DNA Repair by Eukaryotic Nucleotide Excision Nuclease

REMOVAL OF THYMINE DIMER AND PSORALEN MONOADDUCT BY HeLa CELL-FREE EXTRACT AND OF THYMINE DIMER BY XENOPUS LAEVIS OOCYTES*

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Using a human cell-free extract, we have recently shown that thymine dimers are removed from DNA in oligonucleotides 27–29 nucleotides in length (Huang, J. C., Svoboda, D. L., Reardon, J. T., and Sancar, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3664–3668). In this study we find that the excision reaction is dependent on ATP, the excised fragments range in length from 27–32 nucleotides, and have 5'-P and 3'-OH termini. We also found that a thymine-psoralen furan side monoadduct is excised from the DNA with a similar incision pattern, indicating that in humans bulky adducts are removed from DNA by the same enzyme system which hydrolyzes mainly the 22–24th and the 5th phosphodiester bonds 5' and 3', respectively, to the lesion. This incision pattern might be common to eukaryotic excision nucleases as thymine dimers were removed from DNA by the same dual incision pattern by Xenopus laevis oocytes.

Nucleotide excision repair is an important cellular defense mechanism against DNA damage and its consequences, mutagenesis, cellular death, and cancer. Some of the nucleotide modifications, such as base methylation or deamination, base fragmentation, or loss (depurination/depyrimidination), can be corrected by adduct- or lesion-specific enzymes (methyltransferase, DNA glycosylase, AP endonucleases) as well as by the nucleotide excision repair system which is not lesion-specific. Some other nucleotide modifications such as cyclobutane type and 6-4 pyrimidine dimers, thymine-psoralen furan side monoadducts, and a variety of base adducts with polycyclic aromatic hydrocarbons, which are commonly referred to as bulky adducts, can be repaired only by nucleotide excision repair (see Friedberg, 1985; Sancar and Sancar, 1988; Lin and Sancar, 1992). Thus, nucleotide excision repair is of special importance in cellular survival. The significance of this repair mechanism is underscored by the fact that Escherichia coli or yeast cells deficient in nucleotide excision are exquisitely sensitive to the lethal and mutagenic effects of UV and DNA reactive chemicals and that humans with the xeroderma pigmentosum syndrome (which is biochemically characterized by defective nucleotide excision) are extremely prone to sunlight-induced skin cancers (Cleaver and Kraemer, 1989).

Nucleotide excision repair is achieved by removing the modified nucleotide together with several adjacent nucleotides from the damaged strand (excision) followed by filling in the resulting gap by a DNA polymerase (repair synthesis). Genetic analyses have revealed that three genes in E. coli (uvrA, -B, and -C), at least seven genes in yeast (RAD1, -2, -3, -4, -10, -14, and yERCC3), and eight genes in humans (XP-A through XP-G and ERCC-1) are required for the initial incision/excision step (Hoeijmakers, 1991). The molecular details of the repair reaction proper, the excision of the adduct, are well understood only in E. coli in which UvrA, -B, and -C proteins acting in sequential manner remove the damage by incising the 8th phosphodiester bond 5' and the 5th phosphodiester bond 3' to the modified nucleotide (Sancar and Rupp, 1983). Of special interest to us has been the question of whether eukaryotes remove bulky adducts from DNA by incising the damaged strand in a manner similar to that in prokaryotes. Using an in vitro excision repair system consisting of concentrated whole cell extract (Wood et al., 1988; Wood, 1989; Sibghat-Ullah et al., 1989), we have recently reported that human cells excise thymine dimers from DNA by incising the 21-23rd phosphodiester bonds 5' and the 4-6th phosphodiester bonds 3' to the photodimer (Huang et al., 1992; Nichols and Sancar, 1992). In the present study we have further characterized the excision products with regard to cofactor requirement, the precise sites of incision, and the relative frequencies of various incision patterns as well as the nature of the termini produced by the double incisions. In addition, we show that in this system a thymine-psoralen monoadduct is excised in the same manner. Finally, when the thymine dimer substrate was injected into Xenopus laevis oocytes the dimer was removed in 27–29 nucleotide long oligomers identical in length to the major excision products of the HeLa cell-free extract. Taken together, these results lead us to conclude that in eukaryotes bulky adducts are removed by an ATP-dependent excision nuclease whose major incision sites are the 22–24th phosphodiester bonds 5' and the 5th phosphodiester bond 3' to the damaged nucleotide(s).

MATERIALS AND METHODS

Substrates—We used semisynthetic covalently closed plasmid DNAs containing T<<T or T<>HMT at predetermined sites. The thymine dimer substrate pUNC1991-4(T<>T) was prepared by hybridization of the 5' 22P-labeled primer 5'–GCACGT–GACGT–<TAATAGCTC–3' to the single-stranded form of pUNC1991-4 (4303 nt) followed by polymerization of the remaining nucleotides by T4 DNA polymerase (Huang et al., 1992). pUNC1991–

1 The abbreviations used are: T<<T, thymine dimer; T<>HMT, thymine-4'-hydroxymethyl-4,5',8-trimethylpsoralen; nt, nucleotide(s); T4 Pol, T4 phage DNA polymerase; ATPγS, adenosine 5'-O-(thiotriphosphate); RAP, bacterial alkaline phosphatase.

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4 contains the sequence complementary to the primer at different locations separated from one another by 144 nucleotides or more. The T<>T-containing primer was synthesized by the building-block method of Taylor et al. (Taylor et al., 1987; Taylor and Brockie, 1988), and the pUNC1991-4(T<>T) substrate containing 32P radiolabel at the 11th phosphodiester bond 5' to the T<>Ts was prepared as described by Huang et al. (1992).

The psoralen-adducted substrate was prepared in a similar manner. A 13-mer (5'-GCTCGGTACCCGG-3') homologous to the polylinker region of pUC19 (and therefore to that of pUNC1991-4) and containing a HMT furan side monoaduct to the thymine at the 7th position from the 5' terminus was labeled at the 5' end with 32P (Cheng et al., 1988). The fragment was annealed to the single-stranded DNA template, converted to the double strand form, and the RFIV form was purified as in the case of the T<>T substrate (Huang et al., 1992).

Repair Systems—HeLa cell-free extract was prepared as described previously, and repair reactions were done as described (Wood et al., 1988; Sibghat-Ullah et al., 1989) except, when indicated, ATP and an ATP-regenerating system were not included in the reaction mixture. Following the repair reaction the excision products were separated on 10% polyacrylamide sequencing gels (Huang et al., 1992).

Microinjection into X. laevis oocytes was kindly done by Drs. He Xiao-Ping and Brian Kay (University of North Carolina). Fifty nanoliters of pUNC1991-4(T<>T) or of photoreactivated control DNA (about 8,000 cpm each) were injected into each of 50 germinal vesicles. The oocytes were incubated at 22 °C for 60 min. Following incubation the DNA was extracted by treating the homogenized oocytes with 50 µg/ml proteinase K in 2% sodium dodecyl sulfate followed by two phenol extractions (Hays et al., 1990). The DNA was precipitated with ethanol, resuspended in formamide-dye loading solution, and the excision products were separated on a 10% polyacrylamide-denaturing gel.

Analysis of Excision Products—For further analysis of the excision products of both HeLa cell-free extract and Xenopus oocytes, the excision products were located on the gel by autoradiography, the bands were cut out, electroeluted, and precipitated with ethanol. The purified DNA was then treated with bacterial alkaline phosphatase or T4 DNA polymerase 3' to the adduct, or both, and rerun on DNA-sequencing gels with appropriate size markers.

**RESULTS**

**ATP Requirement of Excision Nuclease**—Using the HeLa cell-free extract, Wood et al. (1988) and Sibghat-Ullah et al. (1989) found that repair synthesis with DNA containing bulky adducts was ATP-dependent. Since none of the DNA polymersases require ATP it was argued that the ATP-dependent step must be the incision step. However, direct evidence for this conclusion was lacking and in fact recent evidence strongly indicates that ATP is required for the synthesis reaction *per se* (Nichols and Sancar, 1992; Shivji et al., 1992).

To determine whether ATP is a required cofactor of the excision nuclease, we conducted the excision reaction with and without ATP and with and without dNTPs. The results are shown in Fig. 1. No excision is detected when the reaction mixture lacks ATP or is replaced by the non-hydrolyzable analog ATP-sS. Excision does take place when ATP alone is present and is further stimulated when in addition to ATP, dNTPs are included in the reaction. We conclude that ATP hydrolysis is required for the dual incisions by the human excision nuclease. Further stimulation of excision by dNTPs is due to the fact that, apparently in a manner functionally analogous to the *E. coli* excision nuclease, the human excision nuclease remains bound to DNA following the dual incisions, and it is released from the site only upon the joint actions of repair replication proteins RF-1, RF-2, PCNA, and DNA polymerase 6 or ɛ (Nichols and Sancar, 1992; Shivji et al., 1992; Coverley et al., 1992).

**The Nature of the Phosphodiester Bond Incisions and the Precise Incision Sites**—Previously, we reported that thymine dimers were released in fragments 27–29 nucleotides in length by HeLa cell-free extract (Huang et al., 1992). The sizes of the excision fragments were estimated by comparison with a size marker obtained from spontaneous hydrolysis of a 5' terminally labeled 30-mer. Therefore, implicit in these estimates was the assumption that the excision fragments had a phosphoryl terminus at one end and a hydroxyl terminus at the other. In addition, fragments of lower abundance and ranging in size from 26 to mononucleotides were observed and ascribed to degradation products of the primary excision fragments by nucleases expected to be present in human whole cell-free extracts.

To determine more precisely the fragment sizes, the incision sites 5' and 3' to the adduct, and whether the incisions were at the 5' O-P or the P-O 3' bonds, we conducted an excision assay on a preparative scale, isolated each fragment, and then subjected them to enzymatic analyses separately.

Fig. 2A shows one of the preparative gels used for analyses, and in Fig. 2B we have plotted the relative frequencies of the excision fragments. The data for Fig. 2B were obtained from densitometric scanning of the gel shown in Fig. 2A and six other gels of independently conducted experiments. As apparent, fragments designated 27–29 nt are the most abundant. In the longer size range there is a sudden drop in frequency with fragments 30–32 nt constituting about 4% of the total. In the shorter size range (relative to 27–29-mers), the change...
in size class frequency is gradual, and the sum of size classes designated 17-26 is about equal to the sum of the 27-29-mers. The purified fragments were treated with bacterial alkaline phosphatase (BAP) to remove terminal phosphates or T4 DNA polymerase 3' → 5' exonuclease which is strongly inhibited by bulky adducts (Fuchs, 1984) including T<>T (Doetsch et al., 1985), to identify the chemical nature of the termini and the location of T<>T within the excised fragments. The reaction products were run alongside a sequence ladder generated by the Sanger dideoxy method using a sequencing primer containing a 5' hydroxyl for definitive length assignments. The results obtained with fragments designated 25 through 29 are shown in Fig. 3. Several conclusions can be made from this figure. First, phosphatase treatment results in slower mobility by about 1.5 nt positions for all fragments whether treated with T4 Pol 3' → 5' exonuclease or not. As it is known that the (-2) charge of an extra phosphate leads to faster migration by 1.5-2.0 spaces for fragments 10-30 nucleotides in length (Royer-Pokora et al., 1981), we conclude that the excision fragments carry a phosphate at one terminus only. Second, since T4 Pol 3' → 5' exo which is specific for 3'-OH terminus (Kornberg and Baker, 1991) digests the excision fragments and since phosphatase treatment of T4 Pol 3' → 5' exonuclease products (which are known to have 3'-OH termini) causes the same mobility shift, the excision fragments must have 5'-P termini. It follows that the 3' terminus of the primary excision products must be -OH. Third, by comparing the migration of phosphatase treated fragments with those generated by Sanger dideoxy method, and thus have the same termini, unambiguous size assignments can be made as shown in the figure. Finally, by comparing the migration of phosphatase treated fragments with those treated with both phosphatase and T4 Pol 3' → 5' exonuclease the positions of T<>T in each fragment can be assigned.

The double digestion of the 25-mer (Fig. 3, lane 5) yields mostly 21-mer but also 22-25-mers; the 26-mer upon double digestion similarly yields a ladder extending from 21- to 25-mer (lane 10). These results imply that the 25- and the 26-mer represent heterogeneous populations of DNAs with regard to location of the T<>T. Two trivial explanations for these multiple bands, incomplete digestion by T4 Pol 3' → 5' exonuclease or a minor 5' → 3' exonuclease contaminant in T4 Pol or BAP were excluded because treatment of longer fragments under identical conditions yielded unique products. Therefore, we believe (see below) that this is the consequence of generating these fragments from the primary excision products by exonucleases present in the cell extract. The same heterogeneous size pattern was also observed for other fragments smaller than 25-mer following the digestion with T4 Pol 3' → 5' exonuclease alone or in combination with phosphatase (data not shown). Thus, we consider fragments of 25-mer and smaller to be degradation products. Indeed, inclusion of a 25-mer containing T<>T in the cell-free extract generated a ladder such as seen in Figs. 1 and 2 (Huang et al., 1992).

In contrast, treatment of 27-, 28-, and 29-mers with T4 Pol 3' → 5' exonuclease and phosphatase generated single bands of 23-, 24-, and 25-mers, respectively (Fig. 3, lanes 16, 21, and 26, respectively). There is also a minor 25-mer band generated from the 28-mer. In addition, even though the band in lane 21 is slower than that in lane 26 justifying our size assignment, we note that the migration of the band in lane 21 is somewhat faster than the size marker band in the adjacent length. This could be due to such factors as sequence heterogeneity of the excised fragments, the sequence effect of the size marker, and the presence of T<>T at a terminus. With these minor reservations, then, we conclude that the 27-, 28-, and 29-mers are primary excision products and carry a T<>T four nucleotides from the 3' terminus.

Similar experiments were conducted with 30-, 31-, and 32-mers as well to locate the T<>T relative to the 5' and 3' termini. The results (data not shown) revealed that these fragments were heterogeneous in nature having arisen from different combinations of 5' and 3' incisions. In contrast to the fragments 26 nt and shorter, the 30-32 class fragments were considered to be primary incision products because in numerous excision gels we have analyzed, we have never detected fragments longer than 32 nt. More significantly perhaps is the fact that an analysis of the "repair patch" boundaries revealed that the patch did not extend beyond six nucleotides 3' and 24 nucleotides 5' to the thymine dimer. To summarize, we consider the 27-32 nt long fragments to be primary excision products with the 27-29 nt class representing >95% of the excision reaction. The combinations of 5' and 3' incisions which give rise to the primary excision products are summarized in Fig. 4.

Excision of Psoralen Monoaduct—In prokaryotes, nucleotide excision repair excises all bulky adducts by essentially...
acetate, 66 mM potassium acetate (pH 7.0), and 10 mM magnesium-acetate (Fuchs, 1984). The mixture was incubated at 37°C for 60 min and quenched by phenol. The BAP reactions were carried out at 65°C for 30 min with the same amounts of DNA in 10 ml dye and separating on a 10% polyacrylamide-sequencing gel. The fragment lengths listed above the brackets correspond to the fragments marked as such in Fig. 2 and are based on data in this figure. Lanes 1, 4, 9, 12, 15, 20, 25, and 28 contain a sequence ladder of an unrelated fragment generated by dideoxy sequencing. The numbers on the right margin correspond to the length of the fragments in the sequence ladder. In lanes 6, 17, and 22, the reaction mixtures were incubated in T4 Pol buffer at 65°C to provide adequate control for the double digestion. However, in this buffer extensive degradation by T4 Pol resulted in drastic losses, and only very faint bands were visible in the original autoradiogram which are not seen in this photographic reproduction.

**Fig. 3. Size of the excision products and location of T<>T within each fragment.** Fragments were purified from a preparative gel as shown in Fig. 2A and then treated with T4 Pol or BAP or the combination of the two as indicated. The T4 Pol reaction (10 μl) contained in addition to the radiolabeled DNA 0.1 μg of X174 HaeIII-digested DNA and 0.5 unit of T4 Pol (Boehringer Mannheim) in 33 mM Tris-HCl, pH 8.0, 50 mM NaCl, 10 mM MgCl₂, and 150 units of BAP (Bethesda Research Laboratories). For double digestion, after incubating with T4 Pol, to 5 μl of the reaction mixture 1 μl of 10× BAP buffer, 150 units of BAP were added, the volume was adjusted to 10 μl, and incubation was continued for 30 min at 65°C. All samples were extracted with phenol and lyophilized before dissolving in formamide dye and separating on a 10% polyacrylamide-sequencing gel. The fragment lengths listed above the brackets correspond to the fragments marked as such in Fig. 2 and are based on data in this figure. Lanes 1, 4, 9, 12, 15, 20, 25, and 28 contain a sequence ladder of an unrelated fragment generated by dideoxy sequencing. The numbers on the right margin correspond to the length of the fragments in the sequence ladder. In lanes 6, 17, and 22, the reaction mixtures were incubated in T4 Pol buffer at 65°C to provide adequate control for the double digestion. However, in this buffer extensive degradation by T4 Pol resulted in drastic losses, and only very faint bands were visible in the original autoradiogram which are not seen in this photographic reproduction.

**Fig. 4. Incision patterns of human excision nuclease.** The incision sites derived from Fig. 3 are shown. The 27–29-mers which represent >95% of the primary excision products are indicated by heavy lines and the less frequent 30–32-mers with light lines. The excision fragments used for analysis originate from four positions on the plasmid substrate; 20 nucleotides around the T<>T's are identical in all four positions as shown, and the outside sequences which are unique to each position are labeled N.

the same incision pattern (Sancar and Sancar, 1988). We reasoned that if the T<>T excision activity we observe in HeLa cell-free extracts is a bona fide excision nuclease it should remove other bulky adducts by the same mechanism. We used a psoralen substrate similar to the T<>T substrate with the exceptions that the label was at the 8th phosphodiester bond 5' to the HMT furan side adduct (T<>HMT) and the plasmid contained a single adduct (Cheng et al., 1988). The effect of the HeLa cell-free extract on this substrate is shown in Fig. 5. Fragments of 27–29 nt are removed with the 28-mer being the most prominent. Fragments of 29–32 range are also observed as in the case of the T<>T substrate. For all size classes, the signal is weaker than that of the T<>T substrate. Since the T<>T substrate has four T<>Ts/plasmid while the T<>HMT substrate contains a single adduct and there appears to be a non-linear relationship between the repair signal and the number of adducts/plasmid (Reardon et al., 1991), no statement can be made regarding the relative efficiency of human excision repair on the two DNA lesions. Nevertheless, it is clear from this figure that T<>HMT is excised in essentially the same manner as the T<>T dimer. The low yield of the excision fragment prevented us from determining the location of the excised T<>HMT. However, based on the general excision pattern we predict that it is four to five nucleotides from the 3' terminus, and we propose that the human excision nuclease removes psoralen furan side monoadduct mainly by hydrolysis of the 23rd phosphodiester bond 5' and the 4th or 5th phosphodiester bond 3' to the adduct. More importantly, this activity has the two main characteristics of excision nucleases, ATP dependence and a wide substrate range, revealing that we are dealing with the human excision nucleosome system.

**Nucleotide Excision Repair in Frogs**—In all prokaryotes tested to date nucleotide excision repair is carried out by the same mechanism by excinucleases with extensive sequence homologies (see Lin and Sancar, 1992). It was of interest to
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Figure 5. Excision of HMT-thymine monoadduct. Either 200 ng of pUNC1991-4 (T<>T) or 800 ng of pUNC1991-4 (T<>HMT) were incubated with 120 μg of cell-free extract at 37 °C for 2 h in a standard reaction mixture. The DNAs were then deproteinized and analyzed on a 10% polyacrylamide-sequencing gel. Approximately 25,000 cpm were loaded into each lane. MA, psoralen monoadducted substrate; TT, thymine dimer substrate.

Figure 6. Excision of T<>T by X. laevis oocytes. Fifty eggs were injected with 50 ng (~8,000 cpm) of pUNC1991-4 (T<>T) or its photoreactivated form each. The eggs were incubated at 22 °C for 60 min, and then the DNA was extracted with phenol and precipitated with ethanol. Lanes 1 and 6 contain the 30-mer size marker. Lanes 2 and 3 contained 10,000 cpm of the corresponding DNAs after recovery from the eggs. The remainder of the DNA was separated on a preparative gel, the area spanning 25–30 nt was cut out, DNA was electroeluted, precipitated, and resuspended in T4 Pol buffer. Lane 1, before T4 Pol digestion; lane 2, after T4 Pol 3'→5' exonuclease digestion.

Data are consistent with a mechanism of excision by Xenopus that is initiated by incision of the 22nd-24th phosphodiester bonds 5' and the 5th phosphodiester bond 3' to the T<>T. Furthermore, if the DNA is photoreactivated prior to microinjection the intensity of the 27–29-mers decreases proportional to the level of photoreactivation (Fig. 6, compare lanes 2 and 3) providing confirmatory evidence that this is a true excision repair phenomenon. We conclude that Xenopus removes T<>T from DNA by a mechanism very similar if not identical to the human excision nuclease.

DISCUSSION

The results presented in this paper show that humans and frogs excise T<>T, and in the case of humans a psoralen-thymine adduct as well, in the form of 27–29-mers. These results help provide some answers to the following questions. What is the incision pattern of nucleotide excision nucleases in eukaryotes, and why do the eukaryotes use this particular pattern?
Before discussing these points, however, we need to know whether the excision of T<>T observed in vitro is carried out by an excision nuclease responsible for removing all bulky adducts. There is an excellent agreement between the size of the excised fragments we detect and the repair patch measured in vitro (Edenberg and Hanawalt, 1972; Cleaver et al., 1991) for T<>T and in vitro for 2-(acetylaminofluorene)-guanine adduct (Coverley et al., 1991). These repair patches were not formed in XP cells or in extracts from XP cell lines. Therefore, it is reasonable to conclude that the enzyme which removes T<>T in vitro is the enzyme lacking in XP cells. Furthermore, since these cells are also deficient in repairing DNA damage by UV mimetic agents such as psoralen we feel that it is safe to assume that in our cell-free extract the same enzyme removes both T<>T and HMT-T adducts.

Regarding the incision sites, our analyses of fragments excised from the T<>T substrate by the human enzyme revealed 27-, 28-, and 29-mers as the major excision products and some larger and smaller species at much lower frequencies. While the 30-, 31-, and 32-mer observed at about 4% of the total can only be primary excision products, fragments <26 nt in length appear to have arisen from degradation of the main excision fragments in our crude system. It is interesting that in the Xenopus system almost exclusively 27, 28- and 29-mers are observed presumably because of limited post excision degradation in this system. Treatment of these fragments with T4 Pol 3'→5' exonuclease generates fragments of 23–25 nt mostly in humans and exclusively in Xenopus. Taking the data from the two systems together we propose that the eukaryotic excision nuclease incises the 22-24th phosphodiester bonds (with about equal frequency) 5' and the 5th phosphodiester bond 3' to the damaged nucleotide. However, as the T4 Pol exonuclease analyses of the 30-32-nt fragments has shown in this study and repair patch experiments revealed in the initial work (Huang et al., 1992), in addition to the variability on the 5' side there is some variability of the incision site 3' to the adduct.

Why do eukaryotes remove nearly three turns of one strand of the double helix to eliminate one or two damaged nucleotides? While we recognize that teleological arguments can lead to misleading conclusions, we would like to propose the rationale that was originally proposed for the prokaryotic excision nuclease (Sancar and Rupp, 1983); that, by incising at some distance from the adduct the enzyme has greater flexibility with regard to lesion selection because the subunits need not make any contact with the adduct. In fact in the case of the prokaryotic excision nuclease the incision sites are on the opposite face of the double helix with regard to the damaged nucleotide(s). In the case of the eukaryotic enzyme, the incision sites are at a 90° angle to the adducted base(s) again consistent with the notion that the enzyme can remove damaged bases regardless of the chemical nature of the damage because it need not contact the damaged nucleotide. Why the prokaryotic enzyme removes 13 nucleotides to eliminate a T<>T from DNA while the human enzyme removes more than twice as many nucleotides to achieve the same task we cannot tell. It is hoped that purification of the XP proteins and reconstitution of human excision nuclease will help answer some of these questions.

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