Human chromatin-associated protein extracts were examined for endonucleolytic activity on a defined 132-base pair DNA substrate containing a single, site-specific 4,5,8-trimethylpsoralen plus long wavelength ultraviolet light-induced furan side or pyrone side monoadduct or interstrand cross-link. These extracts produced incisions on both the 3' and 5' sides of each of these lesions. The distance between the 3' and 5' incisions at sites of a furan side monoadduct or cross-link was 9 nucleotides, and at sites of a pyrone side monoadduct or cross-link it was 17 nucleotides. Incisions on the 3' side of both types of furan side and pyrone side adducts were similar and were either at the fourth or fifth phosphodiester bond from the added thymine, depending upon the adduct. However, greater differences were observed between sites of 5' incision. This incision occurred at the fifth and sixth phosphodiester bonds from the added thymine at sites of furan side monoadducts and cross-links, respectively, and at the 13th and 14th phosphodiester bonds at sites of pyrone side monoadducts and cross-links, respectively. Thus, direct analysis of sites of endonucleolytic incision reveals that the location of sites of incision on TMP-adducted substrates depends upon the type of adduct present.

Repair of DNA interstrand cross-links is critical for a number of cellular processes such as transcription and DNA replication and therefore is particularly important for the maintenance of genetic integrity and cellular survival. A nucleotide excision repair mechanism is responsible in both prokaryotes and eukaryotes for the removal of this type of lesion (1–3). The majority of the time is a thymine. If the furan side of the molecule is linked to DNA, then further exposure to UVA light induces the formation of an interstrand cross-link (5–10). However, an endonuclease that specifically incises DNA at sites of these interstrand cross-links has heretofore not been isolated from mammalian cells.

A number of different agents have been shown to produce interstrand cross-links in DNA. One of the most definitive of these, whose reaction with DNA has been well characterized, is psoralen plus UVA light. Psoralens are a group of three-ring heterocyclic furocoumarins that contain two reactive double bonds, a 4',5'-double bond in the furan ring and a 3,4-double bond in the pyrone ring (5–8). Psoralen-DNA adducts are formed in three stages: psoralen first intercalates into the DNA duplex in a noncovalent manner and then, upon photoreaction with UVA light, forms either a furan side or a pyrone side monoadduct with the 5,6-double bond of a pyrimidine, which is recognized by a thymine. If the furan side of the molecule is linked to DNA, then further exposure to UVA light leads to production of an interstrand cross-link (5–10).

In Escherichia coli, the UvrABC nuclease is responsible for repair of psoralen-photoinduced monoadducts and interstrand cross-links. The Uvra, Uvrb, and Uvrc proteins act in concert to excise these lesions by making sequential incisions on both the 3' and 5' sides of the modified nucleotide (11–17). Although repair of psoralen monoadducts and interstrand cross-links has been shown to occur in mammalian cells (18–26), the precise nature of the initial events and proteins involved in removal of these lesions is less clear. We have isolated from the nuclei of normal human cells a chromatin-associated DNA endonuclease complex, pl 4.6, which recognizes and incises DNA containing TMP or 8-methoxypsoralen plus UVA interstrand cross-links and another, pl 7.6, which recognizes and incises DNA containing psoralen monoadducts (22, 23, 25, 27). Each complex contains proteins involved in damage recognition, chromatin interaction, and endonucleolytic incision, and each has been demonstrated to be involved in the repair process by its ability to correct the repair defect in repair-deficient cells when introduced into them via electroporation (28, 29).

In the present study, we describe the construction of a 132-base pair oligonucleotide substrate that contains a centrally placed single, site-specific TMP monoadduct or interstrand cross-link. These substrates are so constructed that endonucleolytic activity on either the furan side or the pyrone side of each type of adduct may be selectively studied. We have used these four substrates to directly examine the pattern of incision produced by human chromatin-associated protein extracts that contain both the endonuclease complexes, pl 4.6 and pl 7.6. Our results show that these extracts make incisions in DNA on...
both the 3’ and 5’ sides of each of the two types of furan side and pyrone side adducts (i.e., monoadducts and cross-links). The incision patterns on each of these four substrates are different. The distance between the 3’ and 5’ incisions made at sites of furan side monoadducts and cross-links is 9 nucleotides, whereas the distance between the 3’ and 5’ incisions made at sites of pyrone side monoadducts and cross-links is 17 nucleotides.

**Materials and Methods**

Chromatin-associated Protein Extracts—Normal human (GM 1989 and GM 3299) lymphoblastoid cell lines (transformed with Epstein-Barr virus) were obtained from the Coriell Institute for Medical Research (Camden, NJ). The cells were grown in suspension culture in RPMI 1640 medium, supplemented with 12.5% fetal calf serum (Grand Island Biological Co.), and harvested under conditions of maximal proliferation (30). Cell cultures were routinely tested for mycoplasma (30).

Cell nuclei were isolated, and the chromatin-associated proteins were separated from the nucleoplastic proteins in a series of steps as described previously (22, 31). Chromatin-associated proteins were dialyzed into 50 mM potassium phosphate (pH 7.1), 1 mM β-mercaptoethanol, and 1 mM EDTA. 0.25 mM phenylmethylsulfonyl fluoride, and 40% ethanol was added, and then passed through a CM Sephadex column and stored at −20 °C (4, 22, 31). Protein concentrations were determined by the Bio-Rad protein assay (Bio-Rad).

DNA Oligonucleotide Synthesis and Purification—The DNA substrate used was the region from position 61 to 200 of the nucleotide sequence of the 5 S RNA gene from *Lytichinus variegatus* (32). This region was synthesized as seven separate oligonucleotides (ML1–ML7) (Fig. 1) (W. M. Keck Foundation Biotechnology Resource Laboratory at Yale University). All oligonucleotides were lightly labeled on their 5’-ends with 30 μCi of [γ32P]ATP (6000 Ci/mmol; Du Pont NEN) using T4 polynucleotide kinase (Pharmacia Biotech Inc.) (33). The full-length products were purified by electrophoresis on 10 or 20% polyacrylamide 7 M urea gels and by subsequent isolation of the bands identified by autoradiography, extracted, and purified as described (33). The purified oligonucleotides were then used in ligation reactions to produce the 132-bp substrates described below (Fig. 2).

Preparation of the 18-Mer Oligonucleotide (ML3). Containing the Furan Side TMP Monoadduct—The region from position 124 to 141 of the nucleotide sequence of the top strand of the 5 S RNA gene from *L. variegatus* (designated ML3) (Fig. 1), which is approximately at the center of the DNA fragment, was used for construction of the furan side TMP monoadduct. ML3 (18-mer) and ML3A (22-mer) were designed to have a single 5’-TpA-3’ dinucleotide sequence at positions 132 and 133, in place of the 5’-Apt-3’ original dinucleotide sequence, since this sequence has been shown to be a hot spot for photoreactivity of thymine with TMP, which photoreacts with the thymine in this sequence (9, 13).

The preparation of the ML3 furan side monoadduct was as follows. 180 pmol of 5’-phosphorylated ML3 and ML3A as tracers, in 1000 μl of 10 mM Tris-HCl, pH 7.6, 0.4 mM EDTA, and 100 mM NaCl and 5 μg/ml TMP (Sigma). The solution was irradiated in 100-μl aliquots with long wavelength ultraviolet (365 nm) light (13 milliwatts/cm2), from a super-high intensity black light lamp (Spectrolite model SB-100, Spectronics Corp.) for 5 min. at 20 °C. The DNA was extracted with chloroform/isoamyl alcohol (24:1) to remove unbound TMP, ethanol-p precipitated, and then electrophoresed on a 12% denaturing 7 M urea polyacrylamide gel so as to separate the strands and isolate the monoadducted ML3 oligonucleotide. Also formed in this reaction was the monoadducted ML3 sequence on the top strand.

For construction of the furan side cross-linked DNA, half of the above furan side monoadducted substrate in 100 μl of 10 mM Tris-HCl, pH 7.6, and 0.4 mM EDTA was irradiated with the UVA light (20 milliwatts/cm2) for 25 min at 20 °C (Fig. 2). The furan side cross-linked substrate was purified by electrophoresis on a denaturing 5% polyacrylamide gel. The strategy for formation of the pyrone side interstrand cross-link was similar to that used for the furan side cross-link. However, in this case, the furan side cross-link was reverted by treatment with hot alkali, which has been shown to specifically cleave the psoralen cross-link at the furan side, whereas the pyrene side monoadduct is relatively resistant to this treatment (34). Half of the above pyrene side cross-linked substrate in 150 μl of 10 mM Tris-HCl, pH 7.6, 0.4 mM EDTA, and 100 mM KOH was heated at 90 °C for 30 min, and the product was purified by a denaturing 5% polyacrylamide gel (Fig. 2). The preparation of the undamaged DNA substrate was similar to this except that it contained an undamaged ML3 sequence on the top strand.

As can be seen in Fig. 1A, the top strand of the DNA substrate (ML8), which is a 136-mer fragment, was assembled from four oligonucleotides: ML1 (20-mer), ML2 (43-mer), ML3 (18-mer), and ML4 (55-mer). Similarly, the bottom strand (ML9), which is also a 136-mer fragment, was assembled from three oligonucleotides: ML5 (45-mer), ML6 (50-mer), and ML7 (41-mer). The preparation of DNA substrates containing site-specific TMP monoadducts and cross-links is outlined in Fig. 2. The top strand (ML8) of the substrate was designed as labeled at the 5’-end of the ML oligonucleotide (thymine at position 81) (Fig. 1) to visualize the furan side incision events. Similarly, the bottom strand (ML9) of the substrate was internally labeled at the 5’-end of ML6 (guanine at position 159) (Fig. 1) to visualize the pyrene side incision events. The full-length labeled top strand (ML8) and bottom strand (ML9) were purified by electrophoresis on a 15% denaturing polyacrylamide gel in order to remove other products. Both ML8 and ML9 had the same mobility on these gels and had migrating along with them the unlabeled complementary ML9 or ML8 strands. The bands corresponding to the full-length ligated products were extracted from the denaturing gel, and the DNA was purified as described (33). These bands represent both the top and bottom strands in their unhybridized forms. Therefore, it was necessary to reolate the hybridized duplex full-length DNA substrates on a non-denaturing 5% polyacrylamide gel. These substrates were 132 bp in length with a 4-base overhang on the 5’-ends. In these substrates, TMP was adducted to a thymine at position 132 in ML8 and at position 133 in ML9 (Fig. 1A). Preparation of the specific ligated, psoralen-monoadducted, and cross-linked substrates is described below. For construction of the furan side monoadducted ML2 (180 pmol) was dephosphorylated with calf intestine alkaline phosphatase and 5’-end-labeled with 1 μCi of [γ32P]ATP (6000 Ci/mmol; Du Pont NEN) using T4 polynucleotide kinase (Pharmacia). ML2 was freed from protein, salts, and unincorporated label by passing through Nensorb 20 nucleic acid purification cartridges according to the manufacturer’s directions (Du Pont NEN). ML2 was then eluted with 50% ethanol and dried in a Speed Vac in a siliconized eppendorf tube. To the dried pellet, 180 pmol of 5’-phosphorylated ML1, ML4, ML5, ML6, and ML7 and 540 pmol of ML3 oligonucleotide containing the furan side TMP monoadduct were added in a total volume of 150 μl containing 10 mM Tris-HCl, pH 7.6, 0.4 mM EDTA, and 25 μM NaCl (Fig. 2). The amount of ML3 furan side monoaducted intermediate was three times that of the other oligomer components, since the efficiency of ligation was dependent upon the amount of ML3 monoadduct in the reaction mixture. The mixture was heated at 95 °C for 3 min, and then the DNA strands were hybridized at 60 °C for 30 min followed by slow cooling to 25 °C. To this mixture 100 units of DNA ligase (Boehringer Mannheim) was added, and ligation was carried out at 12 °C for 90 min, followed by 4 °C ligation. Ligation was found to be essentially complete when carried out at 22 °C for 1 or 2 h. The ligated full-length product was isolated by electrophoresis of the sample through a denaturing 5% polyacrylamide gel. As mentioned above this product also contained the complementary unlabeled full-length bottom strand (ML9). The purity of the duplex DNA was ensured by repurifying this product on a nondenaturing 5% polyacrylamide gel (data not shown). The pyrene side monoaducted substrate in which TMP was added to a thymine at position 132 on ML8 (Fig. 1). The preparation of undamaged DNA substrate was similar to this except that an undamaged ML3 sequence was used for this purpose.

For construction of the furan side cross-linked DNA, half of the above furan side monoadducted substrate in 100 μl of 10 mM Tris-HCl, pH 7.6, and 0.4 mM EDTA was irradiated with the UVA light (20 milliwatts/cm2) for 25 min at 20 °C (Fig. 2). The furan side cross-linked substrate was purified by electrophoresis on a denaturing 5% polyacrylamide gel. The strategy for formation of the pyrone side interstrand cross-link was similar to that used for the furan side cross-link. However, in this case, the pyrene side cross-link was reversed by treatment with hot alkali, which has been shown to specifically cleave the psoralen cross-link at the furan side, whereas the pyrene side monoadduct is relatively resistant to this treatment (34). Half of the above pyrene side cross-linked substrate in 150 μl of 10 mM Tris-HCl, pH 7.6, 0.4 mM EDTA, and 100 mM KOH was heated at 90 °C for 30 min, and the product was purified by a denaturing 5% polyacrylamide gel (Fig. 2). The preparation of the undamaged DNA substrate was similar to this except that it contained an undamaged ML3 sequence on the top strand.
Construction of a 132-bp DNA Substrate Containing Site-
specific TMP Monoaduct or Cross-link—A 132-bp DNA sub-
strate, with a 4-base overhang on the 5'-end of each strand, was
constructed by ligating together seven component oligomers
(ML1–ML7). The ligation of the component oligonucleotides
was indicated by the arrows (Figs. 1 and 2). The numbering
system between the top (ML8) and bottom (ML9) strands refers
to the region of the 5 S rRNA gene from L. variegatus that was utilized.

All of the above substrates were cleaned up with the Nensorb 20
nucleic acid purification cartridges according to the manufacturer’s
protocol (DuPont NEN) and were stored in aliquots at -20°C in 10 mM
Tris-HCl (pH 7.6) and 0.4 mM EDTA.

Assay for Endonucleolytic Incision—For the incision reactions
approximately 100 fmol (concentration determined by In Vitrogen DNA
8
9
Dip Stick kit, version 3.2) of the 132-bp labeled DNA substrate, either
TMP-modified or unmodified, was used as an intermediate in the con-
struction of all of these unique TMP-modified 132-bp DNA
substrates (Figs. 1 and 2). The efficiency of formation of TMP
monoaducts on this intermediate was determined by examin-
ing the ability of TMP-modified ML3 to form interstrand cross-
link when hybridized to complementary ML3A and then irra-
diated with UVA light. It was found that under these
conditions all of the furan side monoaducts were able to form
interstrand cross-links (data not shown).

To construct the 132-bp furan side monoadducted DNA,
TMP-modified ML3 was ligated together with the other six
component oligomers. The upper strand was internally labeled
on the 5'-end of oligonucleotide ML3, containing the furan side
monoadduct of TMP, was used as an intermediate in the con-
struction of all of these unique TMP-modified 132-bp DNA
substrates (Figs. 1 and 2). The efficiency of formation of TMP
monoadducts was confirmed by the use of complementary ML3A
and ML3B. The formation of the furan side cross-link was also very efficient. Greater than 90% of the monoaducted substrate was con-
verted to the cross-linked form (Fig. 3A, lane 1).

For construction of the pyrone side adducted substrates,
TMP-monoadducted ML3 was used in the ligation reactions in
which the bottom strand was internally labeled on the 5'-end of
ML6 (Fig. 2). As was the case for the top strand, the ligation
reaction for the bottom strand occurred with over 50% effi-
ciency. The purity of the duplex DNA was ensured by repurify-
ing this product on a nondenaturing gel (data not shown). To form the furan side cross-linked DNA, the above monoadducted substrate was pho-
toreacted with 365-nm UV light (Fig. 2). The formation of the furan side cross-link was also very efficient. Greater than 90% of the monoadducted substrate was converted to the cross-linked form (Fig. 3A, upper band, lane 1).

RESULTS

Construction of a 132-bp DNA Substrate Containing Site-
specific TMP Monoadducts or Cross-links—A 132-bp DNA sub-
strate, with a 4-base overhang on the 5'-end of each strand, was
constructed by ligating together seven component oligomers
(ML1–ML7) are indicated by arrows. The numbering system between the top (ML8) and bottom (ML9) strands refers to the region of the 5 S rRNA gene from L. variegatus that was utilized.

An 18-mer oligonucleotide (ML3), containing the furan side
monoadduct of TMP, was used as an intermediate in the con-
struction of all of these unique TMP-modified 132-bp DNA
substrates (Figs. 1 and 2). The efficiency of formation of TMP
monoadducts on this intermediate was determined by examin-
ing the ability of TMP-modified ML3 to form interstrand cross-
link when hybridized to complementary ML3A and then irra-
diated with UVA light. It was found that under these
conditions all of the furan side monoadducts were able to form
interstrand cross-links (data not shown).

To construct the 132-bp furan side monoadducted DNA,
TMP-modified ML3 was ligated together with the other six
component oligomers. The upper strand was internally labeled
on the 5'-end of oligonucleotide ML2 (Figs. 1 and 2). The liga-
tion reaction occurred with over 50% efficiency. The purity of the
duplex DNA was ensured by repurifying this product on a
nondenaturing gel (data not shown). To form the furan side
cross-linked DNA, the above monoadducted substrate was pho-
toreacted with 365-nm UV light (Fig. 2). The formation of the furan side cross-link was also very efficient. Greater than 90% of the monoadducted substrate was converted to the cross-linked form (Fig. 3A, upper band, lane 1).

For construction of the pyrone side adducted substrates,
TMP-monoadducted ML3 was used in the ligation reactions in
which the bottom strand was internally labeled on the 5'-end of
ML6 (Fig. 2). As was the case for the top strand, the ligation
reaction for the bottom strand occurred with over 50% effi-
ciency. The purity of the duplex DNA was ensured by repurify-
ing this product on a nondenaturing gel (data not shown). The monoadducted 132-bp substrate was then driven to the cross-linked form by exposure to 365-nm UV light (Fig. 2). Greater than 90% of the monoadducted substrate was con-
verted to the cross-linked form (Fig 3A, upper band, lane 2). The lower bands in Fig. 3A, lanes 1 and 2, may represent
unreacted full-length monoaducted substrates. To form the pyrone side monoaduct, the pyrone side cross-link was reversed by treatment with hot alkali (Fig. 2). The alkali reversal was also very efficient since the cross-linked substrate was completely reversed to the monoaducted form (Fig. 3B).

Identification of Sites of Endonucleolytic Incision at TMP Furan Side Adducts. The human chromatin-associated protein extracts produced incisions on the 132-bp substrate on both the 3’ and 5’ sides of TMP furan side monoaduct. Two bands were produced that represent incisions made at the fifth phosphodiester bond 3’ to the adducted thymine and at the fifth phosphodiester bond 5’ to this same modified base (Fig. 4, lane 1). Bands representing 3’ and 5’ incisions comprise 4.5 and 2.0% of the substrate, respectively. These incisions were not seen on undamaged DNA (Fig. 4, lane 3). In these reactions, salmon sperm DNA was used as a nonspecific competitor DNA. The same results were obtained when two other nonspecific competitor DNAs were used: poly(dI-dC)•poly(dI-dC) and a 260-bp fragment from the 5 S rRNA gene from L. variegatus, which included the sequence for the 132-bp substrate (data not shown). These dual incisions were also observed in the absence of competitors (data not shown). Minor bands were observed that were also present on the undamaged DNA and represent incisions by nonspecific nuclease.

The 3’ and 5’ incisions were reduced (67 and 64%, respectively) upon the addition to the reaction mixture of 100 ng of unlabeled competitor DNA, which contained TMP furan side monoaducts (Fig. 5, lane 1). 3’ and 5’ incisions represent 4.9 and 2.8% of substrate, respectively, compared with lane 2, 3’ and 5’ incisions representing 1.6 and 1.0% of substrate, respectively). Nonspecific cuts were reduced by only 38% (Fig. 5, lane 1 compared with lane 2). Some reduction in nonspecific cuts would be expected since nonspecific nucleases would be acting on the competitor as well as on the substrate DNA. The dual incisions were totally abolished when 500 ng of the competitor was added (lane 4). In several of the figures, the DNA sequence around the damaged sites, when reacted with the protein extracts, appeared to be relatively protected. Since this pattern is seen on both damaged as well as on undamaged DNA (Fig. 4), it may represent sequence-specific protein binding rather than binding by a damage-specific binding protein, particularly since the 3’ and 5’ endonucleolytic incisions are seen only on the damaged and not on the undamaged DNA.

For those substrates in which incisions at sites of interstrand cross-links were examined, the cross-linked DNA was photo-reversed by UVC light before gel electrophoresis. This was done in order to break the interstrand cross-link and allow us to detect potential incisions on both sides of the lesion on the same strand in a single assay (35, 36). Experiments were carried out to make sure that irradiation of the cross-linked DNA with UVC light was not affecting the electrophoretic migration pattern of the incised DNA. Monoadducted DNA, reacted with the human extract and irradiated with UVC light prior to electro-
The presence of 100 ng of unlabeled TMP-adducted DNA; in the presence of 500 ng of unlabeled competitor DNA.

The reaction products were analyzed on a denaturing 6% polyacrylamide gel. The bands indicating incisions 3' and 5' to the adducted thymine are denoted by arrows. The pattern of endonucleolytic incision on this substrate is as follows: lane 1, in the absence of competitor; lane 2, in the presence of 100 ng of unlabeled TMP-modified DNA; lane 3, in the presence of 2000 ng of unlabeled competitor DNA; lane 4, in the presence of 500 ng of unlabeled competitor DNA.

Examination of the ability of the normal extracts to incise DNA containing a furan side interstrand cross-link indicated that dual endonucleolytic incisions were also produced on this substrate (Fig. 4). Incisions occurred at the fourth phosphodiester bond 5' to the adducted thymine and at the sixth phosphodiester bond 5' to this modified base (Fig. 4, lane 2; 3' and 5' incisions, 4.4 and 2.8% of substrate, respectively). For both the furan side monoadduct and cross-link, the distance between sites of incision was 9 nucleotides. These same studies were carried out with similar extracts from HeLa cells, and the same results were obtained (data not shown).

Sites of Endonucleolytic Incision at TMP Pyrone Side Adducts—Incision signals by the protein extracts were not as strong on DNA containing pyrone side adducts. As is shown in Fig. 6, two bands were produced when the extract was reacted with the pyrone side monoadducted DNA substrate. An incision was produced at the fifth phosphodiester bond 3' to the adducted thymine and at the 13th phosphodiester bond 5' to this modified base (Fig. 6, lane 1; 3' and 5' incisions, 2.7 and 1.9% of substrate, respectively; lane 2, 3' and 5' incisions, 3.2 and 2.3%, of substrate, respectively). The extracts did not incise the undamaged DNA substrate (Fig. 6, lanes 3 and 4).

Dual incisions were also produced by the normal extracts on pyrone side cross-linked DNA. However, as noted above, these incisions were produced less efficiently than for the other TMP-adducted substrates. The 3' incision was at the fourth phosphodiester bond from the adducted thymine, and the 5' incision was at the 14th phosphodiester bond from this modified thymine (Fig. 7, lane 1; 3' and 5' incisions, 2.5 and 1.7% of substrate, respectively; lane 2, 3' and 5' incisions, 2.7 and 2.1% of substrate, respectively). The distance between sites of incision on both the pyrone side monoadducted and cross-linked substrates was 17 nucleotides, a greater distance than that between sites of incision on furan side adducted DNA.

All of the incision experiments described above were carried out using two different normal human cell lines. Each experiment was repeated 10–20 times using two to four different extractions from each cell line and three to four different substrate preparations. In all instances the results obtained were always the same. The addition of ATP was not required for any of these incision events.

Effects of Ionic Conditions on Endonucleolytic Incision—The effects of ionic conditions on endonucleolytic incision were examined on the furan side monoadducted 132-bp DNA substrate. Mg2+ (10 mM) was needed for both the 3' and 5' incisions (Fig. 8, lane 1; 3' and 5' incisions, 2.7 and 1.9% of substrate, respectively). Incision signals were much weaker when the Mg2+ was reduced to 2 mM (Fig. 8, lane 2; 3' and 5' incisions, 1.1 and 0.9% of the substrate, respectively) or eliminated (Fig. 8, lane 3; 3' and 5' incisions, 0.4 and 0.5%, respectively). The presence of EDTA inhibited both of the incisions (Fig. 8, lane 4; 3' and 5' incisions, 0.6 and 0.7%, respectively). The addition of Zn2+ to the reaction had minimal influence on the incisions (Fig. 8, lane 5; 3' and 5' incisions, 3.5 and 1.9%, respectively). Mn2+ (10 mM), in the presence of 10 mM Mg2+, slightly enhanced both the 3' and 5' incisions (Fig. 8, lane 6; 3' and 5' incisions, 4.0 and 2.0%, respectively). Incision
incisions in DNA on both the 3' and 5' sides of each of the four types of TMP-thymine adducts. The results show that the human extracts produced interstrand cross-links by human chromatin-associated protein plus UVA light-induced DNA monoadducts and directly examined the incision events involved in repair of psoralen-modified 132-bp substrate, which contained a single site-directed lesion. Utilizing such a uniquely modified 132-bp substrate, we have directly examined the incision events involved in repair of psoralen plus UVA light-induced DNA monoadducts and interstrand cross-links by human chromatin-associated proteins. The results show that the human extracts produced incisions in DNA on both the 3' and 5' sides of each of the four types of TMP-thymine adducts. The distance between the 3' and 5' incisions for furan side monoadducts and cross-links was always 9 nucleotides, whereas the distance between these incisions for the pyrone side monoadducts and cross-links was always 17 nucleotides. This increased distance between sites of incision for the pyrone versus the furan side adducts could be due to the fact that, since TMP is an asymmetric molecule, more distortion may occur in the DNA in the vicinity of the thymine adducted to the pyrone side of TMP as compared with that of the thymine bound to the furan side (7, 9, 10, 37). The sites of incision on the 3' side of both types of furan side and pyrone side adducts were similar; they were either at the fourth or fifth phosphodiester bond from the adducted thymine, depending upon the adduct (Fig. 9). Greater differences were observed, however, in the sites of incision on the 5' side. These incisions occurred at the fifth and sixth phosphodiester bonds from the adducted thymine at sites of furan side adducts and at the 13th and 14th phosphodiester bonds at sites of pyrone side adducts (Fig. 9). Thus, production of a potentially greater distortion in the DNA by the pyrone side adducts appears to have little effect on the site of the 3' incision but to increase markedly the distance of the 5' incision from the adducted thymine, compared with the distance of this incision from the site of furan side adducts.

Svoboda et al. (26) have examined removal of psoralen monoadducts by HeLa whole cell extracts. They utilized as substrate a plasmid DNA containing a single 4'-hydroxymethyl-4,5,8-trimethylpsoralen (HMT) furan side monoadduct. Based on their results using an excision assay, which examined the sizes of DNA fragments excised from HMT-monoadducted DNA after treatment with cell extracts (26), and based on the pattern of excision of thymine dimers from DNA (26, 38), they proposed that fragments of 27–32 nucleotides in length are excised and predicted that the sites of incision occur at the fourth or fifth phosphodiester bond 3' to and 5' to the adducted thymine. Their site of 3' incision is similar to the one we have observed, but their proposed site of 5' incision is significantly farther from the adducted thymine than ours. An important difference, however, between the methods utilized in the present study and those used by Svoboda et al. is that we are looking at sites of endonucleolytic incision directly and they used indirect methods of analysis. For their studies that examined the size of the DNA fragment excised, DNA was labeled at the eighth phosphodiester bond 5'.
to the HMT furan side adduct (26). However, any fragment in which the 5’ incision occurred between the phosphodiester bond and the adduct would not have been detected in their system. Therefore, using this labeling scheme, Svoboda et al. (26) would not have been able to detect the 5’ incision site we describe at the 5th phosphodiester bond from the adduct. Svoboda et al. (26) also predicted sites of incision for HMT monoadducts based on the excision pattern of thymin dimers. In these studies the boundaries of the repair patch synthesized, after excision of the damaged fragment, were examined and used in the determination of the sites of 3’ and 5’ incision (26, 38). By using DNA fragments containing repair patches with phosphorothioate linkages and by analyzing resistance of these linkages to exonuclease III digestion and their sensitivity to cleavage by iodine, they delineated the borders of the repair patch (26, 38). Since whole cell extracts were used for these studies, it is possible that specific as well as nonspecific endonucleolytic and exonucleolytic activities present in these extracts and postincision events could contribute to the size of the DNA fragment excised. The size of this fragment in turn determines the size of the repair patch generated. Thus, these events could account for the larger distance, reported by Svoboda et al. (26), between the sites of the 3’ and 5’ incision. This is in contrast to results obtained when pure proteins are used in this type of excision assay, where a more direct correlation can be obtained between the size of the repair patch generated and the distance between sites of endonucleolytic incision, as has been shown for the UvrABC nuclease (39).

Comparison of the excision pattern produced on psoralen-adducted substrates by the human endonucleases in the present study with that produced by the UvrABC nuclease (11–17) shows that the sites of 3’ incision are fairly similar. Differences were observed, however, in the sites of 5’ incision. The distance between sites of incision on all four types of psoralen-adducted substrates is 12 nucleotides for the UvrABC nuclease, compared with a distance of 9 and 17 nucleotides for the human endonucleases at sites of furan and pyrone side adducts, respectively. Also, in the present study, incisions at the pyrone side TMP cross-link were not produced as efficiently as those at the furan side cross-link. This result is analogous to those from the UvrABC system in which the pyrone side cross-linked substrate was not found to be efficiently incised in one study (14) and was not found to be incised at all in another (11). Studies indicated that UvrABC incision on the pyrone side of the cross-linked substrate may depend upon whether or not a three-stranded intermediate is present (16, 17). These incisions have also been shown to be sequence-dependent (14, 15). It is possible that a similar situation may play a role in the incision of a pyrone side cross-linked substrate by the human repair endonuclease(s).

The present data show that incisions both 3’ and 5’ to a TMP adduct are produced on the 5’-labeled DNA substrate. This means that the 3’ incision occurred in the absence of or independent of the 5’ incision; otherwise, the 3’ incision would not have been detected. This indicates that the 3’ and 5’ incision events are coupled. The 5’ incision, in turn, could represent either a 5’ incision event or the generation of a 5’ incision accompanied by a 3’ incision. Uncoupling of dual incisions at sites of DNA adducts has also been observed for the UvrABC nuclease at sites of pyrimidine dimers (40), psoralen adducts (12, 15), and CC-1065 adducts (41).

The question arises whether the same or different proteins are involved in repair of the various different types of lesions that can be produced in DNA. The chromatin-associated protein extracts utilized in the present study contain an endonuclease complex, pl 4.6, which has specificity for DNA interstrand cross-links, and another, pl 7.6, which recognizes psoralen monoadducts (22, 23, 25, 42). A damage recognition protein with specificity for interstrand cross-links, which we have identified in these extracts, is thought to be a component of the DNA endonuclease complex, pl 4.6 (4). These results are consistent with the hypothesis that different endonuclease complexes are involved in repair of psoralen monoadducts and interstrand cross-links (23, 25, 42). It is, however, possible that the endonucleases involved in the production of the 3’ and 5’ incisions are the same in each complex but that each complex varies in the type of damage recognition protein present. On the other hand, different endonucleases could be associated with these complexes. The system described here, which specifically examines the initial incision events, combined with the use of human cell lines deficient in specific aspects of the damage recognition and incision step of the repair process, should greatly facilitate evaluation of this multifaceted and interactive process and of the proteins involved in these interactions.

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REFERENCES
1. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, pp. 191–316, American Society for Microbiology Press, Washington, D.C.
2. Grossman, L., Caron, P. R., Mazur, S. J., and Oh, E. Y. (1988) FASEB J. 2, 30715.
3. Van Houten, B. (1990) Microbiol. Rev. 54, 18–51
4. Hang, B., Yeung, T., and Lambert, M. W. (1993) Nucleic Acids Res. 21, 4187–4192
5. Ben-Hur, E., and Song, P.-S. (1984) Adv. Radiat. Biol. 11, 133–177
6. Vigny, P., Gaboriau, F., Voturiez, L., and Cadet, J. (1985) Biochimie 67, 317–325
7. Cimino, G. D., Gamper, H. B., Isaacs, S. T., and Hearst, J. E. (1985) Annu. Rev. Biochem. 54, 1151–1193
8. Vigny, P., Gaboriau, F., Voituriez, L., and Cadet, J. (1985) Biochimie 67, 317–325
9. Yeung, A. T., Jones, B. K. and Chu, C. T. (1988) Biochemistry 27, 3204–3210
10. Kumaresan, K. R., Ramaswamy, M., and Yeung, A. T. (1992) Biochemistry 31, 6774–6783
11. Van Houten, B., Gamper, H., Holbrook, S. R., Hearst, J. E., and Sancar, A. (1986) Proc. Natl. Acad. Sci. U.S.A. 83, 8077–8081
12. Van Houten, B., Gamper, H., Hearst, J. E., and Sancar, A. (1986) J. Biol. Chem. 261, 14135–14141
13. Yeung, A. T., Jones, B. K., Capraro, M., and Chu, C. T. (1987) Nucleic Acids Res. 15, 4957–4971
14. Jones, B. K., and Yeung, A. T. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 8410–8414
15. Jones, B. K., and Yeung, A. T. (1990) J. Biol. Chem. 265, 3489–3496
16. Sladec, F. M., Munn, M. M., Rupp, W. D., and Howard-Flanders, P. (1989) J. Biol. Chem. 264, 6755–6765
17. Cheng, S., Sancar, A., and Hearst, J. E. (1991) Nucleic Acids Res. 19, 657–663
18. Kaye, J., Smith, C. A., and Hanawalt, P. C. (1980) Cancer Res. 40, 5399–5404
19. Bredberg, A. (1982) Acta Dermato-Venerol. Suppl. 104, 1–40
20. Gruenert, D. C., and Cleaver, J. E. (1985) Cancer Res. 45, 5399–5404
21. Averbeck, D. (1989) Photochem. Photobiol. 50, 859–862
22. Lambert, M. W., Fenkart, D., and Clarke, M. (1988) Mutation Res. 193, 65–73
23. Parrish, D. D., and Lambert, M. W. (1990) Mutation Res. 235, 65–80
24. Reardon, J. T., Spielmann, P., Huang, J.-C., Sastry, S., Sancar, A., and Hearst, J. E. (1991) Nucleic Acids Res. 19, 4623–4629
25. Parrish, D. D., Lambert, W. C., and Lambert, M. W. (1992) Mutation Res. 273, 157–170
26. Svoboda, D. L., Taylor, J. S., Hearst, J. E., and Sancar, A. (1993) J. Biol. Chem. 268, 1931–1936
27. Lambert, M. W., and Parrish, D. (1989) DNA Repair Mechanisms and Their Biological Implications in Mammalian Cells (Lambert, M., and Laval, J., eds) pp. 295–324, Plenum Press, Inc., New York
28. Tsongalis, G. J., Lambert, W. C., and Lambert, M. W. (1990) Carcinogenesis 11, 499–503
29. Tsongalis, G. J., Lambert, W. C., and Lambert, M. W. (1990) Mutation Res. 244, 257–263
30. Okorodudu, A. O., Lambert, W. C., and Lambert, M. W. (1982) Biochem. Biophys. Res. Commun. 106, 576–584
31. Lambert, M. W., Lee, D. E., Okorodudu, A. O., and Lambert, W. C. (1982) Biochim Biophys. Acta 699, 192–203
32. Simpson, R. T., and Stafford, D. W. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 51–55
33. Maxam, A. M., and Gilbert, W. (1980) Methods Enzymol. 65, 499–560
34. Yeung, A. T., Dinehart, W. J., and Jones, B. K. (1988) Biochemistry 27, 6332–6338
35. Shim, S. C., and Kim, Y. Z. (1983) Photochem. Photobiol. 38, 265–271
36. Thompson, J. F., and Hearst, J. E. (1983) Cell 32, 1355–1365
37. Tonic, M. T., Wertman, D. E., and Kim, S. H. (1987) Science 238, 1722–1725
38. Huang, J.-C., Svoboda, D. L., Reardon, J. T., and Sancar, A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 3664–3668
39. Sibghat-Ullah, Sancar, A., and Hearst, J. E. (1990) Nucleic Acids Res. 18, 5051–5053
40. Yeung, A. T., Mattes, W. B., Oh, E. Y., and Grossman, L. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 6151–6153
41. Selby, C. P., and Sancar, A. (1988) Biochemistry 27, 7184–7188
42. Lambert, M. W., Tsongalis, G. J., Lambert, W. C. Hang, B., and Parrish, D. D. (1992) Mutation Res. 273, 57–71

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