Incorporation of Biotin-Labeled Deoxyuridine Triphosphate into DNA during Excision Repair and Electron Microscopic Visualization of Repair Patches†

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Received March 26, 1985

ABSTRACT: Biotin-labeled deoxyuridine triphosphate (BiodUTP) has the potential to be a useful affinity probe for studies on DNA repair, if it can be incorporated into DNA repair patches and does not inhibit subsequent steps in the excision repair pathway. We have synthesized BiodUTP by an improved procedure and have used permeable normal human fibroblasts to determine the effect of substituting BiodUTP for thymidine triphosphate on several steps in the excision repair pathway: incision, polymerization, ligation, and nucleosome rearrangement. The results demonstrate that BiodUTP is efficiently incorporated into repair patches and has little or no effect on the repair process. The presence of BiodUMP in ligated repair patches has been used to visualize the repair patches by electron microscopy following incubation with ferritin-labeled avidin. This approach has been used to estimate the maximum size of repair patches induced by ultraviolet radiation.

Nucleotide analogues that can act both as affinity probes and as substrates for DNA polymerases have many potential uses, including the visualization and isolation of newly replicated or repaired sites in DNA and chromatin. Langer et al. have synthesized 5-(allylamino)biotin 2′-deoxyuridine triphosphate (BiodUTP)[1] and have found that it is a substrate for several purified DNA polymerases, including eukaryotic polymerases α and β (Langer et al., 1981). In normal human fibroblasts, polymerases α and β mediate the DNA repair synthesis induced by UV radiation, *N*-methyl-*N*-nitrosourea, and bleomycin (Dresler & Lieberman, 1983). These observations, together with the fact that avidin, a glycoprotein isolated from egg white, binds tightly to biotin ($K_{dis} = 10^{-15}$) and recognizes the biotin group of BiodUTP even when it is present in double-stranded DNA, suggest that BiodUTP might be useful for studying DNA excision repair (Langer et al., 1981). We have synthesized BiodUTP by a modification of a published method (Langer et al., 1981) and have used permeabilized normal human fibroblasts to determine the effect on the excision repair pathway of substituting BiodUTP for dTTP. Our results show that BiodUTP is efficiently incorporated into repair patches and that the presence of BiodUMP in repair patches has no effect on subsequent steps in the repair process (i.e., ligation and nucleosome rearrangement). The presence of BiodUMP in the repair patches has allowed the direct visualization of DNA repair patches by electron microscopy using ferritin-conjugated avidin, which permitted an estimation of the maximum size of repair patches induced by ultraviolet radiation.

Materials and Methods

Measurement of the Incorporation of BiodUTP or dTTP into Poly(dA-dT) by Escherichia coli Polymerase I. The rate of incorporation of [^{3}H]dATP into insoluble material at 37 °C was determined in the presence of dTTP (100 pmol) or BiodUTP (100 pmol) or neither. In addition to these substrates, the reaction mixture contained the following components in a volume of 90 μL: 0.01 A_{260} unit of poly(dA-dT), 0.9 μmol of dAMP, 9 μmol of Hepes buffer, pH 7.4, 50 pmol of [^{3}H]dATP (0.5 μCi), and 0.38 Richardson unit of *E. coli* DNA polymerase I. At each time point, aliquots were removed and spotted on squares of Whatman 3MM filter paper which had been wetted with 200 μL of 2% sodium pyrophosphate. The squares were washed (3 × 15 min) with a solution of 5% Cl_3CCOOH and 1% sodium pyrophosphate, rinsed once with 95% ethanol, and finally washed (1 × 15 min) with 95% ethanol. The radioactivity in the dried filter was then determined.

Cell Culture. Human diploid fibroblasts (AG1518; Institute for Medical Research) were passed into plastic tissue culture plates, prelabeled with [^{14}C]dThD (Amersham Corp., 50–60

†This work was supported in part by a grant to the Department of Pathology, Washington University Medical School, from the following companies: Brown & Williamson Tobacco Corp., Philip Morris Inc., R. J. Reynolds Tobacco Co., and United States Tobacco Co.

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[1] Abbreviations: BiodUTP, 5-(allylamino)biotin 2′-deoxyuridine triphosphate; K_{dis}, dissociation constant; BiodUMP, 5-(allylamino)biotin 2′-deoxyuridine monophosphate; dTTP, thymidine triphosphate; UV, ultraviolet; dATP, deoxyadenosine triphosphate; Cl_3CCOOH, trichloroacetic acid; dAMP, deoxyadenosine triphosphate; Tris, 2-amino-2-(hydroxymethyl)-1,3-propanediol; EDTA, ethylenediaminetetraacetic acid; dCTP, deoxycytidine triphosphate; BrdUrd, bromodeoxyuridine; CsTFA, cesium trifluoroacetate; PBS, phosphate-buffered saline; dThd, thymidine; Exo III, exonuclease III; PCA, perchloric acid; SN, staphylococcal nuclease; AAdUTP, 5-(3-aminoallyl)-2′-deoxyuridine 5′-triphosphate; HPLC, high-performance liquid chromatography; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; TLC, thin-layer chromatography; bp, base pair(s).

0006-2960/85/0424-5729$01.50/0

mCi/mmol, 20 nCi/mL) for 1 week, and grown to confluence.

Damaging of Cells with UV Radiation. In most experiments, cells were damaged with UV radiation by removing the medium from the culture plate and exposing the cells at 37 °C to radiation from a G15T8 germicidal lamp at a flux of 1.2 W/m^2. UV fluxes and doses were measured by using an Ultraviolet Products J225 ultraviolet meter. In some cases, however, cells were first made permeable, then spread as a suspension 1 mm in thickness in a plastic dish, and irradiated with UV light at 4 °C as described (Dresler et al., 1982).

Excision Repair in Permeable Cells. The permeable cell system of Dresler et al. was used (Dresler et al., 1982). The cells were harvested, suspended in 10 mM Tris (pH 7.6 at 37 °C), 4 mM $MgCl_2$, 1 mM EDTA, 3 mM dithiothreitol, and 250 mM sucrose, and placed on ice for 30 min. Small portions (0.05–0.1 mL) of this permeable cell suspension were mixed at 4 °C with 0.5 volume of a concentrated reaction mix to give the following final concentrations: 40 mM Tris (pH 7.6 at 37 °C), 8 mM $MgCl_2$, 15 mM KCl, 5 mM ATP, 3 μM dCTP, 3 μM dGTP, 3 μM dATP, 40 μCi/mL [α-^{32}P]dCTP (Amersham Corp., 410 Ci/mmol), 3 μM dTTP or BiodUTP, 167 mM sucrose, 2 mM dithiothreitol, and 0.67 mM EDTA. Samples were placed at 37 °C for the times indicated, and the reaction was stopped by adding 5 mL of cold 10 mM Tris (pH 7.6 at 37 °C), 320 mM sucrose, and 0.5% Triton X-100. For measurement of repair synthesis, the DNA was precipitated with Cl_3CCOOH and collected on glass fiber filters, and radioactivity was determined by liquid scintillation counting. Repair synthesis is the difference between incorporation in corresponding irradiated and unirradiated samples. Under the reaction conditions used, repair synthesis determined by this technique has been shown to be equivalent to values obtained by using the BrdUrd density gradient technique (Dresler et al., 1983).

Purification of DNA from Permeable Cells Using Cesium Trifluoroacetate Density Gradients. The permeable cells were washed 3 times with 10 mM Tris (pH 7.6 at 37 °C), 320 mM sucrose, and 0.5% (v/v) Triton X-100, then resuspended in 2 mM Tris (pH 7.4 at 22 °C), 1 mM EDTA, and 1% sodium *N*-lauroylsarcosine, and sheared 10 times through a 20-gauge needle. The insoluble material was pelleted, and the supernatant was added to cesium trifluoroacetate (CsTFA). The CsTFA was adjusted to a density of 1.60 g/cm^3 at 22 °C and centrifuged at 50000 rpm in a Beckman VTi65 vertical rotor for 10 h. The gradients were fractionated from the bottom, and the absorbance at 260 nm and the refractive index of the 0.2-mL fractions were determined. The DNA banded at a density of 1.60. The peak fractions were pooled, and the DNA was precipitated with ethanol.

Measurement of DNA Strand Breaks. DNA strand breaks were measured by the alkaline elution method (Kohn et al., 1976). Fibroblasts (ca. 2×10^6 cells), prelabeled with [^{14}C]dThd, were damaged and incubated as described and then harvested by gentle scraping in 2 mL of ice-cold PBS containing 0.02% EDTA. One milliliter of cell suspension was added to 20 mL of ice-cold PBS containing an internal standard consisting of ca. 5×10^5 L1210 cells which had been labeled for ca. 16 h with 25 nCi/mL [^{3}H]dThd and irradiated with 1000 or 2000 rad of γ radiation, depending on the required range of the assay. The cells were collected on a poly(vinyl chloride) filter (Millipore, BSWP 025 00), washed with 5 mL of PBS, lysed with 4 mL of 2 M NaCl, 40 mM EDTA, 0.2% *N*-lauroylsarcosine, pH 10, and then incubated with 1 mL of 0.5 mg/mL proteinase K in lysing buffer for 20 min. This was followed by 5 mL of 20 mM EDTA, pH 10, and then the DNA was eluted with 20 mM EDTA adjusted to pH 12.0 with tetrapropylammonium hydroxide, at a flow rate of 20 or 50 mL/100 min for the 1000- and 2000-rad assays, respectively. The radioactivity of the eluted DNA was determined by liquid scintillation counting. Standard curves were constructed by irradiating fibroblast monolayers with γ rays so that data could be expressed in rad equivalents or as DNA strand breaks per cells, using the conversion factor of three breaks per rad per cell (Kohn et al., 1976).

Measurement of Repair Ligation Using Exonuclease III. Unligated repair patches should have free 3′-OH ends and therefore be sensitive to exonuclease III, a 3′–5′-exonuclease. To prepare DNA for digestion, nuclei were incubated with proteinase K (0.5 mg/mL) in 10 mM Tris (pH 7.8 at 20 °C), 250 mM sucrose, 0.1 mM $CaCl_2$, and 10 mM EDTA at 47 °C for 2 h. The samples were extracted 3 times with isoamyl alcohol–chloroform (1:24), and the DNA was precipitated with ethanol and dissolved in Exo III digestion buffer (50 mM Tris, pH 8.0 at 37 °C, 5 mM $MgCl_2$, 10 mM β-mercaptoethanol, and 0.5 mg/mL nuclease-free bovine serum albumin) overnight, at 4 °C on a rotating wheel. All manipulations of the DNA were designed to minimize shearing. The DNA was digested at 37 °C with 0.2 unit of Exo III (BRL) per microgram of DNA, and samples were removed at several times during the 50-min digestion period for determination of PCA-soluble ^{3}H (repair label) and ^{14}C (bulk label) radioactivities which were each expressed as a fraction of the total radioactivity in the DNA being digested. The release of ^{14}C from bulk DNA, which probably resulted from digestion at random breaks produced by shearing during isolation of the DNA, was linear and was usually less than 5% of the total at the end of the digestion. The release of ^{14}C from the DNA of unirradiated cells and that from the DNA of irradiated cells were not significantly different, indicating that continued digestion of bulk DNA 5′ to repair patches or at incised sites which lack repair patches does not contribute significantly to the ^{14}C release. The release of ^{3}H was biphasic, with a rapid initial phase followed by a late slow linear phase. It was assumed that the late slow phase of ^{3}H release resulted, like ^{14}C release from bulk DNA, from Exo III digestion at random breaks not related to the repair process. Therefore, the fraction of repair-incorporated nucleotides present in unligated repair patches was determined by extrapolating the second phase of the ^{3}H release to zero digestion time. Under our conditions, replicative synthesis represented less than 10% of the total ^{3}H incorporation, and therefore, no correction was made for the release of replicative ^{3}H by Exo III.

Preparation of Nuclei and Digestion with Staphylococcal Nuclease. Cells were harvested; nuclei were prepared and digested with staphylococcal nuclease (SN) (Worthington Biochemical Corp.) as described previously (Smerdon et al., 1976) for evaluation of the extent of nucleosome rearrangement.

Purification of Ferritin–Avidin Conjugate. Ferritin conjugated to avidin (Sigma) was separated from the aggregates by sedimentation on a sucrose gradient (5–28%) in 1 mM Tris buffer, pH 7.4, 4 mM NaCl, and 0.25 mM EDTA in a SW41 rotor (Beckman) for 1 h at 38000 rpm.

Purification of DNA–Avidin–Ferritin Complex. Control DNA or BiodUMP–DNA (0.3–0.5 μg) was incubated for 1 h at 30 °C with a 10-fold excess of purified avidin–ferritin conjugate in 10 mM Tris buffer, pH 7.4, containing 100 mM NaCl and 0.2 mM EDTA. The excess of unbound label was removed by gel filtration on a Sepharose 4B (Bio-Rad) column (0.8×15 cm) equilibrated with the same buffer.

Electron Microscopy. Adsorption of the specimens onto carbon-coated nickel grids (400 mesh) was performed either by the amylamine procedure (Dubochet et al., 1971) or by the modified Kleinschmidt method (Davis et al., 1971). The grids were rotary shadowed with tungsten at an angle of 7° in an Edwards evaporator equipped with an electron gun (EVMO52, Balzers). The thickness of the metal deposition was monitored on a quartz thin-crystal monitor (QSG201D, Balzers). The grids were examined in an Hitachi H600 electron microscope equipped with a video system (CV152 Sofretect, Paris). The magnification was calibrated by a carbon grating replica (Fullam, Schenectady, NY).

Results and Discussion

Synthesis of AAdUTP. 5-(3-Aminoallyl)-2′-deoxyuridine 5′-triphosphate (AAdUTP) was synthesized by starting from dUTP essentially as described (Langer et al., 1981). The palladium catalyst was removed by filtration and passage through a Dowex-50 (200–400 mesh) column. HPLC analysis (Whatman ODS-3 reversed-phase column, 0.1 M $(NH_4)H_2PO_4$, pH 5.1, mobile phase) demonstrated two major peaks and three minor ones. One of the major peaks (approximately 50%) was identified as AAdUTP on the basis of its UV and NMR spectra and its chromatographic behavior on HPLC and TLC compared to a sample of AAdUTP provided by Dr. Alex Waldrop.

Synthesis of BiodUTP from AAdUTP. The reaction conditions of Langer et al. were modified (Langer et al., 1981) in order to increase the yield of BiodUTP. *N*-(Hydroxysuccinimido)biotin (0.44 mmol, Sigma) was dissolved in dimethylformamide and added to AAdUTP (0.4 mmol) in sodium bicarbonate buffer (140 mM, pH 8.2). The reaction mixture was left to stir at room temperature for 2 h. An additional 0.22 mmol of *N*-(hydroxysuccinimido)biotin was added, and the reaction mixture was stirred for a further 1.5 h. We found that the reaction conditions used by Langer et al. (0.1 M sodium borate, pH 8.5) produced a very low yield of BiodUTP, probably as a result of rapid hydrolysis of *N*-(hydroxysuccinimido)biotin caused by the buffer. With their conditions, no *N*-(hydroxysuccinimido)biotin could be detected by TLC after a 2-h incubation at room temperature, even in the absence of AAdUTP. Under our reaction conditions, more than 50% of the *N*-(hydroxysuccinimido)biotin was present after a 2-h incubation in the absence of AAdUTP, and the final yield of BiodUTP from the complete reaction mixture was 40%.

The BiodUTP was purified by HPLC using a Whatman Magnum 9, ODS-3, reversed-phase column starting with a mobile phase of $(NH_4)H_2PO_4$ (30 mM, pH 5.1, 2 mL/min) followed by a mobile phase of water which eluted the BiodUTP. The BiodUTP was 95% pure as determined by analytical HPLC and contained no detectable AAdUTP, HgdUTP, or dUTP. The major impurity contained the biotin group since it gave a positive reaction (orange color) with *p*-(dimethylamino)cinnamaldehyde in ethanolic sulfuric acid (McCormic & Roth, 1970) and was probably BiodUDP on the basis of its retention time relative to BiodUTP on an anion-exchange HPLC column. The overall yield of BiodUTP from dUTP was 20%.

Elemental analysis was consistent with the following formula: $C_{22}H_{30}N_5O_{16}P_3SNa_4 \cdot 2H_2O$. Anal. Calcd for $C_{22}H_{30}N_5O_{16}P_3SNa_4 \cdot 2H_2O$: C, 30.25; H, 3.92; N, 8.02. Found: C, 30.12; H, 3.75; N, 7.80.

Incorporation of BiodUTP into Poly(dA-dT) by E. coli DNA Polymerase I. As a test of the BiodUTP synthesized by our procedure, its efficiency as a substrate for DNA polymerase I from *E. coli* was determined. The rate of incorporation of [^{3}H]dATP into Cl_3CCOOH-insoluble material at 37 °C was determined in the presence of dTTP (100 pmol) or BiodUTP (100 pmol) or neither. The initial rate of incorporation of [^{3}H]dATP in the presence of BiodUTP was 30% of the rate in the presence of dTTP, while the rate in the presence of only [^{3}H]dATP was 1% of the control rate. These results are in agreement with published results (Langer et al., 1981).

Table I: Incorporation of dTTP or BiodUTP into the DNA of Permeable Human Fibroblasts in Response to UV Radiation[a]

expt no.	exptl condn	rel repair synthesis	substrate
1	intact cells irradiated, incubated 20 min, and then permeabilized	1.0	dTTP
		0.79	BiodUTP
		0.041	dTTP, unirradiated
2	intact cells irradiated, incubated 20 min in presence of hydroxyurea and aphidicolin, and then permeabilized	1.0	dTTP
		0.78	BiodUTP
		0.12	dTTP, unirradiated
3	cells permeabilized and then irradiated	1.0	dTTP
		0.98	BiodUTP
		0.29	dTTP, unirradiated

[a] In experiment 1, confluent normal human fibroblasts prelabeled with [^{14}C]dThd were damaged with 10 J/m^2 UV radiation and incubated at 37 °C for 20 min at which time the number of DNA single-strand breaks (incised and partially polymerized sites) was at a maximum (ca. 2400 breaks per cell as determined by alkaline elution). The cells were then permeabilized, washed to remove endogenous deoxyribonucleotides, and incubated with [^{32}P]dCTP, dATP, dGTP, and either dTTP or BiodUTP, all at 3 μM, for 15 min at 37 °C. The amount of repair synthesis, measured as the ^{32}P to ^{14}C ratio in Cl_3CCOOH-insoluble material, was then determined. In experiment 2, intact cells were damaged with UV radiation as in experiment 1 and were then incubated with inhibitors of repair synthesis (10 mM hydroxyurea plus 7.7 μM aphidicolin) for 20 min at 37 °C. These inhibitors caused a rapid accumulation of DNA single-strand breaks (incised and partially polymerized sites; ca. 7500 sites per cell). The cells were then permeabilized, washed, and incubated as in experiment 1. In experiment 3, cells were permeabilized, irradiated with 100 J/m^2 UV radiation at 4 °C, and incubated at 37 °C for 20 min in the presence of [^{32}P]dCTP, dATP, dGTP, and either dTTP or BiodUTP, all at 3 μM, and the amount of repair synthesis was determined.

To determine whether the biotin group was present in polymer synthesized in the presence of BiodUTP, the efficiency of binding of the polymer to avidin–agarose was determined. Poly(dA-dT) (0.005 A_{260} unit), synthesized in the presence of [^{3}H]dATP and either dTTP or BiodUTP as described above, was incubated with 1 unit of avidin–agarose (1 unit binds 4 nmol of biotin) in buffer A (150 mM NaCl, 1 mM EDTA, and 50 mM Tris, pH 7.2). After 10 min on ice, the mixtures were centrifuged, the supernatants were removed, the avidin–agarose pellets were washed 3 times with buffer A, and the Cl_3CCOOH-insoluble radioactivity in the incubation supernatants and washes was determined. Of the radioactivity in the polymer, 94% synthesized in the presence of BiodUTP was bound to avidin–agarose, compared to 0% of the radioactivity in the polymer synthesized in the presence of dTTP. This rules out the possibilities either that contaminants in the BiodUTP caused the incorporation of [^{3}H]dATP or that the biotin group was lost during the polymerization reaction.

Effect of BiodUTP on Polymerization and Incision during UV-Induced DNA Repair in Permeable Cells. The purpose of the experiments in Table I was 2-fold: first, to determine whether BiodUTP could substitute for dTTP in the DNA repair synthesis reaction in permeable cells, when incision had occurred in intact cells, and second, to determine the effect

of BiodUTP on the incision step, by irradiating permeable cells and then allowing all steps of the excision repair process to occur in the presence of the reaction mixture containing BiodUTP. Because the incision step is rate limiting under these conditions (Dresler et al., 1982), the rate of repair synthesis in such an experiment is an effective measure of the rate of incision at sites of damage.

In experiment 1, intact cells were irradiated with UV radiation, incubated at 37 °C for 20 min, at which time a maximum number of incised and partially polymerized sites were present (ca. 2400 sites per cell, as determined by alkaline elution), and then permeabilized to allow exogenous deoxyribonucleoside triphosphates to be incorporated during the repair synthesis step. This tested the effect of BiodUTP on the synthesis step, almost entirely independent of the incision step. Experiment 2 differed only in that hydroxyurea and aphidicolin were added to the intact cells to inhibit repair synthesis during the 20-min preincubation and thus to increase the number of incised sites (7500 sites per cell) available for repair synthesis following permeabilization. In experiment 3, the cells were permeabilized and then irradiated with UV radiation so that the polymerization reaction was completely dependent on the incision which occurred in the permeable cells. Under these conditions, the incision step is rate limiting. (Dresler et al., 1982); thus, the effect of BiodUTP on repair polymerization under these conditions is a measure of its effect on incision.

The results in Table I show that when repair polymerization was made independent of incision (experiments 1 and 2), BiodUTP was slightly less efficient (ca. 80%) than dTTP as a substrate for the polymerization reaction. The efficiency with which BiodUTP substituted for dTTP was independent of the number of incised sites present at the beginning of the polymerization reaction, over the range of 2400–7500 sites per cell. The results from experiment 3, in which the polymerization reaction was used as an assay for the rate-limiting incision step, demonstrate that BiodUTP had no effect on the incision step. Comparison of the results of experiments 1 and 2 with those of experiment 3 suggests that when large numbers of incised sites were present (i.e., when incision was not rate limiting as in experiments 1 and 2), BiodUTP was slightly less efficient as a substrate for the polymerization reaction but when incision was rate limiting, as in experiment 3, BiodUTP did not reduce the overall rate of repair synthesis.

Purification of DNA from Permeable Cells and Testing for the Presence of Biotin. By use of cesium trifluoroacetate isopycnic density gradient centrifugation, DNA was purified from cells which had been damaged with 10 J/m^2 UV radiation, incubated 20 min at 37 °C, permeabilized, and then allowed to perform repair synthesis in the presence of [^{3}H]-dCTP (30 Ci/mmol), dATP, dGTP, and either dTTP or BiodUTP, as described under Materials and Methods. The efficiency of binding of DNA containing biotin-labeled repair patches to avidin–agarose was then determined. The DNA was sonicated to an average size of ca. 300 base pairs and incubated with avidin–agarose in 150 mM NaCl, 1 mM EDTA, and 50 mM Tris, pH 7.2, at a ratio of 2 μg of DNA, containing ca. 0.3 pmol of biotin, to 1 unit of avidin–agarose, which is sufficient to bind 4 nmol of biotin. After 10 min on ice, the mixtures were centrifuged, the supernatants were removed, the pellets were washed 3 times, and the Cl_3CCOOH-insoluble radioactivity in incubation supernatants and washes was determined. Of the DNA from permeable cells which had performed repair synthesis in the presence of BiodUTP, 60% was bound to the avidin–agarose, compared to 0.9% of the DNA from permeable cells which had performed repair synthesis in the presence of dTTP. This suggests that the biotin was not removed either by the permeable cells or during the DNA isolation procedure. The finding that only 60% of the repair-incorporated [^{3}H]dCMP was bound to avidin–agarose may indicate that not all of the repair patches contained BiodUMP or that more than one biotin was required per repair patch to ensure tight binding to the avidin–agarose.

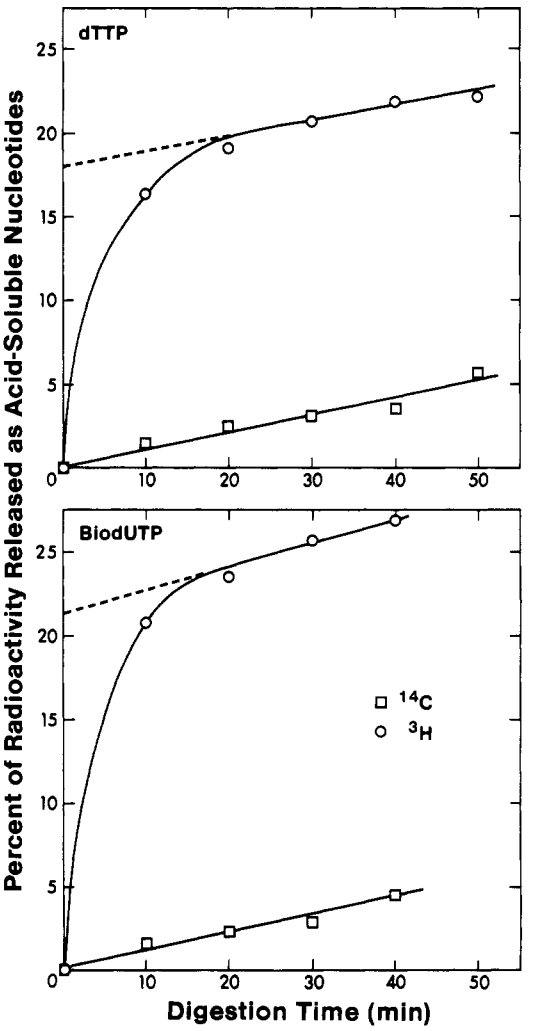

FIGURE 1: Ligation of repair-incorporated nucleotides during DNA excision repair in permeable cells in the presence of dTTP or BiodUTP. DNA purified from permeable cells which had performed DNA repair in the presence of dTTP (top panel) or BiodUTP (bottom panel) was digested with exonuclease III, and the release of PCA-soluble nucleotides from repair patches [^{3}H (○)] and bulk DNA [^{14}C (□)] was determined. The final slope of the ^{3}H digestion curve was extrapolated back to zero time to determine the fraction of repair-incorporated nucleotides present in unligated patches.

Ligation of Repair Patches Containing BiodUMP in Permeable Cells. Confluent human fibroblasts, prelabeled with [^{14}C]dThd, were damaged with 10 J/m^2 UV radiation, incubated 20 min at 37 °C, permeabilized, and allowed to perform DNA repair in the presence of [^{32}P]dCTP and either dTTP or BiodUTP. After the reaction was stopped, DNA was purified and digested with Exo III to determine the percent of repair-incorporated nucleotides present in unligated repair patches (Materials and Methods). Samples were removed at several times during the digestion for determination of the PCA-soluble ^{32}P (repair label) and ^{14}C (bulk label) radioactivity (Figure 1). Extrapolation of the final slope of the ^{32}P release to zero time gave the value for the fraction of repair-incorporated nucleotides present in unligated patches.

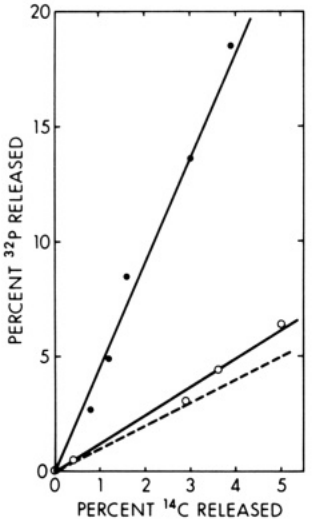

FIGURE 2: Nucleosome rearrangement during DNA excision repair in permeable cells in the presence of BiodUTP. Permeable cells were damaged with UV radiation, incubated for 10 min in a reaction mixture containing 0.1 μM [^{32}P]dCTP, dGTP, dATP, and BiodUTP (●), and then chased for a further 50 min in a reaction mixture containing 3 μM dCTP, dGTP, dATP, and BiodUTP (○). Nuclei were prepared and digested with SN, and the release of PCA-soluble nucleotides from repair patches (^{32}P) and bulk DNA (^{14}C) was measured. The dashed line indicates equal sensitivity of repair and bulk nucleotides, i.e., nucleosome rearrangement completed.

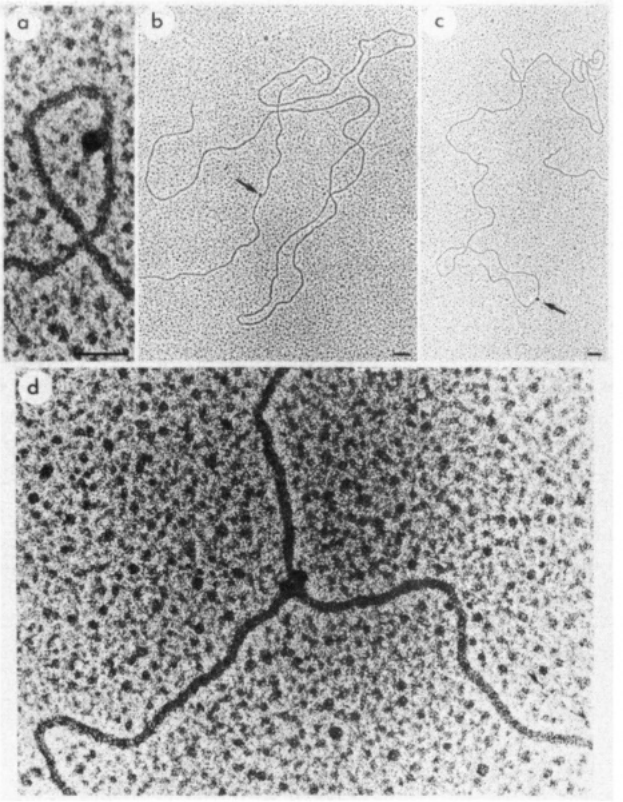

FIGURE 3: Electron microscopic visualization of biotin-labeled repair patches and a replication fork using avidin–ferritin. DNA was purified from permeable cells which had performed UV-induced DNA repair synthesis in the presence of BiodUTP. The DNA was visualized by using the Kleinschmidt cytochrome *c* method. (Panels a–c) Repair patches bound by avidin–ferritin; (panel d) replication fork bound by avidin–ferritin.

Of the repair-incorporated nucleotides, 18% were present in unligated patches after incubation of the permeable cells for 15 min when dTTP was a substrate, compared to 22% when BiodUTP was a substrate. Since a minimum of 60% of the repair-incorporated radioactivity was associated with biotin (see above), we conclude that the presence of BiodUMP in the repair patch had little or no effect on the repair ligation reaction.

Nucleosome Rearrangement in Permeable Cells When BiodUMP Is Present in the Repair Patches. DNA excision repair in eukaryotes involves transient changes in chromatin structure at the level of the nucleosome. Repair-incorporated nucleotides are initially highly sensitive to SN, but with time, they acquire approximately the same nuclease resistance as nucleotides in bulk chromatin. This process is known as nucleosome rearrangement (Smerdon & Lieberman, 1978). To determine if nucleosome rearrangement would proceed in the presence of BiodUTP, confluent fibroblasts, prelabeled with [^{14}C]dThd, were permeabilized, irradiated with 100 J/m^2 UV radiation, incubated for 10 min at 37 °C in the presence of 0.1 μM [α-^{32}P]dCTP, dGTP, dATP, and BiodUTP, washed, and then chased for 50 min in the presence of 3 μM dCTP, dGTP, dATP, and BiodUTP. Nuclei were prepared and digested with SN, and the release of PCA-soluble ^{32}P-labeled (repair label) and ^{14}C-labeled (bulk label) nucleotides was determined, as described (Dresler et al., 1982). As shown in Figure 2, when nuclei prepared immediately following the pulse period were digested with SN, the repair-incorporated nucleotides were released much more rapidly than bulk DNA. After the 50-min chase, repair-incorporated and bulk nucleotides were released at essentially the same rate. Thus, nucleosome rearrangement proceeded rapidly and completely in the presence of BiodUTP. Comparison of these results with those of Dresler et al. shows that the rates of rearrangement observed in the presence of BiodUTP and dTTP were virtually identical (Dresler et al., 1982).

Cleaver's laboratory has recently reported that permeable normal human fibroblasts do not perform nucleosome rearrangement during DNA excision repair (Kaufman et al., 1983). It is clear from the present work and previous work (Dresler et al., 1982) that nucleosome rearrangement occurs rapidly and essentially to completion in the permeable cell system developed by Dresler et al. We cannot explain the discrepancy between our results and those from Cleaver's laboratory, although we note that the chase conditions used by Cleaver's group are significantly different from those which we employ, suggesting that the discrepancy may be the result of technical problems.

Electron Microscopic Visualization of Biotin-Labeled Repair Patches. Using avidin conjugated with ferritin and electron microscopy, we attempted to detect biotin-labeled repair patches. Preliminary experiments were performed by using calf thymus DNA in order to find conditions under which the avidin–ferritin did not bind to nonbiotinated DNA and under which the avidin–ferritin did not form large aggregates (Materials and Methods). To visualize repair patches, cesium trifluoroacetate density gradients were used to purify DNA from cells which had been damaged with 10 J/m^2 UV radiation, incubated 20 min at 37 °C, permeabilized, and then allowed to perform repair synthesis in the presence of either dTTP or BiodUTP. The DNA was then incubated with a 10-fold excess of avidin–ferritin, and the unbound avidin–ferritin was removed by chromatography on Sepharose 4B (Materials and Methods). The DNA was spread by the Kleinschmidt cytochrome *c* technique, rotary showed with tungsten, and examined by electron microscopy (Materials and Methods). As shown in Figure 3, panels a–c, avidin–ferritin was bound to DNA which contained repair patches labeled with biotin. Approximately 25% of the DNA molecules

(average length ca. 3×10^4 bp) were labeled with at least one avidin–ferritin. No avidin–ferritin was bound to DNA from UV-damaged permeable cells incubated with dTTP. The Dubochet DNA spreading technique, which does not use cytochrome *c* (Materials and Methods), also permitted the visualization of biotin-labeled repair patches (not shown).

The finding that repair patches were rapidly ligated in permeable cells in the presence of BiodUTP (see above) indicates that most of the patches were full length and suggests that visualization of repair patches can provide a direct estimate of their size. A single avidin–ferritin molecule spans ca. 80 bp, suggesting that a maximum of two avidin–ferritin molecules can bind in tandem to a repair patch having a length of 80 bp. Since all of the 100 avidin–ferritin molecules examined were bound to the DNA as monomers, this argues that the repair patches were less than 80 bp long, consistent with values obtained by other methods such as the bromodeoxyuridine photolysis and the bromodeoxyuridine density shift method [for a review, see Hanawalt et al. (1979)].

Although the experimental procedure used in this study was designed to minimize replicative DNA synthesis through the use of confluent cells and low concentrations of deoxyribonucleoside triphosphates in the permeable cells, some replicative synthesis still occurred (see Table I), and Figure 3d demonstrates that replication forks can be labeled with BiodUTP.

Electron microscopic visualization of repair patches should be useful for determining the distribution of repair patches in DNA. This possibility is of particular interest because of recent indirect evidence (Cohn & Lieberman, 1984) indicating that at early times following UV damage, repair patches are nonrandomly distributed in the DNA of human fibroblasts.

In conclusion, we have examined the effects of BiodUTP on the process of DNA excision repair following UV damage to human fibroblasts. This nucleotide analogue did not significantly reduce the rate of incision of DNA at sites of damage in permeable cells, and BiodUMP was readily incorporated during repair synthesis, producing repair patches which could be isolated by using avidin–agarose. The presence of BiodUMP in the repair patches had little or no effect on ligation of repair patches or on nucleosome rearrangement. The presence of BiodUMP in the repair patches permitted the electron microscopic visualization of repair patches, using avidin–ferritin conjugates. This approach has the potential for visualizing repair patches in chromatin. Also, BiodUTP may be a valuable tool for the isolation of chromatin proteins associated with repair patches.

ACKNOWLEDGMENTS

We thank Dr. Alex Waldrop, Department of Microbiology, University of Virginia School of Medicine, for his suggestions concerning the synthesis of BiodUTP, for the sample of AAdUTP, and for the UV and NMR spectra of AAdUTP. We thank The University of Alberta Cancer Research Group for providing laboratory space and equipment and Herta Unger and Bonnie Gowans for technical assistance.

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