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Human nucleotide excision nuclease removes thymine dimers from DNA by incising the 22nd phosphodiester bond 5′ and the 6th phosphodiester bond 3′ to the photodimer

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ABSTRACT **By using a human cell-free system capable of nucleotide excision repair, a synthetic substrate consisting of a plasmid containing four thymine dimers at unique locations, and deoxyribonucleoside 5′-[α-thio]triphosphates for repair synthesis, we obtained DNA fragments containing repair patches with phosphorothioate linkages. Based on the resistance of these linkages to digestion by exonuclease III and their sensitivity to cleavage by I_2, we were able to delineate the borders of the repair patch to single-nucleotide resolution and found an asymmetric patch with sharp boundaries. That the repair patch was produced by filling in a gap generated by an excision nuclease and not by nick-translation was confirmed by the finding that the thymidine dimer was released in a 27- to 29-nucleotide oligomer.**

Although there are many biochemical pathways for repairing DNA damage, nucleotide excision is probably the most important. This pathway, in contrast to others, is not damage-specific as it removes all covalently modified nucleotides from DNA (1, 2). Nucleotide excision entails the removal of the damaged nucleotide(s) in the form of an oligomer (3, 4), "repair synthesis" (5, 6) to fill in the single-stranded gap, and eventual ligation to restore the intact duplex. In *Escherichia coli*, (A)BC excinuclease initiates nucleotide excision by incising the damaged strand in a precise manner on both sides of the adduct: the eighth phosphodiester bond 5′ and the fifth phosphodiester bond 3′ to the adduct (7, 8). Two other mechanisms that involve nicking 5′ or 3′ to the adduct by an endonuclease followed by exonucleolytic removal of the damaged base can be envisioned. Which of these three mechanisms is employed in eukaryotes in general and in humans in particular is not known.

Recently, an *in vitro* nucleotide excision repair assay for human cells has been described (9, 10). This assay measures the nicking of, or the incorporation of radiolabeled nucleotides into, an externally added damaged DNA by whole cell extracts (9–11). The assay has been used to characterize several aspects of excision repair in humans (12–15). However, the assay system is relatively inefficient, resulting typically in the removal of 0.5–1% of the adducts (10, 16). As a consequence many of the biochemical techniques employed to determine the incision modes of nucleases are of limited use in this system. In this study we have combined two methods, site-specific insertion of modified nucleotides and phosphorothioate chemistry, to overcome the limitations of low repair efficiency. This approach has enabled us to determine that thymine cyclobutane dimers (T◇T) are removed by a human excision nuclease that incises on both sides of the photodimer.

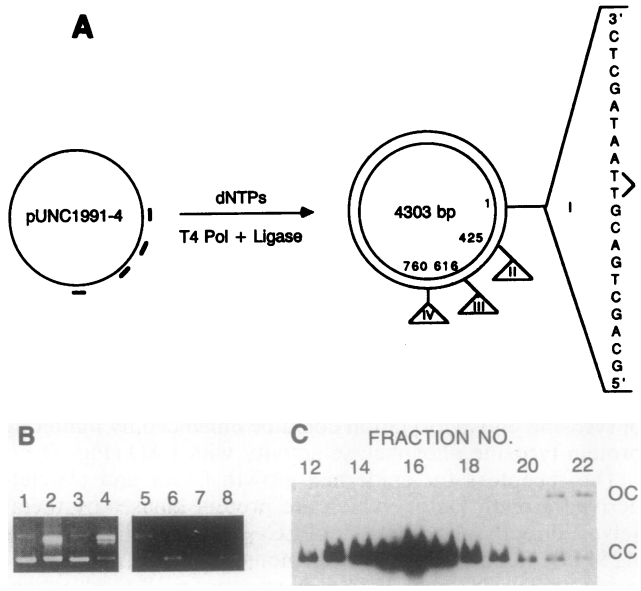

FIG. 1. Substrates for nucleotide excision repair. (*A*) Substrate preparation. pUNC1991-4 contains sequences complementary to the 20-mer(T◇T) inserted in four locations chosen to contain the -CTC sequence on the 3′ side of the insert. The 20-mer(T◇T) was annealed to the single-stranded form of pUNC1991-4 and converted to double-stranded DNA by primer elongation. Locations of fragments I–IV are shown. (*B*) Substrate for repair synthesis assay. Substrate containing 20-mer(T◇T) or 20-mer(T-T) was purified through two CsCl/ethidium bromide gradients and analyzed on agarose gels. Lanes: 1–4, ethidium bromide present during electrophoresis; 5–8, stained with ethidium bromide after electrophoresis; 1, closed circular (CC) form of pUNC1991-4(T-T); 2, open circular (OC) fraction of the same DNA; 3, pUNC1991-4(T◇T) covalently closed circle; 4, open circular fraction of the same DNA preparation; 5, ccDNA(T-T); 6, same as lane 5 plus *M. luteus* gyrase; 7, ccDNA(T◇T); 8, same as lane 7 plus *M. luteus* gyrase. (*C*) Substrate for excision assay. Terminally labeled 20-mer(T◇T) was annealed to the template and converted to double-stranded plasmid. The covalently closed circles were purified through two CsCl/ethidium bromide gradients. Autoradiogram of the fractions (left to right) collected from the bottom of the tube (second gradient) and then analyzed on an agarose gel containing ethidium bromide is shown.

MATERIALS AND METHODS

Substrate. We constructed a plasmid containing four T◇Ts at predetermined sites. The plasmid was derived from pUNC1989 (17) by inserting four 20-nucleotide (nt) segments complementary to our primer by site-specific mutagenesis (18). The location of the insertions was chosen such that a *Xho* I site (CTCGAG) was generated at the 3′ junction of the

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Abbreviations: T◇T, thymine cyclobutane dimer; exoIII, exonuclease III; T4 Pol, T4 DNA polymerase; ccDNA, covalently closed DNA; CFE, cell-free extract; dNTP[αS], deoxyribonucleoside 5′-[α-thio]triphosphate; nt, nucleotide(s).

inserts. The construct containing the four inserts was named pUNC1991-4.

To prepare substrate, the primer 5′-GCAGCTGA-CGT◇TAATAGCTC-3′ (19) at 308 nM was annealed to 7.7 nM single-strand pUNC1991-4 and converted to duplex with T4 DNA polymerase (T4 Pol; Boehringer Mannheim) and ligated with T4 DNA ligase. In a typical reaction, 50–80% of the DNA was converted to covalently closed DNA (ccDNA) duplex molecules. The ccDNA was purified through two consecutive CsCl/ethidium bromide gradients. Control plasmid was prepared in an identical manner, except the primer contained a T-T instead of T◇T in the middle. Alternatively, the control DNA was obtained by photoreactivating the plasmid substrate with *E. coli* DNA photolyase (1). Substrate containing internal label was prepared in the same way except the primer was 5′-end-labeled with [γ-^{32}P]ATP (7000 Ci/mmol; 1 Ci = 37 GBq; ICN). Substrate prepared in this manner contained a terminally labeled 25-mer contaminant, apparently produced by self-priming of the 20-mer. Although this contaminant did not interfere with routine excision assay, it was necessary to remove it from substrates used to analyze the excision product. This was accomplished by gel filtration of the DNA sample through a 2-ml column of Sepharose 4B in the presence of 7 M urea prior to purification by density gradients.

Repair Synthesis. Repair synthesis was conducted with HeLa cell-free extracts (CFEs), uniquely modified DNA, and deoxyribonucleoside 5′-[α-thio]triphosphates (dNTP[αS]s) as described (9, 10). After deproteinization, the DNA was digested with *Hae* III to obtain restriction fragments carrying the repair patches when indicated.

Incision Sites and Patch Size. We employed three approaches to identify the incision site(s) of the human excision nuclease and the boundaries of the repair patch.

(*i*) The excision assay (10, 13). DNA containing a ^{32}P label at the 11th phosphodiester bond 5′ to the T◇T was incubated with the CFE for 2 hr. After deproteinization (10), the DNA was precipitated and then analyzed on a sequencing gel.

(*ii*) The 3′ boundary of the repair patch. Repair synthesis was conducted with all four dNTP[αS]s (each at 20 μM) and either [α-^{32}P]dCTP or [α-^{32}P]dATP (4 μCi per reaction mixture) as the radioactive label. To determine the 3′ end of the patch, appropriate restriction fragments were isolated and the DNA was treated with exonuclease III (exoIII) as described (20, 21).

(*iii*) The 5′ boundary of the repair patch. Phosphorothioate linkages can be selectively cleaved by heating DNA with iodoethanol (22, 23) or I_2 (24). In our experience, cleavage by I_2 is more reproducible and, therefore, it was used exclusively in this study. Restriction fragments carrying the patch were cleaved with *Xho* I, which incises near the 3′ border of the patch (see below) to generate fragments >100 base pairs (bp) long. Provided that the patch is not >50 nt long, cleavage of such fragments with I_2 would generate a ladder (patch) on a sequencing gel that extends from the 3′ end to the 5′ terminus of the patch. The smallest fragment of this ladder is a measure of the distance from the 5′ end of the fragment to the 5′ end of the repair patch.

RESULTS

Preparation of Substrate. The human CFE system for nucleotide excision repair (9, 10) gives a reasonable signal in a repair synthesis assay but yields only a marginal signal in a nicking assay (10). Our repeated attempts to obtain a repair synthesis signal strong enough for patch-size analyses of DNA uniquely modified with a single psoralen adduct or a T◇T were unsuccessful. We have observed a nonlinear response of repair synthesis signal to the number of adducts in DNA that is nearly negligible with about one T◇T per pBR322 but that becomes linear with the number of T◇Ts after about three photodimers per plasmid (25). Therefore, we decided to prepare a substrate containing four T◇Ts at predetermined sites for our studies.

Fig. 1*A* shows the strategy for constructing the substrate. It is essential that the synthetic substrates purified in this manner be as nick-free as possible before they can be used in

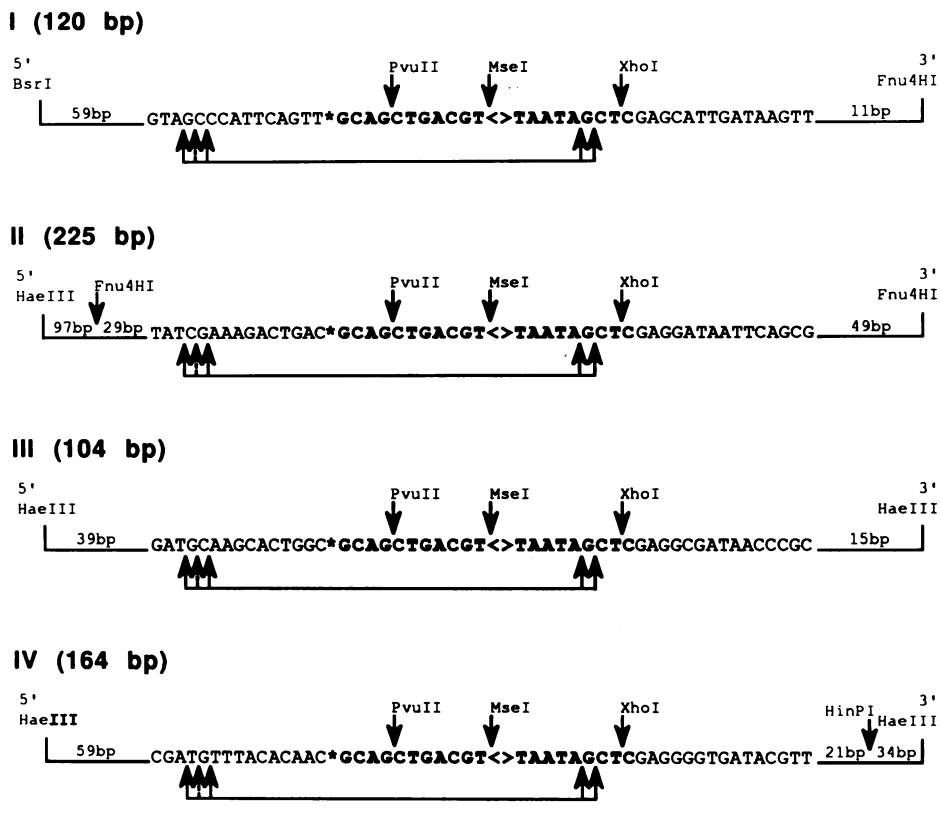

FIG. 2. Fragments used for analyses of the repair patch. The 50-bp sequence surrounding the T◇T in each fragment is shown. The primer sequence is in boldface type, and the locations of restriction sites used in the various analyses are indicated. An asterisk indicates the position of the ^{32}P label in the substrate for the excision assay. The brackets indicate the incision sites and repair patches as determined by exoIII digestion, iodine cleavage, and the excision assay.

repair assays. Therefore, after primer elongation and ligation, the DNA was purified through two CsCl/ethidium bromide gradients and, typically, from a standard reaction mixture, we obtained ≈5 μg of DNA containing <2% nicked molecules (Fig. 1*B*) and 3 μg of internally labeled DNA with <1% nicked molecules (Fig. 1*C*).

Specific Incorporation of Label into Damaged Fragments. Nonlabeled DNA purified as described in the previous section was used in repair synthesis with HeLa CFE and a [α-^{32}P]dNTP radiolabel. The DNA was then digested with *Hae* III, and the fragments were separated on a 5% native polyacrylamide gel. The autoradiograph of the gel (data not shown) revealed preferential labeling of fragments bracketing the T◇T sites when compared to control DNA prepared either by primer elongation using 20-mer(T-T) or by photoreactivating ccDNA synthesized with 20-mer(T◇T). These data provided evidence that T◇T leads to repair synthesis in a restricted area flanking the dimer site, in agreement with the results Wood and coworkers (12, 14) obtained with a plasmid substrate containing a single acetylaminofluorene guanine adduct.

The 3′ Boundary of the Repair Patch. Repair synthesis was conducted in the presence of all four dNTP[αS]s plus [α-^{32}P]dCTP. The *Hae* III fragments carrying the repair patches were isolated and processed to identify the 3′ border of the repair patch. These fragments, with or without cleavage with an appropriate restriction endonuclease at an asymmetric location with respect to the termini (Fig. 2), were digested with *E. coli* exoIII and the products were analyzed on sequencing gels. This 3′ → 5′ exonuclease stops at T◇T, 1 nt 3′ to the photodimer (20), and does not hydrolyze phosphorothioate linkages (21, 26). As a consequence, it is expected to stop at the 3′ border of the repair patch.

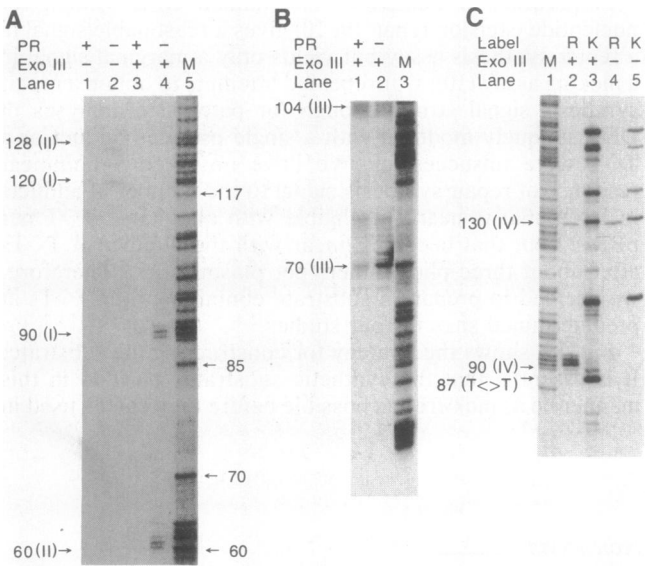

FIG. 3. 3′ boundary of the repair patch. After repair synthesis with all four dNTP[αS]s plus [α-^{32}P]dCTP, restriction fragments were isolated, treated with exoIII, and analyzed on 8% polyacrylamide sequencing gels. In the lanes marked lane 1, PR and +, prior to repair synthesis, the plasmid was photoreactivated completely (*A*) or incompletely (*B*) to obtain control DNA. Lane M contains size markers (the Sanger adenine reaction of an unrelated fragment). In *C*, fragments were labeled by repair synthesis (R) or by kinase treatment (K) and digested with exoIII. (*A*) Repair patches I and II. A mixture of 120-bp (*Bsr* I–*Fnu*4HI) and 128-bp (*Fnu*4HI) fragments carrying patches I and II, respectively, was digested with exoIII as indicated (+). The locations of the full-length fragments and the most prominent of the exoIII-generated fragments are marked by arrows on the left margin (sizes are in nt). Because the distances of T◇Ts (and of the repair patches) from the 3′ termini are quite different in the two fragments, the exoIII stop sites could be ascribed unambiguously to their corresponding restriction fragments. This assignment was confirmed by testing purified fragments individually (data not shown). (*B*) Repair patch III. The 104-bp (*Hae* III) fragment carrying patch III was used for analysis. (*C*) Repair patch IV. To provide a direct comparison between the stop site of exoIII at T◇T and the 3′ border of the repair patch, the same 130-bp (*Hae* III–*Hin*PI) fragment was labeled by repair synthesis (R) or by kinase treatment (K) at the *Hae* III terminus. ExoIII digestion of the two DNAs reveals stop sites at 90 and 87 nt for R and K, respectively. The other bands seen in lanes K are due to contaminating fragments and their exoIII degradation products, whose presence does not interfere with our analysis.

Fig. 3 shows the results of exoIII digestion of the four fragments containing radiolabel and phosphorothioate linkages within the repair patch. In all cases, a major stop site at the fourth nucleotide 3′ to the dimer was observed, which means that the fourth phospho(thio)diester bond is resistant to the enzyme and that the repair patch extends four nucleotides 3′ to the photodimer site. In addition, two other stop sites 5 and 6 nt 3′ to the T-T are seen in three out of four fragments, suggesting some variability at the 3′ border of the repair patch.

The 5′ Boundary of the Repair Patch. Phosphothiodiester bonds are susceptible to preferential cleavage with iodine (22–24). Fragments (shown in Fig. 2) containing radiolabel (^{32}P) and phosphorothioate linkages within the repair patch were digested (with the exception of fragment I) with *Xho* I to obtain essentially 3′-labeled fragments carrying the repair patch. Cleavage of such fragments with I_2 would generate a ladder with the number of bands equal in number to the patch size and with the smallest fragment having a length equal to the distance from the 5′ end of the strand to the 5′ border of the patch. Because of the labeling method, the intensity of the fragments is expected to decrease with fragment size if one-hit cleavage is employed. Since the size of the smallest fragment is crucial to our analysis, we carried out exhaustive I_2 cleavage. The results are shown in Fig. 4. As expected, the long fragments in the patch are underrepresented. The shortest unambiguously visible fragments were 64(I), 131(II),

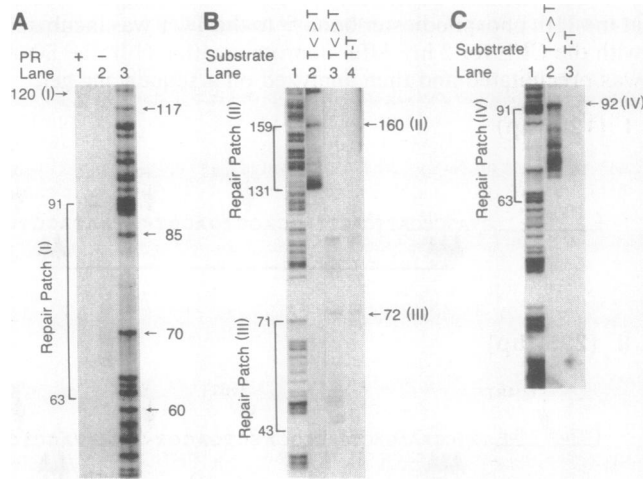

FIG. 4. 5′ boundary of the repair patch. Repair synthesis was carried out with the plasmid substrate or control DNAs prepared by photoreactivating (PR) the plasmid or synthesis with nondimer 20-mer(T-T) primers. After repair synthesis with all four dNTP[αS]s plus a radiolabeled dNTP, appropriate restriction fragments were isolated and cleaved with iodine. (*A*) Repair patch I. The label was [α-^{32}P]dCTP and the 120-bp (*Bsr* I–*Fnu*4HI) fragment was used for analysis. (*B*) Repair patches II and III. The repair label was [α-^{32}P]dATP and the 160-bp (*Hae* III–*Xho* I) and the 72-bp (*Hae* III–*Xho* I) fragments were used for patches II and III, respectively. (*C*) Repair patch IV. [α-^{32}P]dATP was the repair label and the 92-bp (*Hae* III–*Xho* I) fragment was treated with iodine. The faint bands seen in *B* and *C* are the full-length fragments due to incomplete digestion by *Xho* I. Note that the patch boundaries (in brackets) are based on data from exoIII digestion, iodine cleavage, and the excision assay; numbers represent distances from the 5′ end of the strands carrying the repair patches. Molecular sizes (in nt) are indicated.

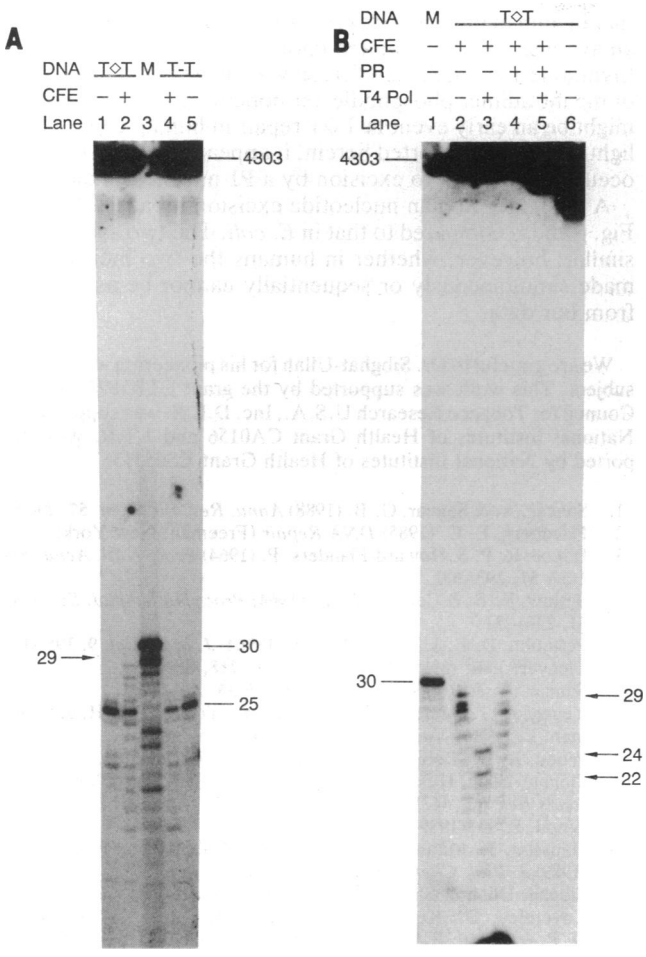

FIG. 5. Excision assay. (*A*) Plasmids containing a ^{32}P label at the 11th phosphodiester bond on the 5′ side of the T◇Ts were treated with CFE for 2 hr, and the DNA was deproteinized and loaded onto a 12% polyacrylamide sequencing gel with appropriate size markers (M). As a control, the same DNA preparation was subjected to photoreactivation (T-T) prior to the repair synthesis reaction. The 25-mer contaminant present in all sample lanes is generated from excess primer during second-strand synthesis. When treated with T4 Pol, this contaminant is degraded to 12- to 13-mers (data not shown). (*B*) Location of T◇T in the excised fragment. The substrate (free of 25-mer contaminant) was treated with CFE and after deproteinization was subjected to the following treatments. Lanes: 2, no further processing; 3, incubated with T4 Pol for 1 hr; 4, mixed with 100 nM *E. coli* photolyase and photoreactivated (PR) with 100 camera flashes; 5, photoreactivated DNA was incubated with T4 Pol for 1 hr (27); 6, substrate not treated with CFE. Positions of the excision products are indicated by arrows, and sizes are indicated in nt. The source of the faint band at 31-mer position in T4 Pol-treated samples (lanes 3 and 5) is not known; however, it must originate from T4 Pol action on the plasmid substrate because it is not seen when purified excision products are treated with T4 Pol 3′ → 5′ exonuclease (data not shown).

44(III), and 63(IV) nt long. As such fragments must have a radiolabeled nucleotide 5′ to the cleaved phosphothiodiester bond to be detectable, these cleavages suggest that the repair patch must extend 21(I), 24(II), 24(III), and 23(IV) nt 5′ to T◇T. Because of the inherent limitations of this approach, it was not possible to locate the 5′ boundary more precisely, although it was narrowed down to a 4-nt region.

Analysis of label distribution into restriction fragments originating from the repair patch area provided additional support for a repair patch of relatively rigid boundaries. Only the repair patch in fragment IV (Fig. 2) was analyzed in this manner. When repair synthesis was carried out with [α-^{32}P]dATP, essentially all of the label was incorporated into the T◇T strand of the fragment, and 90%, 70%, and 67% of the label in this strand was incorporated 5′ to the incision sites of *Xho* I, *Mse* I, and *Pvu* II, respectively. The values expected for a repair patch of 29 nt (Fig. 2) are 100%, 67%, and 56%, respectively. Similarly, when the repair patch was labeled with [α-^{32}P]dCTP, 96% and 67% of the radiolabel was incorporated 5′ to the incision sites of *Xho* I and *Pvu* II, respectively, compared to predicted values of 100% and 67%.

Excision Assay. The results presented so far are consistent with a precise mode of incision by an excision nuclease that hydrolyzes the phosphodiester bonds 21–24 nt from the 5′ side and 4–6 nt from the 3′ side of the T◇T. If such is the case, then, a 27- to 30-mer carrying the T◇T should be released. To test for such an activity, the plasmid substrate containing ^{32}P label at the 11th phosphodiester bond 5′ to the T◇Ts was incubated with the CFE and the products were analyzed on a sequencing gel.

The results shown in Fig. 5*A* reveal that a series of fragments 27–29 nt long were released from T◇T-containing DNA (lane 2) but not from DNA that was photoreactivated prior to incubation with CFE (lane 4). Whether only the 29-mer or all three fragments were primary excision products could not be ascertained from this experiment because even a contaminating 25-mer was partially degraded to smaller species (compare lanes 4 and 5). To locate the T◇T in the excised fragment and thus more precisely define the 5′ incision site, we prepared ccDNA free of the 25-mer contaminant, conducted the excision assay, and then treated the excised DNA with T4 Pol 3′ → 5′ exonuclease (which acts on both single- and double-stranded DNA and stops at the photodimer; ref. 27) before and after photoreactivation treatment (Fig. 5*B*). The excision product consisted of three major bands of 27–29 nt followed by a fainter ladder extending down to a mononucleotide (lane 2). Treatment with T4 Pol generated two major species of 22 and 24 nt (lane 3). Photoreactivation does not appreciably change the pattern of the excision product (lane 4), indicating that the intradimer phosphodiester bond is intact but makes DNA totally susceptible to degradation by T4 Pol as expected (lane 5). Combined with the 3′ boundary of repair patch, these results suggest that the 5′ incision is at the 21st to 23rd phosphodiester bond and that the T◇T is located 4 or 5 nt away from the 3′ terminus of the excised fragment.

DISCUSSION

It is known that human cells remove T◇Ts as oligonucleotides (28) and that nucleotide excision generates repair patches of 20–30 nt, as determined by density labeling (29–31), by the bromodeoxyuridine photolysis methods (32) *in vivo*, and by radiolabeling of restriction fragments of singly adducted DNA substrates in an *in vitro* system (12, 14). However, it is unclear from these studies whether nucleotide excision repair is accomplished by an endonucleolytic incision to one side of the adduct followed by adduct removal with an exonuclease or an excision nuclease mechanism that incises on both sides of the adduct in a precise manner. The results presented here indicate that human cells remove T◇T and perhaps other bulky adducts by an excision nuclease enzyme system. The main findings in support of this conclusion are recapitulated below.

(*i*) We detect relatively sharp 3′ and 5′ boundaries for the repair patch. The case is most obvious for the 3′ boundary where the fourth, fifth, and to a lesser extent the sixth phospho(thio)diester linkages 3′ to T◇T are resistant to exoIII. Since it is known that phosphothiodiester bonds confer partial nuclease resistance to neighboring phosphodiester bonds (26), the resistance of the sixth phosphodiester bond could be due to a proximity effect. Thus the repair patch extends 4–5 nt 3′ to the T◇T site. The 5′ boundary as determined by iodine hydrolysis of the phosphorothioate

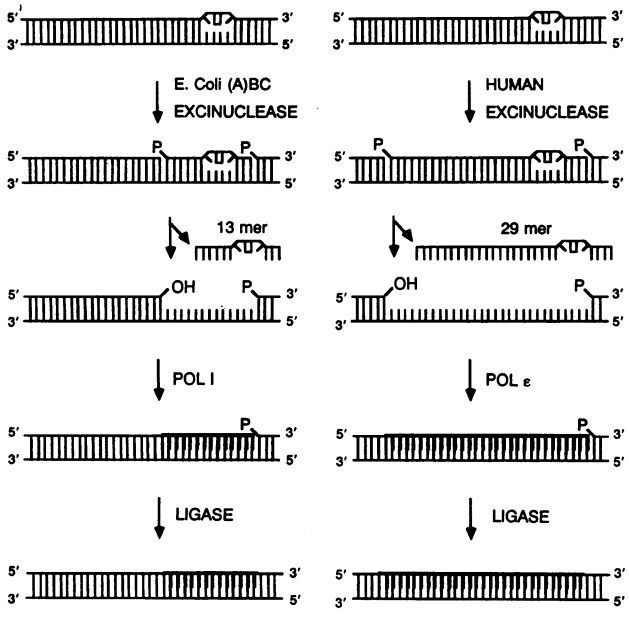

FIG. 6. Model for excision repair in *E. coli* and humans. Filling-in by polymerase ε in humans is based largely on inhibitor studies and is subject to some uncertainty (35, 36). Similarly, the relation of xeroderma pigmentosum genes to human excision nuclease remains to be determined.

linkage is not as precise as the 3′ boundary. However, this imprecision is intrinsic to the method and does not necessarily indicate variability in the 5′ incision site. Even with these limitations, however, the iodine cleavage places the 5′ boundary of the patch beyond 20 nt 5′ to T◇T. (*ii*) In analysis of repair patches labeled with two nucleotides, the distribution of the radioactive label within the repair patch is entirely consistent with sharp patch boundaries.

(iii) The most compelling evidence for an excision nuclease action, however, is our detection of 27- to 29-mers containing a T◇T in the excision assay. Admittedly, in the excision assay in addition to the 27- to 29-mers, a "ladder" of smaller fragments is observed. However, since a ladder was also generated from a contaminating DNA fragment, it is most likely that the smaller-size species detected in the excision assay resulted from degradation of the primary excision product by nonspecific nucleases. Furthermore, treatment of the 27- to 29-mers with T4 Pol 3′ → 5′ exonuclease generates a 24-mer and a 22-mer with a T◇T at the 3′ terminus providing unambiguous evidence that at least in a fraction of the molecules the 3′ incision was made at the 6th phosphodiester bond 3′ to the T◇T. When the sizes of the excision products before and after T4 Pol 3′ → 5′ exonuclease digestion are considered in light of the 3′ boundary of the repair patch, the following incision pattern is consistent with all available data: the 21st or 23rd phosphodiester bond 5′ and the 5th or 6th phosphodiester bond 3′ to the T◇T. Thus, the 29-mer is produced by incisions at the 23rd and 6th phosphodiester bonds 5′ and 3′, respectively, and the corresponding incisions for the 28-mer would be at the 23rd(22nd) and 5th(6th) phosphodiester bonds and for the 27-mer would be at the 21st(22nd) and 6th(5th) phosphodiester bonds.

Treatment of the excised fragments with photolyase made it susceptible to degradation to mononucleotides by T4 Pol. However, neither photolyase treatment nor direct photoreversal of T◇T at a wavelength of 254 nm (data not shown) affected the migration of the 29-mer, indicating that the intradimer phosphodiester bond is intact. It has been found that in human fibroblasts excised T◇Ts are in fragments with an average size of 3.7 nt and photoreversal of T◇T liberates thymidine and TMP; therefore, it was suggested that cleavage of the intradimer phosphodiester bond by a specific nuclease might be an early event in T◇T repair in human cells (33). In light of findings reported herein, it appears that this cleavage occurs subsequent to excision by a P1 nuclease analog (34).

A model for human nucleotide excision repair is shown in Fig. 6 and is compared to that in *E. coli*. The two systems are similar; however, whether in humans the two incisions are made simultaneously or sequentially cannot be ascertained from our data.

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