

Processing *in vitro* of an abasic site reacted with methoxyamine: a new assay for the detection of abasic sites formed *in vivo*

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ABSTRACT

In this study we demonstrate that the different substrate recognition properties of bacterial and human AP endonucleases might be used to quantify and localize apurinic (AP) sites formed in DNA *in vivo*. By using a model oligonucleotide containing a single AP site modified with methoxyamine (MX), we show that endonuclease III and IV of *E. coli* are able to cleave the alkoxyamine-adducted site whereas a partially purified HeLa AP endonuclease and crude cell-free extracts from HeLa cells are inhibited by this modification. In addition MX-modified AP sites in a DNA template retain their ability to block DNA synthesis *in vitro*. Since MX can efficiently react with AP sites formed in mammalian cells *in vivo* we propose that the MX modified abasic sites thus formed can be quantitated and localized at the level of the individual gene by subsequent site specific cleavage by either *E. coli* endonuclease III or IV *in vitro*.

INTRODUCTION

Apurinic/aprimidinic (AP) sites are common DNA lesions that are produced both spontaneously and after exposure to a variety of DNA-damaging agents. The hydrolysis of the glycosyl bond between deoxyribose and purines or pyrimidines occurs at a significant rate at neutral pH and is accelerated by certain types of DNA damage, such as alkylation of the base. Furthermore, DNA glycosylases excise a variety of damaged DNA bases and form AP sites as intermediates in DNA repair (1). AP sites are 'non-informational' DNA lesions (2,3) with potential cytotoxic and mutagenic effects (4,5). Excision repair processes remove AP sites very efficiently *in vivo*. A single-strand break (ssb) is first introduced on the 5' side of a base-free deoxyribose-phosphate residue which is excised, most likely by a DNA deoxyribophosphodiesterase (6), to generate a single nucleotide gap. The replacement of the missing nucleotide by DNA

polymerase followed by chain joining by DNA ligase restores the integrity of the DNA. As a consequence of this efficient repair system, the AP site is a short-lived lesion *in vivo*.

Analysis of mutational spectra, both spontaneous as well as DNA damage induced, strongly implicates AP sites as common intermediates in mutagenesis. Many physical and chemical agents (i.e. ionizing radiation, bulky chemicals, alkylating agents) induce the same types of mutations as those induced by heat and acid-generated AP sites, namely GC-TA and AT-TA transversions (as in the case of depurination) and GC-AT transitions (as in the case of depyrimidination) (7). Prokaryotic polymerases are blocked at AP sites in template DNA *in vitro* and the SOS response is required for mutagenesis *in vivo* (8). In contrast, eukaryotic enzymes appear to be able to replicate past AP sites *via* preferential incorporation of dAMP (9,10,11).

It is of interest to develop assays to quantify and localize AP sites formed *in vivo* at the level of the gene and to correlate their occurrence with specific types of mutational events. Many attempts have been made in the past to assay AP site formation, but detection of AP sites is still limited to indirect assays, such as alkaline sucrose gradients and alkaline elution (12). These assays exploit the alkali lability of AP sites to quantitate them as DNA ssb produced at a given time after DNA damage. A direct assay of AP sites based on the specific reaction of methoxyamine (MX) with the aldehyde group of deoxyribose residues has been successfully applied to the quantitation of AP sites in DNA *in vitro* (13,14). We have shown that AP sites produced *in vivo* after alkylation damage react specifically with MX and become resistant to alkaline cleavage (15). AP sites are also a component of the mutagenic and cytotoxic response to alkylation damage in mammalian cells (P. Fortini et al., manuscript submitted).

In this study a synthetic oligonucleotide containing a single AP site is used as a model to explore the biochemical characteristics of the adduct formed upon reaction with MX. Using this model substrate in conjunction with purified bacterial or human AP endonucleases and with human cell-free extracts, we have

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demonstrated that the different substrate specificities of these AP endonucleases might be exploited to quantify and localize sites of base loss in DNA *in vivo*. A new assay for the direct detection of AP sites formed *in vivo* at the level of the gene is proposed.

MATERIALS AND METHODS

Enzymes

E. coli endonuclease IV and uracil-DNA glycosylase were purified by published procedures (16,17). *E. coli* endonuclease III was kindly supplied by Dr. C. Jones, ICRF, UK. HeLa AP endonuclease was a generous gift of Dr. A.M. Pedrini, Institute of Biochemical and Evolutionary Genetics, Pavia, Italy. T4 polynucleotide kinase was purchased from Bio-Rad and Sequenase from Pharmacia.

Chemicals

Methoxyamine hydrochloride was purchased from Sigma. Stock solutions were prepared in water and stored at -20°C .

Cell extracts

Cell extracts were prepared from human HeLa cells following the procedure of Li and Kelly (18). Briefly, spinner cultures of HeLa cells were grown in Eagle's minimal essential medium supplemented with 10% foetal calf serum. 1×10^{10} cells were harvested in mid-log phase by centrifugation at $1000 \times g$ for 5 min. The cell pellet was rapidly washed in 500 ml of ice-cold hypotonic buffer with 250mM sucrose followed by 500 ml of ice-cold hypotonic buffer alone (20mM Hepes-KOH, 5mM KCl, 1.5mM MgCl_2 , 0.5mM dithiothreitol, pH 7.5). The washed cell pellet was suspended in hypotonic buffer and the cells allowed to swell on ice for 30 min. Cells were then lysed with 10 strokes of a tightly fitting pestle in a Dounce homogenizer. After 30 min incubation on ice, the lysate was centrifuged for 20 min at $10,000 g$, at 0°C . The supernatant was collected and NaCl was added to a final concentration of 100mM. The extracts were centrifuged at $100,000 g$ for 1h, at 0°C . The supernatant was removed, aliquoted, quickly frozen in ethanol/dry ice and stored at -70°C . The protein concentration of the extracts is approximately $10 \mu\text{g}/\mu\text{l}$.

Preparation of oligonucleotide substrates

Oligonucleotides containing a single AP site. 30-mer oligonucleotides were prepared containing either cytosine or uracil at position 13 (5'-TACGGATCGCAG C/U TGGGTGTA-GGGAAGTTGG-3'). These oligonucleotides were 5' end-labeled as already described (19) and annealed to their complementary strands. The single AP site was produced in the duplex oligonucleotide by enzymatic removal of the uracil residue. 5' (^{32}P)-end-labeled oligonucleotide (270 fmoles) was incubated with 2×10^{-6} U (17) uracil-DNA glycosylase for 30 min at 37°C in 70mM Hepes buffer (pH 7.8), 10mM dithiothreitol, 1mM EDTA (final volume 20 μl).

Oligonucleotides containing a single MX-modified AP site. Modification of the AP site with MX was carried out essentially by the method of Talpaert-Borle' and Liuzzi (13). Oligonucleotides containing a single AP site were incubated with 30mM MX for 30 min at 37°C in the same buffer used for digestion with uracil-DNA glycosylase. The oligonucleotides were ethanol precipitated and resuspended in 10mM Tris-HCl (pH 7.5) and 1mM EDTA.

Oligonucleotide assays

Determination of AP-endonuclease activity. Endonuclease activity was monitored on denaturing polyacrylamide gels by the extent of formation of the 12-mer cleavage product from the oligonucleotides containing either the AP site or the MX-modified AP site. Incubation with *E. coli* endonuclease III was performed in 10mM Tris-HCl, (pH 7.5), 1mM EDTA, 0.1mM KCl, 1mM mercaptoethanol. The incubation buffer for *E. coli* endonuclease IV was 0.2M NaCl, 50mM Hepes-KOH, (pH 7.8), 1mM EDTA, 0.1mM dithiothreitol, 50 $\mu\text{g}/\text{ml}$ BSA. Both enzymatic digestions were carried out at 30°C for 30 min. HeLa AP endonuclease digestion was performed in 100mM Hepes-NaOH, (pH 8.25), 3mM MgCl_2 for 30 min at 37°C . The amounts of AP endonucleases used in this study were comparable by a factor of two. When cell extracts were used, HeLa cell extract (10 μg) was incubated with the labelled oligonucleotides for 1h at 37°C . When necessary, MX was added to the incubation mixture.

The endonuclease-treated samples were then deproteinized by treatment with proteinase K, phenol extracted and ethanol precipitated. The oligonucleotides were resuspended and resolved on 12% denaturing polyacrylamide gels (0.75 mm thick). Electrophoresis was carried out in TBE buffer at room temperature at 350 V for 1h. The gels were dried and DNA visualized by autoradiography.

Termination of DNA synthesis

Preparation of single-stranded template containing AP sites and MX-modified AP sites. Uracil containing single-stranded DNA was prepared according to published procedures (20) with some modifications. *E. Coli* CJ 236 *dut*⁻, *ung*⁻ cells were infected at a cell density corresponding to OD₆₀₀ of 0.3 with M13mp18 bacteriophage at a multiplicity of infection of <0.2 . After 5h, the supernatant was collected and digested with RNase (150 μg) for 30 min at 20°C and then precipitated with 1/4 volume of 3.5M ammonium acetate, 20% PEG 8000 for 30 min on ice. The pellet of phage particles was resuspended in 200 μl high salt buffer (300mM NaCl, 100mM Tris-HCl, pH 8.0, 1mM EDTA) and DNA was purified by several extractions with phenol, phenol/chloroform and chloroform/isoamyl alcohol. DNA was precipitated with 7.8M ammonium acetate and 2.5 volumes of ethanol. A 10 μg sample of uracil-containing M13 DNA was incubated with 10^{-5} U (17) of uracil-DNA glycosylase in a 20 μl reaction mixture containing 70mM Hepes-KOH (pH 8), 10mM dithiothreitol, 1mM EDTA. AP sites were modified with MX by 30 min incubation with 30mM MX at 37°C . The presence of AP sites after uracil-DNA glycosylase digestion as well as their modification with MX was monitored on alkaline gels. Alkaline gels were prepared by dissolving 1% agarose in 50mM NaCl, 4mM EDTA. The running buffer was 30mM NaOH, 2mM EDTA. DNA samples were resuspended before loading in 0.5M NaOH, 25% glycerol, 0.025% bromocresol green. The gels were run for 2h at 60 V after a pre-run of 30 min at 50 V.

DNA synthesis on AP and AP-MX DNA templates. DNA polymerase reactions on AP and AP-MX DNA templates were carried as described by Sagher and Strauss (10). Briefly, 1 μg DNA was annealed to a 17bp DNA primer (3ng). Primed M13 DNA was used to synthesize up to the lesion with 0.4U/ μl Sequenase in a buffer containing 20mM Hepes-KOH (pH 7.8), 5mM DTT, 10mM MgCl_2 , 12 μM ^{35}S dATP and the other three deoxynucleoside-5'-triphosphates (300 μM). After 15 min at 20°C , dATP (267 μM) was added to the reaction mixture to

complete DNA synthesis and the incubation was prolonged for a further 15 min at 20°C. The reaction was stopped by adding 95% formamide, 20mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol. Aliquots of the DNA samples were then run on a 8% denaturing polyacrylamide gel in TBE buffer. Electrophoresis was carried out at 2000 V for 2h.

RESULTS

Reaction of MX with AP sites produced *in vitro*

Compounds containing an -ONH₂ group such as MX are known to react with the aldehyde group on C'₁ of an AP site without degrading DNA (21). M13 bacteriophage was grown on an *ung*⁻ (uracil glycosylase deficient) *dut*⁻ (deoxyuridine triphosphatase deficient) *E. coli* mutant. As a consequence of the growth in this strain, the M13 genome contains uracil instead of thymine residues. Uracil-containing DNA was treated with uracil-DNA glycosylase to produce AP sites. This substrate is alkali-sensitive, since AP sites are labile at alkaline pH. As shown in Fig. 1, a smear of DNA molecules of different length is produced on alkaline agarose gels (Fig. 1, lane B). In contrast, the uracil-DNA glycosylase digested substrate treated with 30mM MX (lane C) migrates as the untreated, uracil-containing M13 DNA (lane A). The presence of some linear single-stranded DNA in the MX-reacted sample indicates that a minor percentage of AP sites did not react with MX before the alkaline treatment. Therefore, the addition of MX on the aldehyde group at an AP site *in vitro* inhibits the alkaline rupture of the adjacent phosphodiester bond.

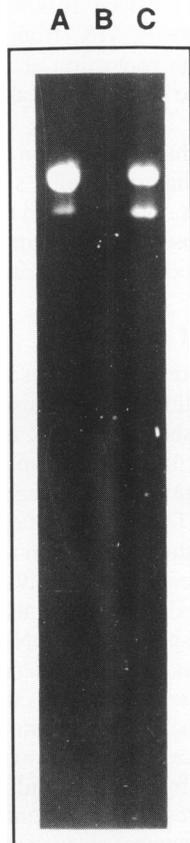


Fig. 1. Reaction of MX with AP sites produced *in vitro*. Alkaline gel electrophoresis of : (A) uracil-containing M13 DNA; (B) after incubation with uracil-DNA glycosylase (2×10^{-5} U) for 30 min at 37°C; (C) as in (B) followed by 30 min treatment with 30 mM MX, at 37°C. The form which migrates faster than the single-stranded DNA is single-stranded linear DNA.

Substrate recognition of oligonucleotides containing AP sites or MX-modified AP sites by bacterial AP-endonucleases

To further explore the structure of the abasic site modified by the formation of an alkoxyamine adduct at the C'₁ aldehyde in DNA, we utilized a synthetic DNA substrate containing a single AP site. The oligonucleotide containing the uracil residue at nucleotide position 13 was 5' end labeled and annealed to the complementary strand. AP sites were produced by incubation with uracil-DNA glycosylase, and were modified by exposure to MX. The extent of cleavage by different AP-endonucleases was monitored on denaturing polyacrylamide gels by the formation of the expected 12-mer cleavage product. As shown in Fig. 2, both *E. coli* endonuclease III and endonuclease IV are able to cleave the AP site containing oligonucleotide leading to the formation of a 12-mer cleavage product (lanes 3 and 5, respectively). Endonuclease III has both a DNA N-glycosylase activity and an AP lyase activity, i.e. cleaves 3' to the AP site, while endonuclease IV is a class II AP endonuclease and cleaves 5' to the AP site. The cleavage product of endonuclease III (lanes 3,4) migrates slightly slower than the cleavage product of endonuclease IV (lanes 5, 6), because the latter is shorter by a deoxyribose 5'-phosphate residue. The nicking of AP sites by these bacterial endonucleases is only slightly inhibited by the reaction of MX with the aldehyde group of the AP site (lanes 4 and 6). Therefore, the presence of the alkoxyamine adduct does not significantly affect either the β -elimination reaction catalysed by endonuclease III or the hydrolytic cleavage of the phosphodiester bond by endonuclease IV.

Substrate recognition of oligonucleotides containing an AP site or MX-modified AP site by HeLa AP-endonuclease

The substrate recognition by a partially purified HeLa AP-endonuclease was also tested. This enzyme, which has been

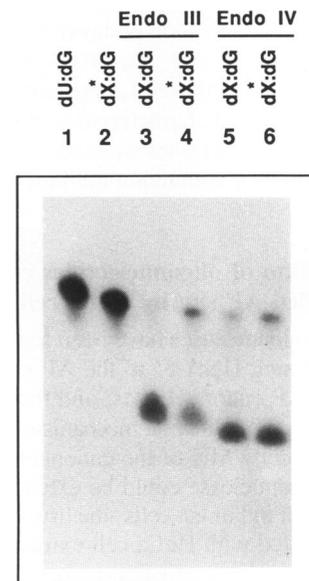


Fig. 2. Substrate recognition of oligonucleotides containing AP sites or MX-modified AP sites by bacterial AP-endonucleases. Lane 1: duplex dU:dG; lane 2: duplex dX*:dG (X* = AP site modified with MX); lane 3: duplex dX:dG (X = AP site) after 30 min incubation with endonuclease III of *E. coli*; lane 4: duplex dX*:dG after 30 min incubation with endonuclease III; lane 5: duplex dX:dG after 30 min incubation with endonuclease IV of *E. coli*; lane 6: duplex dX*:dG after 30 min incubation with endonuclease IV.

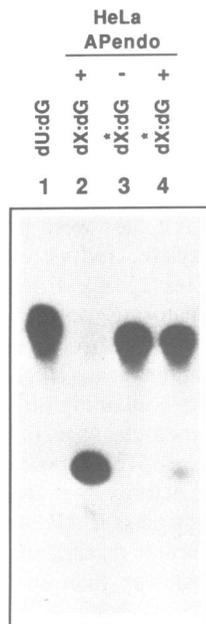


Fig. 3. Substrate recognition of oligonucleotides containing AP sites or MX-modified AP sites by HeLa AP-endonuclease. Lane 1: duplex dU:dG; lane 2: duplex dX:dG after 30 min incubation with partially purified HeLa AP-endonuclease; lane 3: duplex dX*:dG; lane 4: duplex dX*:dG after 30 min incubation with partially purified HeLa AP-endonuclease.

previously described and purified to apparent homogeneity (22), is Mg^{2+} -dependent and hydrolyzes DNA 5' to the AP site. As shown in Fig. 3, cleavage of the AP site by the HeLa AP-endonuclease produces the expected 12-mer oligonucleotide (lane 2), while almost complete inhibition of cleavage is observed when the AP site is modified with MX (lane 4). The MX-modified AP site containing oligonucleotide is shown in lane 3. Therefore, the reaction of MX with AP sites renders them no longer substrates for cleavage by the HeLa AP-endonuclease.

The substrate recognition characteristics of both bacterial and the mammalian AP endonucleases were also confirmed by using as substrate plasmid DNA containing multiple AP sites (data not shown).

Substrate recognition of oligonucleotides containing an AP site or MX-modified AP site by HeLa cell extracts

Two types of AP endonucleases have been found in mammalian cells: those hydrolyzing DNA 5' to the AP site, which are the major mammalian AP endonucleases, and those cleaving DNA 3' to the AP site via a β -elimination mechanism. In order to assess whether the inhibition by MX of the endonucleolytic activity of the 5' HeLa AP endonuclease could be extended to all the AP-endonucleases present in human cells, the uracil-containing DNA substrate was incubated with HeLa cell extracts in the presence of MX.

As shown in Fig. 4, the combined action of uracil-DNA glycosylase and AP endonuclease in extracts from HeLa cells cleaves the uracil containing substrate (lane 2). The cleavage product comigrates with the product of class II AP-endonucleases (data not shown) confirming that the major mammalian cellular AP endonucleases hydrolyze DNA 5' to the AP site. A dose-

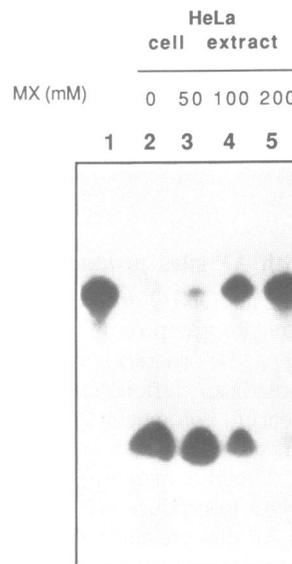


Fig. 4. Substrate recognition of oligonucleotides containing AP sites or MX-modified AP sites by HeLa cell extract. Lane 1: duplex dU:dG; lane 2: after digestion with 20 μ g of HeLa cell extracts; lane 3: after digestion with HeLa cell extracts in the presence of 50mM MX; lane 4: after digestion with HeLa cell extracts in the presence of 100mM MX; lane 5: after digestion with HeLa cell extracts in the presence of 200mM MX.

dependent inhibition of cleavage is observed in the presence of increasing concentrations of MX (from 50 to 200 mM) (lanes 3 to 5) with a complete protection at 200 mM MX (lane 5). Therefore, MX is able to block specifically the cleavage by HeLa AP endonucleases.

Indirect evidence of this phenomenon was previously obtained by treating Chinese hamster cells simultaneously with an alkylating agent and MX. A decrease in the number of detectable alkali-labile sites was observed suggesting an interaction between MX and the AP sites produced by alkylation damage *in vivo* (15).

Assay for detection of AP sites

We have previously shown that MX is able to modify AP sites formed *in vivo* (P. Fortini et al., manuscript submitted). In this study we have further shown that MX modification of AP sites inhibits their cleavage by mammalian AP endonucleases but leaves them still susceptible to cleavage by both endonuclease III and IV of *E. coli*. These observations could potentially be useful for detection of the introduction and removal of AP sites formed *in vivo* at the level of the individual gene. Mammalian cells treated with DNA damaging agents in the presence of MX would accumulate AP sites in their DNA. This modified DNA could then be isolated and AP sites detected by specific cleavage with *E. coli* endonuclease III or IV. In order to test the feasibility of this assay, the oligonucleotide containing a single uracil residue was incubated with HeLa cell extracts in the presence of 200mM MX (Fig. 5). The cell-free extracts did not significantly cleave the MX-reacted AP sites (lane 3), confirming the inability of mammalian AP endonucleases to recognize this modified substrate. The sample was then subjected to proteinase K digestion and ethanol precipitation. The purified DNA was incubated with *E. coli* endonuclease III (lane 4) or endonuclease

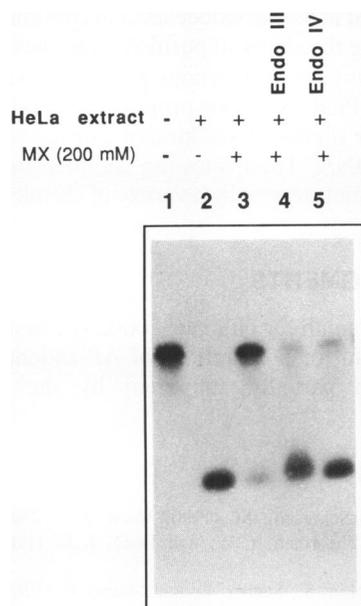


Fig. 5. Model assay for detection of AP sites produced *in vivo*. Lane 1: duplex dU:dG; lane 2: after digestion with 20 μ g of HeLa cell extracts; lane 3: after digestion with HeLa cell extracts in the presence of 200mM MX; lane 4: as in lane 3, followed by 30 min incubation with endonuclease III of *E. coli*; lane 5: as in lane 3, followed by 30 min incubation with endonuclease IV of *E. coli*.

IV (lane 5) and the digestion products were detected on denaturing gels. Both *E. coli* enzymes generated a fragment of the size expected from cleavage at the AP site. Thus, inhibition of mammalian AP endonucleases by MX modification is indeed highly efficient and likely to be sufficient to allow AP sites to accumulate in mammalian cells in the presence of MX. Furthermore, the reaction product of MX and AP sites is sufficiently stable to allow rescue of the modified DNA and its analysis by cleavage *in vitro* by endonuclease III or IV.

Termination of DNA synthesis on templates containing AP sites or MX-modified AP sites

AP sites block DNA replication by a number of different DNA polymerases *in vitro* (10). We were interested in determining whether the pattern of chain termination observed with single-stranded DNA templates containing MX-reacted AP sites was similar to that of AP site-containing DNA. DNA from bacteriophage M13 grown on *E. coli* CJ 236 *dut*⁻*ung*⁻ and containing AP sites introduced by *in vitro* removal of uracil residues, was modified by treatment with 30mM MX and used as a template for DNA synthesis by Sequenase. The products of synthesis were analysed on sequencing gels. Figure 6 confirms that the majority of the sites of arrested DNA synthesis are opposite adenine residues when the template DNA contains AP sites (lane 6) (10). A similar pattern of termination of synthesis was obtained with MX-modified AP DNA (lane 7). When control DNA (lane 5) was used as a template for Sequenase, a few minor termination bands were observed but they were rarely located at the positions seen with the AP DNA. It appears therefore that AP sites and AP sites modified with MX are equivalent blocks to DNA synthesis.

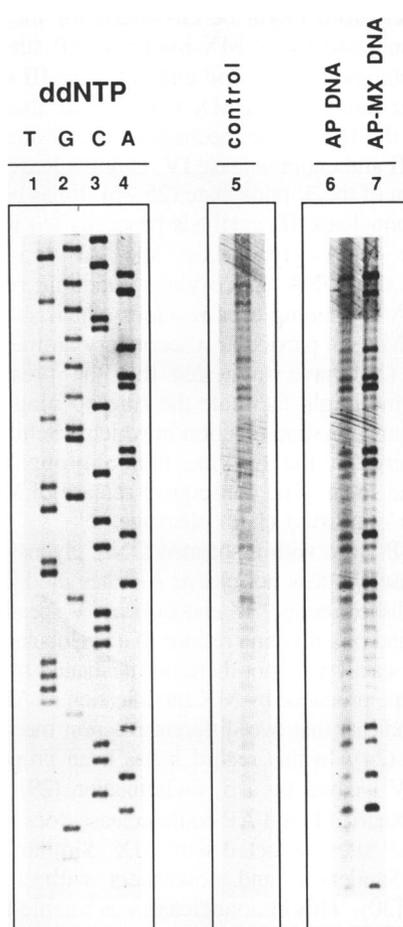


Fig. 6. Polyacrylamide gel pattern of termination of DNA synthesis on an AP-DNA and AP-MX DNA template by Sequenase. Synthesis occurred on AP (lane 6), AP-MX (lane 7) or control uracil-containing (lane 5) DNA templates (0.05 pmol) catalyzed by sequenase (3 units). ddNTP channels (lane 1 to 4) are sequence standards synthesized with sequenase and dideoxynucleotides on control DNA template.

DISCUSSION

In this paper we propose an assay for AP site determination based on the different substrate specificities of mammalian and bacterial AP endonucleases on DNA templates containing AP sites modified with MX.

We have shown that *E. coli* endonucleases III and IV are highly tolerant to the alkoxyamine modification of the C'₁ aldehyde, while the major HeLa AP endonuclease(s) do not cleave this substrate. Our use of a model substrate containing a single AP site modified with MX has provided additional information on the enzymatic properties of *E. coli* endonucleases III and IV and the major AP endonuclease of HeLa cells. The major *E. coli* AP endonuclease, exonuclease III has been shown to cleave AP sites converted to O-alkylhydroxylamine residues (23). Our data indicate that this property is shared by the minor activity endonuclease IV. Both enzymes are Type II AP endonucleases which cleave 5' to the AP site and they also share a number of accessory activities (24). Kow and Wallace (26) hypothesized that the presence of MX facilitates the ring opening of the sugar which creates space for binding and nicking by exonuclease III.

A similar mechanism might be envisaged for the cleavage by *E. coli* endonuclease IV of MX-modified AP sites.

Our data obtained with *E. coli* endonuclease III show that the phosphodiester bond 3' to a MX residue can also be cleaved. In contrast to the hydrolytic mechanism of AP site cleavage of exonuclease III and endonuclease IV, endonuclease III catalyses a β -elimination of the 3' phosphate (25,26). It has been proposed (26) that endonuclease III catalysis proceeds *via* the sequential action of its DNA glycosylase and AP lyase activities. Interestingly, the DNA glycosylase function recognizes as substrate DNA containing urea residues which share structural features with MX, in particular a secondary amine group. Kow and Wallace (26) have suggested that the presence of this secondary amine might facilitate the ring opening of the sugar leading to a transimination reaction in which a Schiff base would be formed between the enzyme amino group and the C', aldehyde of the sugar. The consequent release of MX would be followed by a concerted chain cleavage.

A second AP lyase with associated DNA glycosylase activity, T4 endonuclease V, does not cleave AP sites modified with MX (27; our unpublished results). T4 endonuclease V specifically cleaves the glycosyl bond of a thymine residue in a cyclobutane pyrimidine dimer (28). Its catalysis is not therefore facilitated by a secondary amine of the type produced by MX modification of AP sites. These observations indicate that two different reaction mechanisms exist for AP lyases (24). In this regard it has been proposed that T4 endonuclease V cleaves by a β , δ -elimination (29).

A partially purified HeLa AP endonuclease does not recognize as substrate AP sites adducted with MX. Similar results were obtained by Sanderson and coworkers with a bovine AP endonuclease (30). This endonuclease was unable to cleave the abasic site when a urea residue or an alkoxyamine group was attached to the C', position of the abasic site. Moreover, inhibition of cleavage at AP sites by MX is also observed with HeLa cell extracts suggesting that other forms of AP endonuclease in HeLa cells are either present in very low levels or are also inhibited by MX modification of AP sites.

The interest in quantitation of DNA damage at gene level has been heightened by the accumulation of evidence of heterogeneous distribution and repair of DNA damage in mammalian genome (31). Quantitation of pyrimidine dimers in defined sequences has been achieved using T4 endonuclease V (32). Data from these assays have led to the suggestion that UV photodimers are repaired preferentially in active genes in both bacterial and mammalian cells (33,34). Attempts have been made to develop a quantitative method to assay the introduction of AP sites into DNA. These lesions have been detected in specific sequences by their conversion to alkali-labile sites (35) and by a ³²P end-labelling assay (36). The first method has been proposed as a general tool to quantify abasic sites at the gene level, but the detection limit of this assay is defined by the rate of resealing of the AP sites *in vivo*, which is a fast process. We propose the use of MX to convert AP sites formed *in vivo* into lesions which are resistant to cleavage by the endogenous mammalian AP endonuclease(s). The abasic sites reacted with MX can then be quantitated in Southern blots of specific restriction fragments of isolated DNA following specific cleavage with *E. coli* endonuclease III or IV. This approach should allow the quantitative detection of AP sites formed within cells *in vivo*.

In addition, our data indicate that MX-modified AP sites are effective blocks to DNA replication *in vitro* and are equivalent to unmodified AP sites in this regard. Such modified AP sites

might be localized at the nucleotide level in episomally maintained DNA by assaying the ability of purified molecules to block DNA synthesis *in vitro* or in endogenous genes by a variation of the ligase-mediated PCR (37). Our proposed methods should be of general value for studies of any kind of damage which produces abasic sites in DNA. These sites are common intermediates in cytotoxicity and mutagenesis by a variety of chemical and physical carcinogens.

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