

# The lyase activity of bifunctional DNA glycosylases and the 3'-diesterase activity of APE1 contribute to the repair of oxidized bases in nucleosomes

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## ABSTRACT

The vast majority of oxidized bases that form in DNA are subject to base excision repair (BER). The DNA intermediates generated during successive steps in BER may prove mutagenic or lethal, making it critical that they be 'handed' from one BER enzyme to the next in a coordinated fashion. Here, we report that the handoff of BER intermediates that occurs during the repair of naked DNA substrates differs significantly from that in nucleosomes. During BER of oxidized bases in naked DNA, products generated by the DNA glycosylase NTHL1 were efficiently processed by the downstream enzyme, AP-endonuclease (APE1). In nucleosomes, however, NTHL1-generated products accumulated to significant levels and persisted for some time. During BER of naked DNA substrates, APE1 completely bypasses the inefficient lyase activity of NTHL1. In nucleosomes, the NTHL1-associated lyase contributes to BER, even in the presence of APE1. Moreover, in nucleosomes but not in naked DNA, APE1 was able to process NTHL1 lyase-generated substrates just as efficiently as it processed abasic sites. Thus, the lyase activity of hNTHL1, and the 3' diesterase activity of APE1, which had been seen as relatively dispensable, may have been preserved during evolution to enhance BER in chromatin.

## INTRODUCTION

Reactive oxygen species, produced endogenously during normal oxidative metabolism, generate some 30 000 oxidative lesions in the DNA of every human cell every day (1,2). These lesions include oxidized bases that either mis-pair during replication, making them mutagenic, or block elongation by replicative DNA polymerases, making them cytotoxic. The base excision repair (BER) pathway is the principle enzymatic pathway used by cells to remove and replace

oxidatively damaged DNA bases (3,4), and certain mutations in BER enzymes have been linked to an increased risk of cancer (5–7). Base excision repair requires the stepwise action of multiple enzymes. It is critical these enzymes act in a coordinated fashion, because the DNA intermediates produced during repair are just as deleterious as the initial lesion, and sometimes more-so.

The DNA glycosylases that discover and excise damaged bases share certain properties that are thought to help coordinate BER. Specifically, these glycosylases bind tightly to their DNA products and, as a result, exhibit biphasic kinetics when assayed in substrate excess reactions *in vitro*. The addition of AP endonuclease 1 (APE1), which catalyzes the second step in BER, increases glycosylase turnover, most likely by eliminating product inhibition and stimulating product release. These observations have led others to hypothesize that successive steps in BER are coordinated through a 'passing of the baton', or 'substrate channeling' mechanism in which enzymes in the pathway bind their products until they are displaced by the next enzyme in the BER pathway (8–11). Such a mechanism would not only limit the accumulation of cytotoxic repair intermediates but also help sequester the intermediates once they form.

In eukaryotes, BER occurs in a chromatin milieu. The fundamental unit of chromatin is the nucleosome which consists of 147 base pairs of DNA wrapped ~1.7 times around a histone octamer, which contains two each of four histone proteins, H2A, H2B, H3 and H4 (12). Nucleosomes restrict or interfere with the binding of enzymes and regulatory factors to DNA and are integral to maintenance of gene silencing. This may be reversed by region or gene-specific recruitment of chromatin modifiers and remodelers. These agents not only facilitate transcription but also DNA replication, nucleotide excision repair and the repair of double-strand DNA breaks. Chromatin modifiers and remodelers may also facilitate BER in eukaryotes but this has not yet been conclusively demonstrated (13–15). Our lab has shown that each step in short patch BER of oxidized bases in nucleosomes can be recapitulated *in vitro*, without the aid of chromatin remodelers, and without irreversibly moving or disrupting the host nucleosome (16).

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However, we and others have also demonstrated that the efficiency of repair of lesions in nucleosomes varies considerably with their position relative to the underlying histone octamer. Base lesions on the surface (outward facing) of the nucleosome are processed fairly readily by glycosylases and AP-endonucleases. Base lesions situated such that the incoming glycosylase/AP endonuclease would clash with the histone octamer (inward facing) are also processed, but at a reduced efficiency. This is possible because BER enzymes are able to exploit intrinsic, dynamic properties of nucleosomes, notably the periodic, spontaneous partial unwrapping of DNA from the histone octamer (17–21).

In this study, we set out to investigate how the properties of the human DNA glycosylase NTHL1 (hNTHL1) and APE1 that help coordinate repair of lesions in naked DNA may also help coordinate BER in nucleosomes. We found that like naked DNA, NTHL1 is slow to release its product in nucleosomes. We also confirmed earlier reports that, in reactions with naked DNA the lyase reaction catalyzed by NTHL1, is completely bypassed when APE1 is present (22). Surprisingly however, APE1 failed to completely bypass the lyase activity of hNTHL1 during the repair of nucleosomes. Moreover, in nucleosomes the product of the NTHL1 lyase activity proved to be as robust a substrate for APE1 as is an abasic site. This is in stark contrast to naked DNA, where the NTHL1 lyase product is a poor substrate, and indicates that the lyase activity of hNTHL1 is not detrimental to subsequent steps in BER. These observations support the hypothesis that, by nicking the DNA backbone, hNTHL1 increases the steric flexibility of DNA in nucleosomes, thereby facilitating the subsequent steps in BER. The lyase activity of the glycosylase and the 3' diesterase activities of APE1 are slow on naked DNA and were previously thought to be relatively dispensable, but may have been retained during evolution, to allow for the introduction of a single-stranded break early in BER that may be critical for efficient BER in some sterically challenging nucleosome contexts.

## MATERIALS AND METHODS

### Proteins

Human NTHL1, human APE1 and *Xenopus laevis* core histones were expressed in *Escherichia coli* and purified as described (23–25). Final hNTHL1 concentrations indicated in figures refer to the active fraction, as determined by a Schiff-base trap assay (26).

### DNA and nucleosome substrates

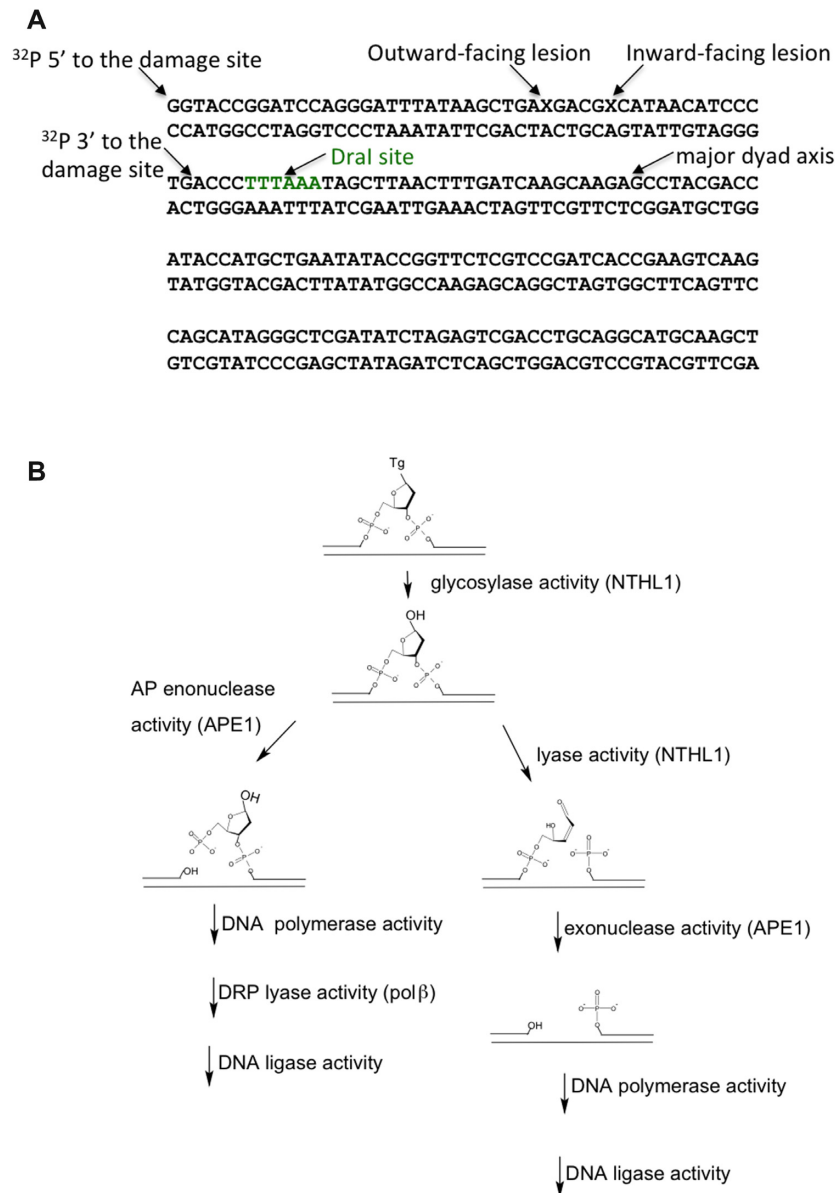
Both nucleosomal and naked DNA substrates consisted of a 184 bp blunt-ended DNA fragment containing the 5S rDNA nucleosome positioning sequence from *L. variegatus* (16) (Figure 1A). Each DNA molecule contained a single Thymine glycol (Tg), tetrahydrofuran (F) or Polyunsaturated aldehyde (PUA) residue, positioned so that its minor groove, when packaged in a nucleosome, would face either toward (inward-facing) or away (outward facing) from the histone octamer. The 5S rDNA assembles into nucleosomes in a single dominant translational position relative to the histone octamer with two other minor variations which differ from the dominant nucleosome by a single helical turn

(10 bp) in either direction. Thus in all cases the lesions are rotationally identically with respect to the face of the histone octamer (inward or outward-facing) but may vary in their distance from the dyad axis by 10 bp increments (16). DNA substrates used in Figures 2–5 and 7 were constructed and purified as in (17) (23). Briefly a 48 bp oligonucleotide containing a single lesion was 5' end labeled with <sup>32</sup>P, and then annealed to a synthetic 184 bp complement (IDT). VENT DNA polymerase (NEB) was used to extend from the <sup>32</sup>P-labeled oligonucleotide, thereby generating a 184 bp blunt-ended DNA fragment. PUA-containing substrates were generated by incubating 7.5 nM of the Tg-containing DNA substrate with 20 nM NTHL1, for 20 min in 50 mM HEPES (pH 8) and 100 mM NaCl. NTHL1 was removed by phenol-chloroform extraction, and the DNA was ethanol precipitated, resuspended and was immediately reconstituted into nucleosomes. DNA substrates used in Figure 6 were generated using the same Tg containing 48 bp oligonucleotide as above. A second 136bp oligonucleotide, identical to the downstream sequence, was 5' end labeled with <sup>32</sup>P. Both were annealed to a 184 NT complement and ligated, using HIFI Taq ligase (NEB) at 40°C. Full-length DNA products were purified using 8% polyacrylamide denaturing gels, then heated 95°C and annealed by slow cooling to room temp. To assemble nucleosomes, these dsDNAs were combined in a 1:19 molar ratio with nucleosome length unlabeled carrier DNAs generated from chicken chromatin digested with micrococcal nuclease (16). DNA was then combined with histone octamers previously reconstituted from recombinant *X. laevis* histones and purified by gel filtration (25,27). This mixture was then subjected to slow salt reduction dialysis, as in (14). Nucleosomes were fractionated using 5% non-denaturing polyacrylamide gels, visualized and reconstitution efficiencies quantified, as in (16). Substrates with reconstitution efficiencies ≥ 95% were used in reactions (Supplementary Figure S1).

### Glycosylase/ AP endonuclease assays

Nucleosomes or naked DNA substrates (constructed as outlined above) were assayed in reaction buffer (final concentration = 25 mM HEPES (pH 8.0), 100 mM NaCl, 5 mM MgCl<sub>2</sub>, 1.2 mM EDTA, and 0.05 mg/ml BSA), at 37°C. Reactions were initiated by the addition of hNTHL1 and/or APE1 (enzymes were premixed on ice). Aliquots removed at varying times were quenched in 150 mM NaOH and heated to 95°C for 2 min, to cleave DNA at hNTHL1-generated abasic sites. Samples were then mixed with two volumes of FE (80% formamide with 20mM EDTA), heated again to 95°C for 2 min, and fractionated using 15% denaturing polyacrylamide gels. In reactions with PUA (polyunsaturated aldehyde)-containing substrates, the addition of NaOH was omitted and aliquots were quenched directly in FE. Following electrophoresis, substrates and products were visualized and quantified using phosphorimetry. Results from 3 independent experiments were graphed as a function of time with error bars indicating standard deviation.

To distinguish between the AP-lyase activity of NTHL1 from the AP endonuclease activity of APE1, DNA substrates were <sup>32</sup>P-labeled at a site 3' to the damage site and



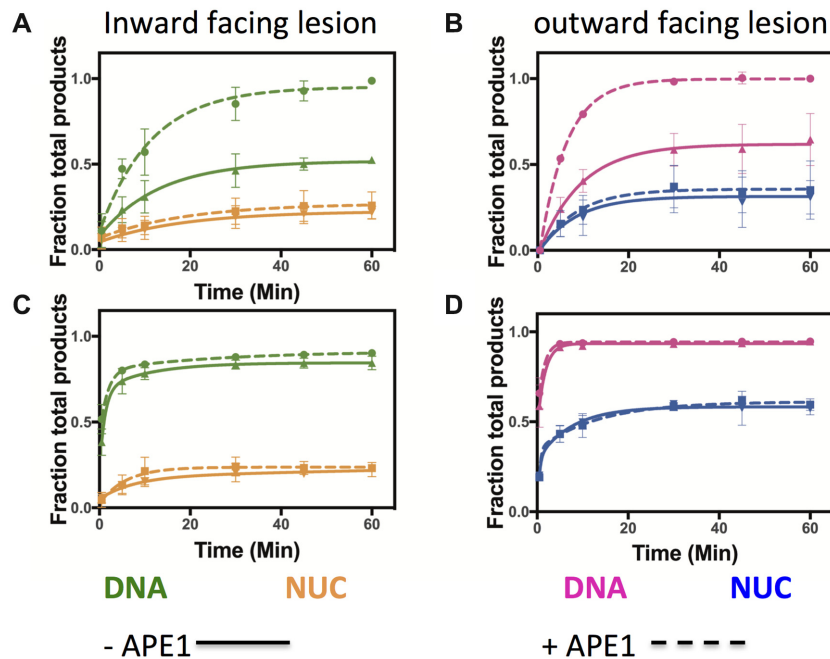
**Figure 1.** (A) The 5S rDNA sequence from *L. variegatus* used to generate nucleosomes with discretely positioned lesions. (B) Enzymatic steps during base excision repair (BER).

reconstituted into nucleosomes as described above (see also Figure 1). Enzyme reactions were conducted exactly as described above except that, after 5 min of reaction, 3  $\mu$ l of the reaction mixture was added to 10  $\mu$ l of 200 mM NaBH<sub>4</sub>. Incubating the sample for 20 min in the presence of NaBH<sub>4</sub> helped stabilize the sugar residue on the 5' end of the APE1 product (28). The DNA was then purified by extraction with 1 volume of tris-buffered phenol and 1 volume of chloroform, and ethanol precipitated. The DNA was suspended in a DRA1 restriction digestion buffer (1X cut smart buffer, 20 U DRA1 (NEB)), incubated with DRA1 for 30 min at 37°C, and quenched with 2 volumes of FE, as before. DNA was fractionated using 20% denaturing polyacrylamide gels, and visualized using phosphor-imagery.

## RESULTS

### APE1 stimulates hNTHL1 activity on naked DNA but not on nucleosomes

On naked DNA substrates, APE1 stimulates NTHL1 product production under multiple-turnover conditions by allowing it to bypass the slow lyase reaction (APE1 processes the AP-site directly) and by eliminating the product inhibition provided by the AP-site (Figure 1B) (22). Stimulation of a glycosylase in the presence of an AP endonuclease is quite common and is thought to constitute a coordination mechanism for BER (29–33). This property is thought to allow for control of glycosylase excision in that it cannot make AP sites faster than an AP endonuclease can process them



**Figure 2.** APE1 stimulates the hNTHL1-mediated excision of oxidized bases from naked DNA but not from nucleosomes. Nucleosomes containing inward (yellow)- or outward (blue)-facing Tg residues, and their respective naked DNA controls (inward green, outward pink), were incubated with hNTHL1, in the presence or absence of APE1. Reactions contained 2.25 nM Tg substrate and 0.2 nM (A and B) or 2 nM hNTHL1 (C and D). Reactions that also contained 0.2 nM APE1 are indicated with a dotted line trace and control reaction with no APE1 are indicated with solid traces. Aliquots taken at the times indicated were processed, and reaction products visualized and quantified, as described in the Methods. Error bars represent standard deviation of three independent experiments.

(8). No direct protein-protein interactions between NTHL1 and APE1 have been detected which is common amongst glycosylases, however APE1 may be able to reduce NTHL1 affinity for its product and thus ‘displace’ it from the AP-site through steric distortion of the DNA (34,35).

To determine if these same properties that help coordinate BER in naked DNA do so in nucleosomes we conducted BER reactions with either 2 nM or 0.2 nM NTHL1 on either naked or nucleosomal Tg-containing DNA (2.25 nM) in the presence or absence of APE1 (0.2 nM). In the case of the nucleosomes the Tg lesion was either inward (Figure 2A and C) or outward (Figure 2B and D) facing with respect to the histones octamer. When the concentration of NTHL1 is far below the substrate concentration (multiple turnover conditions), we observe that product production is enhanced for naked DNA in the presence of APE1 but is unchanged on nucleosomes (Figure 2A and B). When the substrate concentration is closer to the concentration of NTHL1, the extent of product production is quite similar for both nucleosomal and naked DNA in the presence or absence of APE1 (Figure 2C and D).

These findings for naked DNA are consistent with previous observations (22). The failure of APE1 to detectably stimulate product production in nucleosomes under multiple turnover conditions suggests that in nucleosomes the rate-limiting step for NTHL1 is not product release (as with naked DNA) but rather lesion discovery and binding (18). On nucleosomes steps later in the mechanism (product release, lyase-bypass) may be stimulated in the presence of APE1 however this stimulation will be masked in these as-

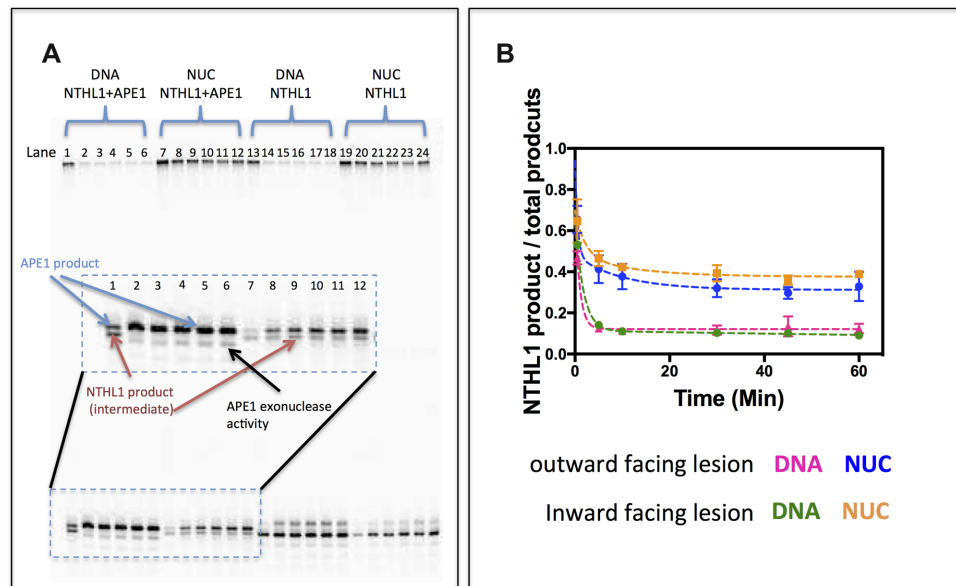
says by the overall rate-limiting step earlier in the mechanism (substrate discovery and binding).

#### **BER intermediates produced by hNTHL1 accumulate and persist during repair in nucleosomes but not during repair in naked DNA**

Any stimulation of the latter steps in the mechanism of hNTHL1 (dissociation or relief of product inhibition) provided by APE1 on the nucleosomes to coordinate BER may have been masked by the overall rate-limiting step for base excision on nucleosomes (discovery and binding to Tg). Therefore to investigate the possible coordination of BER on nucleosomes we must examine directly the contents of the reaction at any given time and compare DNA intermediates generated during the repair of naked or nucleosomal substrates. As depicted in Panel B of Figure 1, the DNA fragments one might see in reactions containing both hNTHL1 and APE1 are: (i) Tg-containing, substrate DNA, (ii) AP-site-containing DNA (produced by the hNTHL1 glycosylase, and a substrate for both the hNTHL1 lyase and the AP endonuclease activity of APE1), (iii) polyunsaturated aldehyde-containing DNA (PUA, the NTHL1 lyase product and a substrate for the 3’ diesterase activity of APE1) and (iv) a DNA fragment containing a 3’OH (the product of the APE1 endonuclease and diesterase activities). Any DNA containing an AP-site or a PUA would constitute an ‘intermediate,’ signifying that repair had been initiated by NTHL1 but not further processed by APE1.

To determine if DNA repair intermediates accumulate during BER in nucleosomes, we conducted reactions sim-





**Figure 3.** DNA intermediates generated during the hNTHL1-initiated repair of Tg-containing nucleosomes accumulate to a greater extent than in reactions with naked DNA substrates. (A) A representative gel showing the various products produced in reactions containing 2.25 nM Tg substrate, in the naked or nucleosomal forms and 2 nM hNTHL1, in the presence and absence of 0.2 nM APE1. (B) Graph showing the abundance of intermediates as a fraction of total product, as a function of time for reactions containing 2.25 nM Tg substrate, in the naked or nucleosomal forms with 2 nM hNTHL1 and 0.2 nM APE1. Error bars represent standard deviation of three independent experiments.

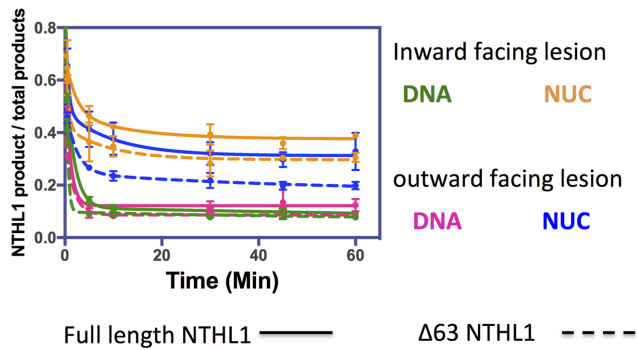
ilar to those described in Figure 2 with 2.0 nM NTHL1  $\pm$  0.2 nM APE1 on substrates containing 2.25 nM Tg in either a naked DNA or inward or outward facing nucleosomal context. Aliquots from the reactions were taken at varying time points, and quenched in base. The treatment with base converts hNTHL1-generated AP-sites and polyunsaturated aldehydes to single-stranded breaks, producing a DNA fragment with a 3'-phosphate. The AP endonuclease and 3' diesterase activities of APE1 produce a DNA fragment with a 3'-OH. Figure 3A demonstrates that these DNA's can be separated from one another by electrophoresis, even though they differ only slightly in charge and mass. Figure 3A shows more rapid and extensive processing of lesions by hNTHL1 in naked DNA reactions as compared to nucleosome reactions which is entirely consistent with previous studies from our lab (16). Close inspection of the naked DNA reaction products produced by hNTHL1 in the presence of APE1, reveals the presence of an NTHL1 product at the 30 sec timepoint (lane 1, Figure 3A). However, this does not persist, as it is rapidly processed by APE1. The same hNTHL1 product is also evident early in the nucleosome reactions (lane 7, Figure 3A) but, unlike that seen with the naked DNA, much of it persists for the entire reaction time course. APE1 products do accumulate, but much more slowly than in the naked DNA reactions. Figure 3B shows a quantitation of intermediates (NTHL1 products) as a fraction of total product(s) for reactions containing naked DNA or nucleosomes. Clearly, more of the hNTHL1 intermediate accumulates during processing of lesions in nucleosomes than in DNA indicating that there is considerable initiation of repair by the glycosylase that is not further processed by APE1. Thus, the mechanism that is thought to coordinate production of APE1 substrates with

the capacity of APE1 to process those substrates may operate less efficiently in nucleosomes than in naked DNA

Interestingly Figure 3B shows that inward and outward facing lesion nucleosomes have nearly equivalent fractions of intermediates present. This may indicate that the lack of APE1 processing may not be due to the steric availability of the lesion alone. If these intermediate-containing nucleosomes were free in solution we would expect that APE1 would be able to preferentially process the outward-facing lesions relative to the inward-facing lesions (36). This observation led us to hypothesize that the intermediates present in the nucleosome reactions that resist APE1 processing may be sequestered at the NTHL1 active site.

#### Repair intermediates in nucleosomes are sequestered by NTHL1

The packaging of DNA into nucleosomes alters its structure and restricts its conformational flexibility. These alterations may affect the relative binding of NTHL1 and APE1 to AP sites in nucleosomes, as compared to those in naked DNA. Differences in relative binding affinity could in turn account for the results in Figure 3. This may be because NTHL1 binds better to its product in nucleosomes when compared to naked DNA, or that APE1 is severely inhibited by the nucleosomal structure and is less adept at 'displacing' NTHL1 in nucleosomes. To address these two potential factors that may contribute to the persistence of the intermediates in the nucleosome reactions, we conducted assays like those in presented in Figure 2 however we used a N-terminal truncation of the NTHL1 protein. This N-terminal truncation is known to dissociate from its product faster when compared to WT. We also conducted experiments like those in Figure 2 with increasing amounts of APE1 (see next section)



**Figure 4.** More efficient processing of DNA intermediates by APE1 during the repair of Tg-containing nucleosomes in reactions containing a  $\Delta 63$  truncation mutant of hNTHL1 instead of full-length hNTHL1. Graph showing the abundance of intermediates as a fraction of total product, as a function of time, in reactions containing 2.25 nM Tg substrate, 0.2 nM APE1 and 2 nM  $\Delta 63$  hNTHL1 N-terminal truncation (dotted line traces). For ease of comparison, the same graph shows data from Figure 3B for reactions with full length NTHL1 (solid line traces). Error bars represent standard deviation of three independent experiments.

to determine if APE1 at higher concentrations can ‘keep up’ with NTHL1 to avoid the build-up of intermediates. If the failure of APE1 to efficiently process NTHL1-generated intermediates in nucleosomes is due to its inability to displace NTHL1 from its product, deletion of the NTHL1 N-terminus ought to increase the efficiency of APE1 processing.

Figure 4 shows data from reactions using the NTHL1- $\Delta 63$  mutant (Figure 4, dotted lines). For ease of comparison, the same graph shows data from Figure 3B for full length NTHL1 (Figure 4, solid lines). In reactions with naked DNA substrates, intermediates generated by NTHL1- $\Delta 63$  were quickly processed, as before. Once again, more of the NTHL1- $\Delta 63$  product accumulated and persisted in reactions with the nucleosome substrates. However, APE1 processed more of the product generated by NTHL1- $\Delta 63$  than by full length NTHL1. In reactions containing  $\Delta 63$ -NTHL1, outward facing lesion reactions have less intermediates present when compared to the inward facing lesion whereas in WT reactions, inward and outward facing lesions produced more similar levels of intermediates. This may be an indication that in  $\Delta 63$ -NTHL1 reactions, NTHL1 has more rapidly dissociated from its product and more of the intermediate-containing nucleosomes are free in solution. Therefore access by APE1 is governed more by lesion position in the nucleosome (inward versus outward) rather than the sequestration by NTHL1 when compared to WT NTHL1. These data support our inference that NTHL1 sequesters the products it generates during repair of lesions in nucleosomes.

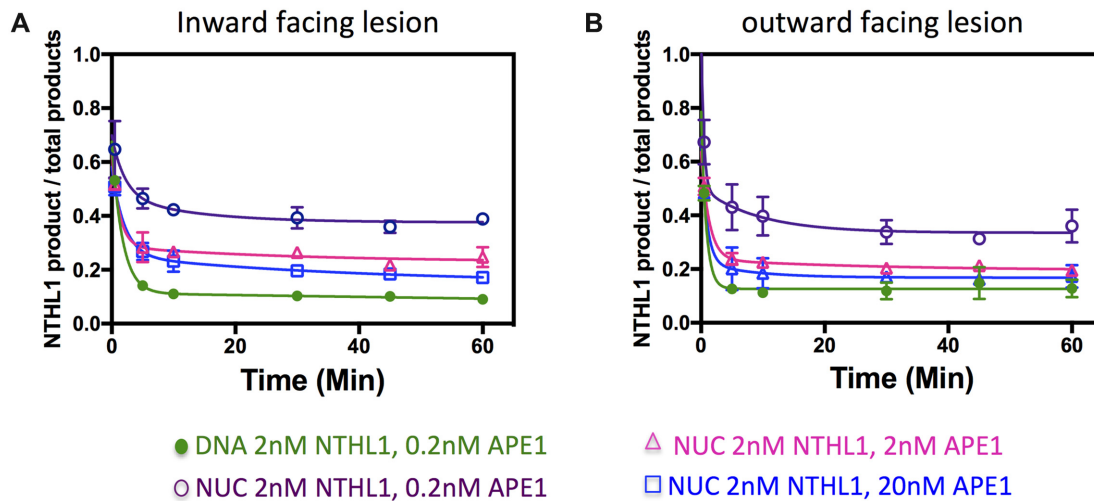
#### APE1 is severely inhibited by the structural constraints of the nucleosome

A second factor that could contribute to repair intermediates accumulating and persisting in the nucleosome reaction under the same condition in which they were quickly processed in naked DNA, is the inhibition of APE1 by the steric effects of the nucleosome structure. In general all DNA pro-

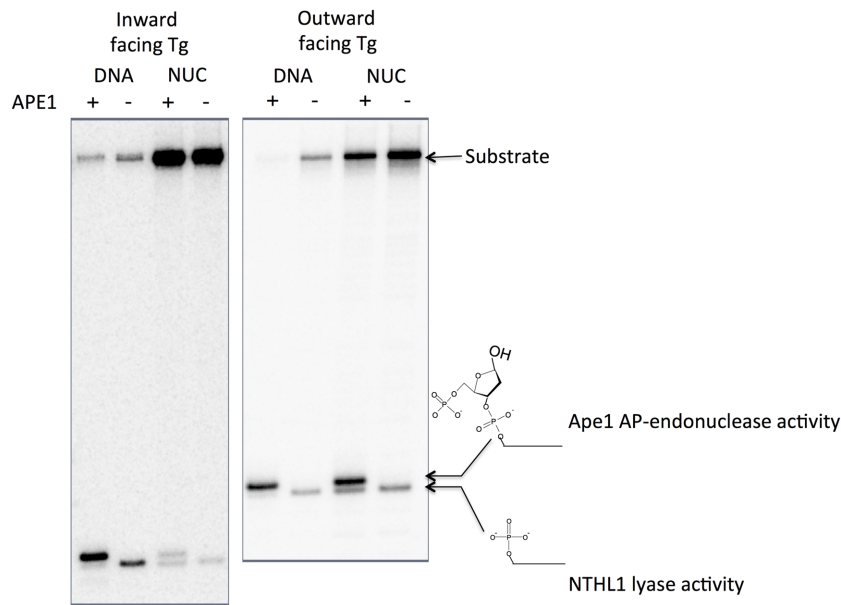
cessing enzymes are somewhat inhibited by the steric effects of the nucleosome and some are more severely inhibited than others. Also different DNA lesions may adopt a conformation in nucleosomes that is more or less amenable to processing when compared to naked DNA. This may also cause great disparities in certain enzymes when comparing activity on naked DNA and nucleosomes (37). To determine how these effects on APE1 may be affecting coordination of repair on nucleosomes, we conducted reactions identical to those in Figure 2, but with 10- to 100-fold higher concentrations of APE1. Figure 5 shows that higher concentrations of APE1 decreased the concentration of intermediates persisting in the nucleosome reactions. However in comparison to naked DNA reactions, 100-fold more APE1 was needed to process virtually all the NTHL1-generated product in nucleosomes containing outward-facing lesions. For nucleosomes with inward-facing lesions, significant amounts of NTHL1-generated intermediates persist, even in reactions containing a 100-fold more APE1. These results indicate that nucleosome structure suppresses APE1 binding (or enzymatic activity) to a far greater extent than it does NTHL1. This factor may account for some of the lack of repair coordination in nucleosomes under conditions in which repair is well coordinated in naked DNA.

#### The DNA lyase activity of NTHL1 contributes to the processing of lesions in nucleosomes, even in the presence of APE1

As described earlier, AP-sites generated during BER reactions with naked DNA substrates are processed exclusively by APE1 (22). However, the poor activity of APE1 in nucleosomes led us to ask if AP sites generated by NTHL1 in nucleosomes are processed by the NTHL1 lyase activity. To determine which enzyme, NTHL1 or APE1, processes AP-sites in nucleosomes, it was necessary to contend with the fact that the product of the NTHL1 lyase can be further processed by APE1 to generate the same product as direct APE1 processing of the AP-site (Figure 1B). We therefore  $^{32}\text{P}$ -labeled our substrate at a position 3' to the lesion site. Direct processing of the AP site by APE1 would leave a sugar residue attached to the newly generated 5' end at the lesion site, which we could stabilize using  $\text{NaBH}_4$  (28). But if the AP site was first processed by NTHL1, the newly generated 5' end would contain only a terminal 5' phosphate (See Figure 1B). Such a small difference in charge/molecular weight was only detectable by gel electrophoresis after cleaving the DNA (isolated after treatment of nucleosomes with NTHL1 and APE1) at a DRA1 site not far from the lesion site (see Figure 1A). Figure 6 shows DRA 1-cleaved DNA products from reactions like those presented in Figure 2 but conducted with appropriately labeled, lesion-containing nucleosomes, and quenched with  $\text{NaBH}_4$  after 5 min. As with the other reactions shown in this study, the overall fraction of lesions processed was highest for naked DNA substrates, lower for outward-facing nucleosomal substrates and lower still for inward-facing nucleosomal substrates. Inspection of the gel shown in Figure 6 indicates that AP-sites generated in BER reactions with naked DNA were processed exclusively by APE1, as expected (22). By contrast, products from both NTHL1 and APE1 are evident in reactions with nucleo-



**Figure 5.** The processing of DNA intermediates generated during the hNTHL1-initiated repair of Tg residues in nucleosomes is enhanced to a greater extent by increasing APE1 concentrations in reactions with outward-facing Tg lesions than for inward-facing Tg lesions. Reactions contained 2.25 nM Tg substrate, 2 nM NTHL1, and either 0.2, 2.0 or 20 nM APE1. Aliquots were taken at various time points and analyzed as before. The abundance of intermediates, as a fraction of total product, was graphed as a function of time. Error bars represent standard deviation of three independent experiments.



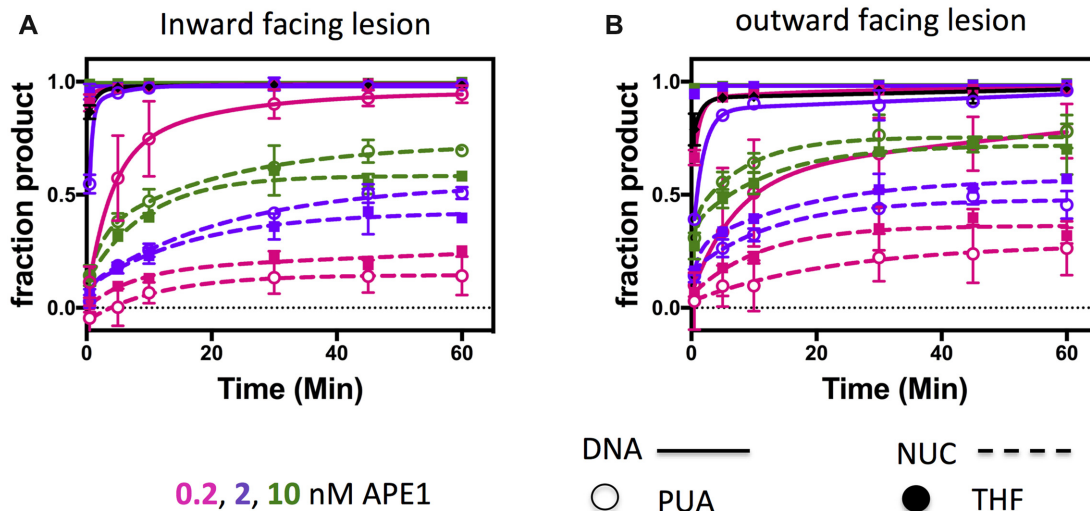
**Figure 6.** The DNA lyase activity of hNTHL1 contributes to the processing of lesions in nucleosomes, even in the presence of APE1. To distinguish between APE1- and hNTHL1-mediated strand incision activities, Tg-containing DNA and nucleosomal substrates were labeled with  $^{32}\text{P}$  at a position 3' to the lesion site (see Figure 1). 2.25 nM of the resulting substrates were incubated with 2 nM NTHL1, in the presence or absence of 0.2 nM APE1. Reactions were halted after 5 min, and samples were processed and reaction products fractionated and analyzed as described in the Methods. The Figure shows products separated on 20% denaturing polyacrylamide gels. Naked DNA lanes contained 20% as much sample as the nucleosomal DNA lanes equalize relative intensities of the product bands to aid comparison of band migration. Product bands resulting from either APE1 activity or NTHL1 activity are indicated (see also Figure 1B).

some substrates. Both enzymes contributed equally to the processing of AP sites generated during repair of inward-facing lesions in nucleosomes, whereas outward-facing lesions were more frequently processed by APE1.

#### APE1 processes both AP-sites and $\alpha$ - $\beta$ unsaturated aldehydes with similar efficiency in nucleosomes but not in naked DNA

In reactions with naked DNA, the AP endonuclease activity

of APE1 is more than 100-fold more efficient than is its 3'-diesterase activity, which is used in processing PUA residues (38). Given that the NTHL1 lyase is significantly more involved in the repair of oxidative lesions in nucleosomes than it is in naked DNA, it was important to determine if this extreme difference in relative activities is also evident during the repair of lesions in nucleosomes. Figure 7 shows the relative activity of APE1 on tetrahydrofuran and 3' polyunsaturated aldehyde in reactions with naked versus nucleo-



**Figure 7.** Tetrahydrofuran and polyunsaturated aldehyde (PUA)-containing DNA's are equally robust substrates for APE1 when packaged in nucleosomes. Reactions contained 2.25 nM substrate containing either a PUA (open symbols) or tetrahydrofuran (closed symbols) residue, and 0.2, 2 or 10 nM APE1. Solid line traces indicate data from DNA reactions and dotted line traces indicate data from nucleosome reactions. Reactions were quenched and samples processed and analyzed, as described in the Methods. Error bars represent standard deviation of three independent experiments.

somal substrates. In stark contrast to its activity on naked DNA, APE1 processed the two substrates at similar rates in nucleosomes. Specifically, tetrahydrofuran was the more favored substrate, in both inward and outward facing contexts, in reactions with relatively low APE1 concentrations. At higher concentrations of APE1, PUA was a somewhat better, inward-facing substrate when compared to tetrahydrofuran.

The data presented here indicate that both the glycosylase and lyase products of NTHL1 are processed by APE1 with similar efficiency in nucleosomes. This is in stark contrast to studies with naked DNA substrates. Our observations suggest that the nicking of DNA by the NTHL1 lyase may contribute to BER in nucleosomes by increasing DNA flexibility at the lesion site.

## DISCUSSION

The stepwise BER of oxidized bases DNA requires the coordinate activity of multiple enzymes. Numerous studies of BER, in cells and *in vitro* using naked DNA templates, suggest that the properties of BER enzymes themselves account for the observed coordination of individual steps in BER (29–33). Nucleosomes, however, limit access to oxidized bases and repair intermediates, to varying extents depending on their helical and translational positions relative to the underlying histone octamer (16–18,23,36,39–41). That these structural impediments may differentially affect enzyme efficiency led us to investigate the impact of nucleosomes on the coordination of successive steps in BER. We have found that, in multiple turnover reactions, APE1 does not stimulate the activity of NTHL1 toward substrates in nucleosomes as it does on naked DNA substrates. We also found that DNA repair intermediates accumulate to a much greater extent during repair of lesions in nucleosomes than they do in naked DNA. Use of an NTHL1 variant that exhibits reduced affinity for its DNA product significantly re-

duced the accumulation of repair intermediates. Thus, despite its reputation as one of the most robust of the BER enzymes, APE1 may find it ‘difficult’ to dissociate NTHL1 from its product in nucleosomes. This inference is consistent with our finding that 100-fold more APE1 was needed to substantially reduce the abundance of repair intermediates generated during the NTHL1-initiated repair of relatively accessible (outward facing) lesions in nucleosomes, as compared to the amount needed in reactions with naked DNA. Even a 100-fold excess was insufficient to process all of the product generated by NTHL1 during the repair of inward-facing lesions.

Collectively, the above results strongly suggest that, in cells, NTHL1 binds tightly to the AP site it generates during repair of lesions in nucleosomes. While this tight binding may limit the access of APE1 to AP sites, it also effectively sequesters NTHL1-generated products, which may prevent them from triggering adverse repair responses. Viewed in this fashion, elements that are central to coordinating successive steps in BER are preserved: while nucleosomes alter rate-limiting steps in BER, and present severe impediments to binding of APE1, NTHL1 turnover and processing of additional lesions may still be largely APE1-dependent.

On naked DNA substrates AP-sites generated by bifunctional glycosylase are quickly processed by APE1, bypassing the slower lyase activity (22,31). This has led many to hypothesize that the lyase activity observed *in vitro* may be dispensable *in vivo* since APE1 is ubiquitous in the nucleus. The differences in NTHL1-APE1 dynamics between nucleosomes and naked DNA led us to ask if the NTHL1 lyase activity might contribute to BER in chromatin. We have shown here that NTHL1 lyase does in fact act during repair of lesions in nucleosomes, even though it is completely bypassed by APE1 in parallel reactions conducted with naked DNA substrates. Our observations suggest that the nicking of DNA by the NTHL1 lyase may contribute to BER in nucleosomes by increasing DNA flexibility at the lesion site.



In reactions with naked DNA substrates the AP-endonuclease activity of APE1 is >100 fold more efficient than the 3' diesterase activity used to remove the 3' PUA product of the NTHL1 catalyzed lyase reaction. If the 3' PUA product were an equally poor substrate for APE1 in nucleosomes, the NTHL1 lyase activity might actually prove detrimental to efficient BER. We therefore tested the ability of APE1 to process both NTHL1 products in nucleosomes. Surprisingly APE1 processed both AP and 3' PUA substrates with relatively equal efficiency. Thus, the lyase activity of NTHL1 is not detrimental to, and may enhance, subsequent steps in BER.

### Future directions

In most cells, roughly half of the oxidative lesions produced under normal metabolic conditions are AP sites; even more AP-sites are generated by monofunctional glycosylases. APE1 is thought to process most of these, although human cells contain a second enzyme, APE2, which also possesses an AP-endonuclease activity (38). Based on phenotypic evidence it has been hypothesized that APE2 is not involved in BER (42,43). However, given our discovery that APE1 is significantly less adept at promoting turnover of a DNA glycosylase in during repair of lesions in nucleosomes, it may be worth re-visiting the possibility that APE2 acts in BER, perhaps specifically during repair of lesions in nucleosomes. A possible alternative is that AP sites that form in nucleosomes may sometimes be processed by an AP-lyase associated with one of the several bifunctional glycosylases present in human cells. This possibility might also apply to a recent study in which AP- or tetrahydrofuran-containing nucleosomes were incubated with cell extracts. Results indicated that some of the AP-sites were processed by a lyase activity in the extract (44). Principal among these potential AP-lyases are the bifunctional glycosylases which can process AP-sites and are constantly searching the genome for lesions.

### SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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