Samples were heated at 90 °C for 10 min prior to being loaded on a 15% denaturing polyacrylamide gel.

RESULTS AND DISCUSSION

Design and Synthesis. We had previously designed a building block for the production of phosphate-terminated single strand breaks based on *o*-nitrobenzyl photochemistry which was to release a 5′-phosphate upon 366 nm irradiation and then the 3′-phosphate by a β-elimination reaction (*20*). Though the 5′-phosphate was released as designed, the 3′-phosphate was not released spontaneously at neutral pH and required hot piperidine treatment to go to completion. To solve this problem, another building block was designed and synthesized that would release a 3′-phosphate photochemically and a 5′-phosphate by a β-elimination reaction. When this building block was used in tandem with the other building block, both the 5′- and 3′-phosphates were directly released upon irradiation (*21*). Because two different building blocks must be used in tandem to produce a phosphate-terminated single nucleotide gap, they may not be useful for in vivo applications in which the strand break precursor must elude repair systems. With this in mind, we designed a single building block to achieve the same goal (Figure 1).

The synthesis of the building block **1** is outlined in Figure 2. We had previously shown that aldol reaction between 2-nitrobenzaldehyde and 2′-nitroacetophenone followed by reduction with sodium borohydride produces the diol **4** in 48% yield (*18*). The diol **4** was monoprotected by the dimethoxytrityl group in 73% yield, and the product **5** was converted to the phosphoramidite building block **1** in 91% yield. The building block was then used to incorporate the caged single strand break site-specifically into a 21-mer and 27-mer by standard automated DNA synthesis.

Photocleavage Experiments. Irradiation of the 5′-end-labeled 21-mer with 365 nm light for 32 min at pH 7.0 afforded a major radioactive band (97%) which was presumed to be the 3′-phosphate-terminated 12p-mer (Figure 3a). In accord with this expectation, treatment of the reaction mixture with T4 polynucleotide kinase, which has 3′-phosphatase activity (*22*), converted the original band to one that comigrated with authentic dephosphorylated 12-mer. Exposure of 3′-end-labeled 22-mer with 365 nm light for 32 min at pH 7.0 resulted in the formation of a major band (95%) which comigrated with the expected 5′-phosphorylated 9-mer (Figure 3b). The lower mobility bands formed during irradiation presumably correspond to the intermediates in the sequential photolysis pathway (Figure 1).

Construction of a Duplex Circular Vector Containing a Site-Specific Strand Break. The site-specific caged strand break containing DNA plasmid was constructed (Figure 4) by standard oligodeoxynucleotide-directed mutagenesis techniques which have been successfully used to incorporate

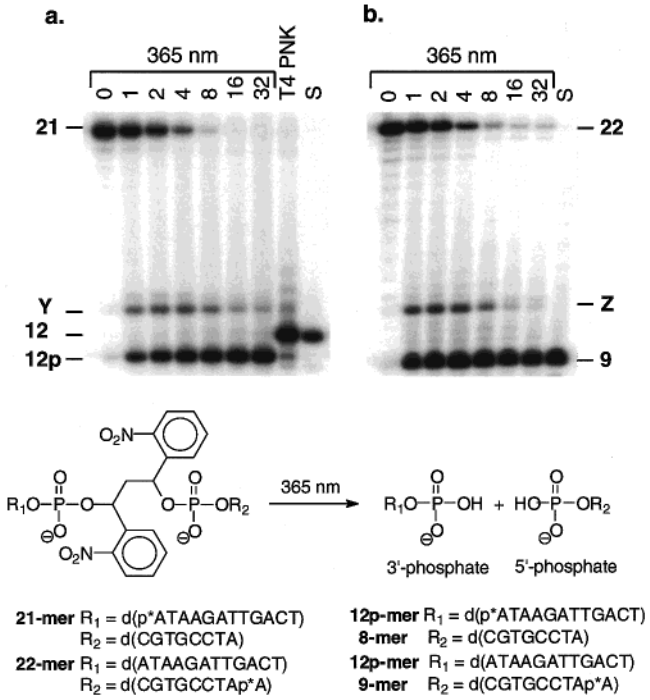

FIGURE 3: Photocleavage of 40 nM (a) 5′-end-labeled 21-mer or (b) 3′-end-labeled 22-mer. Irradiation with 365 nm light was conducted at 0 °C with a hand-held UV lamp, and aliquots were taken at the indicated times in minutes. Lane S: authentic d(p*ATAAGATTGACT) in panel a or d(p*CGTGCCTAA) in panel b. Lane T4 PNK: 3′-phosphatase activity of T4 polynucleotide kinase was used to remove the 3′-phosphate of the 12p-mer. Bands Y and Z refer to the intermediates shown in Figure 1.

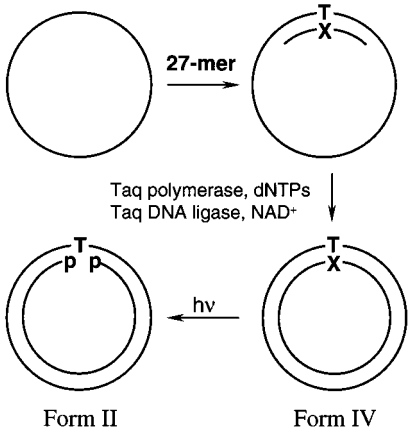

FIGURE 4: Construction of vectors containing site-specific strand breaks by a caged strand break approach.

DNA damage into replicative form bacteriophages (*13*−*16*). To this end, 5′-phosphorylated 27-mer containing the caged single strand break was annealed to the single strand form of pIES/DraI and extended with the Stoffel fragment of Taq polymerase in the presence of dNTP's and Taq ligase and NAD^+ to complete the synthesis of the complementary strand. This process generates a relaxed closed circular DNA, otherwise known as form IV DNA. Because only covalently closed circular duplex molecules can be supercoiled in the presence of ethidium bromide, the desired ligated product (form IV DNA) could be easily separated from unligated products (form II DNA) by agarose gel electrophoresis in the presence of ethidium bromide. Irradiation of the plasmid with 365 nm light for 8 min produced the expected strand

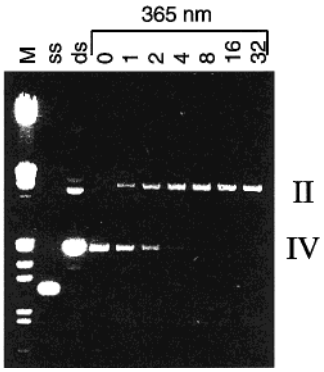

FIGURE 5: Photoactivated conversion of the relaxed closed circular plasmid DNA to single strand break-containing DNA. Irradiation with 365 nm light was conducted at 0 °C with a hand-held UV lamp, and aliquots were taken at the indicated times in minutes. Lane M: λ DNA/*Eco*RI + *Hin*dIII marker. Lane ss: single strand pIES(+)/DraI. Lane ds: double strand pIES(+)/DraI. Roman numerals refer to the position of the relaxed closed circular DNA (form IV) and open circular (form II) DNA.

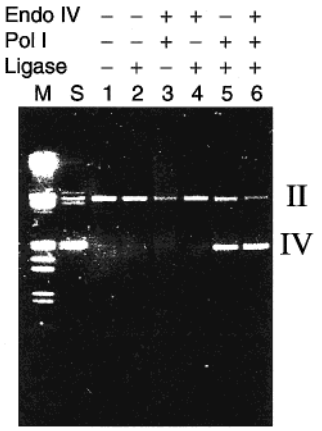

FIGURE 6: In vitro repair of the single strand break in the vector DNA. Lane M: λ DNA/*Eco*RI + *Hin*dIII marker. Lane S: double strand pIES(+)/DraI. Roman numerals refer to the position of the relaxed closed circular (form IV) and open circular (form II) DNA. All reactions were run for 30 min at 37 °C.

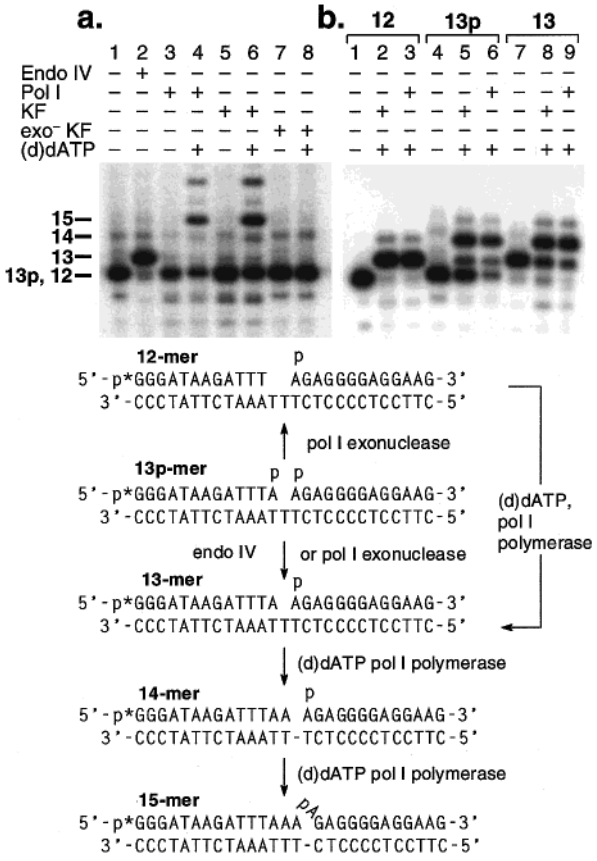

FIGURE 7: Enzymatic reactions on the single strand break-containing 27-mer DNA duplex. All reactions were run for 15 min at 22 °C. The reactions in panel a were carried out with dATP and those in panel b with ddATP.

In Vitro Repair of the Strand Break-Containing Vector. It was originally thought that the repair of single strand breaks consisting of a single nucleotide gap flanked by a 5′-phosphate and either a 3′-phosphate or 3′-phosphoglycolate required three enzymatic steps (*23*, *24*). First the blocking 3′-terminal must be removed, followed by filling of the gap by a polymerase and a dNTP, and finally sealing of the nick by a ligase. For *E. coli*, exonuclease III or endonuclease IV could perform the first step of the repair followed by polymerase I to fill the gap and *E. coli* DNA ligase to seal the nick (*1*, *10*). To help verify that we had produced the expected phosphate-terminated single nucleotide gap in the vector, we subjected the photocleaved vector to various combinations of the *E. coli* enzymes, endonuclease IV, polymerase I, and DNA ligase, and analyzed the reaction mixtures by agarose gel electrophoresis in the presence of ethidium bromide (Figure 6). As expected for a phosphate-terminated single nucleotide gap, DNA ligase or DNA ligase and endonuclease IV were unable to close the gap. Unexpectedly, the break was repaired by polymerase I and *E. coli* DNA ligase in the absence (approximately 46%) as well as in the presence of endonuclease IV (approximately 58%), suggesting that the 3′-phosphate terminus can be processed by polymerase I in contrast to the results of an earlier study (*17*). In that study, 3′-phosphate- and 3′-phosphoglycolate-terminated 7-mers, $(dA)_7$, were found not to be substrates for polymerase I in the presence of dNTPs and a poly(dT) template.

Action of Polymerase I and KF on 3′-Phosphate Termini. To confirm that polymerase I was capable of processing DNA containing a terminal 3′-phosphate, high-resolution gel studies were carried out on the repair of the break formed by irradiating the 27-mer with 365 nm light in the presence of its complementary strand containing a T opposite the caged strand break. As can be seen from Figure 7, both polymerase I and the Klenow fragment which lacks the 5′ → 3′ exonuclease domain were able to extend the 3′-phosphorylated 13-mer to a major product corresponding to the 15-mer in the presence of dATP and in the absence of endonuclease IV (panel a, lanes 4 and 6). In contrast, the 3′ → 5′ exonuclease deficient Klenow fragment could not (panel a, lane 8), suggesting that the exonuclease was required for

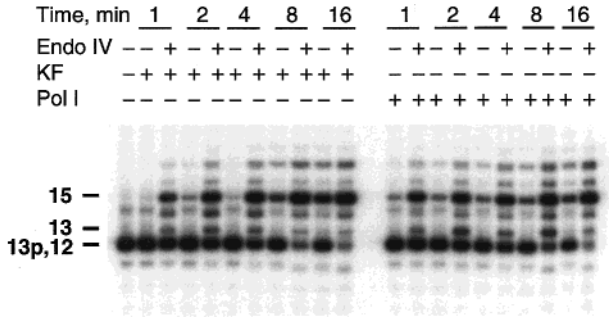

FIGURE 8: Time−course study (22 °C) of primer extension by polymerase I or KF in the presence or absence of endonuclease IV on the single strand break-containing 27-mer DNA duplex. The reactions were run for the indicated number of minutes.

processing the terminus. When the reactions with KF and polymerase I were carried out in the absence of dATP (panel a, lanes 3 and 5), no significant band corresponding to the dephosphorylated 13-mer was observed. This would at first appear to indicate that the 3′-OH terminus needed for primer extension is being produced by 3′ → 5′ exonucleolytic cleavage of the 3′-phosphorylated nucleotide and not by hydrolysis of the 3′-phosphate group. The 12-mer product of exonucleolytic removal of pdAp from the 13p-mer cannot be detected independently, however, because it does not migrate sufficiently differently from the 13p-mer in the gel system used (panel b, lanes 1 and 4).

To gain further evidence for how the polymerases were processing the 3′-phosphorylated end, we investigated the action of the polymerase on the single strand break substrate with ddATP in place of dATP. If the reaction is proceeding through exonucleolytic removal of pdAp from the 13p-mer to give the 12-mer, it would only be extended to the 13-mer in the presence of ddATP, but if it is proceeding through dephosphorylation of the 13p-mer to produce the 13-mer, it would be extended to the 14-mer in the prescence of ddATP. Surprisingly, we find that the 14-mer is the major product of the reaction and that the 13-mer is the minor product (panel b, lanes 5 and 6), suggesting that the major pathway does not involve exonucleolytic removal of the 3′-phosphorylated nucleotide. This is somewhat puzzling in light of the fact that we did not detect the dephosphorylated intermediate in reactions in which the dATP was omitted (panel a, lanes 3 and 5), which would suggest that the presence of the dATP is required for the phosphatase activity. Further study will be required to fully elucidate the mechanism of this reaction.

Though polymerase I and KF can convert the 3′-phosphate-terminated 13p-mer into a suitable substrate for primer extension, the addition of endonuclease IV significantly enhanced the rate of the repair as shown in Figure 8. Whereas extension of the 3′-phosphorylated 13-mer by both KF and polymerase I is half-complete in about 2 min in the presence of endonuclease IV, it is only 40% and 20% complete in 16 min in the absence of endonuclease IV. The failure of the previous study to detect exonucleolytic cleavage of a 3′-phosphate-terminated oligodeoxynucleotide by polymerase I (*17*) was probably due to the short size (a 7-mer) and low concentration of the substrate used and the inefficient nature of the reaction as well as the possible requirement for the presence of a dNTP. The observation that polymerase I and DNA ligase can repair 50% of X-ray-induced strand breaks (*25*) and 20−30% of ^{32}P-induced strand breaks (*26*) suggests that though polymerase I is able to process the 3′-phosphate-terminated gaps, it is not able to process those terminating in a 3′-phosphoglycolate or other types of ends as well.

CONCLUSION

We have been able to cage one of the major types of DNA strand breaks produced by ionizing radiation and AP lyase activity with a readily prepared building block. We have also shown how this building block can be used to readily insert such a break site-specifically and sequence-independently into circular duplex vectors which could be used to facilitate in vitro and in vivo studies of single strand break repair, replication, and transcription. By using substrates prepared from this building block, we were able to demonstrate that *E. coli* polymerase I and DNA ligase alone can repair phosphate-terminated gaps and that 3′-end processing enzymes such as endonuclease IV or exonuclease III are not absolutely required. A phototriggered building block could also be very useful for the construction of double strand breaks and multiply damaged sites (MDS), which might otherwise be hard to prepare by other methods due to their lability.

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REFERENCES

1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) *DNA Repair and Mutagenesis*, ASM Press, Washington DC.
2. Ward, J. F. (1995) *Radiat. Res. 142*, 362−368.
3. Sutherland, B. M., Bennett, P. V., Sidorkina, O., and Laval, J. (2000) *Biochemistry 39*, 8026−8031.
4. Dar, M. E., and Jorgensen, T. J. (1995) *Nucleic Acids Res. 23*, 3224−3230.
5. Dar, M. E., Winters, T. A., and Jorgensen, T. J. (1997) *Mutat. Res. 384*, 169−179.
6. Povirk, L. F. (1996) *Mutat. Res. 355*, 71−89.
7. von Sonntag, C. (1987) *The Chemical Basis of Radiation Biology*, Taylor and Francis, London.
8. Henner, W. D., Rodriguez, L. O., Hecht, S. M., and Haseltine, W. A. (1983) *J. Biol. Chem. 258*, 711−713.
9. Ward, J. F. (1988) *Prog. Nucleic Acid Res. Mol. Biol. 35*, 95−125.
10. Demple, B., and Harrison, L. (1994) *Annu. Rev. Biochem. 63*, 915−948.
11. Harrison, L., Hatahet, Z., and Wallace, S. S. (1999) *J. Mol. Biol. 290*, 667−684.
12. Winters, T. A., Russell, P. S., Kohli, M., Dar, M. E., Neumann, R. D., and Jorgensen, T. J. (1999) *Nucleic Acids Res. 27*, 2423−2433.
13. Preston, B. D., Singer, B., and Loeb, L. A. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 8501−8505.
14. Kodadek, T., and Gamper, H. (1988) *Biochemistry 27*, 3210−3215.
15. Jiang, N., and Taylor, J.-S. (1993) *Biochemistry 32*, 472−481.
16. Ensch-Simon, I., Burgers, P. M. J., and Taylor, J.-S. (1998) *Biochemistry 37*, 8218−8226.
17. Henner, W. D., Grunberg, S. M., and Haseltine, W. A. (1983) *J. Biol. Chem. 258*, 15198−15205.
18. Zhang, K., and Taylor, J.-S. (1999) *J. Am. Chem. Soc. 121*, 11579−11580.
19. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular cloning: A laboratory manual*, Vol. 1, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

20. Ordoukhanian, P., and Taylor, J.-S. (1995) *J. Am. Chem. Soc. 117*, 9570−9571.
21. Ordoukhanian, P., and Taylor, J.-S. (2000) *Bioconjugate Chem. 11*, 94−103.
22. Cameron, V., and Uhlenbeck, O. C. (1977) *Biochemistry 16*, 5120−5126.
23. Kapp, D. S., and Smith, K. C. (1969) *Int. J. Radiat. Biol. 14*, 567−571.
24. Mitzel-Landbeck, L., Schutz, G., and Hagen, U. (1976) *Biochim. Biophys. Acta 432*, 145−153.
25. Laipis, P. J., and Ganesan, A. T. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 3211−3214.
26. Fodor, I., Matvienko, N. I., Zlotnikov, K. M., Tanyashin, V. I., Solonin, A. S., and Bayev, A. A. (1975) *Nucleic Acids Res. 2*, 635−646.

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