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## **Separating substrate recognition from base hydrolysis in human thymine DNA glycosylase by mutational analysis**

Hardeland, U ; Bentele, M ; Jiricny, J ; Schär, P

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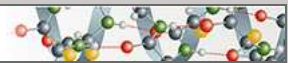
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**Protein Structure and Folding:**  
**Separating substrate recognition from base  
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PROTEIN STRUCTURE  
AND FOLDING



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## Separating Substrate Recognition from Base Hydrolysis in Human Thymine DNA Glycosylase by Mutational Analysis

Ulrike Hardeland, Marc Bentele, Josef Jiricny and Primo Schär

Institute of Medical Radiobiology, University of Zürich and the Paul Scherrer Institute, August Forel Strasse 7, 8008 Zürich, Switzerland.

Corresponding Author: Primo Schär  
Institute of Medical Radiobiology  
University of Zürich and PSI  
August Forel Str. 7  
CH-8008 Zürich  
Tel: +41-1-634 8926  
Fax: +41-1-634 8904  
E-mail: [schaer@imr.unizh.ch](mailto:schaer@imr.unizh.ch)

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## SUMMARY

Human thymine DNA glycosylase (TDG) was identified as an enzyme that can initiate base excision repair at sites of 5-methylcytosine- or cytosine deamination in DNA by its ability to release thymine or uracil from G•T and G•U mismatches. Crystal structure analysis of an *Escherichia coli* homologue identified conserved amino-acid residues that are critical for its substrate recognition/interaction and base hydrolysis functions. Guided by this revelation, we performed a mutational study of structure function relationships with the human TDG. Substitution of the postulated catalytic site asparagine with alanine (N140A) resulted in an enzyme that bound mismatched substrates but was unable to catalyze base removal. Mutation of M269 in a motif with a postulated role in protein-substrate interaction selectively inactivated stable binding of the enzyme to mismatched substrates but not so its glycosylase activity. These results establish that the structure function model postulated for the *E. coli* enzyme is largely applicable to the human TDG. We further provide evidence for G•U being the preferred substrate of TDG not only at the mismatch recognition step of the reaction but also in base hydrolysis, and for the importance of stable complementary strand interactions by TDG to compensate for its comparably poor hydrolytic potential.

## INTRODUCTION

DNA of all organisms is susceptible to modification and damage through the action of a variety of exogenous and endogenous reagents. A prominent form of spontaneous damage arises through hydrolytic deamination of bases carrying exo-cyclic amino groups such as cytosine and 5-methylcytosine. Deamination of cytosine in double-stranded DNA (dsDNA) generates a uracil•guanine mispair and, similarly, deamination of 5-

methycytosine generates a thymine•guanine mispair. *In vitro*, both events occur at appreciable rates, with 5-methylcytosine deamination being slightly faster than that of cytosine (1) and, *in vivo*, both deamination products are mutagenic and will produce C→T transitions upon DNA replication, if left unrepaired. While accurate repair of G•U mispairs is mediated by enzymes that specifically recognize and process uracil in DNA, e.g. uracil DNA glycosylase (UDG) (2), correction of G•T mispairs to G•C base pairs requires a repair function that is able to discriminate between a mutagenic thymine in a G•T mismatch and a normal thymine base-paired with adenine.

A G•T mismatch-specific thymine glycosylase activity was discovered in HeLa cell extracts (3). It was purified to apparent homogeneity (4), and the encoding cDNA was cloned (5). The biochemical properties of this thymine DNA glycosylase (TDG) are compatible with a function of the enzyme in cellular defense against mutagenesis by cytosine and 5-methylcytosine deamination. It is capable of recognizing G•T and G•U mismatches in DNA and initiating their restoration to G•C base pairs through a base excision repair process involving DNA polymerase  $\beta$  (6,7,8). Two bacterial open reading frames with significant homology to the central part of human TDG were discovered, and expression of the *E. coli* homologue produced an enzyme with G•U mismatch dependent uracil DNA glycosylase activity but no G•T mismatch processing activity (9). This protein was therefore named Mug for mismatch-specific uracil DNA-glycosylase. Deletion analysis of the human enzyme revealed that its conserved central domain is sufficient for G•U but not for G•T processing activity and that additional non-conserved amino-acid residues of the N-terminus are required for G•T processing (9).

More recent evidence suggested a wider range of possible substrates and functions for TDG homologues. Both, the *E. coli* and the human enzymes were found to efficiently process a mutagenic cyclic adduct of cytosine, 3,*N*<sup>4</sup>-ethenocytosine ( $\epsilon$ C), that arises in

DNA as a consequence of lipid peroxidation or the exposure to chemical carcinogens such as vinyl chloride (10,11). A vertebrate homologue of TDG was reported to copurify with a 5-methylcytosine DNA glycosylase activity from extracts of chicken embryos and to have weak 5-methylcytosine glycosylase activity when purified as a recombinant protein from overexpressing bacteria (12). Furthermore, a different line of investigation revealed physical and functional interactions of mammalian TDG with retinoid receptors and therefore implicated a role in nuclear receptor mediated control of transcription (13).

The three dimensional structure of *E. coli* Mug was analyzed by X-ray crystallography and resolved at a resolution of 1.8 Å (14). This revealed striking structural similarities between Mug and the functionally-related uracil DNA glycosylases, despite very limited conservation at the amino-acid sequence level. Similar to uracil DNA glycosylase, Mug forms an active site pocket, which penetrates into the core of the enzyme. The inferred catalytic mechanism for Mug suggests that the mispaired base to be released is flipped out of the DNA double helix and accommodated within the active site pocket in a manner that allows the N-glycosidic bond to be hydrolytically attacked by an activated water molecule. Positioning of the water molecule is coordinated by the Asn40 of the highly-conserved putative active site motif GINPGL. Base flipping by Mug is accompanied by intercalation of a three amino acid wedge into the DNA double helix. The less-conserved intercalating residues around Gly143 occupy the abandoned space opposite the guanine and maintain the base-stacking interactions in order to avoid the bases flanking the flipped-out residue collapsing on each other. A notable difference to the mechanism employed by uracil DNA glycosylase (15) is that the Mug residues involved in helix intercalation engage in specific complementary DNA strand interactions in a way that mimics hydrogen bonding to the widowed guanine (14,16).

To test the functional predictions from the Mug crystal structure on the human TDG and to better understand the structural relationship between different TDG homologues, we performed site-directed mutagenesis of critical residues and examined the biochemical activities of the mutant proteins. We were able to separate the DNA glycosylase and the substrate interaction functions in human TDG by mutating the implicated residues Asn140 and Met269. Studying the differential effects of the amino acid substitutions on binding and excision of thymine, uracil and 5-fluorouracil opposite guanine, we found the relative inefficiency of TDG in G•T versus G•U processing to be a consequence of both, a lower G•T binding affinity and a lower catalytic efficiency in thymine release. We further established experimental evidence for the importance of stable complementary strand interactions by human TDG as compensation for its comparably poor hydrolytic potential.

## EXPERIMENTAL PROCEDURES

### *Reagents and oligonucleotides*

All oligonucleotides were synthesized by Microsynth (Switzerland). The substrate oligonucleotides were PAGE purified after synthesis. Restriction enzymes and uracil DNA glycosylase inhibitor (UGI) were supplied by New England Biolabs (USA) and the uracil-DNA-glycosylase (UDG) by Roche Diagnostics (Switzerland). All other chemicals and reagents were purchased from Sigma (Switzerland).

### *Plasmids and expression vectors*

pPRS202b: pQE30 (Qiagen, Germany) containing a BamHI and SalI PCR fragment of full length cDNA encoding human TDG cloned into the respective restriction sites (PCR primers were: BamATG - 5'GCACGTGGATCCATGGAAGCGGAGAACGCG-3');

TAASal - 5'CCGTGTCGACTTTAAGCATGGCTTTCTTCTTCCTG-3'). This cloning strategy generates a 6-histidine-TDG fusion open reading frame downstream of a promoter-operator element consisting of phage T5 promoter and two *lac* operator sequences. pPRS203b: N140A mutant; pPRS204b N140D mutant; pPRS205: M269H mutant; pPRS209: A145S mutant

### *Site-directed Mutagenesis*

*In vitro* mutagenesis of human TDG was performed using the QuickChange™ Site-Directed Mutagenesis Kit from Stratagene (USA) according to the manufacturer's instructions. pPRS202b served as template for mutagenesis and the oligonucleotide primers used to generate the individual mutations were as follows (sense strand sequences shown only):

hsTDG-N140D: 5'-GTCATTATTGGCATAGACCCGGGACTAATGGC-3';

hsTDG-A145S: 5'-CCCGGGACTAATGTCIGCTTACAAAGGGC-3';

hsTDG-N140A: 5'-GTCATTATTGGCATAGCCCCGGGACTAATGGC-3';

hsTDG-M269H-u: 5'-GAAACTCTCTGCTATGTTCATCCATCATCCAGTGC-3'

### *Purification of recombinant TDG proteins*

Expression constructs for the individual TDG variants were co-transformed with the *lacI*-repressor encoded on plasmid pREP4 (Qiagen) into *E.coli* BL21(DE3) cells by electroporation. Transformants were selected on LB-plates containing 100 µg/ml ampicillin, 50 µg/ml kanamycin and 2% glucose after incubation at 30°C. TDG expression cultures of 1 l LB medium containing the same ingredients were inoculated with 25 ml of an overnight culture and incubated at 30°C until an OD<sub>600</sub> of 0.8 was reached. After lowering the temperature of the culture to 20°C over a period of 30 minutes, TDG



expression was induced by the addition of 200  $\mu$ M IPTG and incubation was allowed to proceed at 20°C for a further 4 h. The cells were harvested by centrifugation (Sorvall SLA-3000, 5000 rpm, 4°C, 30 min) and the pellets stored at -80°C. The thawed cell pellets were resuspended in 3 ml/g sonication-buffer (50 mM Na-phosphate pH 8.0, 300 mM NaCl, 10% glycerol, 1 mM imidazol, 10 mM  $\beta$ -mercaptoethanol, 1 mM PMSF) and lysed by sonication on ice (25 x 10 second bursts with intermittent chilling for 10 seconds). After removal of cell debris by centrifugation (Sorvall SS34, 15000 rpm, 4°C, 30 min), 1 ml of sonication-buffer-equilibrated Ni-NTA-Agarose (Qiagen) was added to the crude lysate and incubated for 1 h at 4°C with gentle shaking. The suspension was then packed into a disposable column from which unbound protein was washed out with sonication buffer containing stepwise increasing concentrations of imidazol: 1x15 column-volumes (cv) 1 mM imidazol; 5x5 cv 20 mM imidazol; 1x5 cv 60 mM imidazol. Finally, bound histidine-tagged TDG protein was eluted with 5x1 cv sonication buffer containing 300 mM imidazol. The 300 mM imidazol fractions were pooled and dialyzed overnight at 4°C against binding buffer (50 mM Tris/HCl pH 8.0, 10% glycerol, 5 mM  $\beta$ -mercaptoethanol). After loading the dialyzed fraction onto a 1 ml Resource™Q FPLC column (Pharmacia) and washing with 10 ml binding buffer, bound protein was eluted with a linear gradient of 0-500 mM NaCl in 30 ml. The nearly-homogeneous TDG protein (>98% pure) eluted as a major protein peak in fractions containing approximately 150 mM NaCl. After a last dialysis step against storage buffer (50 mM Tris/HCl pH 8.0, 50 mM NaCl, 10% glycerol, 5 mM  $\beta$ -mercaptoethanol) the pure TDG protein was stored in aliquots at -80°C.

### *Glycosylase Activity Assay*

The enzymatic activity of the recombinant wild-type and mutant proteins was monitored by means of a standardized nicking assay. 60-mer double-stranded

oligonucleotide substrates containing different mismatches were prepared by annealing of an unlabeled upper strand oligonucleotide 5'-

TAGACATTGCCCTCGAGGTACCATGGATCCGATGTCXACCTCAAACCTAGACG

AATTCCG-3' to a 5'-fluorescein-labeled lower oligonucleotide strand 5'-<sup>F</sup>-

CGGAATTCGTCTAGGTTTGAGGTYGACATCGGATCCATGGTACCTCGAGGGCA

ATGTCTA-3', where X = G or A and Y = C, T, U or 5-FU (5-fluorouracil). Strand

annealing was carried out in 10 mM Tris/HCl pH 8.0, 50 mM NaCl with 0.5  $\mu$ M labeled and 1  $\mu$ M unlabeled oligonucleotide by heating to 95°C for 5 minutes and gradual cooling to 25°C over 30 min.

Standardized nicking-reactions were carried out in 20  $\mu$ l total volume in 1x nicking buffer (50 mM Tris/HCl pH 8.0, 1 mM DTT, 0.1 mg/ml BSA, 1 mM EDTA) containing 1 pmol substrate DNA, 1 pmol TDG protein and 0.5 U UGI. The reactions were incubated for 15 min at 37°C. Deviating assay conditions are indicated where appropriate. The reactions were then stopped and the generated AP-sites were cleaved by the addition of 1 N NaOH to a final concentration of 90 mM and heating to 99°C for 10 min. Subsequently, the DNA was ethanol-precipitated at -20°C for 1 h, following the addition of tRNA to a final concentration of 0.4 mg/ml and of sodium acetate (pH 5.2) to 0.3M. It was collected by centrifugation (Eppendorf, 14000 rpm, 4°C, 20 min) and washed in 80% ethanol (-20°C). The dried pellets were resuspended in 10  $\mu$ l of formamide gel loading buffer (90% formamide, 1x TBE), heated for 5 min at 99°C and chilled on ice immediately. The samples were then loaded onto a 15% denaturing polyacrylamide gel (acrylamide : bisacrylamide = 19:1, 1x TBE, 8 M urea) (BioRad, Mini Protean II cell), pre-run for 15 min at 450 V. The gels were run for 5 min at 450 V and subsequently at 250 V until the bromphenol blue dye reached the bottom of the gel. Fluorescein-labeled DNA was visualized using the blue-fluorescence mode of the Storm 860 (Molecular Dynamics) and

analyzed by ImageQuant software (Molecular Dynamics). For kinetic assays, the reactions were performed in larger volumes with an enzyme concentration of 50 nM and substrate concentrations ranging from 50 nM – 25  $\mu$ M. After different time points, samples were withdrawn, stopped by the addition of NaOH and further treated as described above.

#### *Electrophoretic Mobility Shift Assays (EMSA)*

EMSA were performed to measure the DNA-binding ability of wild-type and mutant TDG proteins, using the double-stranded oligonucleotide substrates described above. Substrate containing an AP-site was generated as follows: 10 pmol of duplex DNA containing a uracil opposite G or A at position Y in the labeled oligonucleotide (see above) was incubated with 1 U of uracil-DNA-glycosylase (Roche Diagnostics, Switzerland) in 1x UDG-buffer (50 mM Tris/HCl pH 8.0, 1 mM DTT) in a total volume of 20  $\mu$ l for 2 h at 37°C. Accuracy and completion of AP-site formation was tested by NaOH treatment and denaturing gel-electrophoresis as described above for the nicking assay. Fully- processed substrate DNA was then stored at -20°C and used for EMSA.

In standard EMSA, 4 pmol of TDG protein were incubated in a 10  $\mu$ l reaction mixture containing 1 pmol of labeled oligonucleotide substrate, 10 pmol of unlabeled homoduplex competitor DNA, 50 mM Tris•HCl pH 8.0, 1 mM DTT, 5% glycerol and 1 mM EDTA. After 15 min at 37°C the reactions were loaded immediately onto 6% native polyacrylamide gels in 0.5 x TBE (BioRad, Mini Protean II cell) and electrophoresis was carried out in 0.5 x TBE for 50 min at 100 V at room temperature. The fluorescent probes were visualized using a Storm 860(Molecular Dynamics) in the blue-fluorescence mode and the ImageQuant software (version 1.2) was used for the subsequent quantitative analysis of the fluorescent signals.

In substrate dissociation assays, 2 pmol of TDG protein were preincubated with 1 pmol of labeled substrate in 1x binding buffer and a total volume of 10  $\mu$ l. After 10 min at 37°C, different molar excesses (0, 10 and 20 fold) of unlabeled unspecific competitor (homoduplex) or specific, mismatch-containing competitor substrate was added and the reaction was left to proceed for another 10 min at 37°C. Substrate binding was then analyzed on 6% native polyacrylamide gels as described above.

## RESULTS

*Rationale of Site-Directed Mutagenesis* - The existence of TDG homologues in organisms ranging from bacteria to man documents that the enzyme is of ancient origin and has a highly conserved structure and function. The degree of evolutionary relationship between the catalytic core domains of TDG homologues across the species *E. coli*, *S. pombe*, *D. melanogaster* and *Homo sapiens* is illustrated in Fig. 1. Highlighted are two conserved sequence motifs that build critical parts of the enzyme's active site, as deduced from the crystal structure of *E. coli* Mug. These are the N-terminal GINPGL and the C-terminal NPSGLSR sequences. The amino acid Asn18 in the N-terminal motif is the predicted catalytic residue in Mug (14) and is present in all TDG homologues identified thus far. The structural model suggests a role for this asparagine in activation of a water molecule for the hydrolytic attack of the N-glycosidic bond of the flipped-out base to be released. Residues of the less conserved C-terminal motif NPSGLSR form a structural interface for the protein-DNA interactions involved in the base-flipping/helix-intercalation mechanism proposed for Mug (14). These functions are comparable to those assigned to equivalent motifs in the well characterized uracil DNA glycosylase enzyme (UNG) (15,17) (Fig.1). Yet, the helix intercalating residues of Mug, unlike those of UNG, establish and

maintain specific contacts to the Watson-Crick face of the widowed G in the complementary DNA strand (14,16). Ser23 of Mug is part of a small, flexible helix contributing to the fold of the active site and appears to interfere with the accommodation of the hydrophobic thymine within the catalytic pocket. The equivalent residue in the human enzyme is the smaller and less polar Ala145, a substitution that was proposed to account for the human enzyme's ability to process thymine-containing substrates (16).

To test whether the functional predictions from the Mug crystal structure are applicable to the human TDG enzyme, we mutagenized the putative active site residue Asn140 to Ala (N140A) or Asp (N140D), the presumed DNA binding site residue M269 to His (M269H), as well as Ala 145 to Ser (A145S) according to standard site-directed mutagenesis procedures.

*Expression and Purification of Recombinant TDG Variants-* To facilitate purification, we expressed the wild-type and mutant variants of human TDG in *E. coli* as N-terminal fusions with a 6-histidine-tag. Using a simple two step purification scheme involving metal affinity chromatography (Ni-NTA) and FPLC (Resource Q), we were able to produce 5-10 mg of pure protein from 1 l of culture. Consistent with previous observations (5), we noticed that moderate expression at low temperature (20°C) was required to avoid the formation of inclusion bodies with insoluble protein. Regarding solubility, the proteins carrying mutations at position N140 and A145 behaved as wild-type TDG, whereas the M269H mutant gave rise to a lower amount of soluble protein. All TDG variants showed fractionation properties identical to the wild-type TDG.

*Enzymatic Properties of the TDG Variants* - Human TDG releases thymine and uracil from G•T and G•U mismatches to generate AP-sites opposite G (7). We examined the

ability of the mutant TDG proteins to catalyze this reaction. As substrate we used fluorescein labeled, double-stranded and single-stranded oligonucleotides of 60 base pairs length carrying a target lesion 24 base pairs away from the labeled 5' end. Upon incubation with TDG, we monitored the generation of alkaline sensitive AP-sites by treatment of the reactions with NaOH and separation of the cleaved products from the alkaline resistant substrate fragments in denaturing gels. To analyze the DNA-binding capacity of the mutant proteins, we performed gel retardation assays with fluorescently-labeled oligonucleotide substrates in the presence or absence of specific and unspecific competitor DNA. Because TDG is very strongly product inhibited under *in vitro* assay conditions, we based our comparisons on single turnover kinetics of AP-site formation under standard reaction conditions. The specific conditions applied were equimolar enzyme substrate ratios and incubation at 37°C, the parameters for comparison were total amount of substrate processed ( $P_{\max}$ ) and the time requirement for processing 50% of the plateau levels for each substrate ( $T_{50}$ ). These values are summarized for each TDG variant in Table 1. Enzyme-substrate interactions were assessed as equilibrium binding capacity under standardized EMSA conditions described in 'Experimental Procedures'.

Consistent with previously reported observations (8), we found that purified recombinant wild-type TGD efficiently processed G•U and G•T substrates and very weakly also uracil in A•U base pairs, whereas uracil in ssDNA was resistant to cleavage by the enzyme (Fig. 2A). In the absence of competitor DNA, the wild-type enzyme bound homoduplex DNA nearly as well as the G•T and G•U heteroduplex substrates or an AP-site opposite G (G•AP) (Fig. 4A). Only when substrate binding was assayed in the presence of an excess of homoduplex competitor DNA, did differences in affinities become more evident (Fig. 3A). Under these conditions, substrate binding efficiencies followed the order  $G\bullet AP = G\bullet U > G\bullet T \gg G\bullet C$ .

The N140A mutation abolished the catalytic potential of the enzyme. No G•T or G•U mismatch processing was detectable under standard assay conditions, neither in the presence of a 20-fold excess of enzyme over substrate as shown in Fig. 2B, nor after prolonged incubation times (not shown). Yet, this hydrolytic mutation was still proficient in substrate binding (Fig. 3B). While, in comparison with the wild-type protein, G•C and G•AP binding by the mutant was nearly unaffected, G•U and G•T binding efficiency was decreased, with G•T binding being only marginally stronger than binding to the homoduplex substrate. This mutant thus displayed the following order of decreasing binding affinity: G•AP > G•U > G•T > G•C. The apparently stronger affinity of wild-type TDG for the G•U and G•T substrates is explained by the fact that under standard EMSA conditions, the wild-type enzyme releases the mismatched uracil and thymine bases from these substrates and remains bound to the G•AP sites (our data not shown, (8)).

Mutation of the same site to Asp (N140D) produced an enzyme with reduced catalytic activity. Whereas G•U was still processed ( $T_{50} = 13$  min), the mutant has lost the ability to measurably act on the G•T and A•U substrates (Fig. 2C, Table 1). Even in the presence of a 20-fold excess of enzyme over substrate and after prolonged incubation, no processing of the latter substrates was detectable (data not shown). Substrate binding by the N140D mutant appeared to be slightly destabilized compared to the wild-type protein or the N140A variant, but overall, it showed the same order of binding preference as did the N140A mutant, namely G•AP > G•U > G•T = G•C (Fig. 3C). Binding to G•C and G•T was more affected than binding to G•U and G•AP substrates, which is consistent with the enzyme converting G•U but not G•T substrates to G•AP products that are bound with the highest affinity.

Mutation of M269 to H resulted in an enzyme with reduced glycosylase activity on both G•T and G•U substrates. Fig. 2D shows that a higher than 10-fold excess of the

mutant protein over substrate is needed to yield near wild-type amounts of product under otherwise standard conditions. The substrate specificity as expressed by the ratio between G•U ( $T_{50}$  = 1.0 min) and G•T ( $T_{50}$  = 3.25 min) processing efficiency remained unaffected in this TDG variant (Table 1). A more dramatic effect appeared in the DNA binding assays. No mobility shift was detectable with either substrate indicated in Fig. 3D. Even in the presence of a large molar excess (30 fold) of enzyme over substrate, no interaction was evident under EMSA conditions (not shown). This documents an inability of this mutant protein to stably interact with the DNA substrate, which may account for the general reduction in its uracil and thymine glycosylase activity.

The double mutant N140D, M269H was unable to process or bind any of the substrates used (not shown), indicating that weakening both the catalytic activity and substrate binding capacity of the enzyme has a synergistic effect on its biochemical function.

Mutating Ala145 to Ser (A145S) did not notably change the enzymatic properties of TDG. In particular, we did not observe an effect on the enzyme's ability to process the G•T mismatched substrate (data not shown). Thus, contrary to predictions based on the Mug crystal structure (16), conversion of the equivalent of Ser23 to Ala in the human enzyme cannot adequately account for its acquired ability to process G•T mismatches.

*Insights into DNA interaction modes of human TDG* - TDG has properties of a general DNA binding protein, it efficiently binds different dsDNA substrates ranging from homoduplex DNA to mismatch- and AP-site containing duplexes. The molecular nature of these protein-DNA interactions is largely unknown. While homoduplex binding was not investigated, mismatch binding studies have been complicated by the fact that, under EMSA conditions, TDG readily converts the mismatched substrates to AP sites to which it



remains bound (8,18). Having generated a non-hydrolytic mutant, we were interested to explore substrate recognition and binding by TDG under conditions where no processing occurs. To assess the stability of TDG-substrate interactions, we pre-incubated the wild-type and the mutant proteins with labeled G•C, G•T, G•U and G•AP substrates under conditions where the binding and nicking reactions were completed. We then added non-specific or specific competitor DNA in 10- and 20-fold molar excess and allowed the reaction to proceed. Fig. 4A shows that binding of wild-type TDG to G•C homoduplex DNA was significantly reduced when G•C or G•AP competitor DNA was added. We therefore consider this mode of substrate interaction reversible. In contrast, no reduction in the amounts of protein-DNA complexes was seen when the wild-type enzyme was pre-incubated with either G•T, G•U or G•AP, even after addition of a 20-fold or higher molar excess of specific competitor DNA (Fig. 4A) or after prolonged incubation (not shown). This implies a different mode of DNA interaction, where TDG remains tightly bound to its substrate and cannot be turned over in our experimental system. Taking into account that wild-type TDG immediately processes G•T and G•U mismatches under EMSA conditions, these results document the ability of TDG to stably interact with AP-sites opposite G but do not reflect the substrate binding preference of the enzyme. We therefore performed the same experiments with the hydrolysis-deficient N140A mutant. This TDG variant was indistinguishable from the wild-type enzyme with respect to G•C binding but its interaction with G•T substrate was significantly more labile and reduced to undetectable levels when challenged with a 20-fold excess of specific competitor DNA (Fig. 4B). In contrast, G•U binding was only marginally affected by the addition of specific competitor DNA and the complex with G•AP totally resisted the presence of excess amounts of specific competitor DNA. We therefore conclude that interaction of human TDG with a substrate containing a G•T mismatch is reversible and, thus similar to its mode of

homoduplex DNA interaction, while G•U and G•AP site binding is more specific and highly stable.

*Processing of 5-fluorouracil and its implications on mechanistic properties of TDG -*

Human TDG processes G•T mismatches with a 10 fold lower rate than G•U (8). This can be explained by a lower affinity of the enzyme for the G•T mismatch and/or by a higher chemical stability of the N-glycosidic bond connecting the thymine with the deoxyribose. Whereas our substrate dissociation experiments indeed document a weaker interaction of TDG with the G•T mispair, the latter possibility gains some support from the fact that the 5-methyl group distinguishing thymine from uracil can act as an electron donor and, thus, may exert a stabilizing effect on the glycosidic bond. If so, the contrary might be expected for a uracil carrying a strong electron-withdrawing group at the same position. We were therefore interested to test the efficiency of base hydrolysis with the cytotoxic anti-cancer drug 5-fluorouracil (FU). We found that the wild-type enzyme processed substrates containing FU very efficiently and, interestingly, it did so not only when FU was mismatched with G but also when it was opposite A, or present in a single-stranded oligonucleotide (Fig. 5A, Table 1). The non-binding mutant M269H behaved as the wild-type protein, but was generally less efficient (Fig. 5B, Table 1). Surprisingly, our non-hydrolytic mutant N140A was also able to process the G•FU substrate, but not A•FU and FU in single-stranded DNA (ssDNA) (Fig. 5C, Table1). The fact that this catalytic mutant processed G•FU, while no cleavage was detectable with G•U and G•T (Fig. 2B) supports the view that the 5-fluoro substitution affects the stability of uridine such that a lower activation energy is required to hydrolyze the N-glycosidic bond. This is also in agreement with the effect of the N140D mutation, which has a less dramatically reduced catalytic

potential. This protein failed to process the G•T substrate, showed intermediate activity on G•U and highest activity with the G•FU substrate (Fig. 2C, 5D, Table 1).

Given these results, we pursued to examine whether transient interactions of the fully active wild-type enzyme with an energetically favorable substrate such as FU could be sufficient for base hydrolysis. This would predict that TDG is able to process FU substrate without engaging into stable complementary strand interactions and therefore in a reaction showing enzymatic turnover. To investigate this hypothesis, we performed comparative kinetic experiments with wild-type TDG protein and different ssDNA and dsDNA substrates. Initial rate comparisons of single turnover reactions revealed that TDG released FU faster than U and U faster than T in dsDNA substrates, whereas FU excision from ssDNA proceeded with a lower initial rate (Fig. 6A). However, in contrast to the reactions with double-stranded substrate, TDG appeared to dissociate more easily from the single-stranded AP-sites and showed steady state kinetics of FU processing at higher substrate concentrations (Fig. 6B). We therefore examined the kinetic properties of this reaction and determined its Michaelis-Menten parameters (Fig. 6C). The results substantiated that TDG was able to process the single-stranded FU substrate with a slow but steady turnover ( $k_{cat} = 0.041 \text{ min}^{-1}$ ) and a  $K_M$  of 114 nM. Under standard reaction conditions with an excess of dsDNA substrates, TDG processed less than one molar equivalent of G•U and G•T mismatches. The reactions with the G•5FU substrate also reached plateaus but at higher and substrate concentration dependent product levels corresponding to 1-5 molar equivalents of enzyme (e.g. Fig. 6B). The lack of turnover with the G•U and G•T substrates is explained by the fact that TDG needs to establish specific complementary strand contacts to be able to hydrolyze the mismatched uracil and thymine and then remains bound to the G•AP-site product after base release (Fig. 4A, (14,18)). The limited turnover with the G•FU substrate most likely reflects the ability of TDG to hydrolyze FU from G•FU without engaging in

stable complementary strand interactions. This results in an enzymatic turnover until the concentration of generated G•AP-site products is high enough to inhibit TDG through competitive binding. Taken together, these observations document that destabilization of the N-glycosidic bond can enhance the catalytic activity of TDG and obviate its need for stable interactions with the substrate.

## DISCUSSION

We generated and characterized mutant variants of human TDG protein that explored two structural motifs critical for its catalytic activity and its ability to interact with DNA. The crystal structure of *E. coli* Mug predicts the Asn in the highly-conserved motif GINPGL to be essential for the hydrolytic activity of the protein. Accordingly, we found that mutation of the equivalent Asn of TDG to Ala reduced the enzyme's catalytic potential to undetectable levels. By analogy to the postulated catalytic mechanism for Mug, we conclude that this mutation abolished the activation of a water molecule in the putative active site pocket, which was then not available for efficient hydrolysis of the N-glycosidic bond. Interestingly, G•U as well as G•AP-site binding was largely unaffected in this mutant, but its interaction with G•T mismatched substrate was clearly destabilized. Since in gel retardation experiments with wild-type TDG, AP-site binding rather than G•T or G•U mismatch binding is measured, and assuming that the N140A mutation does not alter the substrate recognition properties of the enzyme, we propose that the mismatch binding capacities of this mutant reflect the genuine substrate preferences of wild-type TDG. This view is consistent with the repeated observation that the wild-type enzyme processes G•T mismatches with a lower efficiency than G•U mismatches (Table 1, (8,9)).

The substrate dissociation characteristics of the N140A mutant revealed that TDG engages in at least two qualitatively different modes of DNA interaction, one being loose and reversible as observed with homoduplex and G•T mismatched DNA, and another being more rigid and resistant to competition as observed with G•U and G•AP substrates. Thus, the thymine of a G•T mismatch might be less well accommodated within the catalytic pocket of the enzyme than the uracil residue of a G•U mismatch. In consequence, the specific contacts to the Watson-Crick face of the mispaired G as evident in the structure of substrate-bound Mug (14,16) could not be properly established with a G•T substrate and, thus, G•T interaction would resemble homoduplex binding rather than G•U and G•AP binding. The structural analysis of Mug suggested that the side chain of Ser23 would clash with the 5'-methyl group of a thymine inside the active site pocket and therefore may account for the G•T discrimination of the bacterial enzyme (16). The equivalent position in the human TDG is occupied by a more hydrophobic alanine (A145), which may more easily facilitate the accommodation of a thymine, although crowding around the 5'-methyl group is still expected to occur. Therefore, the active site geometry of TDG could indeed explain a tolerated but disfavored interaction with G•T mispairs, but our finding that the critical A145S mutation in the human enzyme had no effect on its ability to process G•T substrate is inconsistent with such a mode for thymine discrimination. Also, it should be remembered at this point that the G•T processing activity of TDG was lost through the deletion of 112 N-terminal amino acids (9) without any changes occurring in the active site. This implies that G•T recognition and processing involves regions of TDG other than the immediate proximity of the substrate binding pocket. As the N-terminal extension is absent from Mug, the crystal structure of the bacterial TDG homologue can be of little help here. This phenomenon thus warrants further study.

Mutation of the methionine to histidine in the proposed DNA-interaction motif MPSSSAR generated a protein that failed to detectably bind to its substrate but was still able to hydrolytically process the mismatched substrates. Although more than 10-fold higher enzyme concentrations was needed to achieve wild-type levels of base removal, the substrate specificity of the mutant remained unchanged as compared to that of the wild-type enzyme. This suggests that a rate limiting step in reactions with the M269H mutant is substrate recognition/interaction rather than base hydrolysis, and that the loss of affinity for the substrate can be compensated for by increasing the enzyme concentration. However, the precise role of the mutated methionine in DNA interaction cannot be assigned solely on the basis of our biochemical data. By analogy to the situation in Mug, where the equivalent Asn is an N-terminal coordinate of the helix-intercalating and complementary strand-interacting residues, we propose a similar role for M269 in the human enzyme.

We found that the human wild-type TDG processes the artificial base 5-FU more efficiently than any other substrate tested. *In vivo*, FU is known to inhibit the thymidylate synthase with the cytotoxic consequence of a reduction in the dTTP pool and an increased incorporation of uridine into DNA. The molecular basis of the toxicity of FU is complex, but increased base excision repair activity due to misincorporation of uracil and possibly also FU has been proposed to lead to an accumulation of DNA strand breaks that can trigger cell death (19). Some tumors acquire resistance to FU by an unknown mechanism, but it appears likely that changes in relevant DNA repair functions may be involved (20). Although it is uncertain to what extent FU is incorporated into the DNA of proliferating cells (21), TDG could conceivably contribute to the toxicity of the drug through its ability

to efficiently process FU in a mismatch-independent manner. Thus, inactivation of TDG by mutation could well provide one route for acquiring tolerance to FU.

FU is distinct from uracil or thymine only at the 5 position, where it carries a fluoro substituent instead of a hydrogen or a methyl group, respectively. Whereas uracil DNA glycosylase actively excludes thymine from its catalytic pocket by interference mediated by a strategically-positioned tyrosine residue, no such function is apparent in the Mug structure (14). Although, we have demonstrated that TDG has a lower affinity for a G•T substrate than for a G•U substrate, our results on FU processing show that active site exclusion cannot fully account for its substrate specificity, in particular, it fails to explain why G•FU is more efficiently processed than G•U and why the enzyme excises FU but not U from ssDNA. If the chemical stability of the N-glycosidic bond rather than active site geometry discriminates between the substrates in question, FU would be expected to be released very easily, uracil with intermediate and thymine with lowest efficiency, and this is consistent with our experimental evidence. The fact that wild-type TDG releases FU even from ssDNA supports the view that transient substrate interactions are sufficient for hydrolysis of the N-glycosidic bond if only a low activation energy is required, and this is in agreement with the observation that reducing the catalytic potential of TDG by the mutation (N140A) limits its activity to the energetically most favorable G•FU substrate and brings back the need for a stable interaction with this substrate. It seems a reasonable strategy for an enzyme that hydrolyzes normal bases from DNA to make its action dependent on specific complementary strand interactions. In the case of thymine release by TDG, a sufficiently stable DNA interaction is established only if the opposite base is a guanine, providing thus the substrate discrimination needed to avoid inappropriate and non-specific base hydrolysis. Thus, evolution may have balanced the catalytic power of TDG with its substrate interaction properties in order to achieve the required substrate

specificity. Such requirement may be less important for glycosylases that release damaged bases from DNA, because in such cases, the specificity is achieved through physical recognition of structural irregularities of the inappropriate bases.

Our data establish that the *E.coli* Mug structure is a valid model for human TDG as far as the overall three-dimensional fold of its core domain and, thus, its basic reaction mechanism is concerned. Obviously, the bacterial model is inaccurate when it comes to explaining properties specific for the human enzyme, e.g. the wider substrate spectrum and the activity modulating role of its extra amino- and carboxy-terminal domains. Therefore, the ultimate understanding of the molecular mechanisms involved in substrate recognition, binding and hydrolysis by human thymine DNA glycosylase will have to await structural analysis of this enzyme in interaction with its substrates, and this is where the separation of function mutants characterized in this paper will be instrumental.

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## Footnotes

<sup>1</sup> The abbreviations used are: TDG, thymine DNA glycosylase; UDG, uracil DNA glycosylase; UGI, uracil DNA glycosylase inhibitor; dsDNA, double-stranded DNA; ssDNA single-stranded DNA; SDS-PAGE, SDS-polyacrylamide-gel electrophoresis, FU, 5-fluorouracil; AP-site, apurinic/apyrimidinic site

## Figure Legends

**Fig. 1. Evolutionary conservation of the catalytic domain in TDG homologues.** Shown is a partial amino-acid sequence alignment spanning the core domains of the TDG homologues of human (hsTDG, Accession No. U51166), *Drosophila melanogaster* (dmThd1, EMBL No. AJ277789), *Schizosaccharomyces pombe* (spThp1, EMBL No. AJ277958) and *Escherichia coli* (ecMug; Swissprot No. P43342). Identical residues are shaded and the amino-acid motifs forming the essential parts of the proposed active site pocket are framed. Residues in italics indicate the structural equivalent motifs present in the *Herpes simplex* virus uracil DNA glycosylase (hsvUDG; Swissprot No. P10186). Asterisks indicate the sites mutagenized in the human TDG (N140A, N140D, M269H or A145S).

**Fig. 2 Catalytic activity of wild-type and mutant TDG proteins.** The ability to generate alkaline sensitive sites in standard substrates was assayed for wild-type TDG (A) and the mutant variants N140A (B), N140D (C) and M269H (D) as described in 'Experimental Procedures'. Shown are the results obtained with dsDNA substrates containing either G•C, G•T, G•U or A•U base pairs or ssDNA containing a single uracil (U) at an identical position. The reactions were done in 20 µl volume and in the presence of 1 pmol of substrate DNA. The amounts of enzyme were 1 pmol in (A) and (C), 20 pmol in (B) and 10 pmol in (D). Reaction products were separated from substrates on 15% denaturing polyacrylamide gels. The positions of the 60-mer substrate DNA and 23-mer product fragment are indicated. The slightly shorter migrating fragment in the reaction with ssDNA reflects processing of a G•T mismatch generated in the secondary structure of the single-stranded substrate oligonucleotide.

**Fig 3. Binding of wild-type and mutant TDG proteins to substrate and product DNA.**

Gel retardation assays were performed with wild-type TDG (A) and the mutant variants N140A (B), N140D (C) and M269H (D) as described under EMSA in 'Experimental procedures'. The 10  $\mu$ l reactions contained 1 pmol of labeled substrate DNA as indicated, 10 pmol unlabeled homoduplex competitor DNA and 4 pmol of TDG. Bound fluorescein-labeled DNA was separated from free substrate DNA in 6% native polyacrylamide gels. A representative experiment is shown, with numbers at the bottom of the lanes representing the amounts of bound substrate (%) under steady-state conditions.

**Fig. 4. Substrate dissociation of wild-type TDG and the N140A mutant.** In this substrate dissociation assay, 2 pmol of wild-type or mutant TDG enzyme were preincubated at 37°C for 10 min with 1 pmol of labeled substrate DNA under EMSA conditions. The pre-formed protein-DNA complexes were then challenged by the addition of a 0, 10 or 20-fold molar excess of unlabeled non-specific or specific competitor DNA as indicated, and incubation was continued for a further 10 min at 37°C. The reaction products were separated in 6% native polyacrylamide gels. The positions of the free and bound substrate DNA are indicated and numbers below the lanes represent the amounts of bound substrate (%) in each reaction. Shown is the result of a representative experiment.

**Fig. 5. Processing of 5-fluorouracil and uracil substrates by wild-type and mutant TDG variants.** Generation of alkaline-sensitive sites was assayed with the dsDNA substrates G•U, A•U, G•FU, A•FU and with the ssDNA substrates containing U or FU as indicated. All reactions contained 1 pmol of substrate DNA. The amounts of protein were 1 pmol for the wild-type TDG (A), the N140A (C) and the N140D mutant (D) and 10 pmol

for the M269H mutant (B). Reactions were set up in 20  $\mu$ l volumes, incubated at 37°C for 15 min, and the products were separated on 15% denaturing polyacrylamide gels.

**Fig. 6. Kinetic properties of 5-fluorouracil processing by human TDG.** The time dependent generation of alkaline sensitive sites was assayed by incubation of human TDG with double-stranded 60-mer substrate containing a single G•U (●), G•T (◆) or G•FU (▲) mismatch or ssDNA containing FU (■). (A) Equimolar concentrations (50 nM) of substrate and TDG protein or (B) a five-fold molar excess of substrate over TDG protein (B) were incubated at 37°C and the reactions stopped after the indicated times by the addition of NaOH (see Experimental Procedures). Product formation was monitored and quantified after denaturing gel-electrophoresis and fluorescent scanning. (C) Lineweaver-Burk plot derived from a series of time course experiments with TDG protein and single-stranded FU substrate. The concentrations were 50 nM for TDG protein and 250 nM – 25  $\mu$ M for the ssDNA substrate. The resulting kinetic parameters are:  $K_M = 114$  nM;  $V_{max} = 2.05$  nM  $\text{min}^{-1}$ ;  $k_{cat} = 0.041$   $\text{min}^{-1}$ .

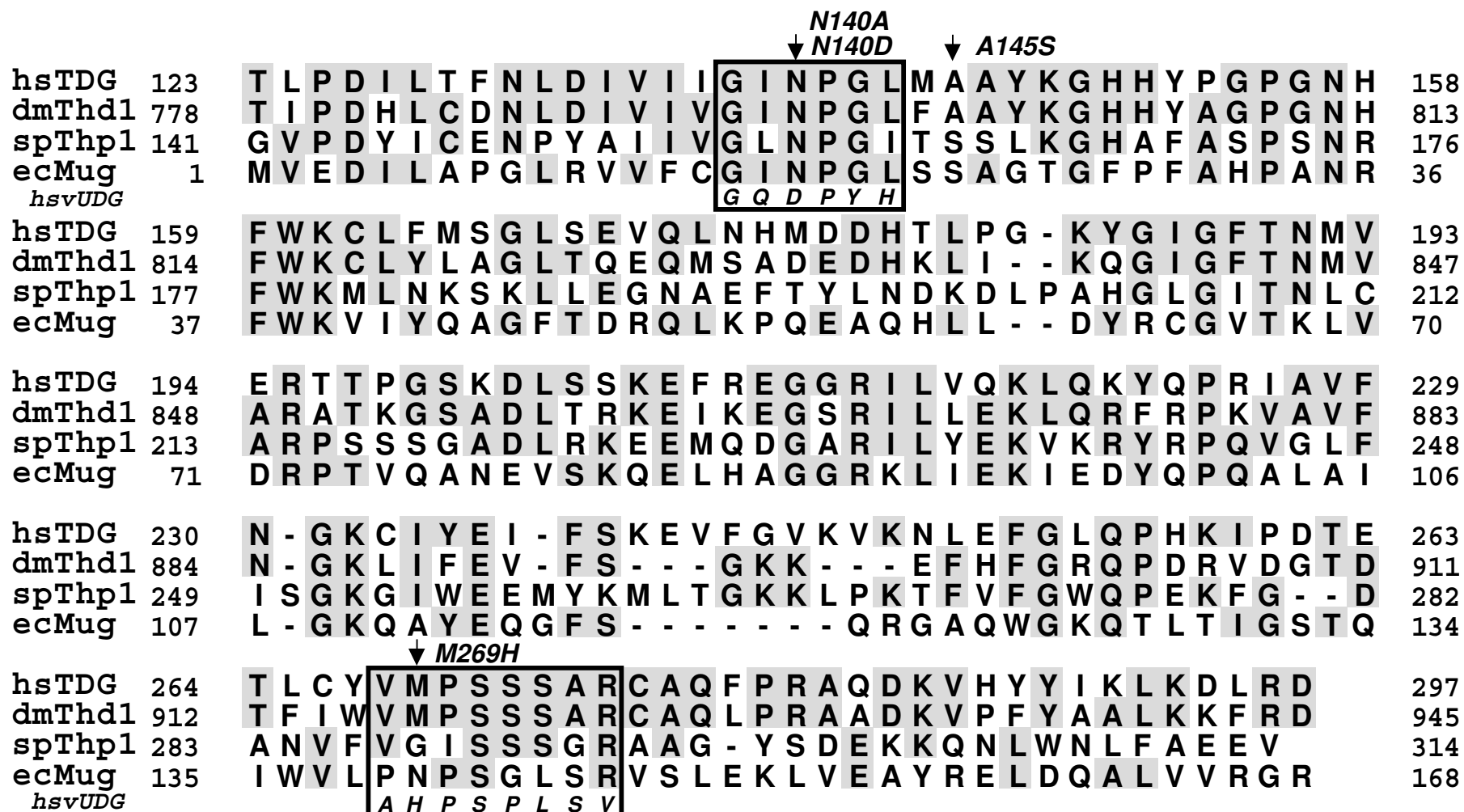
TABLE I

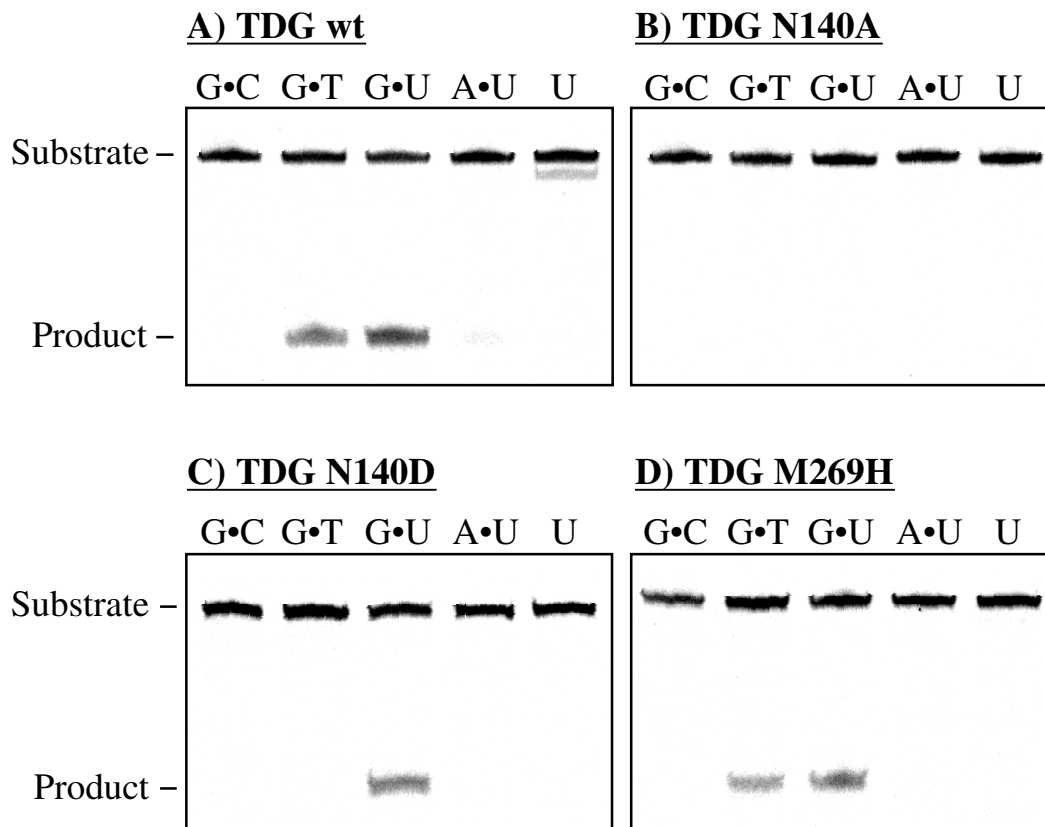
*Quantitative assessment of DNA glycosylase activities of wild-type and mutant variants of human thymine DNA glycosylase*

The generation of alkaline sensitive sites was assayed for wild-type TDG and the mutant variants N140D, N140A and M269H. Standard assays were performed as described in 'Experimental Procedures' with the substrate concentration being 50 nM for all and the TDG concentrations 50 nM for the wild-type, the N140D, and the N140A mutant and 500 nM for the M269H mutant. Reaction products were quantified after denaturing gel-electrophoresis by fluorescent scanning. Shown are the plateau levels of substrate nicking ( $P_{\max}$ ) and the time required for processing of  $1/2 P_{\max}$  ( $T_{50}$ ) for each TDG variant and the substrates as indicated.

| Substrate | TDG        |          | N140D      |          | N140A      |          | M269H      |          |
|-----------|------------|----------|------------|----------|------------|----------|------------|----------|
|           | $P_{\max}$ | $T_{50}$ | $P_{\max}$ | $T_{50}$ | $P_{\max}$ | $T_{50}$ | $P_{\max}$ | $T_{50}$ |
|           | (%)        | (min)    | (%)        | (min)    | (%)        | (min)    | (%)        | (min)    |
| G•U       | 64±1       | 0.55     | 65±1       | 13±1     | <5         | nd       | 43±1       | 1.0      |
| G•T       | 40±2       | 1.90     | <5         | nd       | <2         | nd       | 29±1       | 3.25     |
| A•U       | 11±1       | >13      | <2         | nd       | <2         | nd       | <5         | nd       |
| G•FU      | 90±2       | 0.55     | 80±1       | 1.1      | 40±2       | 9.35     | 49         | 0.9      |
| A•FU      | 54±2       | 2.15     | <2         | nd       | <2         | nd       | >75        | 22.5     |

Fig. 1, Hardeland et al







**Fig. 3, Hardeland et al**

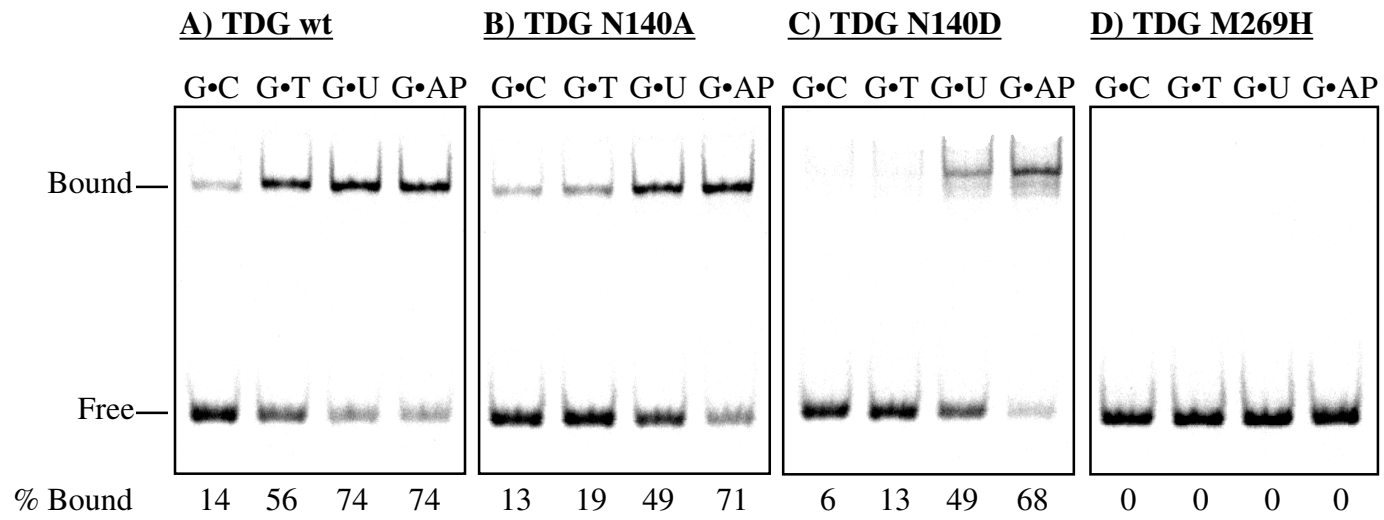
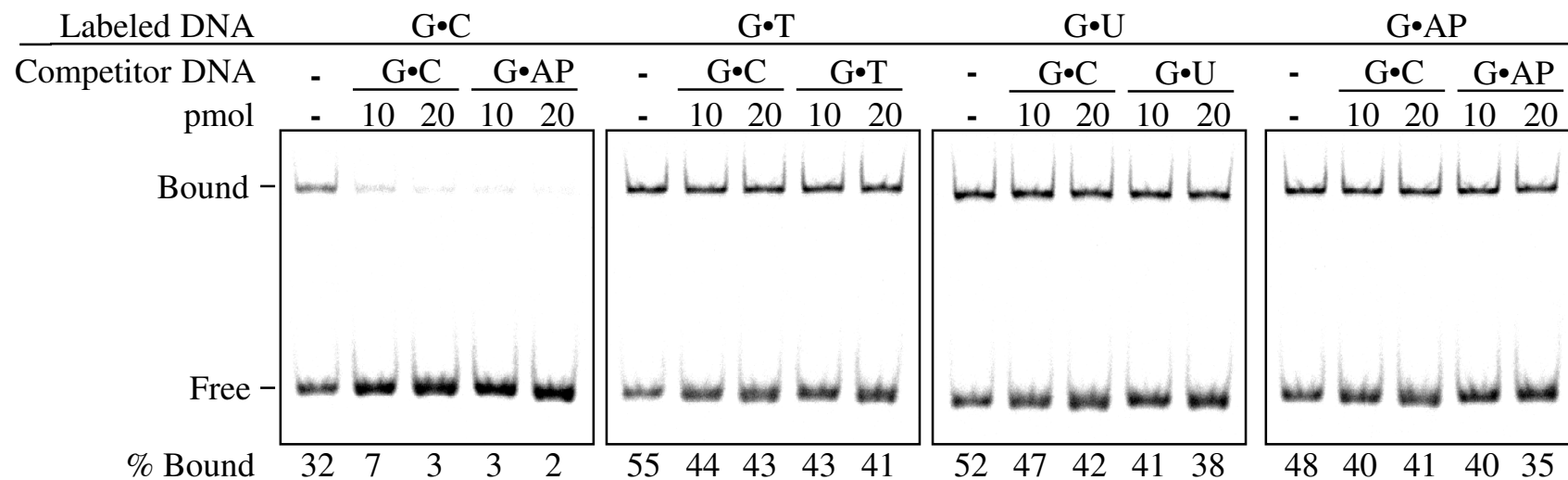
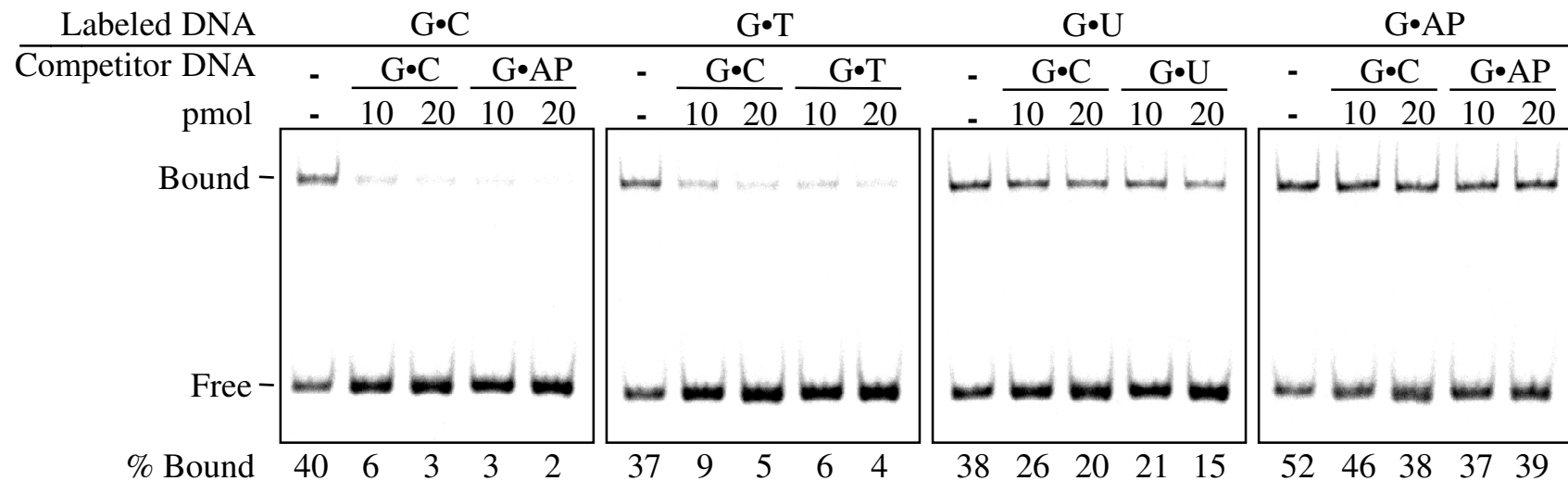


Fig. 4, Hardeland et al

**A) TDG wt****B) TDG N140A**

**Fig. 5, Hardeland et al**

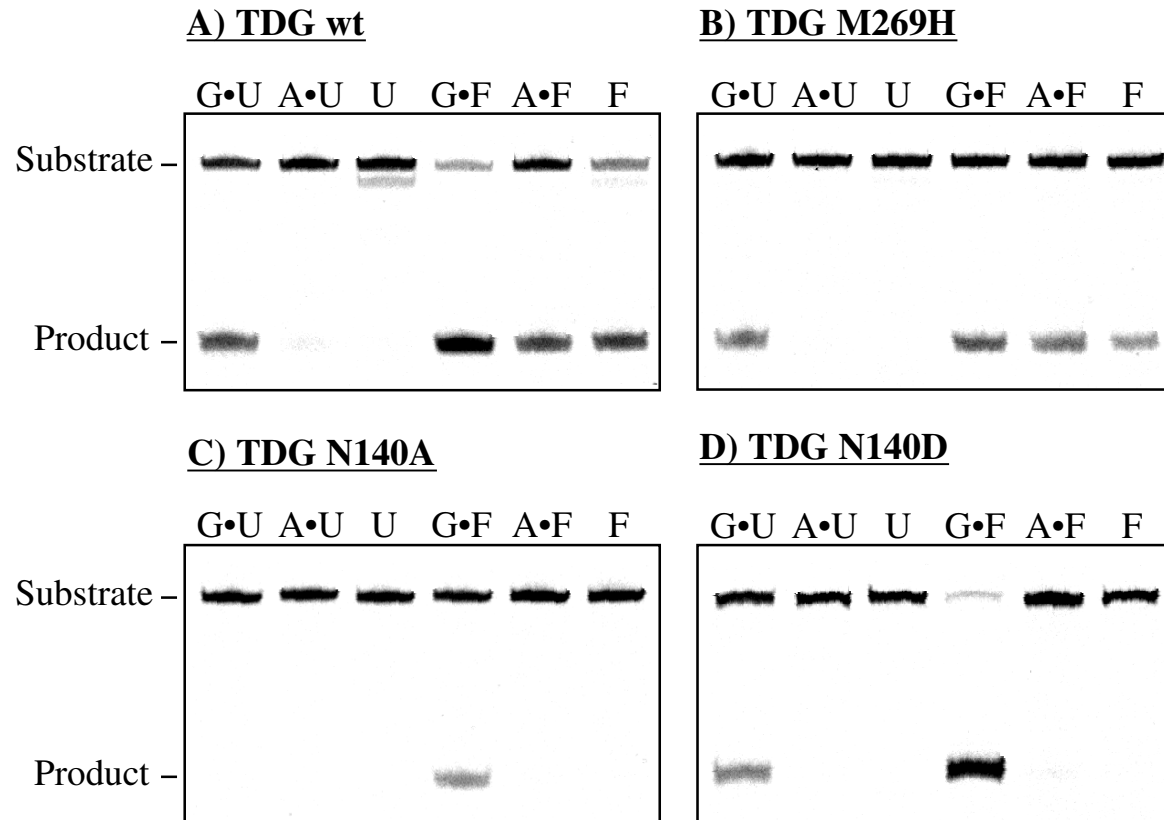


Fig. 6, Hardeland et al

