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Active-Site Clashes Prevent the Human 3-Methyladenine DNA Glycosylase from Improperly Removing Bases

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Summary

The human 3-methyladenine DNA glycosylase (AAG, MPG) removes a diverse array of damaged purines via a nucleotide-flipping mechanism. In the crystal structure of AAG bound to DNA containing 1,N^6 ethenoadenine, an asparagine (N169) occupies the active-site floor, in close proximity to the C-2 position of the flipped-out 1,N^6 ethenoadenine. We engineered site-specific AAG mutants to determine whether N169 prevents normal bases from mistakenly entering the active site. Substituting alanine or serine resulted in mutants that excised substrates at a faster rate than wild-type. Furthermore, these mutants acquired the ability to excise normal guanine within mispairs but not opposite cytosine. The results suggest that AAG can recognize helical deformations, such as mispairs. However, the active site then prevents the mistaken excision of bases, which prevents AAG from acquiring a mutator activity.

Introduction

DNA repair mechanisms actively protect the human genome from continuously occurring alterations. Base excision repair (BER) corrects a wide variety of damaged bases, including base loss [1, 2]. In humans, eight identified DNA glycosylases recognize altered bases and initiate BER by cleaving the glycosidic bond between the base and the DNA backbone [3]. Removal of the altered base produces an apurinic (AP) site, which is repaired through the subsequent steps of BER [4, 5]. The minimal functional steps require cutting the DNA backbone with AP endonuclease or AP lyase, removing the sugar residue via deoxyribophosphatase or phosphodiesterase, inserting the correct base(s) with a DNA polymerase, and sealing the remaining nick in the DNA backbone with DNA ligase [1, 6].

The specificity of human DNA glycosylases ranges from enzymes that target a single substrate, e.g., uracil, to DNA glycosylases that remove a range of modified bases [3]. The human 3-methyladenine DNA glycosylase (AAG, ANPG, MPG) was identified by its ability to remove 3-methyladenine (3-MeA), which can block replication [7, 8]. Additionally, AAG has been shown to excise a range of modified purines, including 7-methylguanine (7-MeG), 1,N^6-ethenoadenine (εA), 2,N^3-ethenoguanine (εG), and hypoxanthine (Hx), although AAG does not excise these substrates with equal efficiency [9–14]. Interestingly, mammalian AAG enzymes appear to excise Hx paired opposite thymine in preference to Hx opposite cytosine, but show little opposing base preference for εA excision [12, 15–17]. The means by which AAG recognizes its substrates is not obvious, as there is no apparent common structural feature among the substrates that the glycosylase can exploit [7, 8].

The crystal structure of AAG bound to an oligodeoxynucleotide containing an εA demonstrates that AAG, like all other known glycosylases, acts by a nucleotide-flipping mechanism. In the active-site pocket, a bound water molecule (activated by glutamate 125) is thought to attack the deoxyribose C-1′ and displace the substrate base [18, 19]. In the active site of AAG, a trio of aromatic amino acids surround the flipped εA; these amino acids likely facilitate the capture and stabilization of target purines in the active site through π cloud stacking interactions [19]. The active-site amino acids likely define substrate discrimination by aiding in the capture of compatible substrates in the binding pocket or by preventing the insertion of bases that are not substrates of AAG, e.g., normal purines. This presents a paradox with AAG and other DNA glycosylases. How do they detect damaged bases amongst vast tracts of undamaged DNA? Substrate recognition occurs in the active site, but what features identify bases as targets for flipping? Substrate recognition is particularly challenging for mammalian 3-methyladenine DNA glycosylases, which recognize a diverse array of substrates. One intriguing consequence of the mechanism is that 3-MeA DNA glycosylases can occasionally make mistakes, i.e., remove normal bases [20–22].

We are interested in determining the mechanism by which AAG recognizes substrates but excludes normal bases from its active site. In the εA/AAG crystal structure, an asparagine (N169) is located at the bottom of the pocket, in close proximity to the C-2 position of the εA (Figure 1A; hydrogen atoms not shown) [19]. When a guanine is modeled into the crystal structure in place of εA, the C-2-amino group clashes with N169, thus providing a plausible mechanism to prevent guanine from occupying the active site (Figure 1B) [19]. 7-MeG, which is removed 100-fold less efficiently than 3-MeA [23], might overcome the steric clash via favorable π stacking interactions. When a serine or alanine is modeled into the N169 position, this increases the space available near the N^2 of guanine (Figures 1C and 1D).

In this study, we examine the role of the active-site amino acid N169 in substrate recognition by determining the biochemical properties of AAG mutants bearing amino acid substitutions at that position. We rationalized that increasing or decreasing the steric bulk of the amino acid at the N169 position could act to decrease or increase the space available for a target base in the active-site pocket. We were also interested in determining electrostatic effects, as some of the substrates of AAG carry formal positive charges. Accordingly, we substituted, by site-directed mutagenesis, the Asn at 169 with an alanine (Ala, A), serine (Ser, S), aspartate (Asp,

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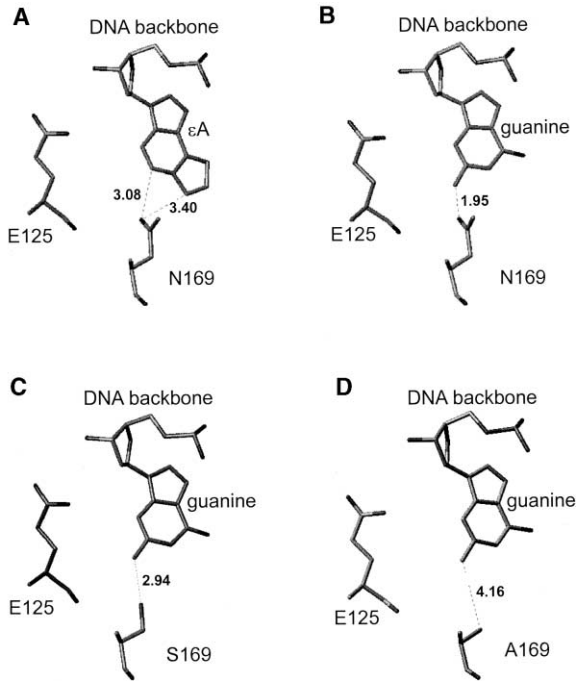

Figure 1. Positioning of N169 in the AAG Substrate Binding Pocket

(A) WT AAG complexed to ϵA-containing DNA. An asparagine (N169) is located in the floor of the binding pocket in close proximity to the flipped ϵA. The calculated distances, indicated in angstroms, do not include hydrogen atoms. Glutamate 125, which is essential for catalytic activity, is shown for reference in the figures.

(B) Guanine modeled in the place of ϵA. When the ϵA is replaced with a guanine, a steric clash occurs between the 2-amino group of guanine and N169.

(C) Serine modeled in the place of the asparagine. The substitution of serine for N169 removes the steric clash by adding an extra angstrom of distance between the guanine.

(D) Alanine modeled in the place of the asparagine. The substitution of alanine for N169 further increases the distance from the guanine, adding two angstroms of distance. These models were generated using TurboFrodo software (v.5.5) and the crystal coordinates [19].

D), and histidine (His, H). The AAG mutants were engineered to determine the steric and electrostatic limitations at N169 that might affect substrate recognition and normal base exclusion. The results underscore the importance of N169 in preventing the mistaken excision of normal guanine.

Results

We initially addressed the issue of steric hindrance at position 169 by examining bond rotation for the wild-type (WT) asparagine using modeling software (TurboFrodo). Each of the six rotamers examined that produced significant movement of the asparagine also created a steric hindrance with the surrounding amino acids in the active site (data not shown). This does not rule out the possibility of a larger-scale reorganization of the active site, but it should be noted that the active-site amino acids, including N169, are in essentially the identical position in the different crystal structures of AAG bound to the abasic transition state mimic and ethenoadenine [18, 19]. We therefore engineered a series of AAG mutants to test the influence of N169 on substrate recognition. The mutants were generated in a full-length *AAG* cDNA in the pYES expression vector, which allows for inducible expression in *S. cerevisiae* upon addition of galactose, whereas glucose represses expression. The full-length cDNAs were sequenced to confirm the changes (data not shown).

DNA Glycosylase Activity in a Model Organism

The N169 AAG mutants were initially screened for their ability to protect 3-MeA glycosylase-deficient RS1 *mag*Δ *S. cerevisiae* from the toxic effects of methyl methanesulfonate (MMS). MMS produces 3-MeA and 7-MeG adducts; 3-MeA is thought to be the toxic adduct because it blocks replication [24]. Yeast expressing the catalytically inactive E125Q AAG were sensitive to MMS, while yeast expressing WT AAG were protected from killing (Figure 2A). AAG bearing a His at 169 appeared to be catalytically inactive, as indicated by the MMS sensitivity of the *S. cerevisiae* expressing the His mutant (Figure 2A). Expressing the N169A or N169S mutants did not restore survival to that provided by WT AAG, suggesting a compromise in glycosylase activity for methylated substrates (Figure 2A). Interestingly, the growth of *S. cerevisiae* expressing the Asp mutant surpassed that of WT, indicating that the Asp mutant provides a better protection against MMS toxicity than WT (Figure 2A). Western blot analysis demonstrated that there was less than 2-fold difference between WT and N169A, N169S, or N169D mutant protein levels under the inducing conditions used for the gradient plate assay; the E125Q and N169H protein levels were ~2.8 and ~2.1-fold higher, respectively, than WT (Figure 2B). Further, these minor differences in protein expression levels do not correlate with survival, indicating that the differences noted in survival are not explained by discrepancies in glycosylase expression.

Binding Properties

In order to further characterize the consequences of mutations at the N169 position, each cDNA coding for the amino acid substitutions was subcloned into a His-tagged expression vector and purified by affinity chromatography (see Experimental Procedures). The proteins were purified as N-terminal Δ54, His-tagged recombinant proteins (data not shown). The N-terminal region of mammalian AAG proteins has been shown to have no effect on either base excision or DNA binding activities for all substrates except 2,N^3-ethenoguanine (ϵG) [13–15, 18, 19, 25]. Electrophoretic mobility shift assays (EMSA) were performed to determine the effect of the N169 substitutions on DNA binding. The DNA probes contained an ϵA substrate and a pyrrolidine (PYR) transition state mimic [26]. Each of the N169 mutants displayed a weakened binding affinity to the PYR-containing DNA compared to WT AAG (Figure 3). The apparent binding affinities of the N169A and N169S mutants (K_{Dapp} = 9.8 and 8.4 nM, respectively) were ~5-fold weaker compared to WT (K_{Dapp} = 1.8 nM) (Figure 3); the His substitution substantially decreased binding to PYR (K_{Dapp} = 51.7 nM) (Figure 3). Substituting the carboxylate

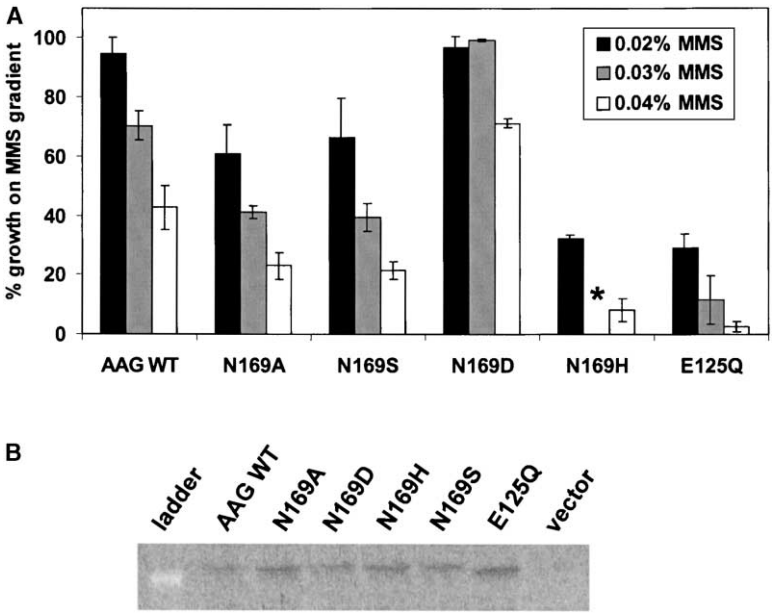

Figure 2. DNA Glycosylase Activity in a Model Organism

(A) The WT and mutant AAG proteins were expressed in *S. cerevisiae* lacking endogenous 3-MeA DNA glycosylase. The yeast were plated under inducing conditions in the presence of increasing concentrations of an MMS gradient, 0.02%, 0.03%, and 0.04%. The experiments were performed three times; error bars represent standard deviation. Asterisk, not determined.

(B) Western analysis of protein induction. Protein extracts were generated from yeast grown under the conditions used in the gradient plate experiments. Expression levels were determined following Western analysis. Yeast harboring the empty expression vector were used as a negative control (vector). Relative protein levels were quantitated by imaging software.

of Asp for the carboxamide of Asn drastically affected binding to PYR; the N169D mutant bound with a K_{Dapp} value of $>$340 nM (an accurate K_{Dapp} value could not be determined) (Figure 3). When a DNA probe containing ϵA was employed, significant binding could only be detected for WT and the N169S mutant (Figure 3A). The binding of the N169S mutant to ϵA was weaker than WT (Figure 3A), while the Ala, His, and Asp substitutions substantially weakened binding to the ϵA substrate (Figure 3A). K_{Dapp} values were not determined for ϵA binding.

Excision of ϵA and Hx substrates

The purified proteins were examined for their ability to release Hx and ϵA paired opposite thymine from oligodeoxynucleotides. A range of protein concentrations between 10–400 nM and short time points were initially examined in order to examine single turnover events, and rates were determined using first-order kinetics (see Experimental Procedures). The results revealed an interesting pattern of biochemical activity for the mutants. As reported previously, the excision rate of Hx by WT AAG was faster than that of ϵA (0.11 min^{-1} and 0.055 min^{-1}, respectively) (Figure 4). The N169A mutant excised Hx at a slightly faster rate than WT AAG (0.17 min^{-1}), while the ϵA excision rate was considerably greater (0.14 min^{-1}), compared to WT AAG (Figure 4). When the N169S mutant was tested for ϵA excision, the excision rate (0.15 min^{-1}) was similar to that for N169A (Figure 4B). However, the N169S mutant excised Hx at a much faster rate (0.35 min^{-1}) than either WT AAG or N169A (Figure 4A). Note that overexpressing the N169S mutant in yeast decreased survival in the presence of MMS (Figure 2A). The N169D protein, which appeared to be more active than WT in MMS-treated yeast, did not excise Hx under the conditions utilized, while ϵA excision was approximately 20-fold worse than WT (data not shown). The N169H mutant, which appeared to be catalytically inactive in yeast, did not excise Hx under the conditions used. Surprisingly, the N169H mutant showed a very weak residual activity for ϵA ($\sim$1000-fold weaker than WT) at extended incubation times (data not shown). The results indicate that the amino acid switches at N169 have altered the activity of AAG; however, these effects appear to be substrate dependent.

Excision of Guanine from Mispairs

We next examined the excision of guanine from G:C, G:T, G:U, and G:A base pairs. Guanine and Hx are identical except for the N^2-amino group present on guanine. The Hx:T and G:T base pairs are wobble base pairs with similar predicted geometry, whereas Hx:C and G:C have similar Watson-Crick geometry [27–29]. Note that AAG demonstrates a preference for Hx paired opposite thymine versus opposite cytosine [12, 15–17]. We therefore wanted to determine whether mutants that lack the steric hindrance of the WT N169 would now tolerate guanine in the active site. Indeed, we detected a significant excision activity for guanine in mispairs by the N169A and N169S proteins (Figure 5). Excision activity of guanine paired opposite uracil, thymine, and adenine was detected with a relative order of G:U $>$ G:T $>$ G:A (Figure 5). The specific activity of the N169S mutant for guanine opposite thymine, uracil, and adenine is $\sim$137-, 104-, and 219-fold weaker, respectively, than for Hx:T. The specific activity of the N169A mutant for guanine opposite thymine, uracil, or adenine is $\sim$150-, 100-, and 233-fold weaker, respectively, than for Hx:T. The aberrant guanine excision activity was not detected for the WT, E125Q, N169D, or N169H proteins (Figure 5 and data not shown). No excision activity for WT, N169A, or N169S was detected when guanine in the same sequence context was paired opposite cytosine (Figure 5). We further investigated the ability of WT and mutant AAG proteins to excise bases from mismatches but did not detect any excision activity for adenine opposite cytosine or guanine (data not shown). Our results agree with a previous report which showed a lack of WT AAG activity for either a G:T or A:C mismatch [15]. We thus conclude

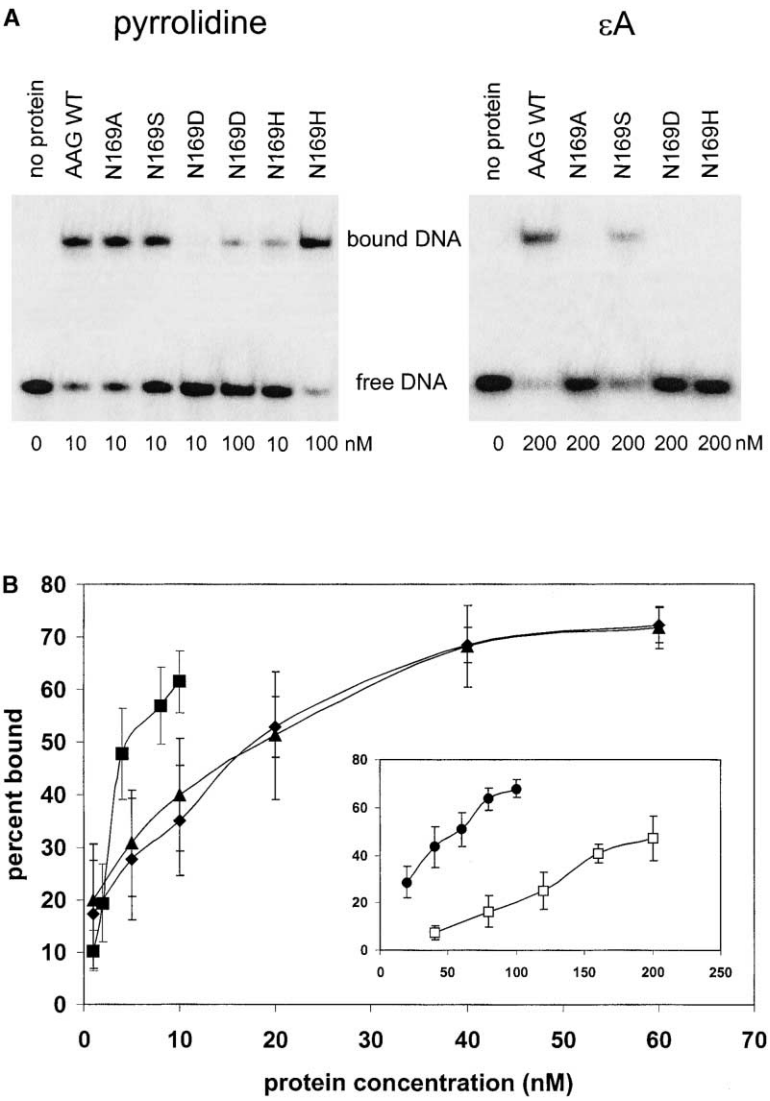

Figure 3. EMSA Assay to Detect DNA Binding of the N169 Mutants

(A) The binding affinities of the purified mutant AAG proteins for the pyrrolidine (PYR) transition state mimic were compared to WT. A representative gel is shown on the left. The glycosylases were incubated at the concentrations listed with oligodeoxynucleotide (1 nM) for 30 min at room temperature. The DNA glycosylases were also tested for their ability to bind to εA-containing DNA. A representative gel is shown on the right. The proteins (200 nM) were incubated with oligodeoxynucleotide (1 nM) for 30 min on ice. Incubation on ice allows DNA binding to occur while inhibiting excision activity.

(B) DNA glycosylase binding to PYR containing oligodeoxynucleotide. The plots show the percentage of bound DNA versus protein concentration. EMSA analysis was performed with increasing concentrations of the glycosylases to determine apparent K_D values for PYR binding of WT AAG (filled squares) and the N169 mutants: Ala (filled diamonds), Ser (filled triangles), His (filled circles), and Asp (open squares). The data are an average of four to eight independent experiments; error bars represent standard deviations for each data point.

that N169 actively prevents the aberrant excision of guanine by WT AAG. Although there was a report that WT AAG can excise 8-oxoguanine (8oxoG) from DNA, more recent reports indicate that AAG does not excise 8oxoG [12, 15]. We tested the ability of the WT, N169A, and N169S mutants to remove 8oxoG paired opposite cytosine; no 8oxoG excision was seen with either the WT or mutant proteins described here (data not shown).

Effect of Base Pairing on Excision of Hx

Because the N169A and N169S mutations significantly increased the excision rates for Hx and εA paired opposite thymine, we next examined whether the increased activity might also alter Hx excision from different base pairs. Hx-containing DNA was annealed to complementary strands that placed a cytosine, uracil, or adenine opposite Hx; excess protein concentrations were used, as above. WT AAG excision of Hx opposite cytosine was significantly slower than Hx:T and followed a linear slope, which was used to estimate relative excision rates (Figure 6A). WT AAG excision of Hx opposite uracil and adenine was similar to that seen for Hx:C (Figure 6A; Table 1). WT AAG prefers thymine opposite the target Hx, as reported previously. The N169A mutant excised Hx opposite cytosine slower than Hx:T, with a reduction that was similar to that seen for WT (Table 1). However, N169A excision of Hx opposite uracil was faster than Hx excision opposite adenine or cytosine and less than 2-fold slower than Hx:T (Figure 6B). The N169S protein, which excises Hx:T faster than AAG WT or N169A, appears to have also lost the ability to discriminate between the base opposite Hx (Figure 6C; Table 1). Thus, reducing the discrimination within the active site increases the catalytic rate and also reduces the discrimination for Hx opposite different bases.

Discussion

DNA glycosylases remove a large array of DNA base damage arising from alkylation, deamination, oxidation, and hydroxylation [1, 2]. DNA glycosylases that excise 3-MeA operate in many different living organisms. Inter-

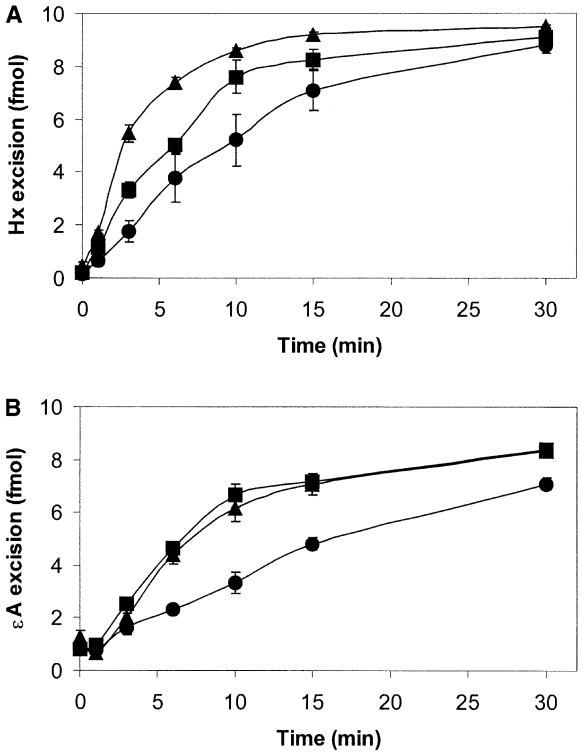

Figure 4. Excision Activities of WT AAG and the N169 Mutants

(A) Glycosylase activity was measured by following the conversion of substrate-containing oligodeoxynucleotides to cleaved products. The plots show femtomoles substrate removed over time. Hx-containing DNA (1 nM) was incubated at 37°C with 200 nM WT (circles), N169A (squares), or N169S (triangles) proteins. Note that the N169D and N169H proteins displayed no detectable activity. The data are an average of three independent experiments; error bars represent standard deviations for each data point.

(B) εA-containing DNA (1 nM) was incubated at 37°C with 200 nM WT (circles), N169A (squares), or N169S (triangles).

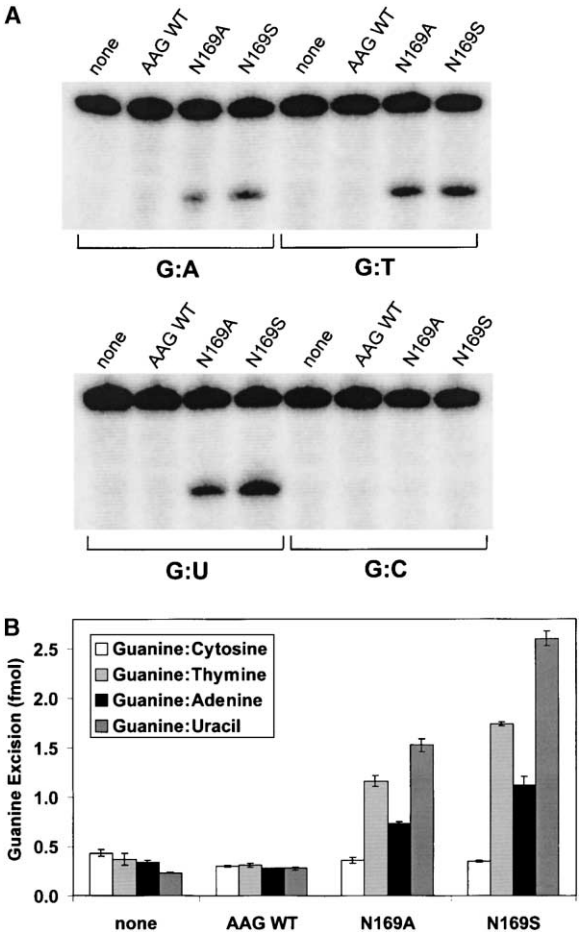

Figure 5. Excision of Guanine from Mispairs by the N169A and N169S Mutant

(A) An oligodeoxynucleotide was annealed to complementary strands that created a G:C pair or a single G:T, G:U, or G:A mismatch. The gels show the excision of guanine following a 2 hr incubation at 37°C with 200 nM protein.

(B) The bar graph shows the excision of guanine in femtomoles. The data are an average of three independent experiments; error bars represent standard deviations.

estingly, genomic analysis has thus far revealed only one such enzyme, AAG, in humans [3]. In addition to 3-MeA, AAG excises Hx, εA, εG, and 7-MeG, which do not appear to share an obvious feature that the glycosylase could exploit as a single means of recognition [7, 8]. The preference of substrate excision for mammalian 3-MeA DNA glycosylases does not correlate with substrate glycosidic-bond stability, as it does for the AlkA/MAG family of 3-MeA DNA glycosylases [20, 30]. Further, the mechanism by which AAG detects substrates amongst vast tracts of undamaged DNA has not been determined.

For AAG and its homologs, multiple factors likely influence substrate recognition, such as the ease with which the target nucleotide is flipped from the DNA helix and the fit of the substrate into the active-site pocket [17, 31]. In this study, we determined the contribution of one such factor, namely the asparagine in the floor of AAG's active site. We rationalized that a series of site-specific mutants at position 169 would allow us to determine whether discrimination against nonsubstrates occurs before or after flipping. Switching N169 to His presumably prevents a substrate base from fully occupying the active site but permits binding to the pyrrolidine abasic site. The binding ability of N169H to the pyrrolidine, albeit compromised compared to wild-type AAG, suggests that N169H can still recognize helical perturbations and attempt to flip nucleotides. It might be possible to trap the N169H mutant in a "preflipped" interaction with substrate-containing DNA, as the presence of the histidine presumably prevents proper/complete insertion of the target base into the active-site pocket.

Reducing the steric interaction of N169 via switches to Ala or Ser produces AAG mutants that excise Hx and εA at a faster rate than WT, thus implying that N169 serves to slow the overall rate of the reaction. N169 seems to enhance the selectivity of the active-site pocket by reducing the chance of inappropriate excision events. Indeed, the N169A and N169S mutants were able to excise undamaged guanine from mispairs. Excision of guanine from a G:T or G:U mispair would be promutagenic, as it would fix the G:C to A:T mutations generated

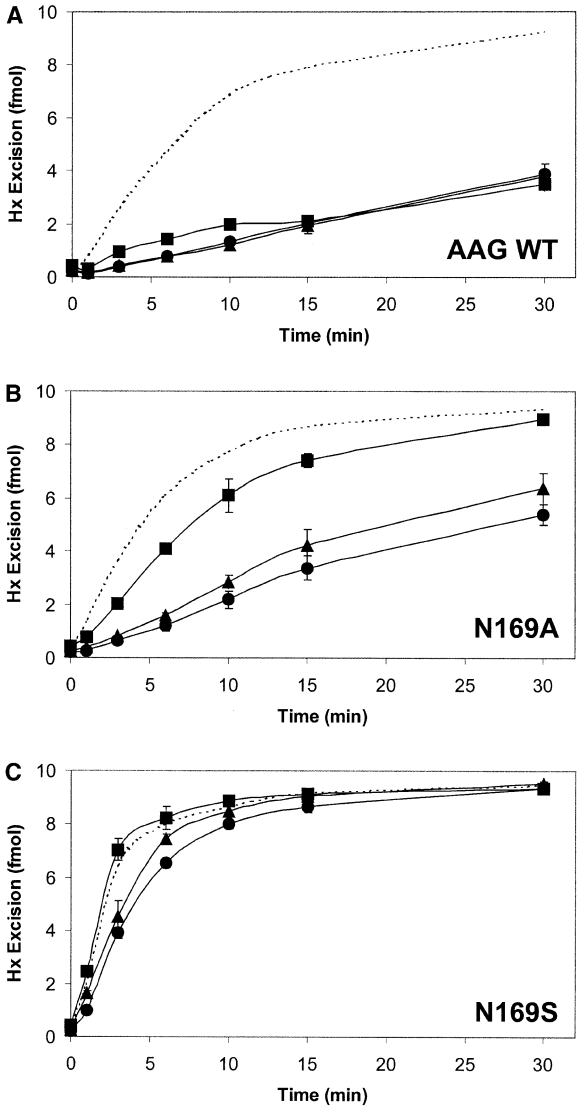

Figure 6. Excision of Hx Opposite Cytosine, Uracil, and Adenine

(A) Incubation conditions were the same as those used to examine the excision of Hx paired opposite thymine (dashed line). Hx containing oligodeoxynucleotides were annealed to complementary strands that placed a cytosine (circles), a uracil (squares), or an adenine (triangles) opposite the adducted base. The oligonucleotides (1 nM) were incubated with 200 nM AAG WT protein. The plots show femtomoles substrate removed over time. The data are an average of two independent experiments.

(B) The oligonucleotides (1 nM), with cytosine (circles), a uracil (squares), or an adenine (triangles) opposite the adducted base, were incubated with 200 nM N169A protein. The dashed line represents the excision of Hx paired opposite a thymine by the N169A protein.

(C) The oligonucleotides (1 nM), with cytosine (circles), a uracil (squares), or an adenine (triangles) opposite the adducted base, were incubated with 200 nM N169S protein. The dashed line represents the excision of Hx paired opposite a thymine by the N169S protein.

by the deamination of 5-methylcytosine or cytosine, respectively. An AAG mutant capable of excising guanine from G:T or G:U mispairs would ironically also compete with DNA glycosylases that specifically remove the mutagenic thymine or uracil opposite guanine [4, 32–34].

Table 1. Excision Rates for the Removal of Hx by AAG WT, N169A, and N169S (min^{-1})

	WT AAG	N169A	N169S
Hx:T	0.11	0.17	0.35
Hx:C	0.0036 (30.6)	0.022 (7.7)	0.18 (1.9)
Hx:U	0.0033 (33.3)	0.11 (1.5)	0.40 (0.85)
Hx:A	0.0011 (100)	0.032 (5.3)	0.23 (1.5)

The table displays the observed excision rates of Hx. The excision rates were determined using first order kinetics and reflect the data presented in Figures 4 and 6. Numbers in parentheses represent the fold decrease in rate, compared to the excision rate of Hx paired opposite thymine, for each enzyme.

In addition to aberrantly excising guanine, the N169S mutant (and N169A to a lesser extent) lost the ability to discriminate between Hx paired opposite thymine versus opposite cytosine. The loss of opposing base selectivity seen for N169S and N169A also supports a role for N169 enhancing the selectivity of the active-site pocket. Guanine and Hx are identical except for the N^2-amino group present on guanine. The Hx:T and G:T base pairs are wobble base pairs with similar predicted geometry, whereas Hx:C and G:C have similar Watson-Crick geometry [27–29]. The results suggest that WT AAG initially recognizes helical deformations, such as mispairs, but does not excise guanine in a mispair because of the steric exclusion provided by N169. The initial recognition of helical deformations provides a reasonable explanation for the observed preference of mammalian AAG enzymes, which excise Hx paired opposite thymine more efficiently than Hx paired opposite cytosine [12, 15–17]. This hypothesis also agrees with the observation that AAG binds but does not excise DNA containing cisplatin intrastrand crosslinks [35]. Two recent reports lend further support to AAG recognizing helical deformations, such as mispairs, then flipping the nucleotide into the active site where verification of the substrate occurs for catalysis. Both reports mention that the ease with which a nucleotide can be flipped likely contributes to the initial recognition process [36, 37]. It remains to be seen how helical deformation and ease of flipping affect the recognition of 3-MeA, which has not been studied in detail due to its instability in DNA.

An Asp mutant was generated to test electrostatic effects, as 3-MeA and 7-MeG carry positive charges, while Hx and ϵA are uncharged. The N169D mutant, with its negatively charged carboxylate group, proved intriguing. When challenged with MMS, yeast expressing the N169D mutant fared better than yeast expressing WT AAG, particularly at higher MMS doses. However, the Asp substitution clearly compromised the biochemical activity for Hx and ϵA. We are currently determining the excision rates of the mutant proteins for 3-MeA and 7-MeG substrates in vitro in order to directly determine the effect of the substitutions on the methylated substrates of AAG. We infer that the active site containing Asp accepts the positively charged 3-MeA, which rescues the *S. cerevisiae* from MMS toxicity. However, it is not clear why *S. cerevisiae* expressing the N169D mutant would fare better than WT when exposed to MMS. Overall, the activity of the expressed mutants and WT inferred from the survival of *S. cerevisiae* exposed

to MMS did not correlate with their biochemical activity determined in vitro for Hx and εA. Previous studies that examined the overexpression of 3-MeA DNA glycosylases note that imbalanced DNA glycosylase activity can become detrimental to the cell [38–41]. One possible explanation for the phenotype of *S. cerevisiae* expressing N169D is that the N169D protein might remove 3-MeA but only poorly removes the more abundant 7-MeG adducts, which are not thought to be directly toxic. Removing toxic 3-MeA while not actively removing 7-MeG would thus avoid overwhelming the remainder of the BER pathway.

Significance

Asparagine 169, located in the floor of the active-site pocket, appears to play an important role in the confirmation of substrates for the human 3-MeA DNA glycosylase (AAG, MPG). Mutants of AAG bearing different amino acid substitutions each displayed some degree of deviation in activity compared to WT. Altering the Asn to serine or alanine increases glycosylase activity for Hx and εA in vitro, but at a cost of acquiring an ability to mistakenly excise guanine from mispairs. Further, the serine and alanine mutants lost the ability to discriminate between Hx paired opposite thymine versus opposite cytosine. The results are consistent with a model in which WT AAG initially recognizes helical deformations, such as mispairs, but active site clashes then prevent the excision of bases where excision would be detrimental. The ease with which a nucleotide can be flipped likely contributes to the initial recognition.

The results indicate that WT AAG excises its substrates at a less than maximal catalytic rate, and the N169 position participates in modulating catalysis. A slower rate of excision may help to enhance substrate specificity by reducing the possibility of mistakenly removing normal guanine. Further, a DNA glycosylase with a slower catalytic rate and a higher affinity for the abasic product likely reduces the chance of producing an unattended abasic site, which is mutagenic and potentially toxic. Our understanding of BER has grown to appreciate the importance of balance and coordination between the steps in the pathway. The proper identification and excision of substrates by DNA glycosylases represent the first step in the quality-control process.

Experimental Procedures

AAG Constructs

The pYES vector containing the WT full-length *AAG* cDNA was a kind gift from Drs. Brian Glassner and Leona Samson. Base pair mutations encoding for substitutions at amino acid 169 of AAG were introduced into the full-length *AAG* cDNA in the pYES expression vector using Stratagene's Quik-Change methodology. The mutant cDNA were sequenced across their entire length to confirm that only the desired amino acid substitution occurred. The pET vector containing the Δ54 truncated and His-tagged *AAG* cDNA was a kind gift from Drs. Ju-Fang Chang and Tom Ellenberger. *AAG* cDNA fragments containing the single amino acid substitutions were subcloned from the pYES vector using internal XmaI sites present in the *AAG* cDNA sequence. Proper orientation of the cDNA sequences in the pET vector was confirmed by digestion with AflIII, which cuts once within the *AAG* cDNA and once in the pET backbone.

Preliminary Screen for DNA Glycosylase Activity

RS1 mag$^-$ yeast were transformed with pYES inducible expression vectors containing the mutated cDNA. Cells were freshly diluted and grown for 3 hr in either glucose (noninducing) or galactose (inducing) minimal media. The transformed yeast were stamped across YPD plates (glucose or galactose) bearing a 0%, 0.02%, 0.03%, or 0.04% MMS gradient. Yeast survival was measured after incubation for 3 days at 30°C. Mutant glycosylase activity was inferred from the survival relative to yeast containing AAG WT (positive control) and the catalytically inactive mutant E125Q AAG (negative control). Generation of the E125Q mutant was reported previously [19]. The results were repeated three to five times for each mutant.

Western Analysis

Yeast carrying the pYES vector lacking the *AAG* cDNA were included as a negative control. The yeast cultures were induced in galactose media under the same conditions used in the MMS gradient plating procedure. Following induction, the yeast were washed in buffer (50 mM Tris [pH 8.3],1 mM EDTA, protease inhibitors), and cells were lysed using glass beads. Protein concentration was determined using Pierce Coomassie Plus Protein Assay. The proteins (50 μg per lane) were separated by SDS-PAGE, then transferred to a 0.45 μm PVDF membrane. The membrane was probed with AAG primary antibody, then anti-rabbit horseradish peroxidase secondary antibody. The secondary antibody was detected using ECL Western blotting detection reagents (Amersham) and a Storm imager (Molecular Dynamics).

Protein Purification

His-tagged WT AAG, E125Q, N169A, N169D, N169H, and N169S were overproduced from the pET vector in *Escherichia coli* C41 strain (a gift from Dr. Tom Ellenberger, Harvard University). Bacteria were grown in a volume of 100 ml LB medium with 50 μg/ml ampicillin. At a cell density of 0.5 (A_{600}), the culture was induced with 0.4 mM isopropylthiogalacto-pyranoside for 18 hr at room temperature. Cells were lysed by sonication on ice in 5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl (pH 7.9), 4 mM β-mercaptoethanol (βME), and a protease inhibitor cocktail (Sigma). After centrifugation, the clear lysate was injected into a His•Bind Quick 900 Cartridge (Novagen), then washed and eluted as described by the manufacturer with slight modifications. The cartridge-purified proteins (concentrations of 4.42 μM, 5.06 μM, 2.74 μM, 3.01 μM, 2.18 μM, and 1.13 μM for AAG WT, N169A, N169D, N169H, N169S, and E125Q, respectively) were stored at −20°C in 50% glycerol, 500 mM imidazole, 250 mM NaCl, 10 mM Tris-HCl (pH 7.9), 2 mM βME, and protease inhibitor cocktail. Protein purification was confirmed by SDS-PAGE analysis, and protein concentrations were determined using the Pierce Coomassie Plus Protein Assay. The cartridge-purified proteins were used to determine protein binding (K_{Dapp}) and initial substrate excision analysis.

An additional large-scale protein purification was performed with WT, N169A, and N169S to determine excision rates. Protein induction was carried out as above in a 1 liter volume. Cells were resuspended in 0.02 M sodium phosphate (pH 7.4), 0.5 M NaCl, and 4 mM βME (binding buffer) including protease inhibitor cocktail, then lysed by sonication on ice. After centrifugation, the clear lysate was loaded onto a 1 ml Ni^{2+} charged HiTrap affinity column using an AKTA FPLC (Amersham). An elution gradient was performed with 0.02 M sodium phosphate (pH 7.4), 0.5 M NaCl, 0.5 M imidazole, and 4 mM βME (elution buffer). The protein began eluting at about 20% elution buffer. Fractions containing purified protein were determined using SDS-PAGE analysis and pooled. The pooled fractions were concentrated and desalted in a Millipore ultrafree-15 centrifugal filter device (10 kDa cutoff). The FPLC-purified proteins (concentrations of 85.0 μM, 39.9 μM, and 12.4 μM for AAG WT, N169A, and N169S, respectively) were stored at −70°C in 50 mM Tris (pH 8.3), 1mM EDTA, 15% glycerol, and protease inhibitor cocktail.

Protein Binding

The DNA binding properties of the WT and mutant glycosylases were evaluated by electrophoretic mobility shift assay (EMSA). The DNA probes contained an εA substrate or a PYR abasic site. PYR-containing DNA acts as a transition state mimic for DNA glycosylases [26]. The sequences of the oligonucleotides are as follows:

5′-GACTACTACATGXTTGCCGACCATT, where X = PYR or εA (Midlands Certified Reagents, Midlands, TX). The PYR was a kind gift from Dr. Tom Ellenberger. The oligodeoxynucleotides containing site-specifically located base lesions were 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP, then annealed to a 5-fold excess of the appropriate complementary strand containing a thymine opposite the adducted base. Complete annealing was confirmed by electrophoresis on a 20% nondenaturing polyacrylamide gel. Incubations were carried out in 50 mM Tris (pH 8.3) and 1 mM EDTA (AAG buffer) for 30 min with a DNA concentration of 1 nM and various glycosylase concentrations. For PYR binding, the incubations were carried out at room temperature, while εA incubations were carried out on ice to inhibit enzyme activity (<3% excision; data not shown). Following the addition of glycerol loading dye, the samples were loaded onto a 10% 0.5× TBE polyacrylamide gel and run at 80V, constant voltage, for 2 hr. Gels were visualized and analyzed using a BIO-RAD Molecular Imager FX and Quantity One software. K_{dapp} values were determined graphically as described [42].

Excision Assays

Glycosylase activity was measured by following product formation using substrate-containing oligodeoxynucleotides. Enzyme concentrations from 10–400 nM and short time points were examined in order to examine single turnover events. The sequences of the ^{32}P-labeled DNA were as follows: 5′-GACTACTACATGXTTGCC GACCATT, where X = Hx or εA (Midlands Certified Reagents, Midlands, TX). Annealing to the complementary strand placed a thymine, a cytosine, a uracil, or an adenine opposite the adducted base. The oligodeoxynucleotides, at a final concentration of 1 nM, were incubated with 200 nM DNA glycosylases at 37°C in AAG buffer for the indicated time points. The resulting AP sites were converted to strand breaks by the addition of NaOH (100 mM final concentration) and incubation at 85°C for 15 min. The full-length and truncated DNA were resolved on a 15% denaturing polyacrylamide gel (8 M urea) run at constant current. Gels were visualized and analyzed using a BIO-RAD Molecular Imager FX and Quantity One software. Excision rates were determined from first-order kinetics using EnzFitter software (Biosoft). Guanine excision for WT and the mutant proteins was determined using 200 nM protein and a 2 hr time point. The oligodeoxynucletide possesses a guanine in position X of the above sequence (PAGE purified from Integrated DNA Technologies, Coralville, IA). The annealed complementary strands placed either a cytosine, a thymine, a uracil, or an adenine opposite the guanine at position X. Assay conditions were similar to above; however, the EDTA concentration in the reaction buffer was increased to 5 mM, and uracil DNA glycosylase inhibitor was included in the experiments containing uracil.

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References

1. Hoeijmakers, J.H. (2001). Genome maintenance mechanisms for preventing cancer. Nature *411*, 366–374.
2. Lindahl, T., and Wood, R.D. (1999). Quality control by DNA repair. Science *286*, 1897–1905.
3. Wood, R.D., Mitchell, M., Sgouros, J., and Lindahl, T. (2001). Human DNA repair genes. Science *291*, 1284–1289.
4. Scharer, O.D., and Jiricny, J. (2001). Recent progress in the biology, chemistry and structural biology of DNA glycosylases. Bioessays *23*, 270–281.
5. McCullough, A.K., Dodson, M.L., and Lloyd, R.S. (1999). Initiation of base excision repair: glycosylase mechanisms and structures. Annu. Rev. Biochem. *68*, 255–285.
6. Parikh, S.S., Mol, C.D., Hosfield, D.J., and Tainer, J.A. (1999). Envisioning the molecular choreography of DNA base excision repair. Curr. Opin. Struct. Biol. *9*, 37–47.
7. Wyatt, M.D., Allan, J.M., Lau, A.Y., Ellenberger, T.E., and Samson, L.D. (1999). 3-methyladenine DNA glycosylases: structure, function, and biological importance. Bioessays *21*, 668–676.
8. Singer, B., and Hang, B. (1997). What structural features determine repair enzyme specificity and mechanism in chemically modified DNA? Chem. Res. Toxicol. *10*, 713–732.
9. Saparbaev, M., and Laval, J. (1994). Excision of hypoxanthine from DNA containing dIMP residues by the Escherichia coli, yeast, rat, and human alkylpurine DNA glycosylases. Proc. Natl. Acad. Sci. USA *91*, 5873–5877.
10. Saparbaev, M., Kleibl, K., and Laval, J. (1995). Escherichia coli, Saccharomyces cerevisiae, rat and human 3-methyladenine DNA glycosylases repair 1,N6-ethenoadenine when present in DNA. Nucleic Acids Res. *23*, 3750–3755.
11. Dosanjh, M.K., Roy, R., Mitra, S., and Singer, B. (1994). 1,N6-ethenoadenine is preferred over 3-methyladenine as substrate by a cloned human N-methylpurine-DNA glycosylase (3-methyladenine-DNA glycosylase). Biochemistry *33*, 1624–1628.
12. Asaeda, A., Ide, H., Asagoshi, K., Matsuyama, S., Tano, K., Murakami, A., Takamori, Y., and Kubo, K. (2000). Substrate specificity of human methylpurine DNA N-glycosylase. Biochemistry *39*, 1959–1965.
13. O'Connor, T.R. (1993). Purification and characterization of human 3-methyladenine-DNA glycosylase. Nucleic Acids Res. *21*, 5561–5569.
14. Saparbaev, M.K., Langouet, S., Privezentzev, C.V., Guengerich, F.P., Cai, H., Elder, R.H., and Laval, J. (2002). 1,N2-Ethenoguanine, a mutagenic DNA adduct, is a substrate of Escherichia coli mismatch-specific Uracil-DNA glycosylase and human alkylpurine-DNA-N glycosylase. J. Biol. Chem. *277*, 26987–26993.
15. Abner, C.W., Lau, A.Y., Ellenberger, T., and Bloom, L.B. (2001). Base excision and dna binding activities of human alkyladenine DNA glycosylase are sensitive to the base paired with a lesion. J. Biol. Chem. *276*, 13379–13387.
16. Saparbaev, M., Mani, J.C., and Laval, J. (2000). Interactions of the human, rat, Saccharomyces cerevisiae and Escherichia coli 3-methyladenine-DNA glycosylases with DNA containing dIMP residues. Nucleic Acids Res. *28*, 1332–1339.
17. Wyatt, M.D., and Samson, L.D. (2000). Influence of DNA structure on hypoxanthine and 1,N(6)-ethenoadenine removal by murine 3-methyladenine DNA glycosylase. Carcinogenesis *21*, 901–908.
18. Lau, A., Scharer, O., Verdine, G., Samson, L., and Ellenberger, T. (1998). Crystal structure of a human alkylbase-DNA repair enzyme complexed to DNA: Mechanism for nucleotide flipping and base excision. Cell *95*, 249–258.
19. Lau, A.Y., Wyatt, M.D., Glassner, B.J., Samson, L.D., and Ellenberger, T. (2000). Molecular basis for discriminating between normal and damaged bases by the human alkyladenine glycosylase AAG. Proc. Natl. Acad. Sci. USA *97*, 13573–13578.
20. Berdal, K.G., Johansen, R.F., and Seeberg, E. (1998). Release of normal bases from intact DNA by a native DNA repair enzyme. EMBO J. *17*, 363–367.
21. Glassner, B.J., Rasmussen, L.J., Najarian, M.T., Posnick, L.M., and Samson, L.D. (1998). Generation of a strong mutator phenotype in yeast by imbalanced base excision repair. Proc. Natl. Acad. Sci. USA *95*, 9997–10002.
22. Posnick, L.M., and Samson, L.D. (1999). Imbalanced base excision repair increases spontaneous mutation and alkylation sensitivity in Escherichia coli. J. Bacteriol. *181*, 6763–6771.
23. Miao, F., Bouziane, M., and O'Connor, T.R. (1998). Interaction of the recombinant human methylpurine-DNA glycosylase (MPG protein) with oligodeoxyribonucleotides containing either hypoxanthine or abasic sites. Nucleic Acids Res. *26*, 4034–4041.
24. Engelward, B.P., Allan, J.M., Dreslin, J.A., Kelly, J.D., Gold, B., and Samson, L.D. (1998). A chemical and genetic approach

together define the biological consequences of 3-methyladenine lesions in the mammalian genome. J. Biol. Chem. *273*, 5412–5418.

25. Roy, R., Biswas, T., Hazra, T.K., Roy, G., Grabowski, D.T., Izumi, T., Srinivasan, G., and Mitra, S. (1998). Specific interaction of wild-type and truncated mouse N-methylpurine-DNA glycosylase with ethenoadenine-containing DNA. Biochemistry *37*, 580–589.
26. Scharer, O.D., Nash, H.M., Jiricny, J., Laval, J., and Verdine, G.L. (1998). Specific binding of a designed pyrrolidine abasic site analog to multiple DNA glycosylases. J. Biol. Chem. *273*, 8592–8597.
27. Case-Green, S.C., and Southern, E.M. (1993). Studies on the base pairing properties of deoxyinosine by solid phase hybridisation to oligonucleotides. Nucleic Acids Res. *22*, 131–136.
28. Cruse, W.B., Aymani, J., Kennard, O., Brown, T., Jack, A.G., and Leonard, G.A. (1989). Refined crystal structure of an octanucleotide duplex with I.T. mismatched base pairs. Nucleic Acids Res. *17*, 55–72.
29. Xuan, J.C., and Weber, I.T. (1992). Crystal structure of a B-DNA dodecamer containing inosine, d(CGCIAATTCGCG), at 2.4 A resolution and its comparison with other B-DNA dodecamers. Nucleic Acids Res. *20*, 5457–5464.
30. Lindahl, T., Sedgwick, M., Sekiguchi, M., and Nakabeppu, Y. (1988). Regulation and expression of the adaptive response to alkylating agents. Annu. Rev. Biochem. *57*, 133–157.
31. Hang, B., Sagi, J., and Singer, B. (1998). Correlation between sequence-dependent glycosylase repair and the thermal stability of oligonucleotide duplexes containing 1,N6 ethenoadenine. J. Biol. Chem. *273*, 33406–33413.
32. Gallinari, P., and Jiricny, J. (1996). A new class of uracil-DNA glycosylases related to human thymine-DNA glycosylase. Nature *383*, 735–738.
33. Hendrich, B., Hardeland, U., Ng, H.H., Jiricny, J., and Bird, A. (1999). The thymine glycosylase MBD4 can bind to the product of deamination at methylated CpG sites. Nature *401*, 301–304.
34. Nilsen, H., and Krokan, H.E. (2001). Base excision repair in a network of defence and tolerance. Carcinogenesis *22*, 987–998.
35. Kartalou, M., Samson, L.D., and Essigmann, J.M. (2000). Cisplatin adducts inhibit 1,N(6)-ethenoadenine repair by interacting with the human 3-methyladenine DNA glycosylase. Biochemistry *39*, 8032–8038.
36. Biswas, T., Clos, L.J., 2nd, SantaLucia, J., Jr., Mitra, S., and Roy, R. (2002). Binding of specific DNA base-pair mismatches by N-methylpurine-DNA glycosylase and its implication in initial damage recognition. J. Mol. Biol. *320*, 503–513.
37. Vallur, A.C., Feller, J.A., Abner, C.W., Tran, R.K., and Bloom, L.B. (2002). Effects of hydrogen bonding within a damaged base pair on the activity of wild-type and DNA-intercalating mutants of human alkyladenine DNA glycosylase. J. Biol. Chem. *277*, 31673–31678.
38. Kaina, B., Fritz, G., Ochs, K., Haas, S., Grombacher, T., Dosch, J., Christmann, M., Lund, P., Gregel, C.M., and Becker, K. (1998). Transgenic systems in studies on genotoxicity of alkylating agents: critical lesions, thresholds and defense mechanisms. Mutat. Res. *405*, 179–191.
39. Coquerelle, T., Dosch, J., and Kaina, B. (1995). Overexpression of N-methylpurine-DNA glycosylase in Chinese hamster ovary cells renders them more sensitive to the production of chromosomal aberrations by methylating agents–a case of imbalanced DNA repair. Mutat. Res. *336*, 9–17.
40. Habraken, Y., and Laval, F. (1993). Increased resistance of the Chinese hamster mutant irs1 cells to monofunctional alkylating agents by transfection of the E. coli or mammalian N3-methyladenine-DNA-glycosylase genes. Mutat. Res. *293*, 187–195.
41. Imperatori, L., Damia, G., Taverna, P., Garattini, E., Citti, L., Boldrini, L., and D'Incalci, M. (1994). 3T3 NIH murine fibroblasts and B78 murine melanoma cells expressing the Escherichia coli N3-methyladenine-DNA glycosylase I do not become resistant to alkylating agents. Carcinogenesis *15*, 533–537.
42. Taylor, J.D., Ackroyd, A.J., and Halford, S.E. (1994). The gel shift assay for the analysis of DNA-protein interactions. In DNA-Protein Interactions, Vol. 30, G.C. Kneale, ed. (Totowa, NJ: Humana Press, Inc.), pp. 263–279.