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Human Base Excision Repair for Preservation of Genomic Stability

Doctoral thesis
for the degree of doctor philosophiae

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Norwegian University of Science and Technology
Faculty of Medicine
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To the memory of my brother Massoud who passed away at young age

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LIST OF PAPERS

List of papers

I: Kavli, B., Sundheim, O., Akbari, M., Otterlei, M., Nilsen, H., Skorpen, F., Aas, P.A., Hagen, L., Krokan, H.E., and Slupphaug, G. (2002) hUNG2 is the major repair enzyme for removal of uracil from DNA, with hSMUG1 as a broad specificity backup. *J. Biol. Chem.*, **277**, 39926-39936.

II: Pena-Diaz, J., Akbari, M., Sundheim, O., Farez-Vidal, M. E., Andersen, S., Sneve, R., Gonzalez-Pacanowska, D., Krokan, H.E., and Slupphaug, G. (2004) *Trypanosoma cruzi* contains a single detectable uracil-DNA glycosylase and repairs uracil exclusively via short patch base excision repair. *J. Mol. Biol.* **342**, 787-799.

III: Akbari, M., Otterlei, M., Pena-Diaz, J., Aas, P.A., Kavli, B., Liabakk, N.B., Hagen, L., Imai, K., Durandy, A., Slupphaug, G., and Krokan, H.E. (2004) Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells. *Nucl. Acids Res.* **32**, 5486-5498.

IV: Akbari, M., Otterlei, M., Krokan, H.E. Different organization of base excision repair of uracil in DNA in nuclei and mitochondria. Manuscript.

V: Kvaløy, K., Nilsen, H., Steinbekk, K.S., Nedal, A., Monterotti, B., Akbari, M., and Krokan, H.E. (2001) Sequence variation in the human uracil-DNA glycosylase (*UNG*) gene. *Mut.Res.* **461**, 325-338.

VI: Akbari, M., Dore Hansen, M., Halgunset, J., Skorpen, F., and Krokan, H.E. (2005) Low copy number DNA template can render polymerase chain reaction error prone in a sequence-dependent manner. *J. Mol. Diagn.* **7**, 36-39.

ABBREVIATIONS

Abbreviations

ADP	adenosine diphosphate
AP-site	apurinic/apyrimidinic site
APE1	AP endonuclease 1
APE2	AP endonuclease 2
ATM	ataxia-telangiectasia mutated kinase
ATR	ataxia-telangiectasia- and Rad-3-related kinase
ATP	adenosine triphosphate
BER	base excision repair
BrdU	bromodeoxyuridine
BSA	bovine serum albumin
CAK	cyclin-dependent kinase-activating kinase
cccDNA	covalently closed circular DNA
CDK	cyclin-dependent kinase
cDNA	complementary DNA
<i>C.elegans</i>	Caenorhabditis
CPD	cyclobutane pyrimidine dimer
CREB	cyclic AMP-response element binding protein
CS	Cockayne syndrome
C-terminus	carboxyl terminus
CTP	cytidine triphosphate
dATP	deoxyadenosine triphosphate
dCTP	deoxycytidine triphosphate
ddNTP	dideoxyribonucleoside triphosphate
dGTP	deoxyguanosine triphosphate
DHU	5,6-dihydro-2'-deoxyuridine
DNA	deoxyribonucleic acid
DNA-PK	DNA-dependent protein kinase
DNA-PK _{CS}	DNA-dependent protein kinase catalytic subunit
dNTP	deoxyribonucleotide triphosphate
<i>D. melanogaster</i>	Drosophila melanogaster
dRp	5'-deoxyribose-5-phosphate
DSB	DNA double-strand break

ABBREVIATIONS

DSBR	double-strand break repair
dsDNA	double-strand DNA
DTT	dithiothreitol
dTTP	deoxythymidine triphosphate
EDTA	ethylenediaminetetraacetic acid
ERK	extracellular signal-regulated kinase
<i>E. coli</i>	<i>Escherichia coli</i>
FADH ₂	flavin adenine dinucleotide (reduced form)
FapyA	4,6-diamino-5-formamidopyrimidine
FapyG	2,6-diamino-5-formamidopyrimidine
5-fU	5-formyluracil
GST	glutathione S-transferase
GTP	guanosine triphosphate
HEPES	N-[2-hydroxyethyl]-piperazine-N'-[2-ethanesulphonic acid]
HIV	human immunodeficiency virus
HMG	high-mobility group
hmU	5-hydroxymethyluracil
H ₂ O ₂	hydrogen peroxide
5-HOC	5-hydroxycytosine
5-HOU	5-hydroxyuracil
HPV	human papillomavirus
HRR	homologous recombination repair
IDL	insertion/ deletion loop
IR	ionizing radiation
kDa	kilo dalton(s)
MAPK	mitogen-activated protein kinase
MBD4	Methyl-CpG-binding protein 4
MEF	murine embryonic fibroblasts
MEK	MAPK/ERK kinase
3-meA	3-methyladenine

ABBREVIATIONS

7-meG	7-methylguanine
MMR	mismatch repair
MMS	methyl methanesulfonate
MNNG	N-methyl-N'-nitro-N-nitrosoguanine
MPG	3-meA-DNA glycosylase (mammalian)
MSI	microsatellite instability
MYH	MutY homolog DNA glycosylase
mtDNA	mitochondrial DNA
NAD ⁺	nicotinamide adenine dinucleotide (oxidized form)
NADH	nicotinamide adenine dinucleotide (reduced form)
NER	nucleotide excision repair
NHEJ	non-homologous end joining
N-terminus	amino terminus
NTH1	thymine glycol DNA glycosylase
O ⁶ -meG	O ⁶ -methylguanine
O ⁴ -meT	O ⁴ -methylthymine
8-oxoG	7, 8-dihydro-8-oxoguanine (8-oxoguanine)
OGG1	8-oxoguanine DNA glycosylase
PARP	Poly(ADP-ribose) polymerase
PCNA	proliferating cell nuclear antigen
PKC	protein kinase C
POL	DNA-dependent DNA polymerase
POL β	DNA polymerase β
POL ϵ	DNA polymerase ϵ
POL δ	DNA polymerase δ
POL γ	DNA polymerase γ
PRR	post-replication repair
Pt-GTG	1,3-intrastrand d(GpTpG) cisplatin-DNA crosslink
RFC	replication factor C
RNA	ribonucleic acid
RNAi	RNA interference
ROS	reactive oxygen species
RPA	replication factor A

ABBREVIATIONS

<i>S. pombe</i>	Schizosaccharomyces pombe
ssDNA	single-strand DNA
SDS	sodium dodecyl sulphate
SSDB	single-strand DNA break
SDBR	single-strand DNA break repair
shRNA	short hairpin RNA
siRNA	small interfering RNA
SMUG1	single-strand-selective monofunctional uracil-DNA glycosylase 1
<i>S. cerevisiae</i>	Saccharomyces cerevisiae
SV40	simian virus 40
TCR	transcription-coupled repair
TDG	thymine-DNA glycosylase
TFIIH	transcription factor IIH
Tg	thymine glycol
TOP	topoisomerase
TTD	trichothiodystrophy
UDG	uracil-DNA glycosylase (family of proteins)
UNG	uracil-DNA glycosylase (name of the gene and its products)
UTP	uridine triphosphate
UV-light	ultraviolet light
XP	xeroderma pigmentosum
XRCC1	X-ray cross complementation group protein 1

INTRODUCTION

Introduction

An organism is a living entity that has developed the ability to store its biological information in form of linear arrangements of four bases in deoxyribonucleic acid (DNA), and has the capacity to transfer this information to its offspring. However, DNA is not stable and the spontaneous damage occurs at high rates under normal physiological conditions. In addition, the genome is under constant attack from endogenous as well as environmental factors that can alter its chemical structure.

Types of DNA damage

Bases in DNA as well as the sugar and phosphate backbone are constantly damaged. Three of the four bases normally present in DNA (cytosine, adenine, guanine), as well as 5-methylcytosine contain exocyclic amino groups. Spontaneous hydrolytic deamination converts these bases to uracil, hypoxanthine, xanthine, and thymine respectively (Figure 1), of which some give rise to mutations during replication.

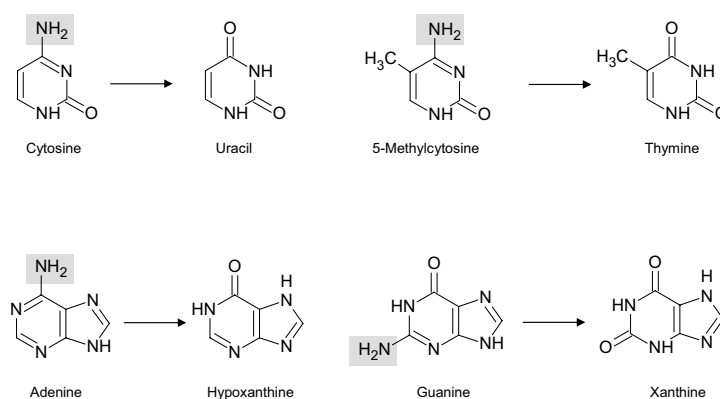


Figure 1. Products formed from the deamination of bases in DNA

Spontaneous loss of bases, mostly purines, occurs at considerable rates that generate the cytotoxic and mutagenic apurinic/apyrimidinic (AP) sites (1). Attack by reactive oxygen species (ROS) is a major source of DNA damage. ROS can generate mutagenic oxidative base lesions (8-oxo-G) and modified bases that can block replication (thymine glycol) (Figure 2), as well as strand breaks (reviewed in (2)). Alkylation lesions in DNA result from endogenous compounds, environmental agents and alkylating drugs. Alkylating agents are electrophilic compounds with affinity for nucleophilic centers in DNA (Figure 3). In general, the ring nitrogens of the bases are nucleophilic, with the N⁷ position of guanine and the N³ position of adenine the most reactive, followed by O⁶ in guanine (3). Alkylated bases can be mutagenic (O⁶-mG) or can block replication (3-meA). Alkylation of oxygen in the phosphodiester linkage results in the formation of phosphotriesters, which apparently are not repaired and are assumed to be relatively harmless (3).

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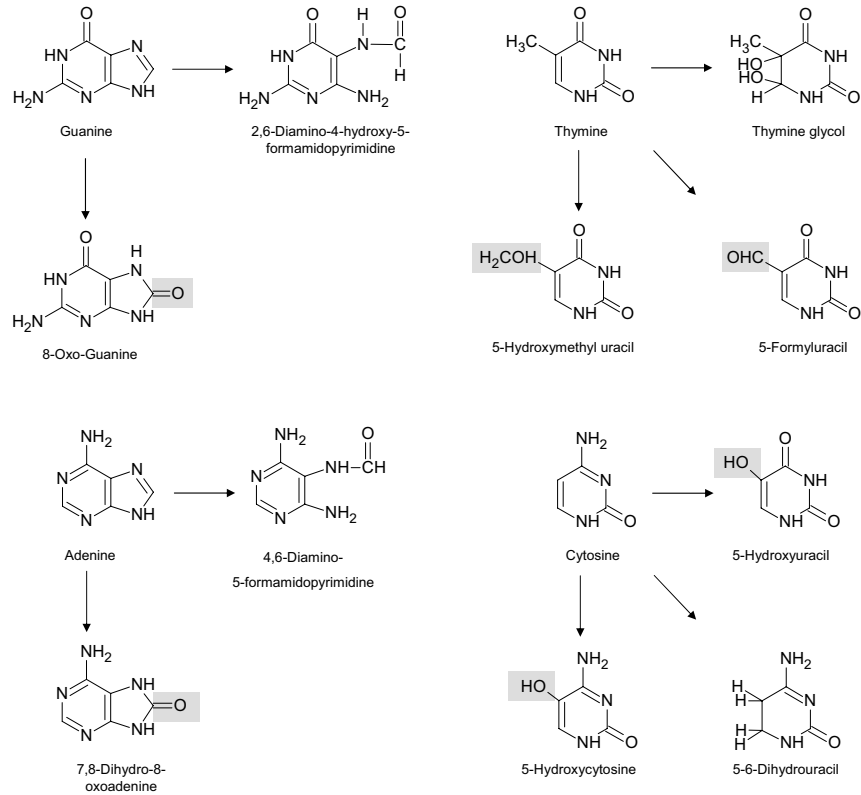


Figure 2. Some common oxidative base damage in DNA

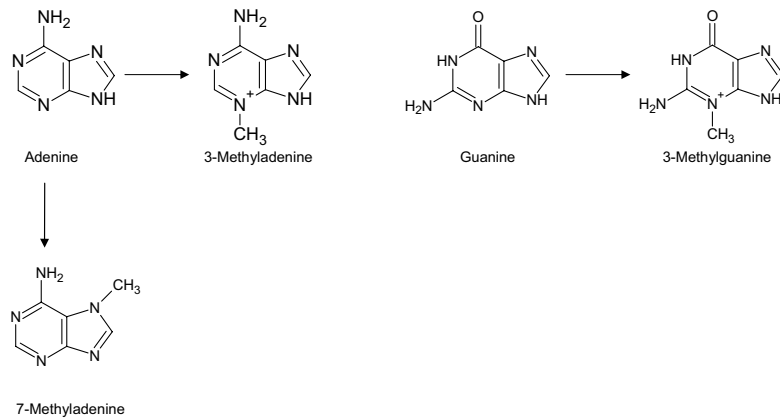


Figure 3. Some products of alkylation damage of bases in DNA

Normal, but mismatched bases, can be introduced in DNA during replication of tracts of short repeated sequences (DNA polymerase slippage) (4). Certain modified bases in the template DNA can cause insertion of incorrect bases by replicative DNA polymerases into nascent DNA, giving rise to mutations. UV-light at 260-310 nm is absorbed by the bases, exciting them, giving rise to bulky adducts, e.g. cyclobutane dimers between adjacent pyrimidines (3). These DNA distorting lesions block DNA replication and transcription and cause strand breaks. High energy ionizing radiation (IR) cause base modifications as well as single- and

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double-strand DNA break (DSB). Single-strand DNA breaks (SSDB) can be converted to DSB when encountered by replisomes (3).

Large scale chromosomal rearrangements frequently occur in human cells, but some specific forms are associated with disease like leukemia. Chromosomal translocations create a fusion gene by bringing together sequences from two genes located in different chromosomes (5).

The consequences of DNA lesions

The types of DNA damage (base damage or strand break), the location of the lesions in the genome (a promoter region, introns or exons, and actively transcribed regions or transcriptionally silent regions), as well as the cell cycle, and type of the cell affect the level of mutagenicity or cytotoxicity of DNA lesions. Damaged bases are by far the most frequent form of DNA lesions (6). One major consequence of DNA base lesions is the introduction of mutations that could result in the alteration of the genetic information. In this respect, a significant group of DNA base lesions are relatively harmless until they are “fixed” as mutations during replication. Hence, DNA repair is particularly important in actively replicating cells. Accordingly, non-dividing human cells are also known to accumulate base damage in their genome (7). Mutations may contribute to the evolutionary diversity of the species (8). The capacity of an organism to evolve is named evolvability or evolutionary adaptability, and includes the capacity to generate heritable mutations through selectable phenotypic variations (9). However, evolutionary favorable mutations are rare events and most of the mutations are neutral or harmful at the cellular level and cause disease and may reduce the biological fitness of the affected individual. The oxidative base damage 8-oxo-guanine can pair with adenine during replication resulting in G:C to T:A transversion mutation. Deamination of cytosine to uracil occurs at a rate of 100 to 500 bases per cell per day causing C:G to T:A transition mutations upon DNA replication (10,11). Therefore, this class of base damage is referred to as mutagenic lesions. Another group of base lesions is considered potentially cytotoxic because they can block DNA replication or RNA polymerase and may result in cell death. Base lesions like uracil that likely do not arrest RNA polymerase may result in mutant transcripts and protein molecules (12,13). However, it should be noted that cells can switch from a replicative DNA polymerase to different damage bypass DNA polymerases that permit replication across lesions in a process known as translesion DNA synthesis. *In vitro* experiments have demonstrated that several bypass DNA polymerases in fact insert correct bases into the nascent DNA across certain lesions in template DNA (14,15). The biological importance of translesion DNA synthesis was demonstrated when xeroderma pigmentosum variant (XP-V) patients were found to carry mutations in the POLH gene that encodes POL η (16), a member of the Y-family damage bypass DNA polymerases.

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How DNA lesions are detected?

A central question in the process of DNA repair is how lesions are detected in the human genome consisting of 3.2×10^9 base pairs. The organization of the chromosomes adds more complexity to this problem. The DNA is wrapped around a core of histone proteins forming beadlike structures of nucleosomes. The nucleosome core comprises 147 base pairs DNA and eight histones, two of each of the four core families- H2A, H2B, H3, and H4. A minimum of another 20 base pairs of DNA stretches between nucleosomes is complexed with the linker histone H1. The string of nucleosomes is folded into a shorter, thicker filament, called a 30-nm fiber. This is further folded into fibers, 100 to 300 nm in diameters, which are organized into loop domains of 15, 000 to 100, 000 base pairs (17) (Figure 4).

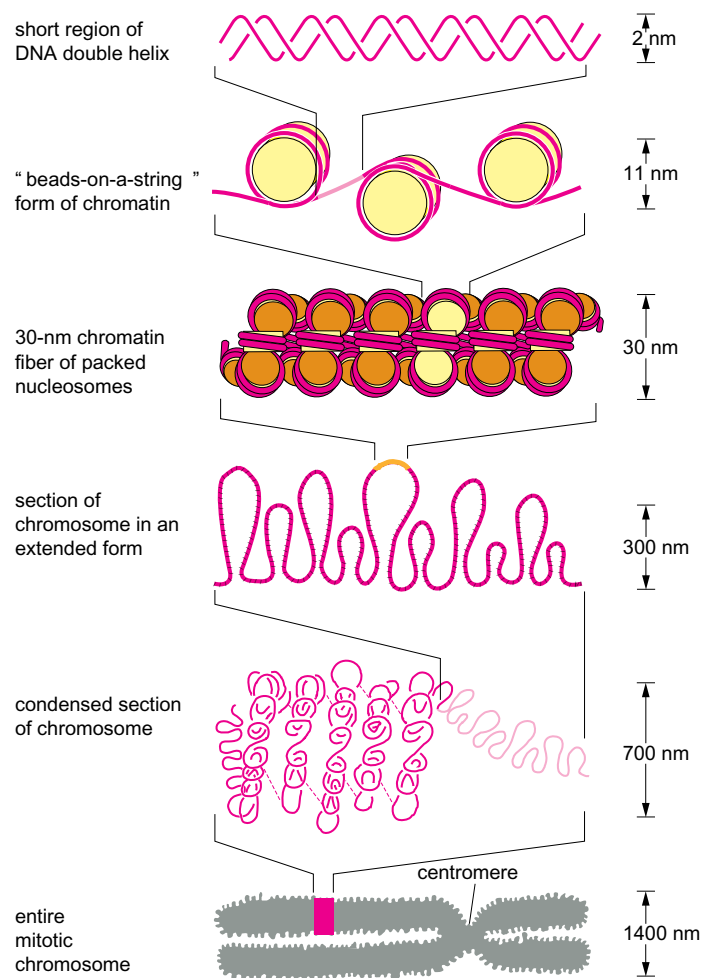


Figure 4. Levels of chromatin packing. (From <http://www.accessexcellence.org> Published by Garland Publishing, The Taylor & Francis Group)

The cellular responses to DNA damage and mechanisms of damage recognition vary considerably depending on the type of damage. Single- and double-strand breaks are cytotoxic, only few DSBs can cause cell death. The cellular response and repair systems handling these types of damage have characteristics distinct from

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excision repair of base lesions and bulky adducts. Higher eukaryotic cells primarily repair DSBs by one of two pathways; nonhomologous end joining (NHEJ) and homologous recombination (HR). NHEJ repairs broken ends with little or no requirement for sequence homology. The ends of the broken DNA are recognized by heterodimer Ku consisting of Ku 70 and Ku 80 proteins. Ku is an abundant molecule at about 4×10^5 molecules per cell (18). The average distance between two Ku molecules is only 4-6 times the Ku diameter which means that there is an average one Ku molecule within 4-6 molecular diameters of a DSB (18). The end-processing (removal of 5' and trimming of 3' overhangs) is carried out by a 469 kDa protein known as the DNA-dependent protein kinase catalytic subunit (DNA-PK_{cs}) in complex with Artemis. The final ligation step is done by XRCC4-LIG4 complex. HR is active in replicating cells and repairs DSBs that occur as a result of collapse of the replication fork. In contrast to NHEJ, HR uses extensive homologous DNA sequences making it in most cases error-free but also a slower repair process. In human cells the main steps in HR are mediated by RPA, Rad51, Rad52, and Rad54. The key protein in HR is Rad51 that forms nucleoprotein filament on ssDNA regions and catalyzes the search for homologous sequences, strand pairing and strand exchange.

Immediately after occurrence of a DSB, a remarkably rapid cascade of protein modifications starts, mainly in the form of phosphorylation. The histone H2AX becomes phosphorylated within one minute (γ - H2AX) in a chain reaction covering several mega bases in either direction of the site of break (reviewed in (19)). This wave of H2AX phosphorylation facilitates rapid recruitment of repair factors. The rapidity of response and availability of DSB repair proteins in the nucleus are excellent configurations distinct for DSB detection and repair. Other proteins that constitute the early cellular responses to DSBs are ataxia telangiectasia mutated (ATM), and ATM-and-Rad3-related (ATR) kinases, and the MRE11/RAD50/NBS1 complex (MRN complex). While ATM seems to be the main kinase that responds to ionizing radiation, the ATR pathway is dominant in the signaling pathway triggered by stalled replication forks or UV-light. ATM is rapidly activated upon induction of a DSB (20) and regulates cell cycle and DNA repair by phosphorylation of key proteins in these systems (reviewed in (21)).

The rapidity of the cellular responses to SSDB shares characteristics of responses to DSB. Poly (ADP-ribose) polymerase-1 (PARP-1) is rapidly and directly activated by SSDB. Upon binding to a SSDB, PARP-1 catalyzes polymer synthesis of ADP-ribose on nearby proteins as well as itself and the levels of polymers can increase more than 100-fold in minutes (22). These events “flag” the position of DNA damage and start a rapid repair process of recruitment of repair proteins mainly through XRCC1-associated repair protein complexes (23,24).

DNA replication and transcription as well as chromatin remodeling complexes facilitate DNA damage recognition and repair. Insertion of normal but inappropriate bases during replication is repaired by

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the post-replicative mismatch repair (MMR) pathway. In *E.coli* the major MMR is the MutHLS pathway. MutS initiates repair by binding to the mismatched bases and activates together with MutL, the MutH endonuclease, which incises DNA at hemimethylated *dam* sites and thereby mediates strand discrimination. In humans MMR can be initiated by the heterodimeric complexes MSH2-MSH6 (MutS α) and MSH2-MSH3 (MutS β). Insertion/deletion loops (IDLs) with up to eight unpaired nucleotides are recognized by MutS β , and base-base mismatches by MutS α . The heterodimer MLH1-PMS2 (MutL α) is the major human MutL activity. No MutH homologue has been identified in eukaryotes, suggesting that strand discrimination in these organisms is different from *E.coli*. (25). Exactly how human MMR distinguishes the newly inserted normal but wrong base from the base in template DNA is not completely understood. However, the ends of Okazaki fragments on the lagging strand as well as PCNA likely play key roles in the process of strand discrimination by human MMR (26).

A number of chemicals as well as UV-light generate bulky base adducts that cause helical distortion. *cis-syn*-cyclobutane dimers (CPDs) and 6-4 pyrimidine-pyrimidone photoproducts (6-4PPs) are formed between adjacent pyrimidines, and constitute the two major classes of lesions induced by UV-light. These lesions are substrate for nucleotide excision repair (NER). The initial damage recognition step of NER involves distinct enzymes and is generally divided into a global genome repair (GGR) and a transcription-coupled repair (TCR) (27). The XPC/hHR23B complex and the UV-DNA damage-binding complex (UV-DDB) comprised of DDB1 and DDB2 subunits, are the initial and damage recognition enzymes in GGR. Upon binding to the DNA lesion, they initiate a repair reaction that involves about 30 enzymes. The initial DNA damage recognition step in TCR involves the action of Cockayne syndrome A and B proteins (CS-A and CS-B) (27) (discussed later).

It appears that certain repair proteins are spatially organized in tight association with the nuclear matrix (28). Thus, aberrant DNA structures may be brought into proximity to repair proteins at fixed sites. Lamin A is a major component of the nuclear lamina and nuclear skeleton. Truncation in lamin A causes Hutchinson-Gilford progerial syndrome (HGPS), a severe form of early-onset premature aging. Very recently, truncated lamin A was found to act dominant negatively to perturb DNA damage response and repair, resulting in genomic instability (29). Although it needs further investigation, spatial organization of DNA repair proteins may be an important cellular mechanism for damage detection and repair.

In a highly compacted DNA-protein complex all cellular processes that use the DNA as a template, including DNA repair require a high degree of coordination between the DNA repair machinery and chromatin modification/remodelling, which regulates the accessibility of DNA in chromatin. Most small base lesions are recognized and removed by DNA glycosylases that start base excision repair. Typically, base excision repair analyses have relied on the use of damaged DNA substrates that are not assembled into nucleosomes

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or chromatin. A few recent *in vitro* analyses have shown that chromatin structure of DNA impedes action of DNA glycosylases and BER (30,31). Thus, factors other than the “core” BER proteins may be required for the efficient repair of base lesions in chromatin. Reports are emerging that rather than stochastic collisions, at least some DNA glycosylases undergo posttranslational modifications as well as protein-protein interactions that channel them to the damaged bases and facilitate damage recognition and repair. Hence, specific association of DNA glycosylases with certain auxiliary proteins, DNA replication proteins and possibly transcription factors as well as chromatin remodeling complexes may facilitate the initial step of base excision repair.

DNA repair

In their historical article in *Nature* in 1953, Francis Crick and James Watson wrote: “It has not escaped our notice that the specific pairing we have postulated immediately suggests a possible copying mechanism for their genetic material”. However, they completely overlooked possible existence of mechanisms for maintenance of the integrity of DNA. In 1974, Crick wrote rather regrettably : “We totally missed the possible role of ... repair although ... I later came to realize that DNA is so precious that probably many distinct repair mechanisms would exist” (32). Since then, various DNA repair mechanisms have been identified that maintain the integrity of DNA (reviewed in (33)). DNA repair pathways function as integral parts of an interconnected network of DNA metabolism mechanisms, cell cycle regulation, cell survival and apoptosis that makes it impossible to present a comprehensive article about human DNA repair pathways without addressing other cellular mechanisms. Even an in-depth coverage of one “DNA repair pathway” in a single article is a challenging task. The focus of the present work is base excision repair (BER) in human cells and I will only briefly address other DNA repair pathways. Several proteins known as BER enzymes are also involved in other DNA metabolism processes. So, throughout this review by a BER protein I merely mean a protein that has a specific function in BER pathway. I will briefly discuss the function of each BER enzyme, but will provide in rather more detail the available reports on protein-protein interactions as well as posttranslational modifications of BER proteins. I will discuss BER in association with DNA replication and transcription, BER in disease, and finally mitochondrial BER and ageing.

Base excision repair (BER)

BER pathways play a major role in counteracting the cytotoxic and mutagenic effects of a broad range of DNA lesions caused by environmental (exogenous) as well as spontaneous (endogenous) factors. Until few years ago, BER was thought to function in a simple and linear pathway in which individual repair enzymes carried out the catalytic reactions independently of one another. In this model, BER started by the

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removal of a damaged base by a DNA glycosylase generating a baseless or an apurinic/aprimidinic (AP) site. This was followed by the incision of DNA at the 5' end of the AP-site by the action of AP endonuclease 1 (APE1), then insertion of the correct nucleotide(s) by DNA POL β or POL δ/ϵ , the removal of 5'-dRP residue by POL β or flap endonuclease 1 (FEN1), and finally end joining and completion of the repair by a DNA ligase (Figure 5). However, we know now that BER pathways are regulated at several levels including

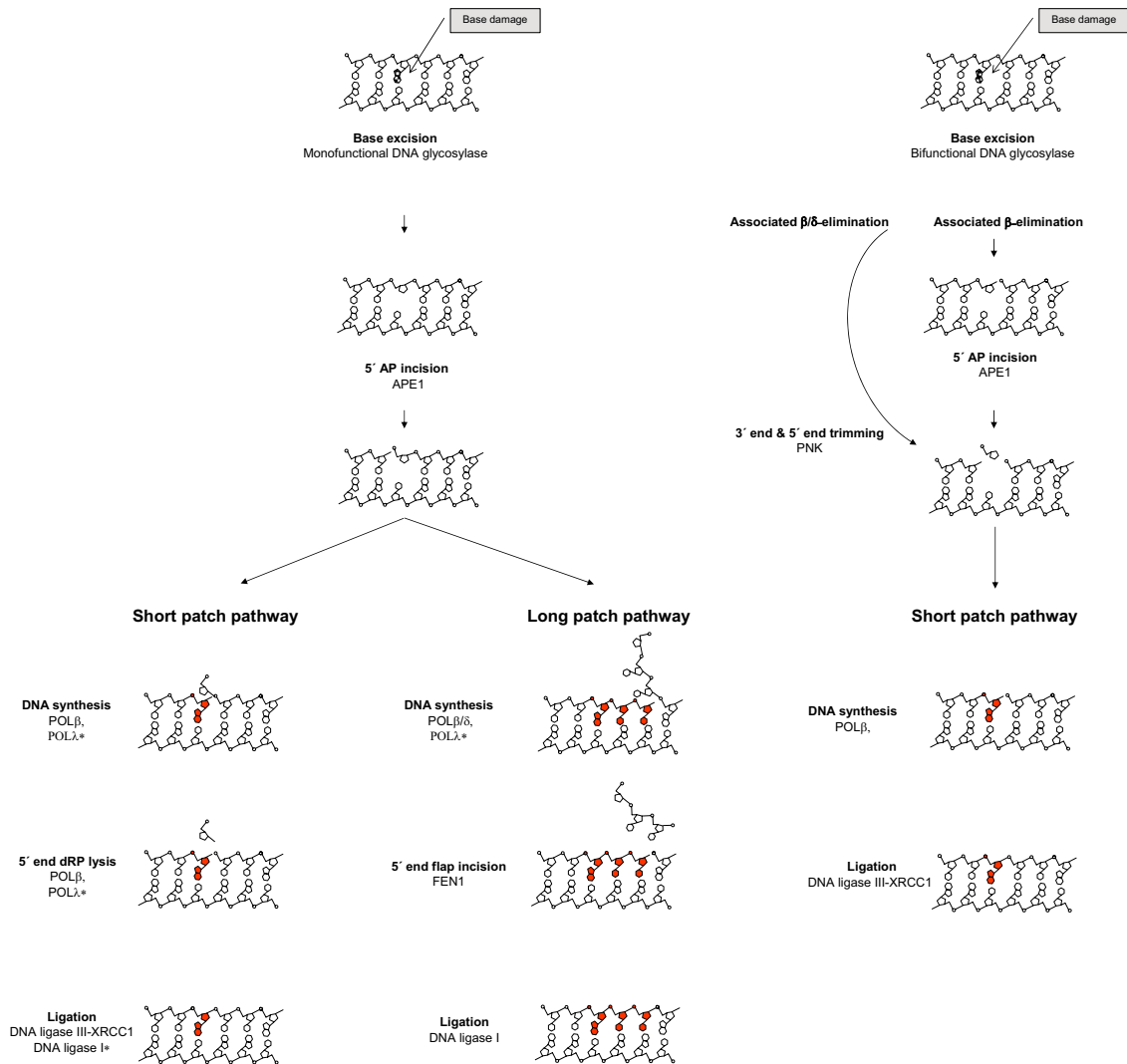


Figure 5. A simplified presentation of BER pathways in human cells. (* Has been reported but needs further investigation).

posttranslational modifications of BER proteins (Table 1) as well as through the action of some auxiliary factors, and specific protein-protein interactions (34). Moreover, the cell cycle regulates the expression of several BER proteins and importantly affects the mechanisms of BER pathways, adding more complexity

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Table1. Proteins involved in human BER.*

PROTEIN	LOCALIZATION	SUBSTRATES, FUNCTIONS	MODIFICATIONS
UNG2	Nucleus	ssU, U/G, U/A), alloxan, isodialuric acid, 5-hydroxyuracil	Phosphorylation (cell cycle specific, UV-light)
UNG1	Mitochondria	ssU, U/A, U/G)	None
SMUG1	Nucleus	ssU, U/A, U/G), isodialuric acid, 5-hydroxyuracil, alloxan, 5-fU, 5-hmU	
TDG	Nucleus	T/G, U/G	Acetylation, SUMOylation
MBD4	Nucleus	U/G, T/G, ϵ C/G (CpG-context)	
MYH	Nucleus, mitochondria	A/8-oxoG, 2-OH-A/G	Phosphorylation (PKC?)
OGG1	Nucleus, mitochondria	8-oxoG/C, associated AP lyase	Phosphorylation
NTH1	Nucleus, mitochondria	Tg, Fapy, dihydroxyuracil, dihydroxyuracil, urea associated AP lyase	
NEIL1	Nucleus	Tg, 5-OHU, 5-OHC, urea, Fapy, 8-oxoG, associated β, δ elimination	
NEIL2	Nucleus	5-OHU, 5-OHC, AP-sites, associated β, δ elimination	Acetylation (p300)
NEIL3	Nucleus		
MPG	Nucleus	3-meA, 7-meG	
APE1	Nucleus, mitochondria	5'-endonuclease at AP-sites, DHU, and redox reaction	Phosphorylation (CK I/II, PKC), acetylation
APE2	Nucleus, mitochondria	Weak 5'-endonuclease at AP-sites	
POL β	Nucleus	DNA polymerase, dRP lyase	Acetylation (p300)
POL δ	Nucleus	DNA polymerase with associated proof reading function	
POL γ	Mitochondria	DNA polymerase with associated proof reading function, dRP lyase	
FEN1	Nucleus	Incises RNA-DNA flaps & DNA flaps, 5'-3'exonuclease	Phosphorylation (cdk1), acetylation (p300)
LIG I	Nucleus	DNA ligation	Phosphorylation (CKII)
LIG III	Nucleus, mitochondria	DNA ligation	
XRCC1	Nucleus	Scaffolds DNA repair proteins	Phosphorylation (CKII)
PARP-1	Nucleus	poly(ADP-ribosyl)ation	
PCNA	Nucleus	Sliding clamp for replication & repair proteins	Ubiquitination, SUMOylation, acetylation
RPA	Nucleus	Binds ssDNA during replication & repair	Phosphorylation

* See the text for references.

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to the function of BER. An example of a highly coordinated BER pathway is the repair of 8-oxoG during DNA replication. 8-oxoG is the most stable and mutagenic oxidative base damage known, that in its *syn*-conformation can pair with adenine during replication resulting in a G:C to T:A transversion mutation. 8-oxoG can also occur in DNA by incorporation of 8-oxodGTP opposite adenine during replication. Hence, repair of A:8-oxoG mispairs during DNA replication requires precise timing for removal 8-oxoG and adenine in the nascent strand to avoid fixation of mutation in DNA (reviewed in (35)).

DNA glycosylases

DNA glycosylases start BER by removing a damaged base from DNA. X-ray crystallographic analysis of several DNA glycosylases complexed with DNA have shown that a general strategy is used by DNA glycosylases. So, upon binding to the damaged base, DNA glycosylases “flip” the aberrant base from DNA into a specific pocket in the active site followed by hydrolysis of the N-glycosylic bond between the damaged base and deoxyribose producing an AP-site in DNA. In human cells, 11 DNA glycosylases are identified so far (Table 1). DNA glycosylases are relatively small monomeric proteins that do not require cofactors for their function (reviewed in (36)). Most DNA glycosylases are monofunctional and only remove the damaged base, while some are bifunctional with an associated AP lyase activity producing a 3'-deoxyribosephosphate residue. The recently discovered DNA glycosylases (NEIL1 and NEIL2) have an associated β/δ elimination function and generate a gap in DNA after removal of the damaged base. The kinetics of DNA glycosylases have been extensively studied and found to vary considerably (37). DNA glycosylases UNG2 and MYH that function in association with DNA replication show higher rates of turnover (37), possibly in order to cope with the speed of DNA synthesis during the replication. DNA glycosylases show varying substrate preference and specificity. UNG is highly specific for removal of uracil from DNA, while 3-meA-DNA glycosylase (MPG) removes several different types of alkylated (Table 1) as well as normal bases.

Uracil-DNA glycosylase (UNG)

In *Ung*^{-/-} mouse embryonic fibroblast cells, the number of steady state level of uracil in DNA was estimated about 3600 uracil residues/diploid genome (38). This number includes uracil from the incorporation during replication and from the deamination of cytosine that occurs at a rate of 100 to 500 bases per cell per day (10,11). Gene-targeted mice deficient in *Ung* displayed a modest rate of mutation in a nontranscribed *lacI* reporter transgene (39), and developed B-cell lymphoma (40). The nuclear (UNG2) and the mitochondrial (UNG1) isoforms of UNG are generated from a single gene by alternative splicing and transcription from different positions in the *UNG* gene (41). UNG2 is the major DNA glycosylase for removal of uracil, both

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from incorporation of uracil during replication (42) and deamination of cytosine which occurs anywhere in the genome (43). UNG1 is constitutively expressed and possesses a unique 35 amino acid N-terminal region that contains a mitochondrial targeting signal. The expression of UNG2 is cell cycle regulated (44-46), and has a unique 44 amino acid N-terminal that contains a nuclear localization signal (41). This region is the target of multiple phosphorylation events (47) and is the site of interaction with PCNA (42) and RPA (48). The function of the phosphorylation modifications is not completely understood. The level of phosphorylated protein peaks during the S-phase of the cell cycle, but apparently none of the phosphorylations alters the enzymatic activity of UNG2 (47). A role for phosphorylation might involve regulation of interaction of UNG2 with PCNA and RPA during the replication. UNG2-associated BER complexes have been reported (49-51). It remains to be seen whether specific phosphorylations of UNG2 may facilitate formation of such complexes. Recently, a role for UNG2 phosphorylation was reported to be the cell cycle dependent degradation of the protein (52).

A recently identified interaction between UNG2 and PPM1D (Wip1) phosphatase is particularly interesting (53). PPM1D is transcriptionally upregulated by p53 following UV-light and IR exposure. UNG2 was phosphorylated at threonines 6 and 126 following UV-light irradiation. The UV-light-induced phosphorylated forms of UNG2 were more active in the removal of uracil from DNA. Dephosphorylation of UNG2 by PPM1D at phosphothreonine 6 reduced activity of UNG2 (53). Although the biological relevance of activation of UNG2 in response to UV-light needs further investigation, these observations demonstrate the regulation of function of UNG2 by transient posttranslational phosphorylation.

A recently discovered role for UNG2 is in immunoglobulin diversification in B-cells (54). Here, UNG2 plays a role distinct from its function in BER by creating AP-sites which are not correctly repaired. Rather, they facilitate induction of mutations in the immunoglobulin genes.

Analysis of the posttranslational modifications of UNG2 as well as specific protein-protein interactions will likely help us understand the mechanisms that regulate the function of UNG2 in such diverse contexts.

Hypoxia is associated with a variety of DNA lesions. Some of the first associations between hypoxia and DNA damage came from earlier studies on the pathophysiology of reperfusion injury. In a reperfusion injury the greatest tissue damage observed after a transient period of decreased blood flow and occurred during the return of blood flow to that region (55). A brief cerebral ischemia enhanced BER including uracil-DNA glycosylase activity (56). *Ung*^{-/-} fibroblasts and primary cortical neurons showed increased cell death when treated with a nitric oxide donor and oxygen-glucose deprivation, respectively (57). *Ung*^{-/-} mice had major increases in infarct size after focal-brain ischemia as compared with the control mice. In *Ung*^{+/+} the mitochondrial UDG activity was increased after cerebral ischemia (57). Increased UDG activity

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might be a result of a general cellular response to ischemia. However, data indicate a role for UNG in repair of oxidatively damaged bases (58,59).

Single-strand-selective monofunctional uracil-DNA glycosylase 1 (SMUG1)

SMUG1 was identified by an *in vitro* expression cloning strategy and screening for enzymes that bound to synthetic DNA glycosylase inhibitors (60). SMUG1 is a member of the UDG family of proteins (61). SMUG1 is found in insects and vertebrates but is not present in *C.elegans*, *A. thaliana*, and yeast (62). SMUG1 was proposed to provide a “backup” activity for UNG (63). Although, both this and later work has provided evidence for a more specific function of SMUG1. So, in addition to uracil, SMUG1 removes the oxidized bases 5-hydroxymethyluracil (5-hmU) and 5-formyluracil (5-fU) (63,64). A more specific role for SMUG1 was demonstrated by identification of a direct interaction between SMUG1 and POL λ for repair of oxidative DNA damage (65). Stable siRNA-mediated silencing of *Smug1* in mouse embryo fibroblasts generated a mutator phenotype (58). The cells were also sensitive to ionizing radiation further supporting a role for *Smug1* in the repair of oxidative DNA lesions (58).

Human thymine-DNA glycosylase (TDG)

TDG is a monofunctional mismatch-specific T/U:G DNA glycosylase that removes thymine or uracil in CpG contexts as a result of deamination of 5-methylcytosine or cytosine, respectively. TDG associates with transcriptional coactivator CREB binding protein (CBP/ p300) and is a substrate for CBP/p300 acetylation (66). The acetylation apparently regulates recruitment of APE1 (66). p300 may play a regulatory role in TDG-initiated BER by easing the topological constraints on chromatin and facilitating recruitment of APE1 to the AP-site produced by TDG. In addition to CBP/p300, TDG interacts with transcription factors; retinoid receptors (RAR/RXR) and the thyroid transcription factor-1 (TTF-1) suggesting a role for TDG in transcription regulation. TDG also interacts with, and is covalently modified by the ubiquitin-like proteins SUMO-1 and SUMO-2/3 (67). TDG strongly associates with AP-sites *in vitro* (68). Interestingly, both forms of the SUMOylation reduced AP-site binding affinity of TDG (67).

A physical and functional interaction between TDG and xeroderma pigmentosum group C protein (XPC) has been reported (69). The interaction enhanced the enzymatic turnover of TDG. *In vitro* experiments suggested that XPC and TDG likely compete for binding to the opposite G, causing dissociation of TDG from DNA and promoting access of APE1 to the AP-site (69).

In vitro experiments have shown that most DNA glycosylases are inhibited by their products, the AP-sites, thus protecting these highly reactive repair intermediates. Reversible protein modifications like

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SUMOylation as well as specific protein-protein interactions might be an important part of regulation of the function of many DNA glycosylases.

Methyl-CpG-binding protein 4 (MBD4)

MBD4 is a member of the methyl-CpG-binding protein family. It contains two DNA binding domains; an amino-proximal methyl-CpG binding domain (MBD) and a C-terminal mismatch-specific glycosylase domain (70). MBD4 removes uracil and thymine when paired with a guanine which occurs as a result of deamination of cytosine and 5-meC, respectively. The optimal substrates for the MBD4 are T:G and U:G mismatches in the context of CpG or 5-meCpG sites (71). So, it appears that the glycosylase function of MBD4 is the preservation of the integrity of genome at CpG sites. MBD4 is a particularly interesting DNA glycosylase. It interacts with FADD (Fas-associated death domain protein) (72), probably providing a mechanism for MBD4 mediated genome surveillance and apoptosis and suggests a role for MBD4 in a functional link between DNA repair and apoptosis. MBD4 also interacts with MLH1 (73) a protein involved in mismatch repair (MMR). Embryonic fibroblast cells prepared from mice lacking functional MBD4 (*Med1*^{-/-}) showed reduced steady-state levels of several MMR proteins, indicating a role for MBD4 in the integrity of MMR system (74).

Human MutY homolog DNA glycosylase (hMYH)

Adenine mispaired with 8-oxoG can arise during DNA replication either by incorporation of an adenine nucleotide opposite an 8-oxoG derived from the direct oxidation in the template strand, or by incorporation of an 8-oxoGTP that results from direct oxidation of dGTP in the nucleotide pool. Both bases in the A:8-oxoG mispairs are mutagenic, which if not repaired correctly will result in G:C to T:A transversion mutation. The 59 kDa hMYH protein interacts with PCNA at the sites of replication and removes adenine from A:8-oxoG mispairs (75,76). In *S. cerevisiae*, which does not have a MYH homolog, mismatch repair is the main pathway for repairing the A:8-oxoG mispairs (77). hMYH is apparently the only DNA glycosylase in humans that directly interacts with APE1 (76). Multiple forms of hMYH have been identified in nuclei and mitochondria (78,79). The glycosylase activity of hMYH but not its DNA binding function, is apparently modulated by posttranslational modifications of the protein (80). Recently, defective phosphorylation of hMYH was found in colorectal cancer cell lines with wild type hMYH alleles (81). In this report, protein kinase C (PKC) was shown to directly phosphorylate hMYH, a process that increased the level of hMYH-dependent repair of A:8-oxoG (81). Biallelic germline mutations of hMYH have been found in patients with familial colorectal cancer with multiple adenomas and carcinomas (82,83), suggesting a role for hMYH in promoting genetic stability and prevention of cancer. The phosphorylation status of hMYH may also

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regulate its binding to APE1, PCNA and RPA (81). hMYH may be phosphorylated in response to DNA damage, because induction of oxidative stress by H₂O₂ stimulates activity of PKC (84).

Human OGG1 DNA glycosylase 1 (hOGG1)

The most stable and mutagenic oxidative base damage known so far is 7, 8-dihydro-8-oxoguanine (8-oxoG). 8-oxoG is formed at a rate of ~1000 lesions/cell/day (85). 8-oxoG does not appear to block replication, but when in *syn*-conformation, it pairs with adenine during DNA replication causing G:C to T:A transversion mutations (2). In human cells hOGG1 removes 8-oxoG from DNA (35). hOGG1 was shown to associate with chromatin and the nuclear matrix during interphase, and the chromatin-bound hOGG1 was found to be phosphorylated on a serine residue *in vivo*, possibly by protein kinase C (86). Since the phosphorylation did not seem to affect the catalytic function of the protein, it might be involved in the subnuclear localization of the protein. hOGG1 interacts with XRCC1 and the interaction stimulated the DNA glycosylase activity of hOGG1 (87).

The human ribosomal protein S3 cleaves AP DNA via a β,δ -elimination reaction and binds to 8-oxoG and AP-sites in DNA. S3 was co-immunoprecipitated with hOGG1. *In vitro* experiments showed higher hOGG1 turnover in the presence of S3 protein (88).

Recently, hOGG1 was found to interact with protein kinases cdk4 (a serine-threonine kinase) and c-Abl (a tyrosine kinase) (89). *In vitro* phosphorylation of OGG1 by cdk4 resulted in a 2.5-fold increase in the 8-oxoG incision activity of OGG1, but the phosphorylation of OGG1 by c-Abl did not affect this activity (89). hOGG1 is a bifunctional DNA glycosylase with associated AP lyase activity. Previously, *in vitro* repair of 8-oxoG was shown to mainly proceed via short patch (90). So, the AP lyase function of hOGG1 was assumed to prevent long patch repair of clustered DNA damage avoiding the generation of DSB. In a later report, using an *in vivo* repair assay, 55-80% of repaired 8-oxoG was found to constitute 2-6 nucleotides long patches (91). Several reports have shown that the AP lyase activity of the enzyme was slower than its glycosylase activity, and hOGG1 remained tightly bound to the AP-site and APE1 enhanced the turnover of the enzyme (92,93). Furthermore, the AP lyase function of hOGG1 was shown to be inhibited by free 8-oxoG and physiological MgCl₂ concentrations (94). These results suggest that the glycosylase and AP lyase functions of the protein are uncoupled. Uncoupled glycosylase/AP lyase functions were also shown for hNTH1, a human DNA glycosylase for removal of oxidized pyrimidines from DNA (95). However, the *E. coli* Fpg and Nth, have coupled glycosylase and AP lyase activity, catalyzing strand incision at about the same rate as base release. So, the biological significance of the AP lyase function of mammalian DNA glycosylases remains unclear and may only be active under specific circumstances and possibly controlled

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by posttranslational modifications of the protein. Alternatively, it may be an evolutionary “spin off” function with little or no biological relevance in mammalian BER.

Human thymine glycol DNA glycosylase (hNTH1)

Thymine glycol (Tg; 5,6-dihydroxy-5,6-dihydro-thymine) is recognized as one of the major oxidative DNA lesions caused by oxidative stress and ionizing radiation. hNTH1, a homolog of the *E. coli* endonuclease III (Nth) is a DNA glycosylase with AP lyase activity that cleaves thymine glycol in DNA. Cells from Cockayne syndrome patients with mutations in the *XPG* gene show reduced global repair of thymine glycol (96). XPG is a structure specific endonuclease. It introduces a nick at the 3' side of a DNA lesion in the dual incision of nucleotide excision repair. XPG interacts with hNTH1 stimulating its DNA glycosylase/AP lyase activity (96,97). Gene-targeted Nth1 mutated mice showed no detectable phenotypical abnormality (98).

Apparently, hNTH1 physically interacts with p53 and PCNA (99). p53 stimulated DNA glycosylase/AP lyase activity of hNTH1 supporting a modulatory role for p53 in BER (100). Interaction of NTH1 and PCNA is particularly interesting. First, thymine glycol blocks DNA replication. Second, the expression of hNTH1 is regulated during the cell cycle with increased transcription during early and mid S-phase (101). Thus, hNTH1 may act “pre-replicative” to avoid replication block by thymine glycol. Such a mechanism has been suggested for SDBR through interaction of XRCC1 with PCNA (102).

DNA glycosylases NEIL1 and NEIL2

DNA damage caused by reactive oxygen species (ROS) generates miscoding as well as blocking lesions that may lead to mutations or cell death. In *E.coli* the DNA glycosylases Nei, Fpg, and Nth initiate BER of oxidative lesions. In humans oxidized base-specific DNA glycosylases named NEIL1, NEIL2, and NEIL3 which belong to the FPG/Nei family, have been identified (103). NEIL1 and NEIL2 possess intrinsic AP lyase activity (103), but unlike OGG1 and NTH1 they carry out β/δ -elimination and generate 3'-phosphate and 5'-phosphate termini. NEIL1 removes oxidative base lesions 5-OHU, 5-OHC, Tg, urea, FapyA, FapyG. NEIL2 shows narrower substrate specificity and is primarily involved in excision of 5-OHU as well as AP-sites. So far, little is known about substrate specificity of NEIL3. The expression of NEIL1 is cell cycle dependent and is enhanced during S-phase, whereas the expression of NEIL2 is not (103), suggesting a role for NEIL1 in replications-coupled BER. NEIL1 stably interacts with POL β and DNA ligase III (104). p300 is a transcriptional co-activator (105) for a number of sequence specific transcription factors, and has an intrinsic histone acetyltransferase (HAT) activity. NEIL2 is acetylated both *in vitro* and *in vivo* by p300, with which it stably interact (106). *In vitro* acetylation of NEIL2 decreases its 5-OHU excision

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activity as well as its AP lyase activity, suggesting a regulatory role of acetylation on the catalytic function of the enzyme (106). Possibly under normal physiological conditions NEIL2 remains in an inactive form as a result of acetylation and becomes activated by deacetylation for instance because of oxidative stress.

3-meA DNA glycosylase (MPG)

Alkylating agents like methyl methanesulphonate (MMS) and the cellular methyl group donor *S*-adenosylmethionine (SAM) generate covalent modifications at ring nitrogen residues of DNA bases, in particular 7-methylguanine (7-meG) and 3-methyladenine (3-meA). Mammalian cells have a single 3-meA DNA glycosylase (MPG) that excises the alkylated base from DNA (36). Whereas 7-meG seems to be a relatively harmless modification, 3-meA is a cytotoxic lesion that blocks both replication and transcription. 3-meA DNA glycosylase activity has been detected in many species, suggesting that it has an important biological function. *Mpg* null mice are viable and develop normally (107). Recently, interaction between MPG and PCNA was reported (108), suggesting a role for MPG in the removal of 3-meA during replication (108). hHR23B binds to MPG and stimulates its activity by increasing the affinity of MPG for substrate (109). hHR23B acts in the recognition of damaged bases in nucleotide excision repair suggesting a role for this protein in the initial DNA damage recognition step of MPG BER. It also appears that MPG interacts with XRCC1 (110). MPG is expressed at low concentrations in most human cells and shows relatively poor turnover *in vitro* (37). So, direct interactions of MPG with these proteins may enhance base damage sensing and removal by MPG and may contribute to increase local concentrations of the enzyme in replication foci.

AP endonuclease (APE)

AP-sites are potentially cytotoxic lesions that can block DNA replication and stall RNA polymerase II during transcription, but they are also mutagenic, causing base substitutions. An estimated number of 9,000-10,000 AP-sites are generated in each cell per day under normal physiological conditions (1,111). In addition to the spontaneous loss of bases from DNA, AP-sites are the intermediate products of monofunctional DNA glycosylases. In humans, AP-sites are incised at the 5'-site by AP-endonuclease I (APE1) generating

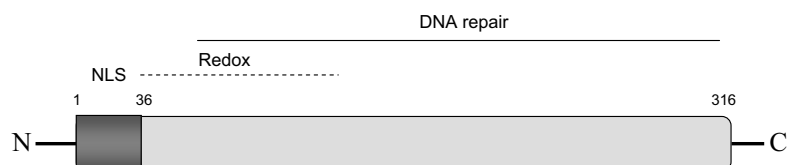


Figure 6. Schematic diagram of APE1. NLS; nuclear localization signal.

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a 3'-OH and a 5'-deoxyribose-5-phosphate (dRP) group flanking the DNA strand break. APE1 (also known as Hap-1, Apex, and Ref-1) is the human homologue of the *E. coli* exonuclease III and has a molecular mass of ~37 kDa. It contains two distinct domains. The N-terminal domain contains the nuclear localization sequence (residues 1-36) and is essential for redox activity, while the endonuclease activity resides in the C-terminal region (Figure 6). Recent studies have shown some overlap in the functional domains. The structure of APE1 bound to AP DNA showed that APE1 uses a rigid, preformed surface to bend the DNA helix by ~35° that enables the enzyme to flip the AP-site out of the DNA and move it into the active site in a MgCl₂ dependent reaction (112). APE1/Ref-1 is expressed ubiquitously, and shows a complex and heterogeneous staining pattern among different tissues (113), even between neighboring cells probably reflecting the different roles of this multifunctional protein (114). The N-terminal domain of APE1 is responsible for the second function of APE1/Ref-1, that is the redox activation of several transcription factors including; Fos, Jun and p53. In addition to its AP endonuclease and redox-regulation function, APE1 exhibits 3' to 5' exonuclease, phosphodiesterase, 3'-phosphatase and RNase H, although these additional functions are much weaker than its AP endonuclease activity. Very recently, APE1 was shown to remove 3'-8-oxoG within a single-strand break (115), that may occur as a result of oxidative DNA damage and IR.

Generation of mice lacking Apex (APE1) has been attempted. However, the homozygous mutant mice (Apex^{-/-}) are embryonic lethal (116), indicating a role for this protein in embryonic development. However, heterozygous mice survive and are fertile (117). In a separate study, Apex^{+/-} mice were shown to display APE haploinsufficiency (118). These mice showed 40-50% reduction in APE mRNA, and protein concentration as well as reduced 5' endonuclease activity in all tissues. However, *in vitro* tests showed significant tissue-specific variations in POLβ dependent BER, likely because of reduced redox function of APE1 in the cells (118). Using an RNAi approach, the endonuclease activity of APE1 was shown to be essential for maintaining genomic integrity of the cell and the absence of this function resulted in the accumulation of AP-sites and apoptosis (119). Expression of yeast Apn1 that lacks the redox function of APE1 reversed these processes indicating involvement of the AP endonuclease function of APE1 in these processes (119). Very recently, conditional APE1^{-/-} MEF cells were established using LoxP-flanking *APE1* constructs and Cre expression by microinjection (120). Removal of *APE1* was found to result in apoptosis within 24 h. A microinjection of mutants lacking either the DNA repair or acetylation-mediated gene regulatory function did not prevent apoptosis. However, co-injection of these mutants or the wild type APE1 rescued the cells from apoptosis, indicating that separate functions of the APE1 are required for cell survival (120). The product of a bifunctional glycosylase is a normal 5'-deoxynucleoside-5'-phosphate, and an abnormal 3'-terminal α,β-unsaturated aldehyde residue that is cleaved by 3'-phosphodiesterase activity of APE1.

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Human POL β is the main repair DNA polymerase in BER. POL β lacks 3' to 5' exonuclease (proofreading) activity. *In vitro* fidelity analyses of POL β using DNA substrates containing U opposite A or G demonstrated an error rate of ≤ 0.3 to $\leq 2.8 \times 10^{-4}$ (121). So, other proteins may act in BER to secure error free DNA repair. Recently, it was shown that APE1 had a DNA exonuclease activity on mismatched deoxyribonucleotides at the 3'-termini of nicked or gapped DNA (122), suggesting a role for APE1 as a potential proofreading factor in BER.

APE1 is a target for cellular defense response. Cytotoxic granules trigger death in target cells via the action of perforin and the granule proteases, the granzymes. Granzyme A was shown to interact with and cleave APE1, thus impairing DNA repair ability of the infected cell and preventing the target cell to recover from the death signal (102).

A repair pathway for removal of oxidative base damage independent of DNA glycosylase was identified in *E.coli* (123). In this nucleotide incision repair (NIR) pathway, Nfo-like endonucleases incise DNA on the 5'-side of various oxidatively induced damaged bases, generating a 3'-hydroxyl end and a 5'-phosphate residue. The 3'-hydroxyl terminus is then a substrate for DNA polymerase I (123). A similar repair pathway was found to be active in human cells (124). Here, APE1 was shown to incise several oxidatively damaged bases in DNA generating 3'-hydroxyl and 5'-phosphate residue. The 3'-hydroxyl end was substrate for DNA polymerase and the 5'-residue was cleaved by FEN1. Because the APE1 initiated NIR removed damaged bases including those formed under IR and anoxic conditions like 5,6-dihydro-2'-deoxyuridine (DHU), a specific role for NIR in removal of these types of lesions was suggested (124).

Overlapping DNA substrate between APE1 and human DNA glycosylase hNTH have been demonstrated and may partly explain the absence of deleterious effects of lack of this glycosylase in *NTH* knockout mice (117).

APE1 is a substrate for phosphorylation by the serine/threonine casein kinases (CK) I and II and protein kinase C (125-127). Although, two of these studies reported conflicting results with regard to the effect of phosphorylation on the endonuclease activity of APE1, they both showed that phosphorylation of APE1 stimulated its redox function. The redox activation of several transcription factors by APE1 provides a rapid cellular response to environmental stress. Although, the level of APE1 in response to oxidative stress was shown to increase, redox activation of transcription factors via upregulation of APE1 is a rather slow response. APE1 is translocated from cytoplasm to the nucleus in response to oxidative stress, probably providing a rapid activation of transcription factors (128).

Helicobacter pylori (*H. pylori*) infection causes inflammation, accumulation of ROS, and oxidative DNA damage in the gastric mucosa. *H. pylori* enhanced APE1 protein synthesis and nuclear accumulation

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in human gastric epithelial cells implicating APE1 in the modulation of the pathogenesis of *H. pylori* infection (129).

APE1 is directly involved in Ca²⁺-dependent downregulation of expression of parathyroid hormone (PTH) by binding to negative calcium response elements (nCaREs) present in the PTH promoter (130). Both *in vitro* and *in vivo* acetylation of APE1 by p300 resulted in down regulation of expression of PTH (131). This suggests that the acetylation is a regulatory mechanism of transcriptional function of APE1. The AP-endonuclease activity of APE1 was not affected by this acetylation (131). Together, these studies show how the function of a multifunctional protein like APE1 is regulated by posttranslational modifications.

A second human AP-endonuclease named APE2 has been identified in humans (132), but shows significantly lower endonuclease activity than APE1 (133). APE2 is localized in both nuclei and mitochondria (134). The nuclear APE2 associates with PCNA, suggesting a role for APE2 in replication-associated BER. The role of mitochondrial APE2 is still unclear, but it may play a role in mitochondrial BER. Recently, APEX2 null mice were generated (135). These mice showed growth retardation and defects in lymphopoiesis. Furthermore, the knockout mice showed a weaker immune response against ovalbumin in comparison with the wild-type mice. However, class switch recombination was found normal in the absence of APEX2 (135).

DNA polymerase β (POL β)

DNA polymerase β is 39 kDa in size and is the smallest eukaryotic DNA polymerase and belongs to X-family DNA polymerases. It contains two specialized domains. An 8 kDa N-terminal domain that possesses a lyase activity, removes the 5'-deoxyribose-5-phosphate (dRp) intermediates generated by APE1 during BER. In addition to its dRp lyase function, the 8 kDa N-terminal has single-strand DNA binding activity. The DNA binding directs the protein to gaps that contain a 5'-phosphate (136). Only when the gap was between one to six nucleotides, POL β filled the gap in a processive manner (136). The larger domain is 31 kDa and has nucleotidyl transferase activity. POL β is the major polymerase in SDBR and BER mainly through single-nucleotide insertion or short-patch BER, but also inserts the first nucleotide in long-patch BER (137,138). POL β together with FEN1 carry out long-patch by strand displacement synthesis (137) and/ or by a "hit and run" mechanism (139).

POL β also plays a role in meiosis events associated with synapses and recombination (140). Knockout of Pol β in mice is embryonic lethal (141), suggesting that Pol β is important for maintaining development. Although Pol β deficient null mice are not viable, the corresponding embryonic cells survive in culture, indicating that Pol β is not essential for cell viability. Cells lacking Pol β are highly sensitive to alkylating agents. However, overexpression of a truncated form of Pol β consisting of the dRp lyase domain

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reversed the methylating agent hypersensitivity in Pol β null cells (142). This indicates that the dRP lyase activity of Pol β and not its DNA synthesis function is essential for repair of methylation-damaged bases. The accumulation of dRP moieties triggers apoptosis in a replication-dependent fashion (143), indicating that the apoptotic signals are generated as a result of conversion of ssDNA breaks to dsDNA breaks during replication and not through detection of dRP moieties. In fact, MMS-induced ssDNA breaks were found to be recombinogenic in Pol β deficient mouse and activated homologous recombination (HR) (144). POL β was reported to become acetylated by p300 (145). The acetylated form showed a reduced dRP lyase activity. POL β interacts with DNA ligase I (146), XRCC1 (147), PCNA (148), PARP-1 (149), and p53 (100), implicating POL β in both short- and long patch BER and SDBR .

DNA polymerases δ and ϵ (POL δ / POL ϵ)

POL δ is a high fidelity replicative DNA polymerase with intrinsic exonuclease (proofreading) activity. POL δ interacts with the sliding DNA clamp PCNA in replication foci and is responsible for DNA synthesis of leading and likely lagging strand. POL δ is also involved in DNA repair synthesis in MMR, NER, and BER. POL δ together with PCNA, FEN1, and DNA ligase I is responsible for the repair DNA synthesis step of long patch BER (150). POL δ -dependent and PCNA-mediated long patch BER is active in replication forks (discussed later) and possibly in repair of modified bases by BER (151). The second replicative DNA polymerase, POL ϵ , may also be involved in long patch BER (152).

DNA polymerase λ (POL λ)

POL λ is a member of the X-family of DNA polymerases and shares catalytic features of POL β , therefore making it a plausible candidate for BER. Recently, a BER deficiency in the POL $\lambda^{-/-}$ cell extract compared with extract from wild-type cells was reported (65). In addition, neutralizing POL λ -antibodies reduced *in vitro* BER in the POL $\beta^{-/-}$ cell extract. POL λ interacts with PCNA (153). Although, this interaction is likely involved in replication-associated DNA lesion bypass (153), a role for POL λ in long patch BER is conceivable. POL λ null mouse fibroblasts were hypersensitive to oxidative DNA damaging agents (154). Moreover, POL λ was found to localize to sites of oxidative DNA lesions and to interact with SMUG1 (154). Thus, POL λ may be important in cellular BER for protection against oxidative DNA damage.

Flap endonuclease I (FEN1)

FEN1 is a structure-specific nuclease that plays an important role in DNA replication and repair. In DNA replication, it is required for Okazaki fragment maturation where it removes the displaced RNA-DNA primers. Mice homozygous for the Fen1 mutation were not viable (155), suggesting that Fen1 is

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important for embryonic development. The proteins identified to physically and/or functionally interact with FEN1 include PCNA (156), WRN (157), RPA (158), APE1 (159), possibly POL β (160), as well as proteins involved in chromatin remodeling (161).

A specific role for FEN1 in BER was demonstrated in FEN1^{-/-} chicken cells (162). The mutant cells were viable, but were hypersensitive to MMS, MNNG, and H₂O₂, but not to UV-light, X-ray, or etoposide (162). In BER, FEN1 is involved in long patch repair. Repair of modified AP-sites that are refractory to the dRP lyase activity of POL β takes place by long patch and involves FEN1, PCNA and POL δ (151). FEN1 was shown to tightly bind to chromatin in response to MMS treatment of the cells (163). This binding did not require the nuclease function of the enzyme, but was strictly dependent on the intact PCNA-binding domain of FEN1 (163). A PCNA-independent scenario that is mediated by POL β and FEN1 have been suggested (137,139). PARP-1 was shown to stimulate FEN1 and POL β probably as part of a repair complex for long patch BER (50). A physical interaction was also identified between APE1 and FEN1 (159). APE1 stimulated endo- and exonuclease activity of FEN1. So, a repair complex composed of APE1, POL β , FEN1, and PARP1 may function in long patch repair of modified AP-sites. This repair complex may function independent of PCNA or its function may be mediated by PCNA through interaction of PCNA with POL β (148).

A way to regulate FEN1 in different biochemical processes is posttranslational modification of the protein. Cyclin-dependent kinase 1 (Cdk1) phosphorylates FEN1 at Ser-187 in late S-phase (164). The phosphorylation reduced FEN1 endo- and exonucleotic cleavage activity of the protein, and abolished interaction with PCNA, but the phosphorylated form retained normal substrate binding ability (164). FEN1 interacts with p300, which allows acetylation of FEN1 at the C-terminus. The acetylation resulted in reduced endo- and exonucleotic cleavage activity because of reduced substrate binding of FEN1 (161). Although these modifications may be important for the role of FEN1 in replication, their possible regulatory effects on the role of FEN1 in BER cannot be ruled out.

DNA ligases I and III

The final step in BER is the formation of phosphodiester bond at the site of repair by a DNA ligase that proceeds in a three-step reaction. First, the enzyme reacts with ATP to form a covalent enzyme-AMP complex through linkage of the AMP moiety to a specific lysine residue. Next, the AMP group is transferred from the enzyme to the 5'-phosphate terminus of a DNA nick. Finally, the enzyme catalyzes the phosphodiester bond formation between the 3'-hydroxyl and 5'-phosphate termini of the nick and releases the AMP. In humans three DNA ligase genes have been identified (*LIG1*, *LIG3*, *LIG4*) with distinct functions, but all

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share a conserved catalytic domain. DNA ligase I and III the products of *LIG1* and *LIG3* genes respectively, function in BER.

DNA ligase I

DNA ligase I has a molecular mass of 125 kDa when measured by SDS-PAGE. The protein is recruited to replication factories during S-phase and is responsible for joining of Okazaki fragments generated during the lagging strand synthesis. The recruitment is directed by interaction with PCNA (165). DNA ligase I was copurified with a high-molecular weight replication complex (166). DNA ligase I was shown to interact with POL β (146). The interaction of DNA ligase I with PCNA and POL β occurs through its non-catalytic N-terminal region, implicating the protein in both short- and long-patch BER. A 180-kDa BER complex containing POL β and DNA ligase I was isolated from extracts of bovine testes (51).

Mutations in *LIG1* in a fibroblast cell line isolated from a patient with immunodeficiency has been reported (167). The cells were defective in the joining of Okazaki fragments (168) and were hypersensitive to the cytotoxic effects of monofunctional DNA alkylating agents like MMS as well as UV-light and IR (169).

The N-terminal domain of the protein (residues 1-216), which is not necessary for its catalytic activity and has no counterpart in the other DNA ligases includes the site of cell cycle specific phosphorylations (170,171). The serine 66 is part of a casein kinase II (CKII) motif and was found to become dephosphorylated in early G1, but phosphorylated through S-phase peaking in the G2-phase (171). The dephosphorylation in G1 required nuclear localization of the protein and a functional PCNA-binding site (171). DNA ligase I interacted with PCNA in G1- and S-phase, but not in G2/M-phases. Moreover, Ser91, Ser76, and Ser91 which are part of the motifs for cyclin-dependent kinases were also found to become phosphorylated in a cell cycle-dependent manner (172).

DNA ligase III

The *Lig3* gene encodes several polypeptides with different cellular functions and subcellular localizations. Two forms of DNA ligase III (α and β) are produced by alternative splicing resulting in proteins with different C termini (173). DNA ligase III α mRNA is expressed in all tissues, and is localized in nucleus and mitochondria (174), whereas the expression of DNA ligase III β mRNA is restricted to male germ cells (173). Apparently the mitochondrial and nuclear isoforms of DNA ligase III α are produced by alternative translation initiation (174). DNA ligase III is absent in *S. cerevisiae*, *D. melanogaster*, and *C. elegans*, suggesting that this gene was a relatively recent evolutionary event in the genome of mammals (175).

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A zinc finger motif at the N-terminus of DNA ligase III distinguishes the products of the *LIG3* gene from other eukaryotic DNA ligases (176) and is closely related to the two zinc fingers located at the N-terminus of PARP-1 (176). The zinc finger was not required for DNA ligase activity, but enabled DNA ligase III to interact with and ligate nicked DNA (177). DNA ligase III in nucleus interacts with the scaffold protein XRCC1 (178), and PARP-1 (179). XRCC1 is required for the stability and normal levels of DNA ligase III in nucleus (178), but not in mitochondria (180).

Auxiliary BER proteins

In recent years, several proteins have been found to modulate BER by direct interaction with BER proteins. Although these proteins are not strictly required for *in vitro* BER assays they are important for BER *in vivo*.

X-Ray Repair Cross-Complementing protein 1 (XRCC1)

XRCC1 has no enzymatic activity and acts as a molecular scaffold repair protein. XRCC1 deficiency is associated with reduced SDBR capacity, increased frequencies of sister chromatin exchange, and hypersensitivity to ionizing radiation, oxidizing chemicals, and alkylating agents (181). *Xrcc1*^{-/-} mice display

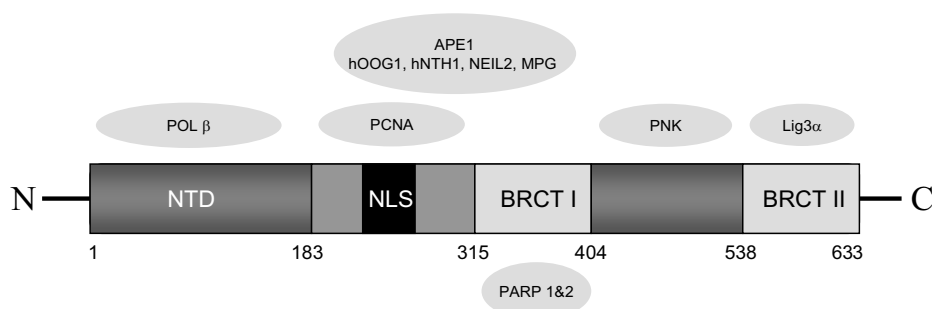


Figure 7. Schematic presentation of XRCC1 and some of its interacting proteins.

embryonic lethality (182). XRCC1 is also implicated in coordinating BER, because it interacts with repair enzymes that are common for SDBR as well as BER such as APE1 (183), DNA ligase III (178), PNK (184), POLβ (147), PARP-1 (147,185), and hOGG1 (87). XRCC1 stimulated the base excision activity of hOGG1 *in vitro* (87). XRCC1 is a phosphorprotein (186) and is phosphorylated *in vitro* and *in vivo* by CK2 (187,188). An increased phosphorylation stimulated interaction of XRCC1 with polynucleotide kinase (PNK) and facilitated recruitment of this protein to chromosomal DNA breaks after H₂O₂ treatment of the cells (187). Interaction of XRCC1 with damaged DNA may occur through direct interaction with PARP-1/2 bound to the lesion (189), and via interaction with POLβ (190). Moreover, XRCC1 was shown to bind tightly to nicked and 1 nucleotide-gapped DNA substrate indicating that it is able to directly recognize and

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bind to DNA lesions (191). XRCC1 rapidly forms foci after H₂O₂ treatment (187,189). It remains to be seen if H₂O₂ induced phosphorylation of XRCC1 would increase recruitment of XRCC1-hOGG1 or other DNA glycosylases to the sites of oxidative DNA damage. Recently, DNA glycosylases MPG, NTH1, and NEIL2 were also found to interact with XRCC1 (110). *In vitro* tests showed that XRCC1 was able to stimulate the enzymatic activity of the DNA glycosylases (110). They also detected uracil repair activity in both XRCC1 and control samples, therefore they concluded that this activity was a result of unspecific interaction (110). However, in a recent report XRCC1 was identified in UNG2-associated repair complexes (49). Whether UNG2 and other DNA glycosylases interact directly or indirectly via a common protein with XRCC1 needs further investigation.

Poly(ADP-ribose) polymerase (PARP)

Poly(ADP-ribose) polymerase-1 (PARP-1) has in recent years received considerable attention as a BER modulator. PARP-1 is ~116 kDa and is an abundant nuclear enzyme found in many eukaryotes, with the exception of yeast. PARP-1 is constitutively expressed at high levels from a promoter with features typically found in housekeeping genes (192). PARP-1 mRNA is present in all tissues with highest levels in testes, spleen, brain, and thymus (193). PARP-1 knockout mice are viable and fertile, but are sensitive to γ -rays as well as MNU and show increased genomic instability and sister chromatid exchanges (194). When bound to DNA, PARP-1 uses NAD⁺ and rapidly catalyzes poly(ADP-ribose) synthesis resulting in covalent modification of itself and other nearby proteins like histones (22). The automodification of PARP-1 results in dissociation of the protein from DNA that is necessary for the DNA repair to proceed. PARP-1 binds to SSDB and nicks in DNA and facilitates recruitment of repair factors through XRCC1, thus, functioning as a SSDB sensor (195). However, the absence of PARP-1 did not prevent SDBR but only reduced the rate of repair 2-3-fold (196). PARP-1 may affect DNA repair indirectly, because PARP-1 affects the regulation of expression of genes (197) and PARP-1 deficient cell extracts showed reduced concentration of FEN-1 and DNA ligase I (196).

A role for PARP-1 in BER was demonstrated by a BER assay in cell extracts from PARP-1 deficient mice. Long patch repair was reduced in PARP-1 deficient mice extract compared with the wild type (198). However, other studies, did not find a major role for PARP-1 in BER (199,200). PARP-1 interacts with XRCC1 (185), POL β (149), and DNA ligase III (179). PARP-1 was shown to be required for the rapid formation of XRCC1 foci at sites of oxidative DNA damage (189). Further characterization of the role of PARP-1 in BER demonstrated that PARP-1, POL β , APE1, and FEN-1 form complex for repair of AP-sites (201). It is possible that PARP-1 modulates the selection of BER pathways. PARP-1 may also protect BER DNA intermediates from degradation during repair (202). Although the main cellular poly(ADP-ribosylation)

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activity is attributed to PARP-1, cells contain several other PARPs. PARP-2 accounts for 10% of the cellular PARP activity. PARP-2 is a 62 kDa protein and is the closest homologue of PARP-1. Poly(ADP-ribose) synthesis activity of PARP-2 as well as different experimental conditions may explain the apparent conflicting results on the essential role of PARP-1 in BER (198-200). PARP-2 forms homo- and heterodimers with PARP-1 and interacts with XRCC1, POL β , and ligase III (203), and enhances BER in association with PARP-1 and XRCC1 (203).

Proliferating cell nuclear antigen (PCNA)

PCNA is a member of the DNA sliding clamp family, which includes the *E.coli* DNA polymerase III β -subunit and the phage T4 gene45 protein. PCNA was originally characterized as a DNA sliding clamp for replicative DNA polymerases and as an essential component of chromosomal replisome. The clamp loader for PCNA is the replication factor C (RFC) complex, an ATPase composed of four subunits (p149, p40, p38, p37, and p36). The homotrimeric PCNA encircles DNA and interacts with many proteins involved in DNA metabolism including DNA repair. The BER proteins that interact with PCNA include; UNG2 (42), MYH (76), MPG (108), POL δ (204), POL ϵ (205), FEN1 (206), DNA ligase I (165), APE1 (159), and POL β (148). PCNA also interacts with APE2 (134). However, a role for APE2 in BER is still unknown. PCNA is thought to be involved in long patch BER, including repair of modified AP-sites by recruiting POL δ , FEN1 and DNA ligase I to the site of lesion (151).

P21 is a cyclin-dependent kinase inhibitor. In response to DNA damage, the expression of p21 is increased by p53. p21 binds to the same domain in PCNA as FEN1, POL δ , and DNA ligase I. Binding of p21 to PCNA was shown to decrease long patch BER (207). PCNA interacting proteins show different binding affinity (208). Therefore, the availability of the proteins as well as their binding affinity are among the factors that regulate interaction of proteins with PCNA. Posttranslational modifications of the PCNA-interacting proteins as well as PCNA itself are additional regulatory mechanisms. Posttranslational ubiquitination (209), SUMOylation (210) and acetylation (211) modifications of PCNA have been reported. Deacetylation was found to reduce the ability of PCNA to bind POL β and POL δ (211). However, it is currently unknown if these modifications may affect BER.

Replication protein A (RPA)

RPA is essential for multiple DNA metabolism processes like DNA replication, recombination, and DNA repair pathways including BER. RPA is a single-stranded DNA-binding protein composed of three subunits of 70-, 32- and 14-kDa (212). RPA binds ssDNA with high affinity and interacts specifically with multiple proteins. Cellular DNA damage causes the N-terminus of the 32-kDa subunit of human RPA

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to become hyper-phosphorylated (213). RPA directly interacts with PCNA, hMYH (76), and UNG2 (42). The fact that these proteins are found in replication foci and RPA was shown to enhance the activity of DNA ligase I (214) and long patch BER (215) indicates that these protein complexes are involved in the replication associated BER pathway.

P53

The tumor suppressor and transcription factor p53 that is inactivated in many human cancer cells is involved in BER (216). The stimulation of BER by p53 was independent of its transactivation function (217), and was likely through direct interactions with BER factors APE1 and POL β (100). Recognition of certain types of DNA lesions by p53 have been previously reported (218,219) and represents one of the several functions of this protein in response to DNA damage. Recently, a role for p53 in sensing oxidative DNA damage was reported (220). 8-oxoG containing oligonucleotides pulled down p53 together with hOGG1 and APE1 (220). *In vitro* analysis showed that p53 enhanced activities of hOGG1 and APE1 (220). So, p53 regulates BER through direct interactions with BER enzymes and likely also by facilitating the recruitment of these enzymes to the site of damage.

Werner syndrome protein (WRN)

WRN is a member of RecQ family of helicases that play central roles in genomic stability of organisms ranging from prokaryotes to mammals. WRN is a bifunctional protein with an additional exonuclease activity. Defects in WRN cause Werner syndrome (WS) which is an autosomal recessive premature aging disease manifested by the mimicry of age-related phenotypes. WRN interacts with POL δ , and RPA (reviewed in (221)). A role for WRN in BER has been investigated. WRN was shown to interact with FEN-1 (157), and APE1 (222) and stimulated strand-displacement DNA synthesis of POL β on a nicked BER intermediate *in vitro* (222).

Heat-shock protein 70 (Hsp70)

Molecular chaperones are proteins that assist the structure formation of proteins *in vivo*. In the mammalian cells, the molecular chaperones Hsp70 and Hsp90 are involved in the folding and maturation of key regulatory proteins, like steroid hormone receptors, transcription factors, and kinases. Hsp70 was found to interact with and stimulate the enzymatic activities of APE1 (223) and POL β (224). The mechanisms of these interactions are currently unknown, but these results extend the protective role of Hsp70 in mammalian cells to BER and genomic stability.

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Rad9-Rad1-Hus1 (9-1-1) complex

Rad9-Rad1-Hus1 (9-1-1) complex is a heterotrimer toroidal molecule that is loaded onto DNA by Rad17-RFC2-5 clamp loader in response to many different genotoxic stresses, including alkylation, UV-light, IR, and replication inhibitors (225). Possible involvement of the 9-1-1 complex in BER have been investigated. In human cells the 9-1-1 complex interacts with FEN1, POL β , and DNA ligase I and modulates their activity (226-228). In *S. pombe*, DNA glycosylase MYH interacts with all subunits of the 9-1-1 complex (229). H₂O₂ treatment increased the interaction between Hus1 and MYH, and correlated with Hus1 phosphorylation. MYH interacts with PCNA. However, this interaction remained unchanged after H₂O₂ treatment (229). So, it appears that cells have developed sliding clamps that interact with the same repair enzymes, but are involved in distinct repair pathways. The exact function of the 9-1-1 complex in BER is an interesting field of research and more BER enzyme partners of this complex may be identified in the near future. These investigations will increase our understanding of the network of the cellular stress response, DNA damage recognition and BER.

BER protein complexes

BER has long been thought to function through sequential interactions of repair proteins with the site of ongoing repair independently of each other. Our conception of the apparent simplicity of BER pathways may be partly because BER is relatively easy to reconstitute *in vitro* with limited number of proteins. However, in recent years, a number of stable interactions not only between the core BER enzymes, but also between BER enzymes (34) and proteins involved in other DNA metabolic processes have been reported suggesting a more complicated scenario (Figure 8). Moreover, isolation of protein complexes from mammalian cell extracts capable of complete BER have been reported (49-51). Formation of a BER complex for repair of AP-sites was suggested as early as in 1991 (230). In this study tetrahydrofuran (synthetic AP-site) containing cccDNA was incubated with cell extract in the absence of dNTPs and the repair proteins that were bound to the DNA were eluted by gel filtration (230). Using a series of experiments they showed a concerted repair of AP-sites that they suggested to be carried out by a repair complex. Few years later, a separate group used affinity column chromatography with POL β protein or POL β antibodies as baits and isolated BER protein complexes from bovine testes (51). The eluted proteins carried out the complete repair of uracil in DNA. Later, the same group incubated oligonucleotides containing tetrahydrofuran with mouse cell extract in the presence of a novel photoaffinity labeling probe to identify the interaction of BER enzymes. Six proteins were strongly labeled including PARP-1, FEN1, POL β , and APE1 (50).

Recently, we used antibodies against the non-catalytic N-terminal domain of UNG2 and isolated UNG2-associated repair complexes (UNG2-ARC) that completely repaired uracil in DNA (49). Western

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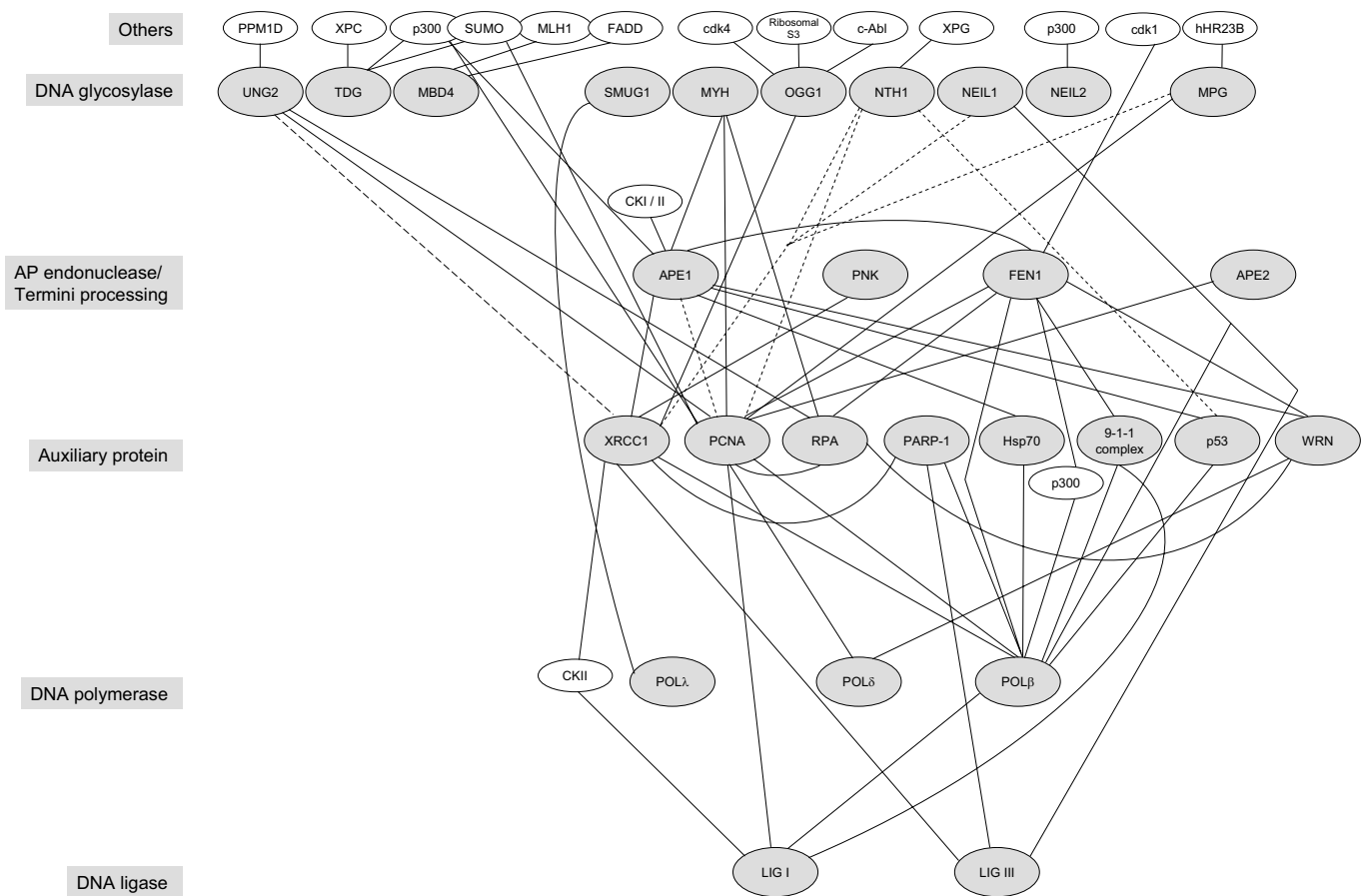


Figure 8. The network of interactions between human BER proteins. (---) Interactions that need further investigation.

analysis showed that the complexes contained UNG2, APE1, POL β , XRCC1, PCNA, and POL δ , suggesting that UNG2-ARC might consist of repair complexes that function in distinct locations on the genome and in different phases of the cell cycle. Hence, UNG2-ARC isolated from growth arrested HaCaT cell extracts showed lower long patch repair compared with the cycling cells. This agrees with DNA replication-associated long patch repair of uracil through direct interactions of UNG2 and PCNA (42). Neutralizing anti-POL β - antibodies significantly inhibited repair DNA synthesis in arrested cells compared with freely cycling cells, indicating POL β as the main DNA polymerase in UNG2-ARC in non-cycling cells (49). Moreover, UNG2-ARC isolated from extract prepared from HeLa cells overexpressing UNG2 showed increased uracil repair compared with the control cell extract, suggesting that the formation of a fraction of complexes might be independent of damage in genomic DNA (49). The N-terminal of UNG2 is phosphorylated at multiple sites (Lars Hagen et al. unpublished results and (47)). It remains to be seen whether modified forms of the protein may affect the formation of UNG2-ARC.

In a recent study, uracil-containing oligonucleotides with 3'-biotinylated end were incubated with cell extracts and the reactions were stopped at different times by the addition of formaldehyde to crosslink

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the bound proteins with DNA. This novel approach identified formation of stable complexes of POL β , XRCC1, and DNA ligase III during the repair of uracil in DNA (231).

Stable single-strand break repair complexes have been identified. Repair of single-strand breaks shares both the mechanistic characteristics of BER as well as the components involved (232). Thus, XRCC1 interacts with several repair enzymes including, POL β (147), DNA ligase III (178), APE1 (183), PNK (184), and PARP-1 (147,185). Direct interaction between XRCC1, and hOGG1 (87), MPG as well as NEIL1/NTH1 (110) supports a role for XRCC1 as a component of some BER complexes (49), and XRCC1-mediated BER complex formation. However, role of XRCC1 in BER and the extent of stable interactions of repair proteins other than DNA ligase III with XRCC1 is still under debate. Recently, repair of several types of lesions in oligonucleotides was investigated in whole cell extract prepared from XRCC1 deficient Chinese ovary hamster cells (EM9) and their wild-type counterpart (233). They found that the ligation step of BER and SDBR was inefficient in the absence of XRCC1, while repair of base lesions as well as AP-site, and gap-filling were unaffected (233). This is somewhat in contrast to an earlier report that XRCC1 deficient cell extract showed reduced AP incision activity (183). XRCC1 is required for the stability of the nuclear DNA ligase III (178). So, the observed low ligation activity in EM9 cells (233) maybe merely a result of low concentration of catalytically active ligase III in the cell extract. In a separate study, using a DNA-protein cross-linking assay as well as gel filtration of whole cell extracts they did not find a stable preassembled complex of Pol β with the XRCC1-ligase III heterodimer (190). However, another group immunoprecipitated preformed XRCC1-POL β complexes in extracts from cycling HeLa cells (188).

Recently, XRCC1 was found to interact with PCNA and to exist as multidimers *in vivo* (234), likely through interactions between the BRCT domains of XRCC1 and those of its partners DNA ligase III and PAPR-1 (235). Several repair enzymes use the same domain in XRCC1 for binding. Interaction between XRCC1 molecules circumvents the problem of the overlapping binding sites and may provide a platform for the buildup of higher order complexes enhancing DNA repair (235).

Probably, BER complexes are highly dynamic in composition and may include preassembled complexes with specific functions as well as complexes formed during sequential accumulation of the repair proteins at the site of damage (236). However, our knowledge on the extent of BER complexes as well as mechanisms that control the buildup of specific complexes is limited. The flexibility of the complexes may be provided by series of posttranslational modifications and protein-protein interactions between the core BER enzymes as well as specific “auxiliary BER factors” that would bring together repair proteins for special repair functions and subnuclear compartmentalization. These include BER during DNA replication, transcription and specific BER complexes that function in different phases of the cell cycle and those that are formed in response to exogenous sources of DNA damage. Hence, a fraction of UNG2 and possibly

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hMYH as well as MPG may be integrated parts of BER complexes with specific subnuclear function during replication as all interact with PCNA (42,76). The BRCT domains of XRCC1 were shown to play a cell cycle (237,238) and a damage specific (186) role, illustrating the dynamic of function of repair proteins. In BER, repair of adenine and 8-oxoG during replication requires a coordinated repair process to avoid generation of mutation (35). Hence, to analyze and to dissect BER complexes may require carefully designed model systems. Probably using DNA glycosylases as bait would help us to study the extent of BER complexes and the mechanisms of their buildup. Transient knockdown of individual BER proteins by expression of short interference RNA (siRNA) is an attractive tool to study BER complexes.

In vitro studies of BER complexes encounter certain challenges. During preparation of cell extracts, the spatial organization of proteins may become disrupted making it difficult to study BER pathways in their original site of function. In addition, nuclear DNA is packed in chromatin, constituting physical barriers for repair proteins to get access to damaged bases (31,239), whereas naked DNA is mostly used in BER analysis. Thus, more *in vivo* tests are needed to clarify significance of BER complexes, as *in vitro* tests maybe highly sensitive to the experimental conditions.

DNA replication associated BER and SDBR

Using fluorescence tagged proteins or antibodies against specific proteins, the cellular DNA replication process can be visualized in form of specific foci, which represent large multiprotein complexes known as replication factories. Using this approach, BER proteins have been found to physically interact with the components of the DNA replication machinery (42,240). During DNA replication and possibly transcription, chromosomal DNA is single-stranded in the surrounding regions (241). The DNA remodeling process provides the suitable conditions for repair proteins to get access to DNA lesions. The significant role of replication-assisted DNA repair was demonstrated in non-replicating cells in which DNA damage was found to accumulate in non-transcribed genes (242). However, we need to distinguish between post-replicative DNA repair that acts on DNA errors produced during DNA replication itself such as incorporation of uracil and adenine opposite 8-oxoG (42,240), as well as normal but mispaired bases, and those which should be preferentially repaired before replication encounters the lesion. Deamination of cytosine results in uracil, and unless repaired would result in C:G to T:A transition mutation during the replication. Moreover, DNA base lesions that can block replication (e.g. thymine glycol and 3-meA) must be preferably removed ahead of replication. Such lesions may be repaired during G1- and S-phase, but before the segment is replicated, and/or ahead of replication. Although a coordinated pre-replicative removal of base lesions has not been identified so far, its existence is possible. The recently identified interaction of XRCC1 and PCNA may provide pre-replicative SDBR and BER systems (34). Importantly, in this study the XRCC1-PCNA

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interaction was found to be independent of exogenous DNA damage, suggesting a ubiquitous DNA replication associated DNA repair. Cell cycle specific role of XRCC1 have been reported. Thus, BRCT domains of XRCC1 play a G1- and S-phase specific roles in BER and SDBR (237,238) as well as DNA replication recovery after MMS treatment (186). MPG removes 3-meA that can block DNA replication. Therefore, direct interaction of MPG with PCNA (108) and maybe also XRCC1 (110), further suggests that a coordinated pre-replicative BER may exist.

Transcription-coupled repair (TCR) BER

Detection and repair of some DNA damage is closely coordinated with the process of RNA transcription. Irradiation of cells with UV-light results in DNA damage, which is primarily in the form of covalent linkage between adjacent pyrimidines. Such photoproducts represent blocks to RNA polymerases. A blocked RNA polymerase II targets components of nucleotide excision repair (NER) to repair the transcribed strand. This process repairs DNA lesions from actively transcribed DNA strand and is known as transcription-coupled repair (TCR). Two major factors of this pathway are Cockayne syndrome A and B proteins (CS-A and CS-B, respectively); so called because their inactivation results in the Cockayne syndrome. A previous report showed that the repair of thymine glycol and 8-oxoG was normal in NER defective (XP) cells (243). However, CS cells including CS-B, XP-B/CS, XP-D/CS, and XP-G/CS lacked TCR of 8-oxoG in a transcribed sequence, despite its efficient repair when not transcribed (243). Since then several reports have implicated CS proteins in the repair of oxidative DNA damage. CSB functions as a chromosome remodeling protein in an ATP-dependent manner (244). Array analysis showed that CS-B cells had a general deficiency in H₂O₂ induced gene expression compared with the wild type cells. Several of these genes were involved in DNA repair, transcription, and signal transduction. This deficiency was related to the ATPase function of the protein (245). The transcription-coupled repair of 8-oxoG did not require DNA glycosylase Ogg1 (246), implying that other DNA glycosylases might be involved. Bubble structures are transiently formed in DNA during the transcription. Of the four DNA glycosylases tested (OGG1, NTH1, NEIL1, and NEIL2) NEIL1 and NEIL2 were able to remove 8-oxoG from bubble DNA *in vitro* (247). So, it appears that TCR of 8-oxoG takes place, although it is still unknown what DNA glycosylases are involved.

Deregulation and inactivation of BER

Knudson's two-hit model of tumorigenesis states that mutation of both alleles of a tumor suppressor gene is needed to trigger tumor formation (248). However, some more recent studies have shown that mutation or loss of a single allele may be sufficient for tumorigenesis without inactivation of the second allele (249-251). This gene-dose effect is called haploinsufficiency, and has also been demonstrated for

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BER proteins Ape1 (118), Pol β (252), and Fen1 (155) in mice. Thus, a reduction in cellular concentration of some BER proteins either as a result of deregulation of gene expression, mutations, or direct inactivation by exogenous agents like certain chemicals or virus infections may result in a mutator phenotype and cancer development. The long time held view that mutagenic chemicals and radiation in the environment increase cancer rates by inducing mutations in normal human cells has been challenged by alternative hypothesis that common environmental risk factors may rather have a favorable selective action on preneoplastic cells previously initiated by spontaneous mutations (253). In support of this view, several recent reports have shown that direct inactivation of DNA repair proteins by environmental agents may account for the carcinogenetic effects of these agents. Cadmium is a natural element in the soil. It is usually found as a mineral combined with other elements such as oxygen, chlorine, or sulfur. Animals given cadmium in food or water had high blood pressure, iron-poor blood, liver disease, and nerve or brain damage (254). Recently, cadmium was found to specifically inactivate mismatch repair (255,256), and hOGG1 (257). The reduced hOGG1 activity might be through inactivation of transcription factor SP1 (258). hOGG1 was also shown to be inhibited by nitric oxide (NO) an inflammatory mediator, through formation of S-nitrosothiol adducts (259). This suggests a synergism between the ability of NO to generate DNA damage and the ability to inhibit repair of such lesions. Both lead and cadmium were reported to inhibit endonuclease activity of APE1 (260). Furthermore, arsenic inhibited poly(ADP-ribosyl)ation, which is mainly mediated by poly(ADP-ribose) polymerase-1 (PARP-1), at very low concentrations (261).

Many types of papillomavirus (HPV) cause benign skin tumors (warts) in their natural hosts. These warts often regress spontaneously, but human genital warts (tumors caused by specific types of papillomavirus, particularly types 16 and 18) may become malignant if they persist for a sufficiently long time. The E6 protein of HPV1, HPV8, and HPV16 was found to bind XRCC1, causing reduced SDBR (262). This report showed selective inactivation of a host DNA repair protein by a DNA tumor virus, likely providing an explanation for the genomic instability seen in cells infected by HPV.

Several studies have reported enhanced oxidative stress in patients with HIV infection. A reduced DNA glycosylase activity and higher levels of oxidative DNA damage in CD4⁺, but not CD8⁺ cells of HIV-infected patients was reported (263). Interestingly, antiretroviral therapy induced increased glycosylase activity in CD4⁺ T cells and normalized 8-oxoG levels (263).

In recent years, the epidemiology of BER capacity implicating single mutations as well as single nucleotide polymorphism (SNP) of BER proteins in human cancer has been the focus of investigation. In this regard, the scaffold protein XRCC1 has received a particular attention. XRCC1 interacts with several BER proteins and is important for the coordination and stimulation of this repair pathway. Two polymorphisms have been rather extensively studied and suggested as biomarkers for cancer susceptibility; the Arg194Trp

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polymorphism which resides in the linker region separating the POL β domain from the PARP interacting domain, and the Arg399Gln polymorphism which resides within the BRCT1 domain (Figure 7) (264). The hOGG1 gene is localized on the short arm of chromosome 3 in a region commonly deleted in lung cancer (265), and Ser326Cys polymorphism in *hOGG1* gene has been suggested to increase lung cancer susceptibility (266). hMYH, hAPE, and hOGG1 repair activities varied in human lung cancer cells because of somatic mutations or SNP (267). The low efficiency of 8-oxoG removal in these cells may be involved in lung cancer (268). Recently, germline mutations in *hMYH* were implicated in colorectal adenoma and carcinoma predisposition (269). MBD4 (also known as MED1, for methyl-CpG binding endonuclease 1) was found to be mutated in human carcinomas with microsatellite instability (270). These mutations were predominantly monoallelic and the majority occurred at a poly-A tract. MBD4 deficiency did not seem to increase mutation or accelerate tumorigenesis in mice lacking MMR (271). So, it appears that mutations in *MBD4* are a consequence rather than a cause of genomic instability. Epidemiological studies of BER polymorphisms and mutations have been thoroughly reviewed (264). Uracil-DNA glycosylase (UNG) is evolutionary highly conserved. The only reported mutation in the coding region of *UNG* gene in human cancer was detected in sporadic human glioma (272), which might be a result of PCR error (273). The human *UNG* gene from 62 different sources including 42 human cancer cell lines was screened and considerable sequence variations were identified, but none in the coding region of the gene (274). A separate group screened 100 samples and found no mutations in the catalytic region of *UNG* (275). However recently, patients with hyper-IgM syndrome (HIGM) were found to be defective in uracil-DNA glycosylase as a result of truncation mutations in the *UNG* gene (275).

Based on the reports so far, it appears that mutations in BER genes in cancer cells are rare events probably because severe defects in BER are incompatible with cell survival. For instance, it is very unlikely that defects in AP-site repair can ever be detected in human cells. However, certain polymorphisms as well as subtle variations in the gene expression and protein function likely modulate susceptibility to endogenous DNA damage and contribute to inter-individual variations in BER function and development of disease. Cancer occurs as a consequence of rounds of genetic instability and mutations and only cells that carry mutations that provide growth advantage and clonal expansion will develop into cancerous cells. Hence, it is possible that neoplastic cells carrying gross mutations in BER genes will not acquire growth advantage and are eliminated by the fierce selection pressure during the development of cancer.

Gene-targeted disruptions of DNA glycosylases in mice have produced none or mild phenotypes and modest levels of genomic mutations (117), with two exceptions: An age-dependent increased B-cell lymphomas in *Ung* knockout mice (40,276), and an increased spontaneous rate of liver and intestinal cancer, concomitant with the accumulation of 8-oxoG residues in mice with both the *Myh* and *Ogg1* genes inactivated

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(277). Functional redundancy and “backup activity” of DNA glycosylases have also been suggested as explanations for these observations. Some base lesions may be tolerated under certain threshold, but rapid environmental challenges may cause harmful effects in the absence of a functional DNA glycosylase (57,58). We should also consider an important role of DNA glycosylases in the preservation of population and species by counteracting gradual accumulation of mutations through generations as discussed before (47).

BER in mitochondria

Mitochondria, which probably evolved from endosymbiotically incorporated organisms, have their own genome. Mitochondrial DNA (mtDNA) is more vulnerable to damage than nuclear DNA (278), probably because of its proximity with the inner mitochondrial membrane which is the site of oxidative phosphorylation and generation of ROS. Thus, higher rates of mutation in mtDNA are expected. In fact, mutation rates of mtDNA were found to be considerably higher than nuclear gene mutation rates (279). Specific mutations in mtDNA have been found to associate with diseases as diverse as diabetes and deafness (280,281), and neurodegenerative disorders including Alzheimer disease (reviewed in (282)). Initially, mitochondria were believed to lack DNA repair activity, because they were unable to repair UV-light induced pyrimidine dimers in their genome (283). Although, subsequent research supported this observation, it became apparent that mitochondria were able to repair certain types of DNA lesions (284). We know now that human mitochondria have BER activity (285) and the mitochondrial isoforms of DNA glycosylases for removal of oxidized base lesions (286) and uracil (41) have been identified (Table 1). Commonly, the mitochondrial DNA glycosylases are generated by alternative splicing from the same gene that encodes the nuclear form. Although it needs further investigation, MMR activity in mitochondria has also been reported (287).

APE1 does not have a mitochondrial N-terminal presequence, but the presence of APE1 in mitochondria was demonstrated by electron microscopy immunocytochemistry and by subcellular fractionation combined with western analysis (288). APE1 was localized to mitochondria after H₂O₂ treatment of the cells (289), possibly to enhance BER of oxidative DNA damage. A role for the redox activation function of APE1 in mitochondria is unknown. A second human AP endonuclease (APE2) with similarity to the *S.cerevisiae* APN2 has been identified (132). APE2 has class II AP endonuclease activity *in vitro*, which is much lower than the AP endonuclease activity of APE1 (133). APE2 has a functional N-terminal mitochondrial localization signal (MLS) and is localized in mitochondria (134). The biological significance of APE2 in mitochondria is currently unknown.

Apparently, POL γ is the only DNA polymerase in human mitochondria (reviewed in (290)). POL γ comprises a catalytic core (POL γ A) in a heterotrimeric complex with two subunits of the processivity factor (POL γ B) (291). The holoenzyme is an efficient and processive polymerase, which exhibits

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high fidelity in nucleotide selection and incorporation with intrinsic 3' to 5' exonuclease proofreading activity (292). POL γ is also able to catalyze the removal of a 5'-deoxyribose phosphate (293). The accessory subunit stimulates the ability of the catalytic subunit to function in BER by enhancing the rate at which POL γ is able to locate damage in DNA, and by stimulating the dRP lyase activity of POL γ A (294). The DNA synthesis step in nuclear BER is via single nucleotide insertion (short-patch) or long-patch through incorporation of several nucleotides. The mitochondrial uracil repair was found to solely occur via short patch BER (295).

The DNA ligase III gene, *LIG3*, encodes a mitochondrial form of the protein using an alternative translation initiation site upstream of the initially identified start site (174). Transfection of human cells with an antisense human DNA ligase III vector reduced the mtDNA content (296). The residual mtDNA had numerous single-strand nicks (296), demonstrating a requirement for DNA ligase III in mtDNA repair. Heterodimerization of DNA ligase III with XRCC1 is necessary for the maintenance of normal cellular levels of cellular DNA ligase III (178). However, Chinese hamster ovary cells lacking XRCC1 (EM9) showed no defect in their mtDNA repair activity, indicating that the function of mitochondrial DNA ligase III is independent of XRCC1 (180).

Mitochondrial BER and ageing

Ageing can be defined as a progressive general weakening of function, resulting in an increasing vulnerability to environmental challenge and growing risk of disease and death. Commonly, theories of ageing involve damage to macromolecules (297). DNA damage from ROS is considered a major cause of ageing, and includes base modifications as well as single- and double-strand breaks. The vast majority of cellular ROS (~90%) can be traced back to the mitochondria implicating these organelles in ageing (reviewed in (298)). A systematic RNAi screen that inactivated over 5600 random *C.elegans* genes that increased lifespan also implicated mitochondria (299). A large number of life span determining genes were identified in this screen; the largest functional genes appeared to include genes that somehow regulate mitochondrial function. Oxidative DNA base modifications, large-scale deletions, and point mutations of mtDNA have been found to increase with age in various human tissues (300,301). These observations has led to the concept of “vicious circle” in which an initial ROS-induced damage, including mtDNA mutations, results in impairment of mitochondrial function that in turn leads to more mitochondrial damage. The significance of maintenance of mtDNA integrity was recently demonstrated in knock-in mice that expressed a proofreading-deficient DNA POL γ (302). These mice exhibited a significantly higher number of mtDNA deletions as well as mutations (302). Interestingly, these mice had a significantly shortened lifespan and displayed a number of age-related phenotypes (302). A gradual reduction in the integrity of mtDNA may be partly attributable to compromised mitochondrial BER by ageing that is the main mitochondrial DNA repair

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system if not the only. In fact, in older mice a large fraction of the mitochondrial Ogg1, and Ung1 were stuck to the membrane in the precursor form, which could not be translocated into the mitochondrial matrix (303). Apparently specific cell types such as neurons are particularly sensitive to the effects of age-related accumulation of mtDNA mutations. An age-dependent decline of DNA repair activity for removal of oxidative base lesions and uracil was found in rat brain mitochondria (304,305). Very recently, it was shown that normal ageing was associated with the activation of a caspase 3-mediated apoptotic pathway (306). Mice with POL γ defect in proofreading function and high mtDNA mutations displayed an early onset of this phenotype (306). Hence, accumulation of mtDNA mutations may contribute to apoptosis-mediated loss of irreplaceable cells and ageing. In support of this, an enhanced mtDNA repair and cellular survival after oxidative stress was seen by targeting hOGG1 to mitochondria (307).

AIMS OF THE STUDY

Aims of the study

Four uracil-DNA glycosylases (UNG, SMUG1, TDG, and MBD4) have been identified in human cells. SMUG1 was suggested to be the main enzyme for removal of uracil from U:G mispairs, while UNG2 was the major enzyme for removal U:A that occur during replication. To further clarify roles of UNG2 and SMUG1 in repair of uracil in human genome we decided to carry out *in vitro* and *in vivo* analysis of these proteins in human cells. This work is presented in paper I.

Trypanosoma cruzi (*T.cruzi*), causes Chagas' disease in humans. A single uracil-DNA glycosylase has been identified in *T.cruzi* (TcUNG). To develop drugs that specifically target *T.cruzi* without adverse affects on human cells is needed. For this purpose we wanted to see if human UNG and TcUNG show any distinct biochemical properties. This work is presented in paper II.

Specific protein-protein interactions as well as formation of multiprotein complexes that carry out specific biochemical actions are rather common in human cells. We were interested to see if UNG2 was able to form complex(es) with other BER proteins for complete repair of uracil in DNA. We decided to use non-neutralizing antibodies against the N-terminal region of UNG2 to immunoprecipitate possible UNG2-associated proteins. For functional analysis of the immunoprecipitates we decided to use BER assay with circular DNA containing uracil at a defined position. For identification of BER proteins in the immunoprecipitates we decided to use western analysis. The results of this work are shown in paper III. Next, we were interested to carry out a similar but slightly modified line of investigation to see if the mitochondrial UNG1 was able to form complex(es) with other BER proteins. This time we used extracts from HeLa cells stably expressing UNG1-EYFP fusion proteins and antibodies against EYFP protein for immunoprecipitation. This work is presented in paper IV. We decided to include analysis of UNG2-EYFP expressing HeLa cells in this study to directly compare this method of immunoprecipitation to the method we used to immunoprecipitate UNG2-associated BER proteins in paper III.

Uracil-DNA glycosylase (UNG2) is an evolutionary highly conserved DNA repair enzyme that removes uracil from U:A and U:G mispairs in DNA. Unrepaired U:G mispairs result to C to T transition mutations during replication. Human tumors contain a high proportion of C to T transition mutations. Hence, theoretically, mutation inactivation of UNG2 can contribute to the accumulation of such mutations in cancer cells. To test this we decided to screen human *UNG* gene for mutations in a number of human cancer cell lines (paper V) as well as in DNA isolated from paraffin-embedded tissue samples from gastric cancer patients (paper VI).

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Summary of the results and general discussion

Paper I (hUNG2 is the major repair enzyme for removal of uracil from U:A matches, U:G mismatches, and U in single-stranded DNA, with hSMUG1 as a broad specificity backup)

N-terminal proteolysis of hUNG2 has impeded the purification of full-length protein. Therefore, most studies on hUNG2 have been carried out on the purified catalytic domain of the protein. In this study purified full-length of hUNG2 and SMUG1 DNA glycosylases were used in comparative biochemical analysis to investigate their role in human BER. This study contains novel results on the biochemical behavior of full-length hUNG2 that varied significantly from earlier results from the purified catalytic domain of the protein. Contrary to the catalytic core of hUNG which is inhibited by MgCl_2 at all concentrations, full-length enzyme was stimulated 10-fold in the presence of 10 mM MgCl_2 . So, the N-terminal region of hUNG2 is needed for the effect of MgCl_2 . The same concentration of MgCl_2 stimulated SMUG1 by 2-fold. MgCl_2 affected the catalytic turnover of hUNG2 on dsDNA, whereas only the affinity of the enzyme was affected on ssDNA (reduced K_m). So, under near physiological concentrations, MgCl_2 turns hUNG2 to an efficient ssDNA selective enzyme. Interestingly, in the presence of physiological concentrations of MgCl_2 the affinity of SMUG1 for ssDNA substrate was reduced. Hence, the term “single-strand selective monofunctional uracil-DNA glycosylase” does not reflect its true function. Another interesting result presented in this study was the effect of AP-sites on the catalytic function of the enzymes. The catalytic core of UNG was known to bind to AP-sites more strongly than to uracil-containing DNA. However, no inhibition by AP-sites was seen for the full-length hUNG2. SMUG1 displayed a somewhat different response. Hence, ss-oligonucleotides containing AP-sites were found to have no inhibitory effect on SMUG1, whereas AP-sites containing ds-oligonucleotides strongly inhibited the catalytic activity. Furthermore, this study showed that SMUG1 had broader substrate specificity than hUNG2. Hence, UNG2 was active on ssU, U:A, U:G, and 5-fluorouracil (5-FU):A, while SMUG1 was in addition active on 5-hydroxymethyluracil (5-HmU):G, and 3,*N*⁴-ethenocytosine (ϵC):G. A fraction of hUNG2 was previously shown to localize in replication foci in the S-phase (42). In this study we used EYFP-hUNG2 and EYFP-hSMUG1 fusion proteins and demonstrated that hUNG2 was distributed in the nucleoplasm outside the S-phase, and a fraction was accumulated in replication foci in the S-phase. Moreover, hUNG2 appeared to be excluded from nucleoli both in the S-phase and outside the S-phase. However, hSMUG1 appeared to be less strictly localized to nuclei, and a substantial fraction was distributed throughout the cytoplasm. SMUG1 also appeared to be abundant in the nucleoli both in replicating and non-replicating cells.

SMUG1 was suggested to represent a major glycosylase against U:G mismatches, the product of cytosine deamination (62). Using neutralizing antibodies against UNG2 we showed that UNG2 constituted

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the major cellular DNA glycosylase activity both against U:A and U:G DNA substrates in human cell extracts. In the presence of both antibodies DNA glycosylase activity on both substrates was completely inhibited. This indicated that UNG2 and SMUG1 constituted the main uracil-DNA glycosylases for repair of U:A and U:G in these cell extracts. Moreover, we found that a complete inhibition of repair of U:A in circular dsDNA substrate was achieved by preincubation of extracts with neutralizing antibodies against UNG2, while antibodies against SMUG1 had no detectable inhibitory effect. Interestingly, repair of U:G in circular dsDNA substrate produced a different result. Therefore, a somewhat delayed but significant repair of U:G was seen in the presence of both antibodies. Possibly a fraction of UNG2 is inaccessible for UNG2 antibodies, which agrees with a previous observation in our lab not included in this paper.

In summary, the results presented in this paper showed that hUNG2 is the main DNA glycosylase for removal of uracil from U:A and U:G. Moreover, the results clearly demonstrate that UNG2 and SMUG1 have common as well as distinct functions in human BER, and act in specific subcellular locations.

Paper II (*Trypanosoma cruzi* contains a single detectable uracil-DNA glycosylase and repairs uracil exclusively via short patch base excision repair)

A few species of *Trypanosoma* are found in the world. From the standpoint of human health, the most important is *Trypanosoma cruzi* (*T.cruzi*), causing Chagas' disease. Chagas' disease affects primarily the nervous system and the heart. Chronic infections result in various disorders, including dementia, megacolon, and megaesophagus, and damage to the heart muscle. Acute infection can be lethal, but the disease usually evolves into a chronic stage, accompanied in 25 to 30% of cases by severe debilitation and ultimately death. In most cases, treatment of symptoms is all that is possible. Present medications can reduce the duration and severity of an acute infection, but are only 50% effective, at best, in eliminating the organisms. Hence, drugs or vaccines that can specifically target *T.cruzi* without adverse effects on the host cells are needed. Uracil DNA glycosylases have a broad distribution and proteins from eukaryotes, prokaryotes, eukaryotic viruses show a high degree of sequence homology. Inactivation of *E.coli* Ung causes a mutator phenotype, but mice deficient in *Ung* show a moderate increase of mutation frequency (39) probably because of presence of alternative uracil DNA glycosylases. Active UNG was previously shown to be essential for replication of herpes simplex virus type 1 (HSV-1) (308). A single uracil-DNA glycosylase has been identified in *T.cruzi* (TcUNG) (309). Hence, developing drugs that can specifically target DNA repair of *T.cruzi* is an appealing thought. To this end, we aimed to investigate the biochemical properties of TcUNG compared with human UNG (hUNG). A striking difference was the effect of MgCl₂. hUNG2 is stimulated about tenfold in the presence of 10 mM MgCl₂ which is attributable to the N-terminal

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domain of hUNG2 (paper I). However, this concentration resulted in nearly 85% inhibition of TcUNG. TcUNG contains an N-terminal presequence of about the same length as hUNG2, but with low apparent sequence similarity with hUNG2. A second obvious difference between TcUNG and hUNG was seen in the inhibitory effects of AP-sites. The purified catalytic domain of hUNG is inhibited by AP-site in the micromolar range. But, the presence of the N-terminal domain eliminates inhibition by AP-sites (paper I). We found that despite the presence of N-terminal presequence in TcUNG, the enzyme was susceptible to inhibition by double-stranded, but not single-stranded AP-containing oligonucleotides.

Depending on the length of DNA repair synthesis, base excision repair (BER) is divided into a short-patch pathway (single-nucleotide insertion) or a long-patch pathway when several nucleotides are inserted. BER in mitochondria seems to occur through short patch repair only (295), despite POL γ the only known DNA polymerase in human mitochondria, being a processive DNA polymerase. Under our experimental conditions, we found that repair of uracil (U:G) in circular double-stranded DNA (cccDNA) by extracts prepared from log-phase as well as stationary phase *T.cruzi* takes place via short-patch. POL β is the main BER DNA polymerase in human cells for short-patch BER. POL β is not inhibited by N-ethylmaleimide (NEM), but efficiently incorporates ddNTPs in DNA, hence inhibiting further DNA synthesis. A human POL β -like DNA polymerase has been identified in *T.cruzi* (310). This DNA polymerase was insensitive to aphidicolin, and NEM, but was markedly inhibited by the dideoxythymidine triphosphates (310). We found that repair of U:G in cccDNA substrate was not inhibited by ddCTP, while NEM completely inhibited repair DNA synthesis displaying a POL δ -like pattern of inhibition. This was somewhat in odds with the dominant short-patch repair we detected in *T.cruzi* extracts which, we expected to involve the reported human POL β -like. Moreover, TcUNG contains a putative PCNA-binding motif and DNA sequence analysis of *T.cruzi* have identified the presence of Pol δ and PCNA both of which are involved in long-patch BER in humans. Analysis of the complete sequence of *T.cruzi* genome identified several human counterpart DNA polymerases including translesion DNA polymerases η , κ , ζ , and Rev1, as well as X-family DNA polymerases proteins λ , μ , and Pol β (311). Recently, human DNA POL λ was found to function in BER (154). Involvement of DNA polymerases other than Pol β in *T.cruzi* BER may also explain the apparent discrepancies we observed here.

The uracil BER analysis we carried out in this study demonstrated certain organ specific biochemical properties distinct for TcUNG that may be beneficial for development of target-specific anti *T.cruzi* drugs.

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Paper III (Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells)

The N-terminal sequence of the nuclear form of uracil-DNA glycosylase (UNG2) is required for targeting (41). This region is the site of interactions with RPA (48) and PCNA (42) as well as a range of phosphorylation modifications (47). Terminal extension with unconserved polypeptide sequence, either at the N- or C-terminus, is quite common among mammalian glycosylases but are absent in *E.coli*. In addition to subcellular targeting, these segments are likely involved in establishment of communications among BER proteins and interactions with auxiliary proteins during replication, transcription and different phases of cell cycle. In this study we used non-neutralizing antibodies against the N-terminal region of UNG2 to immunoprecipitate (IP) UNG2-associated proteins. We showed that the UNG2-IP contained functional proteins and completely repaired uracil in DNA, which we called UNG2-associated repair complexes (UNG2-ARC). Using western analysis we showed that the UNG2-ARC contained UNG2, APE1, POL β , POL δ , PCNA, and XRCC1. Recently, we showed that neutralizing UNG-antibodies completely inhibited repair of U:A but not U:G in circular DNA by human cell extracts (Paper I). In the present study, we showed that anti-UNG-antibodies completely inhibited repair of U:A and U:G DNA substrates by UNG2-ARC. Moreover, we showed that inhibition of UNG did not impair subsequent BER steps by UNG2-ARC and recombinant SMUG1 partially restored BER of U:G, but not U:A by UNG2-ARC. Previously, hUNG2 was proposed to represent the main uracil DNA glycosylase for removal of U:A from incorporation of uracil during the replication (39), while SMUG1 was the major DNA glycosylase for removal U:G from deamination of uracil (62). However, in a later study we demonstrated that at close to physiological MgCl₂ concentrations, UNG2 was the main DNA glycosylase for removal of both U:A and U:G (Paper I). In support of this, later studies showed a more specific role for SMUG1 in removal of oxidative base lesions (58,63,65). Our results presented in this study and in paper I support the view that UNG2 is the main DNA glycosylase for removal of both U:A and U:G in DNA.

We tested role of APE1 in UNG2-ARC and found that antibodies against APE1 completely impaired repair of uracil by UNG2-ARC. This result agrees with APE1 as the major AP endonuclease in human cells. Moreover, we found that UNG2-ARC prepared from extract immunodepleted for XRCC1 showed reduced uracil and AP-site repair as well as the amount of ligated repair products. This result agrees with the interaction of POL β and DNA ligase III with XRCC1, and suggests that association of POL β with UNG2-ARC may occur through interaction with XRCC1.

Possibly, UNG2-ARC consists of repair complexes that are formed in different phases of the cell cycle. To better characterize possible sub-complexes of UNG2-ARC, we isolated complexes from freely cycling and growth arrested HaCaT cells. We found lower long patch repair in growth arrested cells compared with the cycling cells. This agrees with a DNA replication-associated long patch repair of uracil through direct interactions of UNG2 and PCNA. Furthermore, preincubation of UNG2-ARC with neutralizing

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POL β -antibodies significantly inhibited repair DNA synthesis in arrested cells compared with freely cycling cell, indicating that POL β is the main DNA polymerase for repair of uracil in DNA in non-cycling cells.

The buildup of repair complexes like UNG2-ARC depends on the concentration of repair proteins and/or to the rate of damage to DNA. We tested these possibilities using stably transfected HeLa cells overexpressing hUNG2. We found that repair of U:A in cell extract as well as UNG2-ARC increased by nearly 4- and 2-fold compared with control cells, respectively. But, the level of the cellular UDG enzymatic activity was 15-fold higher than in the normal cells. These results imply that UNG2 may be a limiting factor, but not the sole rate-limiting factor in the generation of repair complexes. Importantly, the results show that the formation of at least a fraction UNG2-ARC may be independent of damage in DNA, possibly in form of preassembled complexes.

Conventionally, we think of BER as a linear pathway initiated by a DNA glycosylase with subsequent processing of the damage by interaction of individual proteins with DNA lesion independently of each other. This model was developed mostly on the basis of *in vitro* analysis of naked DNA in cell free extracts or purified proteins. Data started accumulating on the posttranslational modifications and partial interactions of BER proteins, as well as interactions of core BER proteins with proteins that assisted BER, but were not required for *in vitro* conditions. It has been demonstrated that the protein modifications and protein-protein interactions affected BER through regulation of localization of repair enzymes as well as regulation of catalytic activity of individual proteins. Some recent *in vitro* studies demonstrated that BER on chromatin DNA was impeded at several steps (30,31), indicating that core BER enzymes likely need special conditions and additional factors to carry out repair. The results presented in this work and others point to a more complex and organized BER process that is regulated at several levels rather than random collisions of individual repair proteins with damaged DNA. Such interactions will not only make the process of DNA repair more efficient, but also the highly reactive repair intermediates will in this way be better protected from harmful modifications. Moreover, specific interactions can contribute to elevated local concentration of the repair proteins where they are needed.

Paper IV (Different organization of base excision repair of uracil in nuclei and mitochondria) (manuscript)

Mitochondrial uracil-DNA glycosylase (UNG1), and the nuclear form (UNG2) have different N-terminal sequences, but a common catalytic domain (41). Recently, we reported isolation of functionally active UNG2-associated repair complexes (UNG2-ARC) by using non-neutralizing antibodies targeted to the N-terminal region of UNG2 (paper III). In the present study, we tested a new approach for isolation of UNG-associated repair complexes. We prepared cell extracts from HeLa cells expressing UNG1 or UNG2 fused to enhanced yellow fluorescent protein (EYFP) placed C-terminally, and used antibody against EYFP

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to isolate possible UNG-associated repair complexes. We found that UNG2-EYFP immunoprecipitates carried out complete repair of uracil in DNA in agreement with our recent report (paper III). However, we did not detect uracil-BER activity in UNG1-EYFP immunoprecipitates. Moreover, we demonstrated that UNG1-EYFP had catalytic activity similar to UNG2-EYFP, indicating that the fusion proteins were catalytically functional. Very recently, most mitochondrial BER proteins were shown to associate with the inner membrane, independently of mtDNA (312). In our study, we carried out an *in organello* formaldehyde crosslinking of mitochondrial proteins and showed that interactions between UNG1 and other proteins including BER proteins (APE1) may take place. However, these interactions may not be stable, because we were unable to isolate sufficient amounts of stable complexes for complete repair of uracil in DNA. Therefore, it appears that contrary to the nuclear UNG2, the mitochondrial form of the enzyme does not undergo stable protein interactions with other BER proteins. There are some indications that at least certain nuclear repair enzymes are organized in tight association with nuclear matrix (28). Based on our results and others (312) it appears that mitochondrial and nuclear BER take place in more organized and coordinated fashion than previously thought. However, the form of organization differ between these organelles.

Perhaps the most important and time consuming step for analysis of BER complexes is a successful generation of antibodies that do not compete with other proteins for binding and are not inhibitory. Our findings demonstrate a versatile method for functional as well as structural analysis of nuclear BER complexes.

Paper V (Sequence variation in the human uracil-DNA glycosylase (*UNG*) gene)

In this study we PCR amplified regions of the human *UNG* gene covering all exons including exon-intron boundaries, both promoters, and intron V from 42 cell lines derived from tumor tissues, 10 cell lines established from normal tissues and DNA from blood (total of 62 sources). We carried out direct sequencing of the PCR products for possible mutations. We identified a number of alleles variants, but non in the coding region of the gene. We did not find any significant correlation between UDG activity and sequence variants. The frequencies for the variant alleles ranged from 0.01 to 0.23, which corresponds to the identification of one variant allele per 3.8 kb in non-coding sequences of the gene. The sequence variation within NER genes was found to be one variant allele in every 2.3 kb in coding and 1.2 kb in non-coding regions. Thus, apparently there is less sequence variation in the *UNG* gene than in genes for NER.

We found four different sequence variants in promoter B with allele frequencies ranging from 0.01 to 0.21, including a transition (C to T) in a putative binding site for transcription factor AP2, and a T to A transversion within a Yi element. Introducing the substitution in AP2 into chimaeric promoter-luciferase constructs affected transcription from the promoter. We detected this substitution only in cancer cells with

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an allele frequency of 0.1. The T to A transversion in a Yi element and a downstream G to A transition appeared to be linked with an average allele frequency of 0.18 and 0.2, respectively. Introducing these substitutions in concert into promoter B sequence did not influence expression from chimaeric promoter-luciferase construct.

In some cell lines, all identified sequence variants appeared to be homozygous. Two cancer cell lines and one normal cell line showed homozygosity for three substitutions. We carried out analysis of loss of heterozygosity using polymorphic markers; one for intron V in the *UNG* gene, as well as one centromic and one telomeric marker. The cancer cells showed homozygosity for the markers suggesting that they have undergone loss of heterozygosity. The normal cell line was heterozygous for the centromic marker and homozygote for the intron V and the telomeric markers.

Although, we found a number of sequence variation in non-coding regions of the *UNG* gene in cell lines from normal fibroblasts and tumor tissues, none was accompanied by significantly reduced UDG activity. Our results suggest that mutations affecting the function of human *UNG* gene are infrequent in human cell lines.

Paper VI (Low copy number DNA template can render polymerase chain reaction error prone in a sequence-dependent manner)

Human tumors contain a high proportion of C to T transition mutations, a fraction of these may be a result of deamination of cytosine to uracil, which left unrepaired may result in the C:G to T:A mutations during replication. Uracil DNA glycosylase (*UNG*) is an evolutionary highly conserved protein and is the major DNA glycosylase that removes uracil from DNA and counteracts accumulation of such mutations. Previously, a guanine to adenine mutation in exon III of the *UNG* gene resulting in a G143R substitution was reported (272). We conducted mutation analysis of *UNG* gene in DNA isolated from paraffin-embedded tissue samples from gastric cancer patients. In some samples we detected a mutation in exon III identical to the above mentioned mutation. However, we experienced difficulties to reproduce the results, and further experiments showed that the mutation was a PCR artifact, and its occurrence was inversely proportional to the amounts of DNA template in PCR samples. Importantly, we found that these errors occurred at particularly high rate at GC-rich sequences of the gene. This suggests that certain DNA sequences are particularly vulnerable for this type of PCR errors. In an earlier study we screened 62 samples including 42 human cancer cell lines for possible mutations in *UNG* gene (paper V). We found considerable sequence variations, but none in the coding region of the gene. In a separate study, no mutations were found in the coding region of the *UNG* gene from 100 human samples (275). So, it seems that mutations in the catalytic region of the *UNG* gene in human cancers if occur are very rare events and so far no verifiable data is available for such

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mutations. Although, germ line mutations in *UNG* gene have been reported in the patients with hyper IgM syndrome (275). Our results have general implications for mutation analysis of the so-called hot-spots, in particular in samples with low DNA contents like those isolated by laser-capture microdissection.

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Paper I

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Paper II

Trypanosoma cruzi Contains a Single Detectable Uracil-DNA Glycosylase and Repairs Uracil Exclusively Via Short Patch Base Excision Repair

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Enzymes involved in genomic maintenance of human parasites are attractive targets for parasite-specific drugs. The parasitic protozoan *Trypanosoma cruzi* contains at least two enzymes involved in the protection against potentially mutagenic uracil, a deoxyuridine triphosphate nucleotidohydrolase (dUTPase) and a uracil-DNA glycosylase belonging to the highly conserved UNG-family. Uracil-DNA glycosylase activities excise uracil from DNA and initiate a multistep base-excision repair (BER) pathway to restore the correct nucleotide sequence. Here we report the biochemical characterisation of *T. cruzi* UNG (TcUNG) and its contribution to the total uracil repair activity in *T. cruzi*. TcUNG is shown to be the major uracil-DNA glycosylase in *T. cruzi*. The purified recombinant TcUNG exhibits substrate preference for removal of uracil in the order ssU > U:G > U:A, and has no associated thymine-DNA glycosylase activity. *T. cruzi* apparently repairs U:G DNA substrate exclusively via short-patch BER, but the DNA polymerase involved surprisingly displays a vertebrate POL δ -like pattern of inhibition. Back-up UDG activities such as SMUG, TDG and MBD4 were not found, underlying the importance of the TcUNG enzyme in protection against uracil in DNA and as a potential target for drug therapy.

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Keywords: base excision repair; *Trypanosoma cruzi*; uracil analogues; uracil-DNA glycosylase; Ugi

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Introduction

Trypanosoma cruzi is the causative agent of the Chagas disease, a tropical disease affecting 16–18 million people in an area where 100 millions are estimated to be at risk†. Neither effective drugs nor vaccines are available for treatment despite

considerable research efforts.¹ *T. cruzi* undergoes a complex life cycle shuttling between the digestive tract of an insect vector, the blood of a mammalian host and inside the target cells.^{2–4} During its life cycle the parasite is exposed to rigorous environmental changes, and effective protection of the genome against genotoxic stress is likely to be crucial for its survival. Thus, proteins serving DNA-protective functions in *T. cruzi* are considered potential targets for drug therapy. Two such enzymes encoded by the *T. cruzi* genome are deoxyuridine pyrophosphatase (TcdUTPase)⁵ and uracil-DNA glycosylase (TcUNG)⁶ which are both involved in protection against uracil in DNA. The former is responsible for the hydrolysis of dUTP to dUMP and pyrophosphate, and thereby eliminates the triphosphate form of deoxyuridine, and erroneous incorporation of dUMP into DNA. The latter excises uracil occurring either from misincorporation of dUMP or from deamination of cytosine,

Abbreviations used: AP, apurinic/aprimidinic; BSA, bovine serum albumin; HMU, 5-hydroxymethyluracil; LIT, liver infusion tryptose; 8-oxoG, 8-oxo-guanine; UDG, uracil-DNA glycosylase; UNG, uracil-DNA glycosylase belonging to the highly conserved family-1 of UDGs; hUNG2, nuclear isoform of human UNG; Ugi, uracil-DNA glycosylase inhibitor protein from *Bacillus subtilis* bacteriophage PBS2; ϵ C, 3,N4-ethenocytosine; BER, base-excision repair; ss, single-stranded; ds, double-stranded.

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† <http://www.who.int/ctd/chagas/burdens.htm>

and initiates a multistep base-excision repair (BER) pathway.^{7,8} BER can be completed either by short-patch repair (one nucleotide insertion), or long-patch repair (two to eight nucleotides insertion).^{9,10} These mechanisms largely use different enzymes and accessory proteins that may differ from organism to organism. Uracil DNA glycosylases have a broad phylogenetic distribution and proteins from prokaryotes, eukaryotes and eukaryote viruses display a high degree of sequence homology.¹¹ TcUNG also shows high homology at the amino acid level with its counterparts.⁶ Uracil DNA glycosylases form a protein superfamily consisting of five families.^{12,13} Family-1 enzymes (comprising human UNG and *Escherichia coli* Ung) are active against uracil in ssDNA and dsDNA, and recognise uracil in an extrahelical conformation. Family-2 enzymes (comprising human TDG and *E. coli* Mug) are mismatch specific and recognise the widowed guanine in the complementary strand. Family-3 (SMUG) and family-4 (predicted from a wide range of archaea as well as thermophilic and mesophilic eubacteria) enzymes have common active site motifs but their catalytic residues are not conserved. Family 5 has recently been described and comprise an enzyme which also catalyses the removal of hypoxanthine from DNA.¹⁴ The many functional back-up UDG activities in mammals probably explains why homozygous *ung*^{-/-} mice display no immediately apparent phenotype, and only have a moderately increased mutation frequency.¹⁵ However, in organisms that apparently rely on only one UDG, such as several viruses, this gene appears to be essential.^{16,17} Moreover, it has been shown that the intracellular pathogens *Pseudomonas aeruginosa* and *Mycoplasma smegmatis*, which have G+C rich genomes, depend on functional UDG activity for their survival.¹⁸

Human UNG is the best studied enzyme among the family-1 enzymes and accounts for more than 95% of the total UDG activity in human cells *in vitro*.¹⁹ The enzyme is a monomeric protein, not dependent on cofactors and with preference for uracil in ssDNA. hUNG mostly removes uracil faster from U:G mismatches than from U:A matches, but this is sequence dependent, and has no activity against thymine in T:G mismatches. The enzyme is inhibited by micromolar concentrations of certain uracil analogues,^{20,21} and shows dose-dependent and stoichiometric inhibition by the UDG inhibitor protein Ugi encoded by the bacteriophage PBS2.²² Human UNG2 likely has a major function in both post-replicative removal of uracil near the replication fork as well as overall removal of deaminated cytosine residues and certain uracil analogues derived from oxidative damage.^{23–26} Viral members of family-1 UDGs (vaccinia- and herpesviral UDGs) have been shown to be required for viral replication.^{27,28} Thus, cellular and viral UDGs are potential chemotherapeutic targets for cancer and viral diseases, respectively. Several uracil analogues have been tested and are already in use for the treatment of such diseases although

their mechanisms of action are not completely understood.^{29–31}

In contrast, less is known about DNA base excision repair in trypanosomatids. The existence of a gene encoding uracil-DNA glycosylase in *T. cruzi* and AP endonuclease genes in both *Leishmania major* and *T. cruzi* provides molecular evidence for base excision repair of uracil.^{6,32,33} In addition, several DNA polymerases have been partially characterised whereas their role in repair has not yet been defined.^{34,35} In *T. cruzi*, the *udg* gene encoding TcUNG exists as a single copy, and Western analysis of parasite extracts indicates the presence of only one protein species encoded by this gene.⁶ The sequence revealed high degree of homology with UDGs belonging to the family-1 of the UDG superfamily. Recombinant TcUNG protein was expressed and purified. The protein behaved as a monomer in gel filtration chromatography and activity was measured using a qualitative assay dependent on enhanced fluorescence of ethidium bromide when intercalated into double stranded DNA at pH 12.⁶ In the present work we have quantified the contribution of TcUNG to the total UDG activity in *T. cruzi* by using inhibitory antibodies and the protein inhibitor Ugi. The presence of enzymes belonging to the other UDG families is investigated by using alternative substrates. The type of BER (short- or long-patch) is studied and the roles of TcUNG and polymerases analysed. Finally, an extensive biochemical characterisation of recombinant TcUNG is performed, and the data compared to human UNG. In particular, the kinetics of inhibition by a panel of known UNG-inhibitors is investigated to further establish the potential of TcUNG as a target for drug design.

Results

Activity and substrate specificity of TcUNG

When [³H]dUMP:A-containing calf-thymus DNA was used as substrate, maximum TcUNG activity was found at 45 °C, pH 7.5–8.0 and 65 mM NaCl (data not shown). These values are similar to what is observed for the nuclear human UNG2.²⁵ Under standard conditions, the specific activity obtained was 6000 units/mg, which is about two-fold higher than the specific activity of hUNG2. Kinetic analyses revealed that this was mainly caused by a markedly higher affinity of TcUNG (lower K_m) for both ss- and ds-substrates than hUNG2, whereas k_{cat} of TcUNG was somewhat lower than that of the human enzyme for both substrates (Table 1). Excision of U or T by TcUNG from oligonucleotides with variant base partners revealed a substrate preference in the order ssU > U:G > U:A (96, 28 and 16% uracil excised, respectively), while no G:T-mismatch activity was observed (Figure 1). Furthermore, excision of εC and oxoG from dsDNA or HMU from either ssDNA or dsDNA was not detectable (data not shown).

Table 1. Enzyme kinetic parameters of TcUNG and hUNG2

	Substrate	K_m (μM)	k_{cat} (min^{-1})	k_{cat}/K_m ($\text{min}^{-1} \mu\text{M}^{-1}$)
TcUNG	U:A	0.44	82	185
	ssU	0.62	222	358
hUNG2	U:A	3	187	62
	ssU	13	1060	79

Kinetic parameters were analysed from direct linear plots using the method of Wilkinson.

This is similar to that observed for hUNG2²⁵. TcUNG demonstrated no dependence on divalent cations and complete inhibition of activity was observed with 10 mM Co^{2+} , Cu^{2+} , Fe^{3+} or Zn^{2+} . Approximately, 70% activity was retained at 10 mM Ca^{2+} or Mn^{2+} , while 15% residual activity was retained at 10 mM Mg^{2+} . The latter stands in contrast to the about tenfold stimulation of hUNG2 observed in the presence of 10 mM Mg^{2+} .²⁵

TcUNG complements *E. coli* ung mutants *in vivo*

The similarities between the TcUNG and the other Family-1 UDGs, raised the question whether TcUNG was able to complement *E. coli* ung mutants, as shown for human Ung.³⁶ To assess functional activity of the TcUNG in *E. coli*, wild-type (NR8051) and ung mutant cells (NR8052) were transformed with either pETTcung or pET28a empty vector. Mutant frequencies were scored by counting rifampicin-resistant colonies in induced and non-induced cells.³⁶ Induction of TcUNG resulted in a threefold decrease of revertants in NR8052, demonstrating that TcUNG is able to suppress the weak mutator phenotype of these cells. The frequency of rif^R mutations was of 0.77 ± 0.01 and 0.22 ± 0.1 per 10^8 cells for the control and the cells overexpressing TcUNG, respectively.

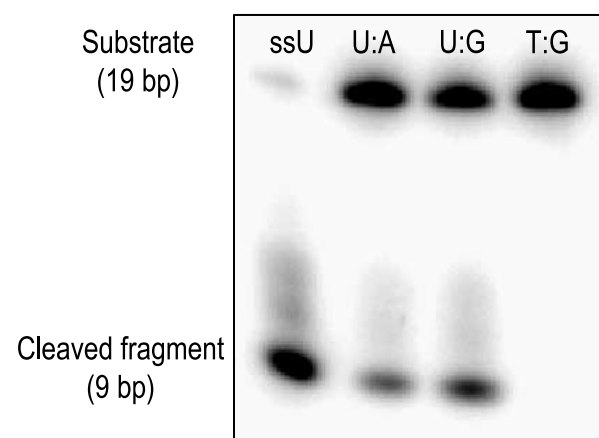


Figure 1. Substrate specificity of TcUNG. Denaturing PAGE of the products of the reaction catalysed by TcUNG with single stranded oligonucleotides containing uracil (lane 1), and double stranded oligonucleotides containing either U:A mismatches (lane 2), U:G mismatches (lane 3) or T:G mismatches (lane 4).

Sequence specificity of TcUNG resembles that of other family-1 UDGs

To study the dependence of local sequence context upon uracil excision, ³²P-labelled dsM13-DNA containing uracil at random positions was used as substrate. Uracil-excision varied about 20-fold at the different positions (Figure 2). Melting temperature (T_m) for the 10 nt sequence encompassing uracil at each position was calculated, and the average values for the ten “best” and ten “poorest” substrates were 29.4 °C and 33.6 °C, respectively.

Uracil position ^a	Sequence 5'-3'	% removal of uracil ^b
238	T G C A T U A A T G A	100
241	A T T A A U G A A T C	85
245	A T G A A U C G G C C	64
233	C C A G C U G C A T T	62
206	C G C T T U C C A G T	61
221	A A A C C U G T C G T	51
160	C C T A A U G A G T G	50
169	T G A G C U A A C T C	48
179	C A C A T U A A T T G	45
226	T G T C G U G C C A G	44
279	C G T A T U G G G C G	44
174	T A A C T U A C A T T	42
197	G T C A C U G C C C G	39
188	T G C G T U G C G C T	39
223	A C C T G U C G T G C	36
237	C T G C A U T A A T G	36
183	T T A A T U G C G T T	31
204	C C C G C U T T C C A	31
193	T G C G C U C A C T G	28
211	T C C A G U C G G G A	26
178	T T A C A U T A A T T	25
164	A T G A G U G A G C T	25
205	C C G C T U T C C A G	25
272	C G G T T U G C G T A	23
310	A C C A G U G A G A C	21
173	C T A A C U T A C A T	19
276	T T G C G U A T T G G	18
182	A T T A A U T G C G T	15
278	G C G T A U T G G G C	15
291	C A G G G U G G T T T	15
271	G C G G T U T G C G T	14
344	C C G C C U G G C C C	14
187	T T G C G U T G C G C	13
350	G G C C C U G A G A G	13
326	A C A G C U G A T T G	9
270	G G C G G U T T G C G	5

Figure 2. Sequence specificity of uracil excision for TcUNG. Sequences are shown in order of decreasing rate of removal. ^a Position number is the distance from the M13 sequencing primer to the uracil residue (bold). ^b Removal of uracil is given as the mean of four independent experiments. Total (100%) removal is considered as the removal of uracil in the band presenting higher intensity.

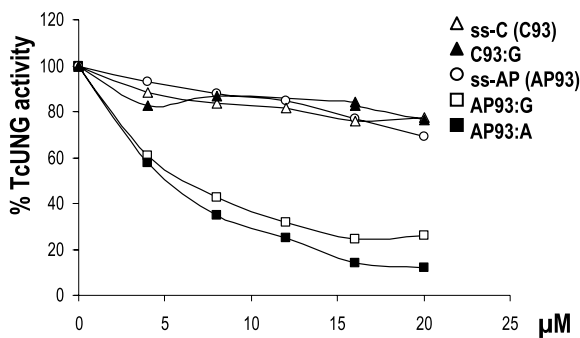


Figure 3. Effect of apyrimidinic sites on TcUNG. The activity of TcUNG against [^3H]dUMP-containing calf thymus DNA in the presence of oligonucleotides containing AP-sites is measured. Filled squares, duplex AP93:A; empty squares, duplex AP93:G; empty circles, ss-AP(AP93); filled triangles, ss-C control(C93); empty triangles, duplex C93:G control. 100% activity is measured in the absence of added oligonucleotides.

The sequence specificity resembled what has previously been reported for the human UNG and *E. coli* Ung proteins.^{19,37–39} Several sequences from which U is removed fastest were common among the three organisms when using the same substrate. Moreover, the sequence preference of TcUNG was in accordance with the consensus sequence for “poor removal” of uracil observed with hUNG and *E. coli* Ung (5'-(G/C)U(T/G)-3' or 5'-UT-3').

Inhibition of TcUNG by uracil and uracil analogues

T. cruzi UNG is subject to product inhibition by uracil with a IC_{50} value of 0.3 mM (data not shown). For the uracil analogues 6-chlorouracil, 6-amino-uracil and 5-fluorouracil, IC_{50} values of 0.1 mM, 2 mM and 5 mM were observed, respectively. A 25 bp oligonucleotide (2'FU141) containing the uracil analogue βFdUrd was previously shown to

specifically inhibit HSV-1 Ung, while hUNG was less affected.⁴⁰ TcUNG proved to be even less inhibited by the human enzyme, retaining about 95% and 80% activity in contrast with the 70% and 40% retained activity found with the recombinant catalytic domain of human UNG (UNG Δ 84) enzyme against ss and ds 2'FU141, respectively.⁴⁰

Product inhibition of TcUNG by AP sites

AP-sites have been demonstrated to be micro-molar product inhibitors for UDGs from different organisms, including the family-1 UNG proteins. Recently, however, it was demonstrated that the N-terminal presequences of the mitochondrial (UNG1) and nuclear (UNG2) of human UNG precluded such inhibition.^{25,41} To investigate the effect of AP-sites on TcUNG, 4–20 μM ss or ds-oligonucleotides containing AP sites or normal bases were included in the standard UDG-assay. More than 50% of the TcUNG activity was inhibited by the presence of 8 μM of the double-stranded AP-containing oligos, increasing to about 80% inhibition at 16 μM . The specificity of the AP-containing oligos was verified by the low inhibition observed with normal G:C-containing oligos. Interestingly, no significant inhibition was observed with the single-stranded AP93 relative to ss controls at the same concentration (Figure 3). A selective product-inhibition of TcUNG by AP-sites in duplex DNA may thus contribute towards the low k_{cat} of the enzyme against double stranded substrates.

Inhibition of TcUNG by the PBS2 inhibitor protein, Ugi

The PBS2-encoded inhibitor protein Ugi is a strong inhibitor that binds stoichiometrically and irreversibly to family-1 UDGs.^{42,43} TcUNG was also found to be stoichiometrically inhibited by Ugi, with an IC_{50} value of 3.1 fmol when using 7.07 fmol enzyme. A plot of $[\text{S}]$ versus $[\text{S}]/V$ shows that the K_m

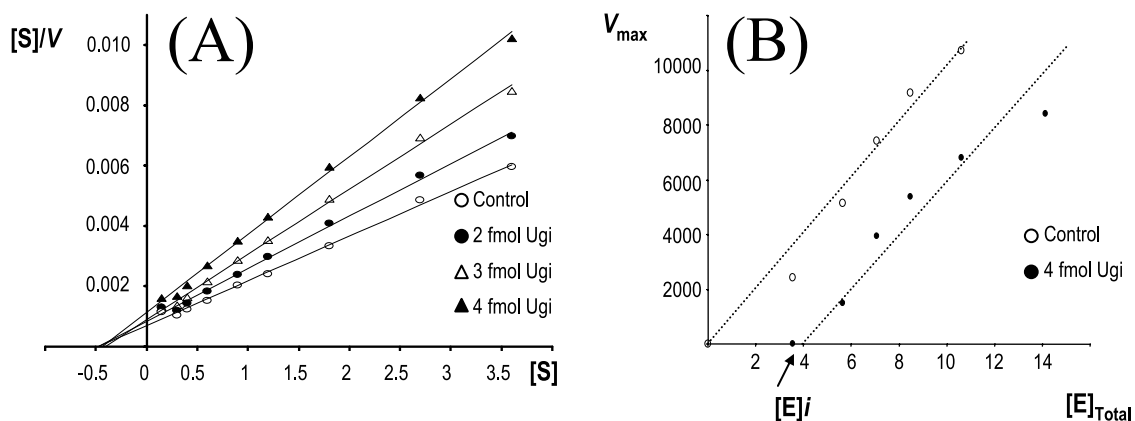


Figure 4. Inhibition of purified recombinant TcUNG by Ugi. (A) Hanes plot of the effects of various concentrations of Ugi protein. (B) Plot of V_{max} versus amount (fmol) of enzyme $[\text{E}]_{\text{Total}}$ added in the presence and absence of Ugi. $[\text{E}]_i$ represents the amount of enzyme titrated by the irreversible inhibitor and controls in (A) and (B) are experiments performed in the absence of Ugi.

is not affected by Ugi (Figure 4(A)) and a plot of V_{max} versus enzyme amount in the presence and absence of Ugi furthermore indicated that the binding was irreversible (Figure 4(B)).

Family-1 TcUNG is the major uracil-DNA glycosylase in *T. cruzi*

The specific UDG activity in cell-free *T. cruzi* extracts was 0.03 unit/mg when measured under standard conditions. To quantify the relative contribution of TcUNG to the total UDG activity in *T. cruzi*, cell free extracts were preincubated with the hUNG-neutralising antibody PU101.¹⁹ In separate experiments, PU101 was shown to neutralise the recombinant TcUNG in a dose-dependent manner, while no inhibition is observed with antibodies toward human SMUG1 or a control antibody (2261) against a region of the mouse UNG1 N-terminal domain with no homology to TcUNG (Figure 5). The UDG activity in *T. cruzi* extracts was strongly inhibited by PU101. Using 1.25 µg antibody a 95% inhibition of the total UDG activity is found, increasing to >99% in the presence of 11.5 µg antibody (Figure 5). In contrast, essentially no inhibition was observed by corresponding amounts of PSM1 or 2261. This strongly indicates that TcUNG is the major, if not only uracil-DNA glycosylase in *T. cruzi* cells.

It could not be excluded, however, that the calf-thymus (A:U-containing) substrate used in these experiments was not optimal for the detection of other potential UDG-activities, such as G:T(U)-mismatch-specific glycosylases corresponding to human TDG or MBD4 (MED1). To investigate this further, *T. cruzi* extracts were subjected to analysis using a panel of oligonucleotides containing U (or T) and HMU with either A or G as the opposite base. An εC:G containing oligonucleotide known to

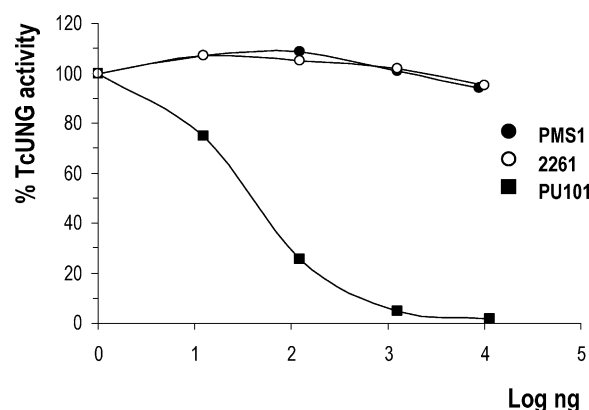


Figure 5. Inhibition of total UDG activity in *T. cruzi* extracts by polyclonal PU101 IgG. Extracts were incubated with various amounts of PU101 IgG (filled squares) directed towards the catalytic domain of hUNG, prior to analysis of UDG activity. The polyclonal IgG PSM1 against human SMUG1 (filled circles) and 2261 control IgG (open circles) demonstrated essentially no neutralisation of UDG activity. The abscissa represents the logarithm of the amount of IgG (in ng) added in each reaction.

be a substrate of hTDG and hSMUG1 was also assayed. The assay was carried out in presence and absence of Ugi. As shown in Figure 6, and by quantitation of damage excision from the various substrates (data not shown), detectable activity is observed with all three uracil-containing substrates. However, no G:T-mismatch or 5-HMU activity is observed. The latter supports the notion that double-strand specific UDGs and SMUG-type activities are absent in *T. cruzi*. The absence of SMUG1 is also verified by western analysis of

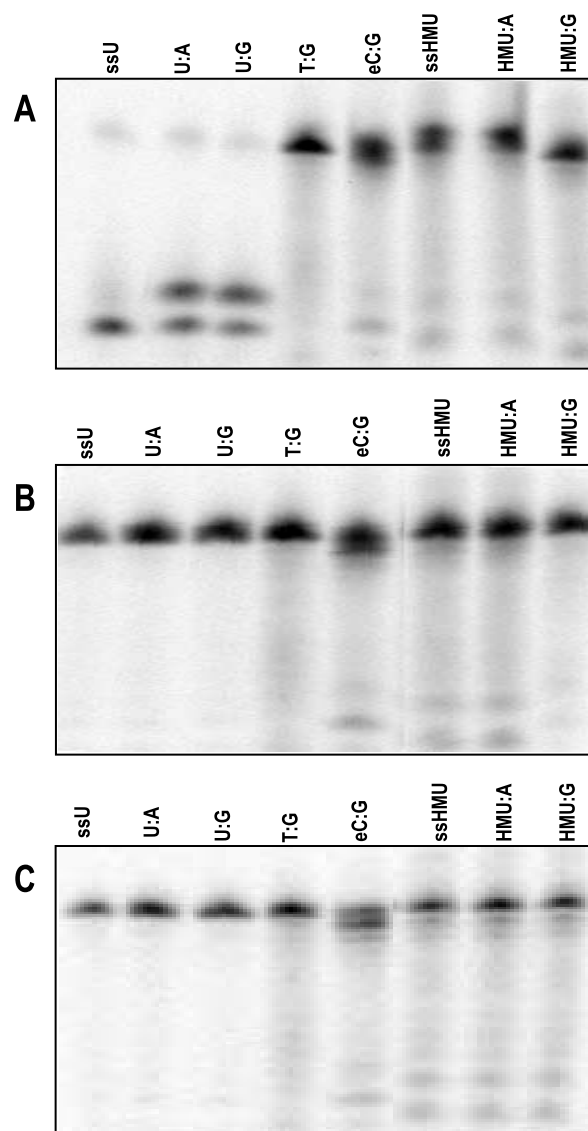


Figure 6. TcUNG is the major *T. cruzi* Uracil-DNA glycosylase. Denaturing PAGE of the ³²P-labelled products of the reaction catalysed by *T. cruzi* cell-free extracts. Single stranded oligonucleotides containing uracil (lane 1) or HMU (lane 7), and double stranded oligonucleotides containing either U:A (lane 2), U:G (lane 3) T:G (lane 4), εC:G (lane 5), oxoG:C (lane 6), HMU:A (lane 8) or HMU:G (lane 9) were employed. Panels A and B correspond to the oligonucleotides treated with *T. cruzi* cell-free extracts in absence and presence of Ugi, respectively. The control panel C corresponds to the substrates incubated in absence of *T. cruzi* cell-free extracts. Gels were run in 0.5x TBE buffer, and quantified after phosphoimaging.

cell-free extracts and probing with anti-hSMUG1, as well as the lack of sequence similarity to SMUG1 proteins of translated EST's in the *T. cruzi* genome database. To date no other UDG-family proteins have been annotated in the *T. cruzi* genome project.

Repair of U:G mismatches in *T. cruzi* cell extracts proceeds via short-patch BER

Repair of cccDNA containing a U:G mismatch at a defined position (Material and Methods) was analysed using *T. cruzi* extracts prepared from cells in both stationary and exponential growth phase. BER was also measured in the presence of the TcUNG inhibitor Ugi, the polymerase δ -type inhibitor *N*-ethylmaleimide (NEM) and ddCTP (Figure 7). ddCTP can be readily incorporated into nascent DNA by the human DNA Pol β , and to a lesser extent by Pol α or Pol δ resulting in the inhibition of elongation of the DNA chain and therefore it is used to detect polymerase β -type activities. Short-patch but no long-patch BER was detected (lane 1, low molecular mass band). Repair is inhibited when TcUNG is inhibited by Ugi (lane 2) and also when the polymerase inhibitor NEM is used (lane 3). ddCTP does not have any effect on repair (lane 4) indicating that the polymerase involved is a polymerase δ -like. We obtained identical results using cell-extracts from parasites in stationary phase (lanes 5–8, respectively). The lower panels corresponding to "total BER" show only the 22 nt repair product indicating that repair was completed and ligation of the final product occurred. The weaker band of high molecular mass DNA (HMW, theoretically 3191 base-pairs) in Figure 7 represents unspecific incorporation in the rest of the plasmid. The increased strength of the corresponding HMW band in the intermediate panels is the result of the contribution of short-patch repair in this band, as expected from the HincII/PstI cleavage pattern.

Discussion

In a previous study the existence of a uracil-DNA glycosylase in the parasitic protozoan *Trypanosoma cruzi* was demonstrated. Cloning of the gene and deduction of the encoded amino acid sequence revealed that the corresponding protein, TcUNG, belonged to the group of highly conserved family-1 UDGs.^{13,19} In the present study a detailed characterisation of the recombinant enzyme and its interactions with different inhibitors is presented, as well as the contribution of TcUNG to the total UDG-activity in *T. cruzi* cell-free extracts. Recombinant TcUNG was enzymatically active, and expression of the enzyme in *E. coli* ung mutants restored the wild-type phenotype. This corresponds to previous findings that human UNG is able to complement *E. coli* ung mutants and indicates a functional homology between the *E. coli*, human and *T. cruzi* enzymes. This is supported by the

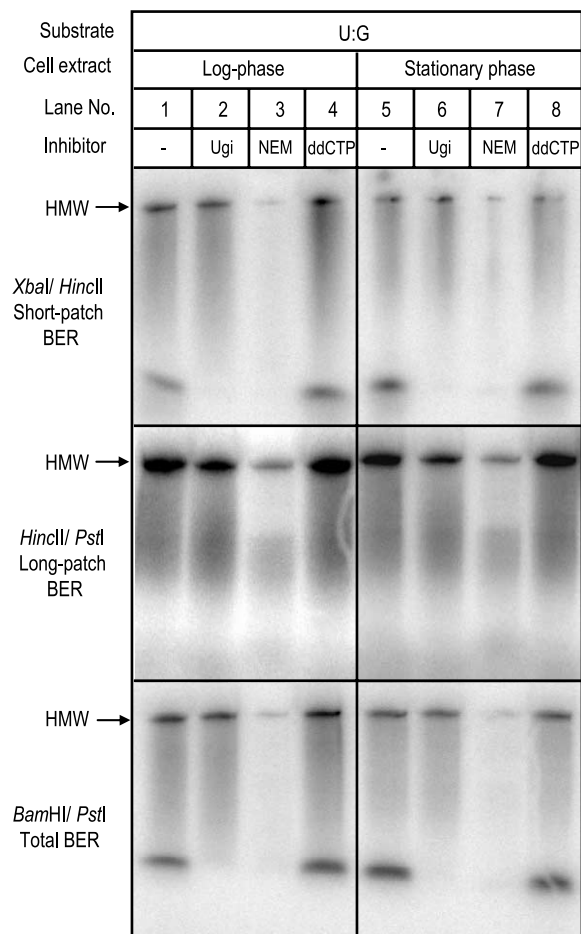


Figure 7. BER assay of U:G repair in *T. cruzi* cell extracts. Mode of BER is analysed by using a cccDNA substrate containing a uracil. In lanes 1–4, cell extract from parasites growing in logarithmic phase is used. In lanes 5–8, cell extract from non-growing parasites is employed. Upper panels show results after digestion of the repair mixture with the restriction enzymes XbaI/HincII, the lower band indicates radioactivity incorporation at the site of damage (one nucleotide insertion). Intermediate panels show results after digestion of the substrate with the restriction enzymes HincII/PstI, the lower band arise from radioactive nucleotide incorporation in positions 3' to the site of damage (several nucleotides insertion). Lower panels show results after digestion with BamHI/PstI, the lower band in this case indicates complete repair and ligation of the substrate. Lanes 1 and 5 correspond to substrate repair in absence of inhibitors, in lanes 2 and 6 the samples were incubated in the presence of Ugi, samples 3 and 7 contain NEM, and samples 4 and 5 contain ddCTP. HMW (high molecular mass DNA).

similar biochemical characteristics of the human UNG2 and TcUNG enzymes with respect to NaCl, pH and temperature optimum. A striking difference between TcUNG and hUNG2 is, however, observed with regard to the effect of Mg^{2+} . Whereas hUNG2 is about tenfold stimulated in the presence of 10 mM Mg^{2+} , this resulted in approximately 85% inhibition of TcUNG. Interestingly, the

Mg²⁺-responsiveness is confined to the N-terminal regulatory domain of UNG2,²⁵ and is also associated with the mitochondrial UNG1 N-terminal domain (unpublished results). TcUNG contains an N-terminal presequence of about the same length as human UNG2 and UNG1 presequences, but with low apparent sequence similarity with the human enzymes (Figure 8). The molecular basics underlying the difference in Mg²⁺-responsiveness and its functional implications, thus remain to be elucidated. Catalytic efficiency of TcUNG against uracil in ss- and dsDNA in the absence of divalent cations is in the same range as hUNG2, and the enzyme has essentially the same substrate preference as previously observed with hUNG2 with respect to the opposing base.²⁵ Moreover, additional activities against εC, 5-HMU T:G mismatches are not associated with the recombinant TcUNG. The absence of 5-HMU glycosylase activity, such as SMUG1, has already been described in several kinetoplastid protozoans, and may rely on the capability of these organisms to synthesise the unusual modified base B-D-glucosyl(hydroxymethyl)uracil, called J. J is synthesised through an intermediary 5-hydroxymethyldeoxyuridine^{44–46} and expression of a SMUG1-like enzyme in protozoa causes DNA damage and interferes with J biosynthesis.⁴⁷

Sequences for good removal do not exhibit a clear (A/T)UA(A/T) pattern found for the calf thymus and *E. coli* enzymes but resemble those obtained with the human enzyme with lower melting temperature values and preferentially two A residues adjacent to the uracil.^{19,37–39} The effects of sequence context have been shown to arise from differences in binding and not catalysis and have been explained as originated by local differences in DNA flexibility in the region surrounding uracil.^{38,48} The very similar catalytic properties of TcUNG and hUNG2 are reflected in the high degree of conservation of the residues involved in binding to DNA and recognition of the uracil among these proteins (Figure 8). Conservation of the residues involved in dUMP-interaction and catalysis among hUNG^{49,50} and TcUNG likely also explains that uracil and uracil analogues used in this study showed IC₅₀ values in the same range as for the human enzyme,²¹ being 6-chlorouracil the most effective inhibitor tested. Likewise, the strong inhibition by Ugi is in agreement with the conservation in TcUNG of residues demonstrated to be involved in Ugi-binding to other UDGs^{22,51–58} (Figure 9). An oligonucleotide containing 2'-fluoro-uridine (2-F-U) was also tested for inhibition of the *T. cruzi* enzyme. This approach was proven to be useful for specific inhibition of the herpes simplex virus UDG over the human UNG.⁴⁰ Remarkably, IC₅₀ values for inhibition of TcUNG with double and single-stranded oligonucleotides were even higher than those for hUNG2 showing possible differences between rates for removal of uracil from the oligonucleotide sequence or specific effects of the 2-F-U among both enzymes.

A pronounced difference in inhibition between

TcUNG and hUNG2 was observed in the presence of AP-sites. We have demonstrated that the catalytic domain of hUNG is inhibited by AP-sites in the micromolar range,⁴⁹ whereas the presence of mitochondrial (hUNG1) or nuclear (hUNG2) N-terminal domains obviates inhibition by AP-sites.^{25,41} The presence of an N-terminal presequence in TcUNG, however, still renders the enzyme susceptible to inhibition by double-stranded AP-containing oligonucleotides. This is also in agreement with the observed stimulation of TcUNG activity in the presence of AP endonuclease from *Leishmania major*⁶ and likely contribute to the preference of TcUNG for ssDNA substrate. Furthermore, the AP-site responsiveness supports the differences in Mg²⁺-responsiveness, in that the N-terminal sequences of TcUNG and hUNG2 confer different functional properties to the two proteins.

Whether TcUNG is responsible for both nuclear and mitochondrial uracil repair in the parasite remains to be determined. A single band detected in Western analysis of *T. cruzi* cell-free extracts indicates the presence of only one TcUNG species, although two or more closely migrating species could not be ruled out. The N-terminal presequence of TcUNG contains motifs in agreement with both nuclear and mitochondrial targeting, and based on known mitochondrial targeting sequences in other early branching eukaryotes, a potential cleavage site for mitochondrial localisation is located at the N-terminal QRT/LL⁶ (Figure 8). Thus, proteolytic cleavage could yield a mitochondrial form lacking only five N-terminal residues compared to the full-length protein. Interestingly, this putative cleavage site is an integral part of a sequence highly similar to a conserved PCNA-binding motif in other eukaryotes (Figure 9).⁵⁹ The PCNA sliding clamp can mediate the interaction of proteins with DNA. One of the proteins known to interact with PCNA is the hUNG2 enzyme. This interaction has been described in post-replicative long-patch BER.⁶⁰ A PCNA homologue has been predicted in *T. cruzi* (TIGR, *T. cruzi* Genome Project,†), and such homologues have also been described in other protozoa like *Leishmania*⁶¹ and *Plasmodium*. In *Plasmodium*, BER seems to occur predominantly as a PCNA-dependent, long-patch BER pathway⁶² and the absence of a short-patch BER pathway may provide opportunities to develop selective chemotherapeutic strategies for damaging the parasites *in vivo*. In *T. cruzi*, despite the putative presence of PCNA and a PCNA binding motif in the TcUNG sequence and that the polymerase involved follows a pol δ-like pattern of inhibition, BER apparently proceeds *via* short-patch BER only. The absence of a polymerase β role in short-patch BER in these parasites constitutes a unique feature in opposition to the well-documented role of this enzyme as part of short-patch BER in vertebrates and other organisms. In addition, Ugi inhibits

† <http://www.tigr.org/tdb/>

protein concentration of the supernatant (extract) was measured using the Bio-Rad protein assay (BSA as standard) prior to snap freezing in liquid nitrogen and storage in small aliquots at -80°C .

Immunological analyses

Cell-free *T. cruzi* extracts and purified proteins were subjected to SDS-PAGE and electroblotted to Immobilon PVDF membranes (Millipore). Primary antibodies were polyclonal rabbit IgGs against either the catalytic domain of human UNG (PU101), the N-terminal domain of the mouse UNG1 (2261), or full-length human SMUG1 (PSM1). Secondary antibody was HRP-conjugated goat anti rabbit IgG (DAKO). Proteins were visualised by ECL-western blotting reagents (Amersham Biosciences, UK). To quantify the contribution of TcUNG to the total UDG-activity in *T. cruzi* cell-free extracts, 1.25 μg total protein was incubated with various amounts of antibodies for 30 minutes on ice prior to the UDG assay as described below.

Bacterial strains, growth media and plasmids

E. coli BL21 (DE3) (Novagen, EMD Biosciences, San Diego, CA) were employed for expressing the TcUNG protein, *E. coli* NR8051 ($\Delta(\textit{pro-lac}), \textit{thi}^{-}, \textit{ara}, \textit{trp9777}$) and NR8052 ($\Delta(\textit{pro-lac}), \textit{thi}^{-}, \textit{ara}, \textit{trp9777}, \textit{ung1}$)⁶⁴ were used for mutation rate analyses. Bacterial cultures were grown in LB-medium with the required antibiotics. The pET expression system and His-bind resin were from Novagen. M13 forward primer and USB Sequenase sequencing kit containing modified T7 DNA polymerase were from United States Biochemical (Cleveland, OH), T4 polynucleotide kinase was from New England Biolabs (Beverly, MA). Synthetic oligodeoxyribonucleotides were from MWG-Biotech AG (Ebersberg, Germany).

Mutator assay

E. coli NR8052 and NR8051 were transformed with the expression plasmids pET28a and pET28-TcUNG containing the TcUNG cDNA. To detect the number of rifampicin resistant bacteria, 200 μl of the appropriate bacterial culture was mixed with 3 ml top agarose, poured on LB-plates containing 100 $\mu\text{g}/\text{ml}$ rifampicin (Sigma) and incubated overnight at 37°C . The colonies were counted and the number of rifampicin resistant colonies per 10^8 viable cells was calculated.

Overexpression and purification of TcUNG

The pET-28a expression vector was employed to clone the coding sequence for the TcUNG gene. *E. coli* BL21 (DE3) cells carrying the target vector pETTcUNG were used for overexpression of the recombinant TcUNG protein carrying an N-terminal $6\times$ His-tag sequence under conditions previously established.⁶ Protein was purified by affinity chromatography with a His-bind metal chelation resin as described.⁶

Measurement of uracil-DNA glycosylase activity

Unless otherwise stated, UDG activity was measured in (referred to as standard conditions) 20 μl of assay mixture containing (final) 62.5 mM NaCl, 20 mM Tris-HCl (pH 7.5), 1 mM EDTA, 1 mM DTT, 0.5 mg/ml BSA and 1.8 μM [³H]dUMP:A-containing calf-thymus DNA substrate (specific activity 0.5 mCi/ μmol). The mixture was

incubated for ten minutes at 30°C and the amount of uracil released was measured as described.¹⁹ One unit of UDG activity was defined as the amount of enzyme releasing 1 nmol of uracil per min at 30°C . Proteins were estimated either by the modified Bradford assay (Bio-Rad) using BSA as standard or by measuring absorbency at 280 nm using the following molar extinction coefficients: TcUNG $\epsilon_{280}=4.3\times 10^4$, UNG $\Delta 84$ $\epsilon_{280}=5.04\times 10^4$, Ugi $\epsilon_{280}=0.83\times 10^4$.

Measurement of Michaelis constants and interaction with inhibitors

Enzyme kinetic constants and inhibition were measured in the presence of 0.1–8.5 μM and 1.8 μM [³H]dUMP-containing calf-thymus DNA substrate (measured as uracil), respectively, and 7.07 fmol of TcUNG. Single stranded substrate was prepared by heating the dsDNA substrate at 100°C for ten minutes and then quenching on ice immediately prior to the assays. Uracil analogues were prepared as 0.1 M stock solutions in homogenisation buffer and a final pH of 7.5. IC₅₀ values were calculated using the Enzfitter software (Biosoft, Cambridge, UK). Catalytic constants were calculated using Enzpack, version 3.0 (Biosoft) using the method of Wilkinson. UNG-Ugi interactions were measured by incubating different amounts of Ugi with 7.07 fmol of either TcUNG or the recombinant catalytic domain of human UNG (UNG $\Delta 84$)¹⁹ in 10 μl homogenisation buffer for ten minutes prior to the UDG-assays. To calculate the amount of TcUNG titrated by Ugi, 1.8 μM of substrate was used together with 4 fmol of Ugi and 2–14 fmol of purified TcUNG.

All oligonucleotides analysed for inhibition of TcUNG were PAGE-purified. A 19 bp duplex oligonucleotide containing 2'-fluoro-uridine (2'-F-U) was prepared by annealing the following oligonucleotides:

2'FU141 5'-CATAAAGTG2'-F-UAAAGCCTGG-3'
141G 5'-CCAGGCTTTGCACTTTATG-3'

Seventy-five micromolar of the single stranded 2'FU141 or 50 μM of double stranded 2'FU141G were used in final reactions of 20 μl .

Oligonucleotides containing AP-sites were prepared from the following.

Target strands:

U93 5'-TGAAATTGUTATCCGCTCA-3'
NormC93 5'-TGAAATTGCTATCCGCTCA-3'

Complementary strands:

93G 5'-TGAGCGGATAGCAATTTCA-3'
93A 5'-TGAGCGGATAACAATTTCA-3'

Ten nanomolar of U93 and NormC93 (control) were incubated with 1 μg of UNG $\Delta 84$ for 30 minutes at 37°C in a final volume of 100 μl and the reactions stopped by heating at 80°C for 15 minutes. This yielded AP93 and C93, respectively. In separate reactions, equimolar amounts of the complementary strands were added prior to heat inactivation. The oligonucleotides were allowed to anneal by slow cooling to room temperature, to yield AP93A, AP93G and C93G, respectively.

Substrate specificity analyses

Oligodeoxyribonucleotides of 19 bp, each containing a

central altered base, were 5' labelled using [γ - 32 P]ATP and T4 polynucleotide kinase. The following oligonucleotides were used:

Target strands.

U141 5'-CATAAAGTGUAAAGCCTGG-3'
 HMU141 5'-CATAAAGTGHMUAAGCCTGG-3'
 ϵ C141 5'-CATAAAGTG ϵ CAAAGCCTGG-3'
 oxoG141 5'-CATAAAGTGoxoGAAAGCCTGG-3'
 T141 5'-CATAAAGTGTAAGCCTGG-3'

Complementary strands:

141A 5'-CCAGGCTTTACACTTTATG-3'
 141G 5'-CCAGGCTTTGCACTTTATG-3'

Duplexes were prepared by mixing equimolar amounts of the appropriate single stranded oligonucleotides followed by heating for two minutes at 65 °C and slowly cooling to room temperature. Each labelled substrate was then mixed with 100 \times excess of the corresponding unlabelled substrate, and used in UDG assays at a final concentration of 2 μ M. Release of uracil was visualised by cleaving the resulting AP-sites with hot piperidine and subjecting the reaction products to denaturing PAGE. After drying and phosphorimaging of the gels, uracil excision was quantified as percent cleaved, labelled fragments. For analysis of substrate specificity of *T. cruzi* cell-free extracts, 1.8 μ g of protein was used in the presence or absence of 4 fmol of Ugi.

Sequence specificity for removal of uracil

Sequence specificity of TcUNG was assayed using [32 P]dUMP-labelled M13-DNA essentially as described.³⁹ Band intensities were quantified after phosphorimaging of the gels.

DNA substrates for BER assay

Covalently closed circular DNA (cccDNA) substrates were prepared essentially as described.⁶⁵ Briefly, 20 μ g of ssDNA (pGEM-3zf+) were annealed to 4.2 μ g of a 5' phosphorylated 22-mer complementary oligonucleotide containing a uracil in a specific position (Figure 10). Synthesis of duplex DNA was carried out in the presence of T4 DNA polymerase, T4 DNA ligase, and T4 gene 32 ssDNA-binding protein at 37 °C for two hours. cccDNA duplex molecules were purified by CsCl gradient centrifugation.

BER assay

The base excision repair mixtures (50 μ l) contained (final) 40 mM Hepes-KOH (pH 7.8), 70 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 2 mM ATP, 20 μ M dATP, 20 μ M dTTP, 8 μ M dCTP, 8 μ M dGTP, 4.4 mM phosphocreatine, 2.5 μ g creatine kinase, 18 μ g bovine serum albumin, 2 μ Ci [α - 32 P]dCTP and [α - 32 P]dGTP, 50 μ g whole cell extract (measured as protein) and 300 ng cccDNA substrate. The repair mixtures were incubated at 30 °C for 60 minutes, stopped by adding (final) 20 mM EDTA and 80 ng/ μ l RNase A (37 °C, ten minutes), and further incubated with (final) 190 ng/ μ l proteinase K and 0.5% SDS (37 °C, 30 minutes). When indicated, BER assays were performed in the presence of either Ugi (100 fmol), 10mM (final), *N*-ethylmaleimide (Sigma) or 25 μ mol (final) ddCTP (Amersham Biosystems). The repair products were purified by phenol/chloroform extraction and ethanol/salt

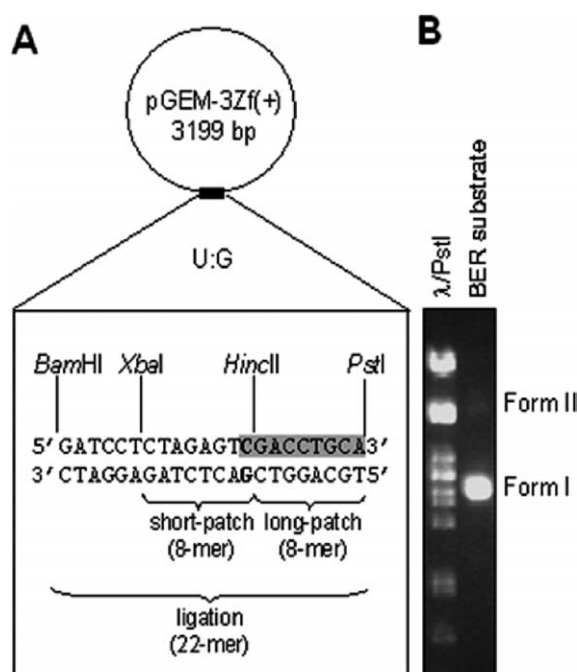


Figure 10. Substrate and strategy for BER studies. (A) As substrate for the BER assay we used double stranded circular covalently closed pGEM-3zf(+) with U/A or U/G in the marked positions 1* and 2* respectively. The cleavage sites for restriction enzymes used for mapping of the repair patches are indicated. The shaded region corresponds to the long-patch. (B) The quality of the substrate in terms of the amount of covalently closed circular plasmid DNA (form I) and nicked circular forms (form II) purified by CsCl/ethidium bromide equilibrium centrifugation. Purity was assessed by running 100 ng of DNA on 1% agarose gel with *Pst*I digested λ DNA as marker.

precipitation. DNA was digested with indicated restriction enzymes (Figure 1), and analysed by electrophoresis in denaturing 12% polyacrylamide gels and phosphorimaging (Fuji, BAS-1800II) of the dried gels. Digestion of the U:A substrate with restriction enzymes *Xba*I/*Hinc*II yielded an eight nucleotide fragment containing label in the one nucleotide at the site of damage only (position 0). Digestion with *Hinc*II/*Pst*I yielded an eight nucleotide fragment that could contain label in positions +1, +3, +4, +6 and +7 (long-patch repair) when using [α - 32 P]dCTP and [α - 32 P]dGTP as radioactive labels. Digestion with *Bam*HI/*Pst*I yielded a 22 nucleotide fragment if ends were ligated, and a 14 nucleotide fragment if the dominant short-patch product was not ligated. The purity of the substrate prepared was estimated by agarose gel electrophoresis (Figure 1). Only DNA substrate containing >95% cccDNA was used in BER assays.

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Paper III

Repair of U/G and U/A in DNA by UNG2-associated repair complexes takes place predominantly by short-patch repair both in proliferating and growth-arrested cells

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ABSTRACT

Nuclear uracil-DNA glycosylase UNG2 has an established role in repair of U/A pairs resulting from misincorporation of dUMP during replication. In antigen-stimulated B-lymphocytes UNG2 removes uracil from U/G mispairs as part of somatic hypermutation and class switch recombination processes. Using antibodies specific for the N-terminal non-catalytic domain of UNG2, we isolated UNG2-associated repair complexes (UNG2-ARC) that carry out short-patch and long-patch base excision repair (BER). These complexes contain proteins required for both types of BER, including UNG2, APE1, POL β , POL δ , XRCC1, PCNA and DNA ligase, the latter detected as activity. Short-patch repair was the predominant mechanism both in extracts and UNG2-ARC from proliferating and less BER-proficient growth-arrested cells. Repair of U/G mispairs and U/A pairs was completely inhibited by neutralizing UNG-antibodies, but whereas added recombinant SMUG1 could partially restore repair of U/G mispairs, it was unable to restore repair of U/A pairs in UNG2-ARC. Neutralizing antibodies to APE1 and POL β , and depletion of XRCC1 strongly reduced short-patch BER, and a fraction of long-patch repair was POL β dependent. In conclusion, UNG2 is present in preassembled complexes proficient in BER. Furthermore, UNG2 is the major enzyme initiating BER of deaminated cytosine (U/G), and possibly the sole enzyme initiating BER of misincorporated uracil (U/A).

INTRODUCTION

Uracil in DNA occurs as a result of deamination of cytosine and incorporation of dUMP during replication. Deamination

of cytosine occurs at a rate of 100–500 per human cell per day, yielding mutagenic U/G mispairs which, unless repaired, result in GC to AT transitions upon replication (1). Incorporation of dUMP during replication results in U/A pairs which are not miscoding, but which may yield cytotoxic and potentially mutagenic abasic (AP) sites (2). Uracil in DNA may also affect transcriptional fidelity (3), as well as binding of transcription factors (4). A recently identified source of uracil in the genome is the enzymatic deamination of cytosine to uracil by activation-induced cytidine deaminase (AID) in the process of somatic hypermutation and antibody class switch in B-cells (5). Uracil is recognized by a uracil-DNA glycosylase (UDG) activity, which cleaves the N-glycosylic bond leaving an AP-site in DNA. Human cells contain at least four types of UDG; mitochondrial UNG1 and nuclear UNG2, SMUG1, TDG and MBD4, which have overlapping substrate specificities (6). Their specific functions are still unclear. Among these glycosylases, UNG proteins are by far the catalytically most efficient (6,7). UNG1 and nuclear UNG2 are both encoded by the *UNG*-gene and have a common catalytic domain, but different N-terminal sequences required for subcellular sorting (8). Upon mitochondrial import the preform of UNG1 is processed to a mature form lacking 29 N-terminal amino acid residues (9). AP-sites in nuclear DNA are repaired by either single-nucleotide (short-patch) base excision repair (BER) or via replacement of several nucleotides (long-patch BER). Short-patch BER requires an AP endonuclease, POL β and DNA ligase III or possibly also DNA ligase I, while long-patch BER depends on flap endonuclease I (FEN-1), and may require proliferating cell nuclear antigen (PCNA), DNA polymerases δ/ϵ and DNA ligase I. POL β has also been suggested to be required for long-patch repair either for insertion of the first nucleotide (10) or for strand displacement (11). An APE1-independent short-patch BER pathway has recently been suggested. Thus, the bifunctional DNA glycosylases NEIL1 and NEIL2 carry out β , δ -elimination after excision of damaged bases generating a one-nucleotide gap flanked by 3'-phosphate and 5'-phosphate termini. The 3'-phosphate terminus may

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subsequently be converted to a 3'-OH terminus by polynucleotide kinase (PNK), thus setting up for short-patch repair (12).

In DNA repair and related processes, there is evidence for the existence of functional multi-protein complexes. Thus, multi-protein complexes involved in eukaryote transcription and replication have been reported (13,14). A group of proteins that associates with DNA repair protein BRCA1 has been identified and named BRCA1-associated genome surveillance complex (BASC). It includes DNA repair proteins MSH2, MSH6, MLH1, ATM, BLM and the RAD50-MRE11-NBS1 protein complex, PCNA and RF-C (15). Furthermore, a multi-protein complex that connects Fanconi anemia and Bloom syndrome has recently been reported (16). Many interactions between the proteins in the initial step of nucleotide excision repair (NER) have also been reported and the existence of a multiprotein repairosome complex was suggested (17), although a later study did not find evidence in support of such a complex (18). Nevertheless, it is evident that many interactions do occur and it may seem likely that the mechanism of NER at least in part relies on formation of complexes (19). There is also evidence for complex formation in BER. Thus, a 180 kDa protein complex that repairs uracil-containing DNA was isolated from bovine testis using DNA polymerase β -affinity chromatography (20). Direct molecular interactions between UNG2 and other BER factors, e.g. RPA (21) and PCNA (22) have also been reported. However, PCNA interacts with multiple partners, e.g. POL δ , POL ϵ , RF-C, DNA ligase I, FEN-1, POL β , APE1 and XRCC1, all of which are involved in long-patch BER, and all, except POL β and XRCC1, in DNA replication (23–25). Most likely PCNA engages in different complexes, but it is also possible that the three binding sites in this homotrimeric protein bind different proteins. POL β also interacts directly with ligase I (26), XRCC1 (27) and APE1 (28). In addition, XRCC1 interacts with DNA ligase III (27,29) with poly(ADP-ribose) polymerase (PARP) through one of its BRCT-modules (30), PNK (31), APE1 (32) and PCNA (24) acting as a scaffold protein in BER and single-strand break repair (33).

Given the plethora of interactions between BER proteins, it seemed likely that it would be possible to isolate a complex for nuclear BER of uracil-containing DNA, using a UNG2-specific antibody or other BER-antibodies. The present work demonstrates the presence and properties of an UNG2-containing protein complex proficient in complete repair of uracil-containing DNA.

MATERIALS AND METHODS

Synthetic oligonucleotides were from MedProbe (Oslo, Norway). [α -³²P]dTTP and [α -³²P]dCTP (3000 Ci/mmol) were from Amersham Biosciences. Primary antibodies against POL δ (mouse monoclonal) were from Transduction Laboratories, Lexington, KY, antibodies against PCNA (ab29, PC10), POL β (mouse monoclonal) and XRCC1 (rabbit polyclonal) were from Abcam Ltd, UK, and antibodies against APE1 (rabbit polyclonal) from Novus Biologicals Inc., Littleton, CO, USA. Rabbit antiserum to APE2 was kindly provided by Magnar Bjørås (Centre of Molecular Biology and Department of Molecular Biology, Oslo). Human recombinant POL β and neutralizing antibodies to POL β were generous gifts from

Dr S. H. Wilson (Laboratory of Structural Biology, NIH). Paramagnetic Protein A-beads were from Dynal, Oslo, Norway. A neutralizing polyclonal rabbit anti-hSMUG1 IgG (PSM1) was prepared as described previously (7). Silver-staining of protein gels was carried out using the ProteosilverTM Plus, Silver Stain Kit (Sigma-Aldrich Co.).

DNA substrates

Covalently closed circular DNA (cccDNA) substrates were prepared essentially as described previously (34). Briefly, 20 μ g of ssDNA [pGEM-3zf(+)] were annealed to 4.2 μ g of a 5' phosphorylated 22mer complementary oligonucleotide containing either a uracil or normal base (molar ratio 1:30) (Figure 1A). Synthesis of duplex DNA was carried out in the presence of T4 DNA polymerase, T4 DNA ligase and T4 gene 32 ssDNA-binding protein at 37°C for 2 h. cccDNA duplex molecules were purified by CsCl gradient centrifugation. The purity of the substrate prepared was estimated by agarose gel electrophoresis (Figure 1B). Only DNA substrate containing >95% cccDNA was used in BER assays. By site-directed mutagenesis of a pGEM-3zf(+) Phagemide, we prepared a G to A mutation to be able to prepare substrates containing U/A and U/G in the same sequence context. For AP-site substrate, uracil-containing cccDNA was incubated with purified catalytic domain of UNG (35) just before use. Complete removal of uracil was confirmed by further incubation of an aliquot of this DNA with purified APE1 protein resulting in complete conversion of form I (supercoiled closed circular DNA) to form II (nicked circular DNA) detected by agarose gel electrophoresis (data not shown). For generation of AP-nick substrate DNA, uracil-containing plasmid was incubated with purified catalytic domain of UNG and purified APE1 protein.

UNG antibodies

Neutralizing anti-UNG-antibody PU101 against the catalytic domain has been described previously (35). Polyclonal anti-UNG2 PU1 was prepared following the same procedure. PU1sub antibody against the N-terminal region of the human UNG2 was prepared by passing PU1 IgG over a matrix containing the recombinant C-terminal catalytic UNG-domain bound to NHS-activated Sepharose (Amersham Biosciences). The IgGs in the flow-through proved to be specific for the UNG2 N-terminal regulatory domain as judged from western analysis (Figure 1C and D), and did not inhibit UNG enzyme activity.

HeLa Tet-On cells (HTO) overexpressing UNG2 (HTO-UNG2)

UNG2 cDNA was cloned into the EcoRI/XbaI sites of vector pTRE and the construct (pTRE-UNG2) co-transfected with pTK-Hyg into HTO cells. Hygromycin resistant clones were selected and subcloned by dilution. The subclone that repeatedly gave the best expression after induction, HTO-UNG2-45, was used in the present study.

Culture of cell lines and preparation of whole cell extracts

HaCaT, HeLa S3 and HTO-UNG2 cells were cultured in DMEM with 10% fetal calf serum (FCS), 0.03% glutamine

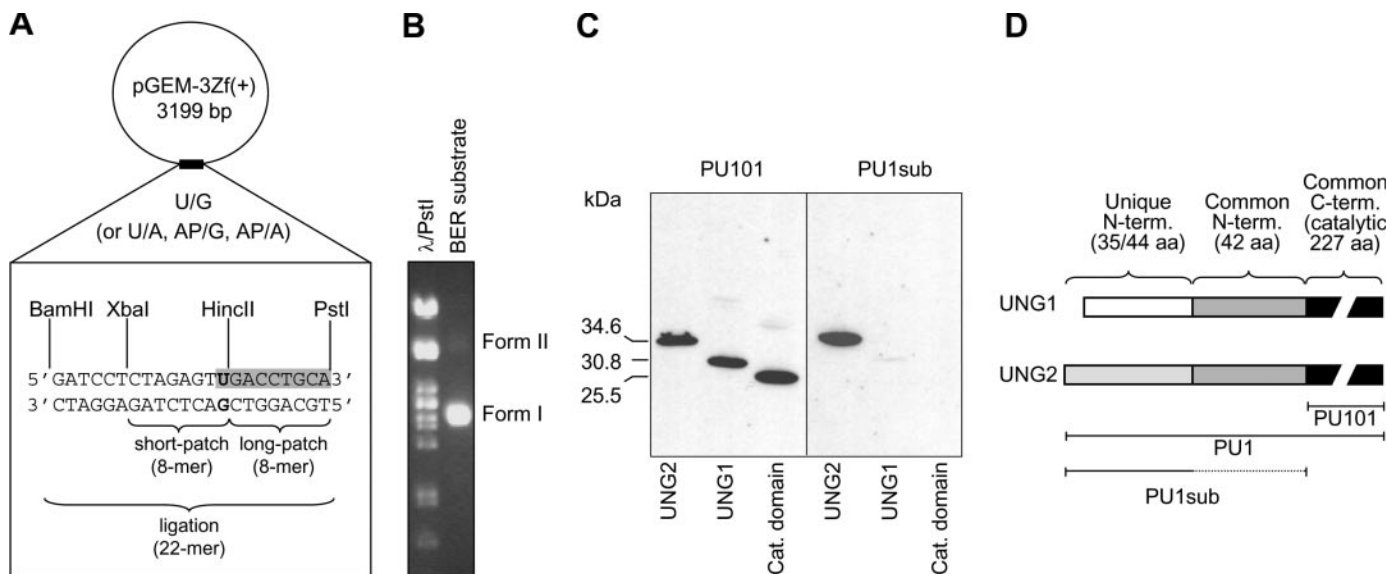


Figure 1. Substrate and strategy for BER studies. (A) As substrate for the BER assay we used double-stranded covalently closed circular pGEM-3Zf(+) with U/G, U/A, AP-site/G or nicked AP-site/A in the same position. The cleavage sites for restriction enzymes used for mapping of the repair patches are indicated. The shaded region corresponds to the repaired patch. (B) The quality of the substrate in terms of the amount of covalently closed circular plasmid DNA (form I) and nicked circular forms (form II) purified by CsCl/ethidium bromide equilibrium centrifugation. Purity was assessed by running 100 ng of DNA on 1% agarose gel with PstI digested λ DNA as marker. (C) Specificity of anti-UNG-antibodies PU101 and PU1sub. Recombinant UNG2, the mitochondrial form UNG1 Δ 29 and the catalytic UNG-domain were separated by polyacrylamide gel electrophoresis and subjected to western analysis using either PU101 or PU1sub, as indicated. (D) Structure of the preform of UNG1, UNG1 Δ 29, UNG2 and the catalytic domain UNG Δ 84.

and 0.1 mg/ml gentamicin at 5% CO₂. Human myeloma cell line JFN-3 was cultured under similar conditions but in RPMI 1640 medium. Peripheral blood lymphocytes were obtained by density gradient centrifugation of buffy coat over LymphoprepTM (Nycomed, Norway). The UNG^{-/-} lymphoblastoid cell line was from patient 2 (36) and carried a Phe251-Ser homozygous mutation. Cells were grown in RPMI 1640, with 0.03% glutamine, 10% heat-inactivated FCS, and 100 U/ml penicillin and 100 μ g/ml streptomycin at 5% CO₂.

Whole cell extracts were prepared essentially as described by Tanaka *et al.* (37). Briefly, cells were pelleted at 215 g and resuspended at 1 \times packed cell volume in buffer I [10 mM Tris-HCl (pH 8.0), 200 mM KCl] and 1 \times packed cell volume of buffer II [10 mM Tris-HCl (pH 8.0), 200 mM KCl, 2 mM EDTA, 40% (v/v) glycerol, 0.5% NP-40, 2 mM DTT, Complete[®] protease inhibitor]. The mixture was rocked at 4°C for 2 h and cell debris was pelleted at 22 000 g at 4°C for 10 min. The supernatant was recovered and protein concentration measured using the Bio-Rad protein assay. Extracts were snap frozen in liquid nitrogen and stored in small aliquots at -80°C.

Preparation of BER complex UNG2-ARC

PU1sub IgGs were covalently linked to magnetic Dynabeads[®] Protein A using dimethyl pimelimidate dihydrochloride (DMP) according to instructions from the manufacturer (DynaL, Norway) with minor modifications: 400 μ g protein from whole HeLa cell extract was mixed with 5 μ l of the antibody-coated beads or otherwise indicated, and kept in suspension under constant and gentle rocking for 4 h at 4°C. The beads were washed three times with 10 mM Tris-HCl, pH 7.5, transferred to a new tube, washed once more in

the same buffer and resuspended in appropriate buffer for further use. For control experiments, we prepared beads linked to the same amount of pre-immune IgG from the same rabbit (pre-immune-IgG), and non-immunized rabbit serum (non-immune-IgG).

BER assay

The BER mixtures (50 μ l) contained (final) 40 mM HEPES-KOH (pH 7.8), 70 mM KCl, 5 mM MgCl₂, 0.5 mM DTT, 2 mM ATP, 20 μ M dATP, 20 μ M dGTP, 8 μ M dCTP or dTTP depending on the isotope used, 4.4 mM phosphocreatine, 2.5 μ g creatine kinase, 18 μ g BSA, 2 μ Ci [α -³²P]dCTP or [α -³²P]dTTP, 50 μ g whole cell extract (measured as protein) or UNG2-ARC (5 μ l beads) or otherwise immunoprecipitated material, and 300 ng cccDNA substrate if not otherwise indicated. For BER assay experiments, the beads were resuspended in 10 mM Tris-HCl, pH 8.0 containing Complete[®] protease inhibitor and 7% glycerol (final). The repair mixtures were incubated at 30°C for the indicated times (usually 60 min), and stopped by adding (final) 20 mM EDTA and 80 ng/ μ l RNase A (37°C, 10 min), and further incubated with (final) 190 ng/ μ l proteinase K and 0.5% SDS (37°C, 30 min). The repair products were purified by phenol/chloroform extraction and ethanol/salt precipitation. DNA was digested with indicated restriction enzymes (Figure 1), and analyzed by electrophoresis in 12% denaturing polyacrylamide gels and phosphorimaging (Fuji, BAS-1800II) of the dried gels.

Western analysis

Proteins were separated on pre-cast 10% denaturing NuPAGE gels (InvitrogenTM, Life Technologies) and transferred to PVDF membranes (ImmobilonTM, Millipore). Primary rabbit

or mouse antibodies were diluted in 5% fat-free dry milk in phosphate-buffered saline (PBS) containing 0.1% Tween[®]-20. Membranes were incubated with the primary antibody for 1–2 h, followed by incubation for 1 h with either peroxidase-labeled goat anti-mouse IgG or peroxidase-labeled swine anti-rabbit IgG (DAKO, Denmark). Membranes were treated with ECL chemiluminescence reagent (ECL[™], Amersham Biosciences) and the bands visualized by exposing the membranes to Hyperfilm[™], ECL[™] (Amersham Biosciences).

RESULTS

The primary objective of this study was to isolate UNG2-containing complexes, if present, by immunoprecipitation, and to examine their ability to carry out complete repair of uracil-containing DNA. For this purpose, we used antibodies attached to magnetic beads as bait. The experimental strategy for the repair studies is outlined in Figure 1. As DNA substrate for BER reactions, we used ccdDNA containing a single uracil, an AP-site, or a nicked AP-site at a defined position, either opposite of A or G (38,39). The BER type (short-patch or long-patch) was examined after recovery of DNA. Digestion of this DNA substrate with the restriction enzymes XbaI/HincII yields an 8 nt fragment that will only contain label in the one nucleotide at the site of damage (position 0) (short-patch BER). Digestion with HincII/PstI yields an 8 nt fragment that may contain label in positions +3, +4 and +7 (long-patch repair) when using [α -³²P]dCTP as radioactive label. Note that while the HincII/PstI cleavage detects long-patch repair exclusively (dCMP incorporated in positions +3 +4 and +7), XbaI/HincII cleavage detects short-patch predominantly, but also the first nucleotide inserted in long-patch repair. The relative contribution of short-patch and long-patch repair is therefore not accurately defined by the assay. Digestion with BamHI/PstI yields a fragment of 22 nt if ends are ligated, and a fragment of 14 nt if the dominant short-patch product is not ligated.

Isolation of functional UNG2-ARC prepared from HeLa extracts

Using PU1sub antibodies attached to paramagnetic beads (PU1sub-beads), we succeeded in isolating complexes proficient in short-patch and long-patch repair of U/G substrate, although short-patch repair was most prominent (Figure 2A). The weaker band of high molecular weight DNA (HMW, theoretically 3191 nt) in Figure 2A represents unspecific incorporation in the rest of the plasmid. The increased strength of the corresponding HMW band in lane 3 is the result of the contribution of short-patch repair in this band, as expected from the HincII/PstI cleavage pattern. As control, we used a substrate with C/G in the same position as U/G. No repair incorporation in the 8 nt fragments was observed with the C/G substrate, and the general HMW background was low, thus the incorporation is damage specific (lanes 2, 4 and 6). We did not succeed in preparing BER-proficient complexes using the other UNG-specific antibodies described in Materials and Methods, or the APE1-specific antibodies (data not shown).

We then examined BER carried out by UNG2-ARC as function of time and amount of UNG2-ARC present in the

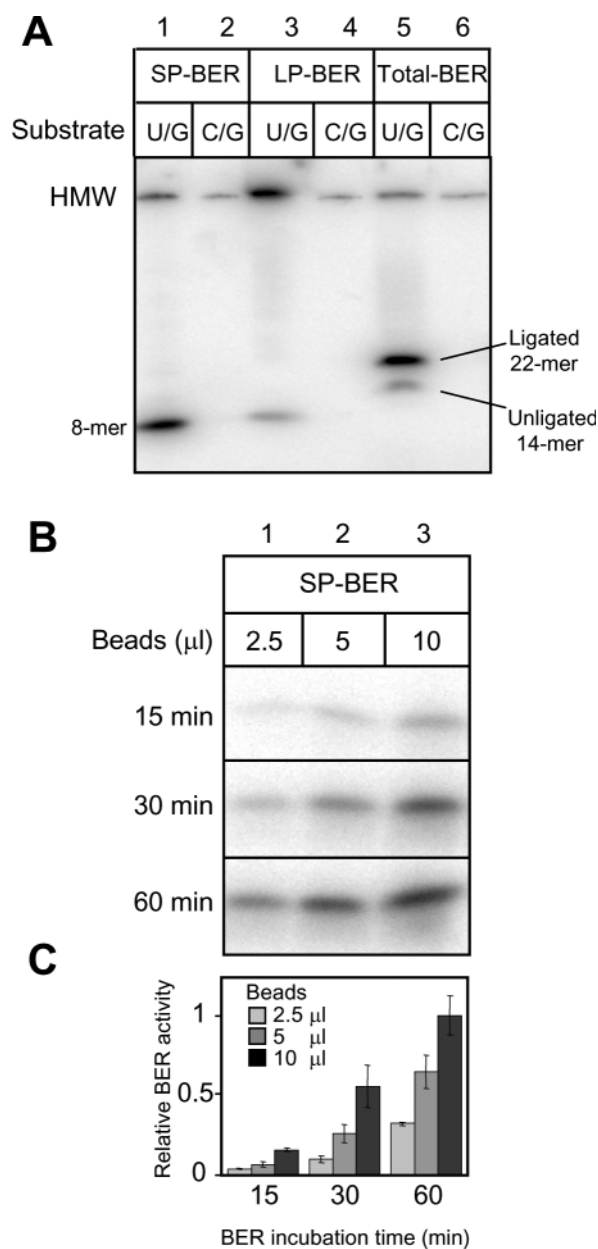


Figure 2. BER carried out by UNG2-ARC. UNG2-ARC was prepared from whole HeLa cell extract and used in BER assays. (A) BER was carried out using U/G substrate or C/G as control and [α -³²P]dCTP as radioactive isotope. After incubation, DNA was purified and digested with restriction enzymes to release short- (SP-BER) and long-patch (LP-BER) repair products as well as total repair products (ligated/unligated products, BamHI/PstI). HMW, high molecular weight band. (B) BER as function of time and input of UNG2-ARC. The indicated amount of beads carrying UNG2-ARC was incubated for specified time periods in BER assay. Short-patch BER products were then analyzed and quantified. (B) Results from a single experiment. (C) Results calculated from four independent experiments with error bars showing SD. Gels were analyzed by phosphorimaging.

incubation. We found an essentially linear dependency of BER with time, and also near-linear dependency on input of beads, except at the highest input of UNG2-ARC. Figure 2B shows results from single experiments, and Figure 2C shows the results calculated from four independent experiments. These results demonstrate that the substrate concentration

used is not a limiting factor and that the BER capacity of the complexes is relatively stable under the incubation conditions used. We found that IgG-binding approached saturation when using 400 μ g protein extract per 5 μ l beads (data not shown), and therefore used these relative amounts of beads and extract routinely for preparation of UNG2-ARC. We also routinely used UNG2-ARC complexes remaining attached to the beads.

We wanted to analyze the proteins captured by PU1sub. For this purpose, we resolved immunoprecipitates by SDS-PAGE and silver-stained the gel (Figure 3A). The lower panel shows BER capacity of the immunoprecipitates. Western analysis demonstrated that PU1sub specifically immunoprecipitated UNG2, POL δ , PCNA and XRCC1 (Figure 3B). We estimated that <0.05% of the total protein in the extracts were attached to the beads (data not shown). In contrast, total UDG activity was clearly reduced by PU1sub from the same extract (Figure 3C, upper panel). The UDG assay measures activities of UNG1 and UNG2, and the two forms are essentially equally active. By western analysis, we found that approximately 50% of total UNG2, but no appreciable UNG1, were captured with the standard input of PU1sub beads (1 \times). When bead input was doubled (2 \times), over 80% of total UNG2 were captured. Therefore, the decreased UDG activity is solely due to extraction of UNG2 by the PU1sub beads (Figure 3C, lower panel). The broad UNG2 band in western analysis (lower panel) is due to different phosphorylated forms of the protein. We also demonstrated that UDG activity is associated with these PU1sub beads (Figure 3D). The weak bands representing repair products seen when using control IgGs (Figure 3A, lower panel) are likely to result from unspecific binding of UNG protein

(Figure 3D), and other repair proteins like POL δ (Figure 3B) to these IgGs. Control experiments demonstrated that essentially equal amounts of UNG2-specific and control IgG were equally efficiently coupled to beads (data not shown). These experiments demonstrate that PU1sub-beads partially deplete extract of UNG2 and associated BER proteins, without measurable reduction in total protein in the extracts. Identification of specific BER factors in the complex by silver staining is complicated by the presence of many proteins in the immunoprecipitate, and the insufficient sensitivity of silver staining relative to the amount extract used in our experiments.

Functional UNG2-ARC isolated using UNG2-specific antibodies contains proteins required for BER of uracil in DNA

Using whole cell extracts of HeLa cells and PU1sub-beads, we isolated UNG2-ARC that repaired U/G substrate, but such a complex could not be prepared using IgGs from pre-immune and non-immunized animals. Neither was repair of AP/G or nicked AP/G substrates observed when using unspecific IgGs for preparing complexes (Figure 4A). This demonstrates the requirement for specific antibodies and supports the view that UNG2-ARC is captured via UNG2. Furthermore, DNase I treatment of the extract did not reduce the yield of functional UNG2-ARC. Thus, the components of UNG2-ARC are not co-precipitated via common DNA molecules (Figure 4A).

In general, results from western analysis supported the functional assays. We detected UNG2, APE1, PCNA, POL δ , POL β and XRCC1 in the UNG2-ARC (Figure 4B).

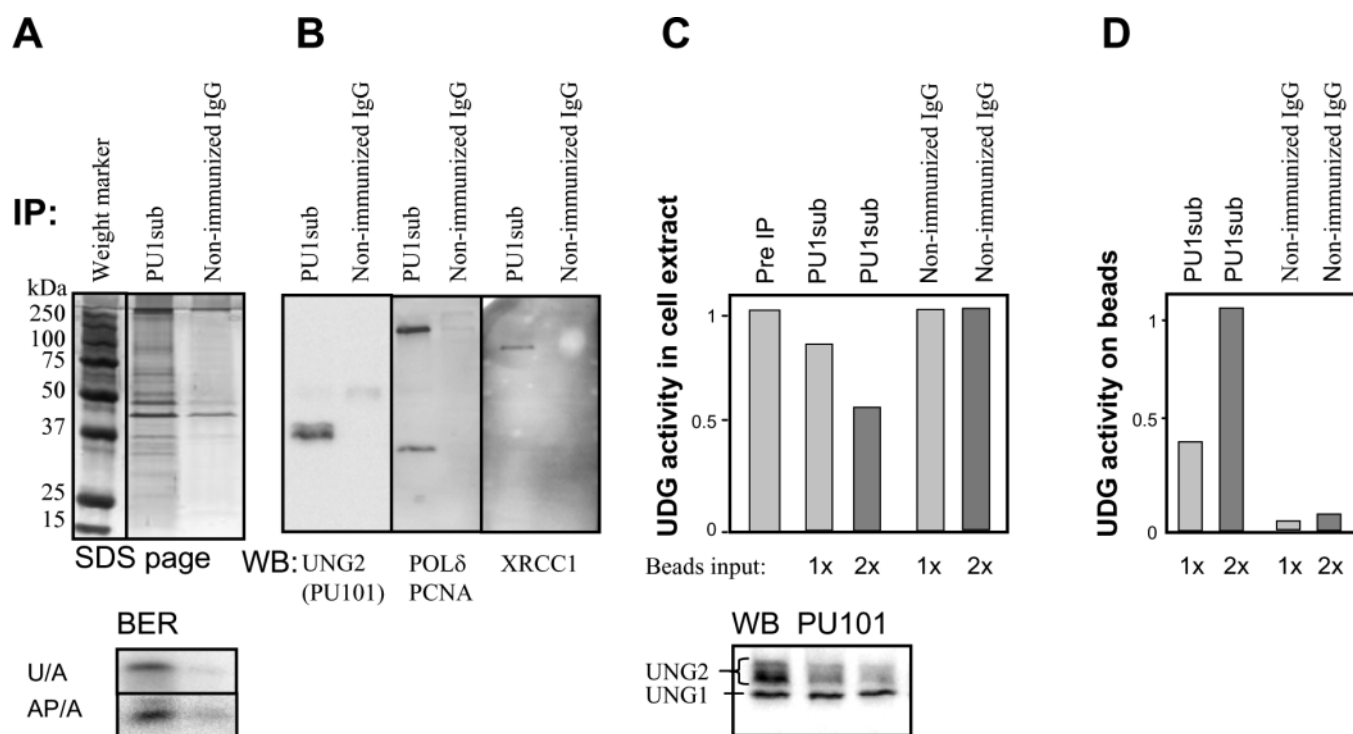


Figure 3. Characterization of UNG2-ARC. (A) UNG2-ARC was immunoprecipitated using PU1sub antibodies and non-immunized IgGs as control. Proteins were resolved on a 12% SDS-polyacrylamide gel and silver-stained (upper panel). The lower panel shows BER capacity of the corresponding immunoprecipitates using U/A- and AP/A substrates. (B) Western analysis of UNG2, PCNA, POL δ and XRCC1 in immunoprecipitates. (C) Reduction of UDG-activity in cell extracts by immunoprecipitation. 'Pre-IP' indicates UDG-activity prior to immunoprecipitation. The lower panel displays the corresponding western analysis of UNG proteins in cell extracts pre-IP and post-IP. (D) UDG activity of PU1sub- and non-immunized IgG beads. 1 \times , 7.5 μ l input of beads; 2 \times , 15 μ l input of beads.

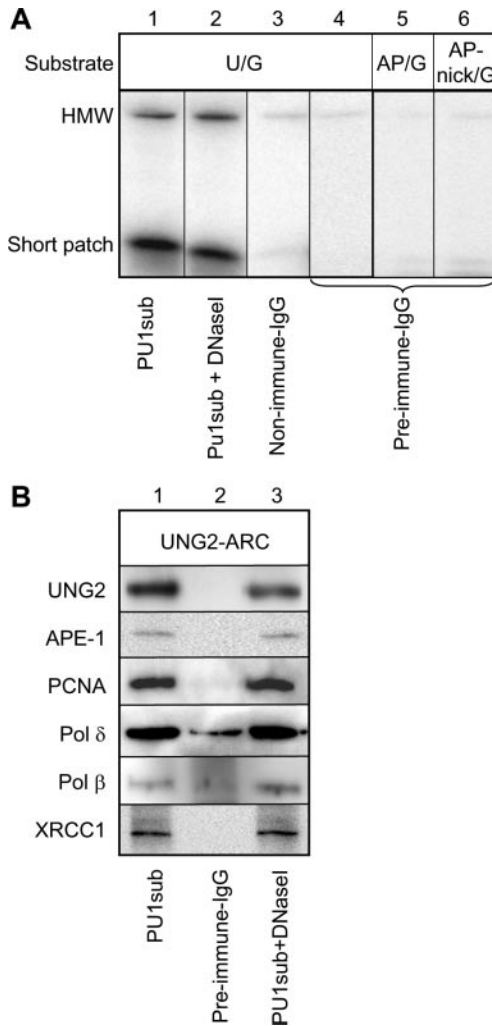


Figure 4. Specificity in isolation of functional UNG2-ARC and western analysis of proteins present. (A) UNG2-ARC was prepared/attempted to be prepared using PU1sub coated beads, beads coated with non-immune IgG, or pre-immune IgG, which were all incubated with 400 μ g HeLa whole cell extracts. The beads were incubated with BER assay mixture containing [α - 32 P]dCTP, and U/G, AP/G or AP-nick/G substrate, as indicated. In lane 2, extract was pretreated with 3 U DNase I at 30°C for 30 min prior to UNG2-ARC isolation. Purified DNA was digested for short-patch analysis. (B) Proteins from beads prepared as in (A) were mixed with denaturing loading buffer, heated at 70°C, separated by electrophoresis, blotted and visualized as described in Materials and Methods.

We did not detect DNA ligase I possibly because of the properties of the antibody, since we only detected a weak band corresponding to DNA ligase I in whole cell extract using the same antibody. We did not investigate the possible presence of DNA ligase III. We also did not detect APE2, SMUG1, POL γ , POL ι , TDG or UNG1 in the UNG2-ARC, although they were all detectable in whole cell extracts (data not shown). The different intensities of the bands do not necessarily reflect the molar ratio of the proteins in the complex, but may merely indicate different properties of the antibodies used for western analysis. As control, we carried out similar experiments using pre-immune IgG linked to beads. No UNG2 was precipitated non-specifically (Figure 3B). The absence of detectable PCNA in the control suggests that

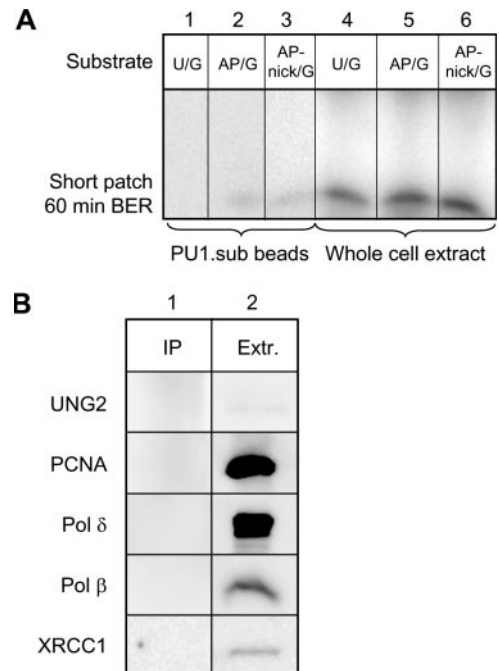


Figure 5. BER and western analysis with UNG $^{-/-}$ human lymphoblastoid cells. (A) Whole cell extract was prepared from UNG $^{-/-}$ human lymphoblastoid cells. Whole cell extract (50 μ g protein) was used in BER assays with [α - 32 P]dCTP and substrates as indicated (lanes 4–6), or ‘UNG2-ARC’ was attempted to be prepared from whole cell extracts using PU1sub antibody, and used for BER assays with substrates as indicated (lanes 1–3). (B) Protein in whole cell extract (lane 2) or from immunoprecipitated ‘UNG2-ARC’ (IP) attempted to be prepared from whole cell extract using PU1sub (lane 1) were subjected to western blot analysis as in Figure 4B.

PCNA is captured via interaction with immobilized UNG2, with which it is known to interact directly (22). Precipitation of POL δ was strongly reduced, but not completely absent with pre-immune IgG (Figure 4B). Pre-treatment of the extract with DNase I did not affect the capture of UNG2, PCNA or POL δ significantly, in agreement with the functional BER assays (Figure 4A).

We also found that proficient UNG2-ARC could not be prepared from an UNG $^{-/-}$ human lymphoblastoid cell line (36), strongly supporting the view that the BER complex is attached via UNG2 (Figure 5A). As an additional control, we successfully prepared proficient UNG2-ARC from peripheral blood lymphocytes, as well as cultured myeloma cells (data not shown). Using whole cell extract from hUNG $^{-/-}$ cells instead of UNG2-ARC, we detected repair of all three DNA lesions, indicating UNG2-independent BER in the extracts (Figure 5A). In agreement with this result, we could not detect any of the BER factors attached to beads coated with PU1sub when we attempted to isolate UNG2-ARC from UNG $^{-/-}$ lymphoblastoid cells (Figure 5B). Furthermore, UNG2 was not detectable in whole cell extracts from these cells, while PCNA, POL δ , POL β and XRCC1 were all identified (Figure 5B).

These experiments demonstrate that isolation of functional UNG2-ARC requires interaction between UNG2 and UNG2-specific antibodies and that complex formation is dependent on protein–protein interactions rather than protein–DNA interactions.

BER activity for uracil-containing DNA in UNG2-ARC and whole cell extracts has preference for U/G over U/A

To further examine the BER process in UNG2-ARC, we used a cccDNA substrate containing uracil opposite adenine (U/A) at the same position as in the U/G substrate. This eliminates possible differences related to sequence dependency of the repair process. As demonstrated in Figure 6, we consistently found that repair of U/G was better than repair of U/A, and was not a result of different quality of the substrate as such. Repair of U/A (upper panel) and U/G (lower panel) by UNG2-ARC was abolished by PU101, while a neutralizing anti-SMUG1 antibody (7) had no detectable inhibitory effect. This is consistent with our failure to detect SMUG1 in the complexes. Furthermore, in the presence of PU101, repair of U/A by whole cell extract after 60 min incubation was essentially undetectable (Figure 6, upper panel), and repair of U/G after 10 min was approximately 80% reduced (Figure 6, lower panel). This suggests that UNG2 is the major glycosylase responsible for repair of uracil-containing DNA in

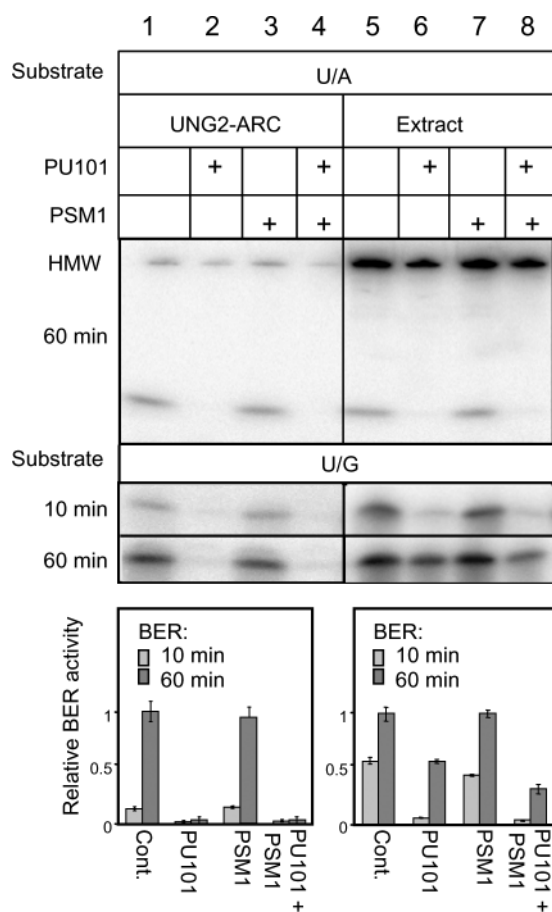


Figure 6. Roles of UNG2 and SMUG1 in short-patch repair of U/A pairs and U/G mispairs. UNG2-ARC or whole cell extract of HeLa cells was incubated with BER assay mixture for 60 min with [α - 32 P]dTTP and U/A (upper panel), or with [α - 32 P]dCTP and U/G substrate (lower panel) in the absence or presence of anti-UNG antibody (PU101) or anti-SMUG1 antibody (PSM1) or both, as indicated. The diagram represents results from four independent experiments where bars indicate SD.

U/A pairs and U/G mispairs. However, whole cell extracts carried out a delayed, but significant repair of U/G after prolonged incubation in the presence of PU101 antibodies (Figure 6, lower panel). In whole cell extracts anti-SMUG1 antibodies alone had no significant effect on BER (Figure 5, lane 6), but in the presence of PU101, a small but further increase in inhibition by anti-SMUG1 antibodies was observed, when compared with PU101 alone (Figure 6, lane 8). These results indicate that UNG2 is the main, if not sole, enzyme for repair of U incorporated opposite of A, and the major enzyme for repair of U/G mispairs resulting from deamination of C. Furthermore, SMUG1 may have a back-up function, but is not the primary enzyme responsible for repair of U/G mispairs in this *in vitro* system. The failure of PU101 antibodies and anti-SMUG1 antibodies to completely inhibit BER when added together may indicate that the two other uracil-DNA glycosylases known, TDG and MBD4, as well as the newly identified DNA glycosylases NEIL1 and NEIL2 (40) may also be considered as potential candidates for the 'delayed' U/G repair. Although TDG has very low turnover of substrate alone, the rate is enhanced by APE1 (41), which is certainly present and active both in whole cell extract and in the complexes. Although unlikely because of the small patch size, we could not formally exclude DNA repair mechanisms other than BER in the whole cell extracts; e.g. nucleotide excision repair (NER) or mismatch repair (MMR). However, MMR repair was excluded as a significant source of repair in this system by using nuclear extracts prepared from MMR-deficient HCT-116 colorectal cancer cells (ATCC CCL-247) in the presence of both neutralizing antibodies, which again did not abolish repair completely (data not shown). Finally, these results indicate that UNG2 is the only uracil-DNA glycosylase present in UNG2-ARC.

Inhibition of UNG2 does not impair subsequent BER steps and recombinant SMUG1 partially restores BER of U/G by UNG2-ARC, but not U/A repair

To test whether inhibition of UNG2 affects subsequent BER steps carried out by UNG2-ARC, we assayed repair of U/G, AP/G and nicked-AP/G DNA substrates in the presence of neutralizing PU101 antibody. The antibodies completely inhibited repair of U/G, whereas AP/G and nicked AP/G substrates were effectively repaired (Figure 7A). Addition of 2 ng of recombinant SMUG1 enzyme (7) to PU101-inhibited UNG2-ARC, partially restored BER capacity for U/G and this restoration was inhibited by neutralizing SMUG1 antibodies, as expected. Next, we tested repair of U/A substrate under similar assay conditions. Neutralizing PU101-antibodies also inhibited repair of U/A completely, while repair of AP/A and nicked AP/A was not affected (Figure 7B). Contrary to experiments with U/G substrate, 2 ng recombinant SMUG1 enzyme did not restore BER of U/A. SMUG1 was previously shown to remove U from U/A pairs, although k_{cat}/K_m is 3.2-fold higher for U/G than for U/A in uracil release assays (7). These findings may in part explain the inability of SMUG1 to complement UNG2 in repair of U/A pairs.

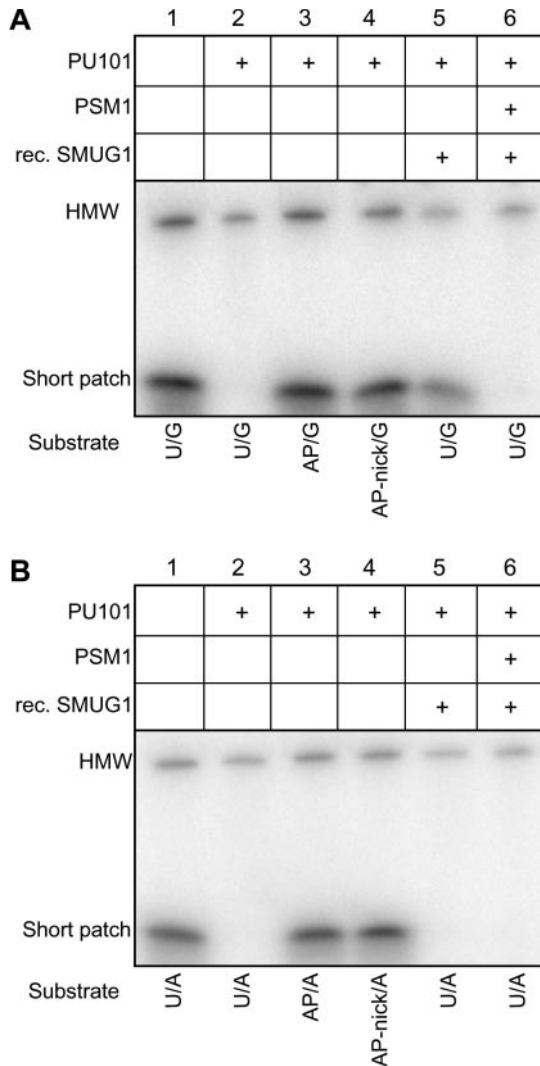


Figure 7. Lack of functional coupling between the glycosylase step and the subsequent steps in short-patch repair. (A) UNG2-ARC was incubated with BER assay mixture with [α - 32 P]dCTP and U/G, AP/G or AP-nick/G as indicated, in the absence or presence of anti-UNG antibody (PU101) and/or anti-SMUG1 antibody (PSM1), and absence or presence of 2 ng recombinant SMUG1 (rec. SMUG1). (B) UNG2-ARC was incubated as in (A), but with [α - 32 P]dTTP and U/A, AP/A or AP-nick/A substrate.

APE1 is indispensable for BER activity and immunodepletion of XRCC1 reduces BER capacity of UNG2-ARC and the yield of ligated repair product

Mammalian cells have two AP-endonucleases, APE1 and APE2, that share homology with *Escherichia coli* exonuclease III (ExoIII). To test the relative contribution of these to the AP-endonuclease activity of UNG2-ARC, we conducted BER assays in the absence or presence of polyclonal anti-APE1 antibodies. We found that APE1 antibodies completely inhibited BER activity of UNG2-ARC (Figure 8A), indicating that APE1 is the only functional AP-endonuclease in UNG2-ARC. Further, we tested the functional role of XRCC1 in UNG2-ARC, and found that polyclonal antibodies to XRCC1 had no direct inhibitory effect in BER (Figure 8A). However, immunodepletion of XRCC1 resulted in markedly reduced

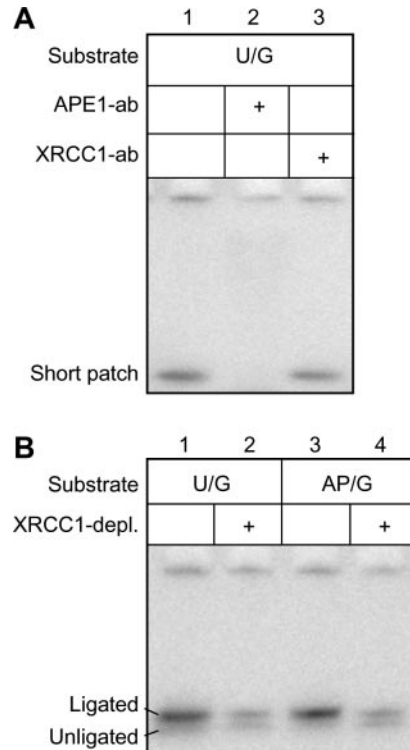
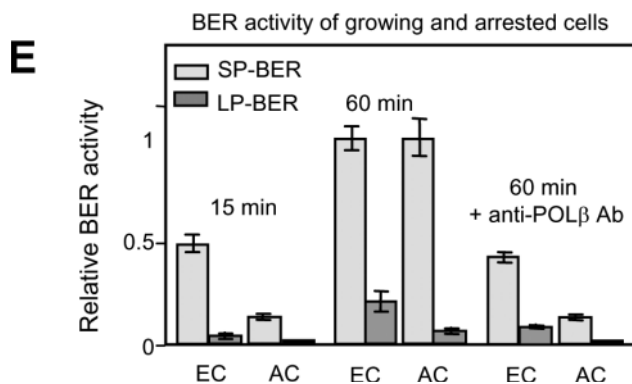
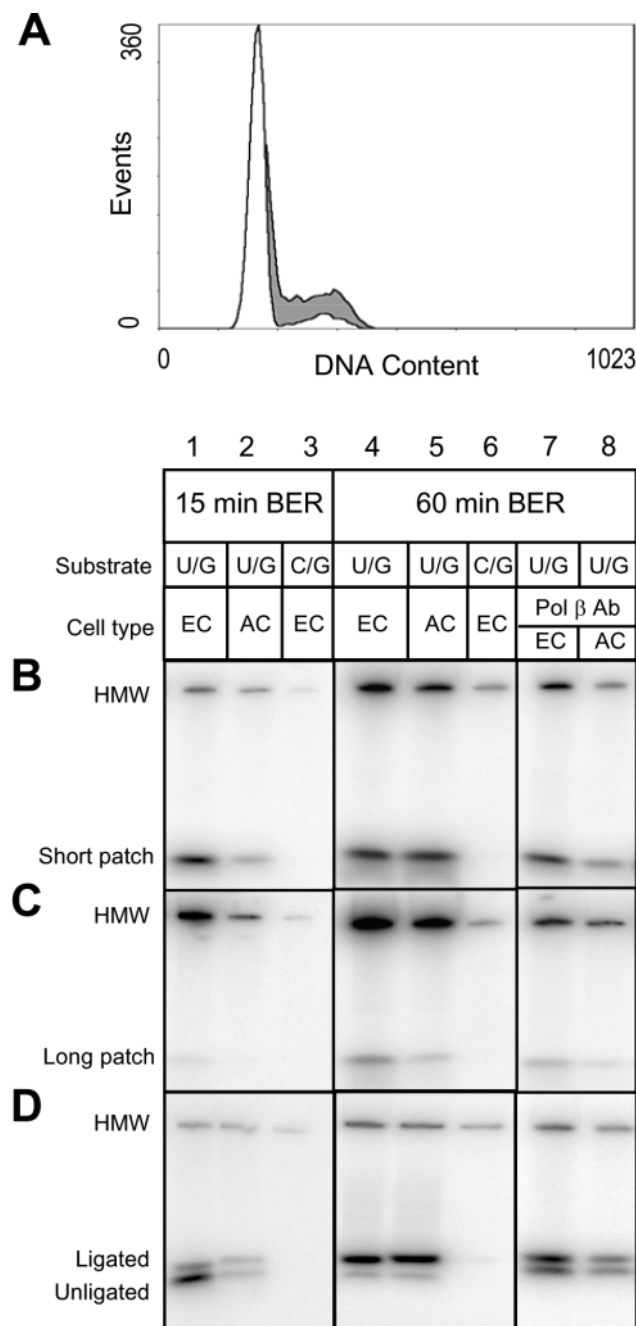


Figure 8. Roles of APE1 and XRCC1 in BER. (A) UNG2-ARC was incubated with BER assay mixture in the absence or presence of APE1 and XRCC1 polyclonal antibodies as indicated. (B) Whole HeLa extracts were incubated with XRCC1-antibody linked beads (lanes 2 and 4) or non-immunized serum IgG-linked beads (lanes 1 and 3) for 4 h at 4°C. The beads were removed and the extracts were further incubated with PU1sub-linked beads. The UNG2-ARC thus captured was subsequently used in BER assay.

BER, and a significant fraction of repaired products remained unligated after XRCC1 depletion (Figure 8B). These results are consistent with the presumed role of XRCC1 as scaffolding protein that interacts with other BER proteins, including POL β , DNA ligase III, and the fact that XRCC1 itself has no known catalytic activity.

UNG2-ARC isolated from growth-arrested HaCaT cells display lower BER activity than UNG2-ARC isolated from exponentially growing cells

To examine BER activity of UNG2-ARC in relation to proliferative status of the cells, we isolated complexes from cell extracts prepared from exponentially growing cells (EC) and growth-arrested (AC) HaCaT cells. The cell cycle status of the cells was determined by FACS analysis (Figure 9A). Using the BER assay, we found a markedly lower BER activity (short- and long-patch) after 15 min in UNG2-ARC isolated from growth-arrested HaCaT cells compared with those prepared from exponentially growing cells (Figure 9B and C, lanes 1 and 2, and diagram E). However, after 60 min incubation the BER process had reached equal levels of short-patch repair in the complexes (Figure 9B, lane 4 and 5, and diagram E), whereas long-patch repair of UNG2-ARC isolated from arrested cells was only 30% of that from growing cells (Figure 9C, lane 5 and 6, and diagram E). This is consistent with the roles of several DNA replication factors in long-patch



BER, implying close association of these DNA metabolizing systems. Furthermore, using the BER assay, we found similar patterns of repair in the corresponding whole cell extracts (data not shown). Addition of neutralizing anti-POL β antibodies to the BER mixture resulted in stronger inhibition of repair activity of UNG2-ARC prepared from the growth-arrested cells compared with that from growing cells (Figure 9B and C, compare lanes 4 and 5 with lanes 7 and 8, respectively, and diagram E). This indicates that POL β is the predominant repair DNA polymerase in the UNG2-ARC isolated from arrested cells. Adding 3-fold higher amounts of neutralizing anti-POL β antibodies to the repair reaction had no additional inhibitory effects (data not shown). In a previous study, inhibition of POL β was found to reduce long-patch by nearly 75% in cell extract, indicating involvement of POL β in this pathway (42). This agrees well with our results demonstrating reduction of long-patch BER when POL β activity was inhibited by neutralizing antibodies (Figure 9C, lanes 7 and 8). This indicates that either a fraction of long-patch repair in UNG2-ARC is initiated by POL β (10,43) or that POL β may synthesize repair patches longer than one nucleotide (11).

The capacity of BER activity of cell extracts and UNG2-ARC is rate-limited not solely by the cellular content of UNG2

Base excision repair proteins has been suggested to be sequentially recruited to the site of repair, implying that repair complexes are generated in proportion with damage to DNA (44). Alternatively, the buildup of repair complexes such as UNG2-ARC could depend on the concentration of repair factors. For this purpose, we prepared extracts from ordinary HeLa cells and stably transfected HeLa cells overexpressing UNG2 (HTO-UNG2-45). We found 15-fold higher level of UDG enzymatic activity in induced cells compared with non-induced cells (data not shown). To examine uracil BER capacity in extracts and UNG2-ARC, we used U/A substrate because repair of this lesion is completely dependent on functional UNG, in contrast with U/G repair (Figure 6). The rate of uracil repair was increased 4-fold in cell extract prepared from induced cells compared with non-induced cells at 5 and 10 min incubation (Figure 10, lanes 1–6 and the panel below). The increase in BER capacity of UNG2-ARC isolated from UNG2-overexpressing cells was smaller (1.5- to 2.5-fold, lanes 7–10

Figure 9. BER activity in proliferating and non-proliferating cells. UNG2-ARC was isolated from whole extract of exponentially growing HaCaT cells (EC) as well as from whole extract of high-density and growth-arrested HaCaT cells (AC) that did not replicate DNA. (A) Comparison of DNA content distributions as analyzed by flow cytometry analysis of propidium iodide stained HaCaT cells. Grey histogram; exponentially growing proliferating cells, white histogram; growth-arrested non-proliferating cells. Fluorescence intensity is on the linear axis, while the vertical axis indicates the relative number of stained cells. UNG2-ARC was isolated from the extracts and used in BER assays with U/A substrate and [α - 32 P]dTTP isotope and incubated for indicated time periods in the absence (lanes 1–6), or presence (lanes 7–8) of neutralizing POL β antibodies. The repair products were purified and digested with restriction enzymes to release short-patch (B) and long-patch (C) repair products as well as total repair products (ligated/unligated products, BamHI/PstI) (D). Results calculated from two independent experiments, each in duplicate, for BER kinetics analysis of exponentially growing cells relative to growth-arrested HaCaT cells after 15 min incubation and in the presence and absence of anti-POL β antibodies after 60 min BER incubation (E).

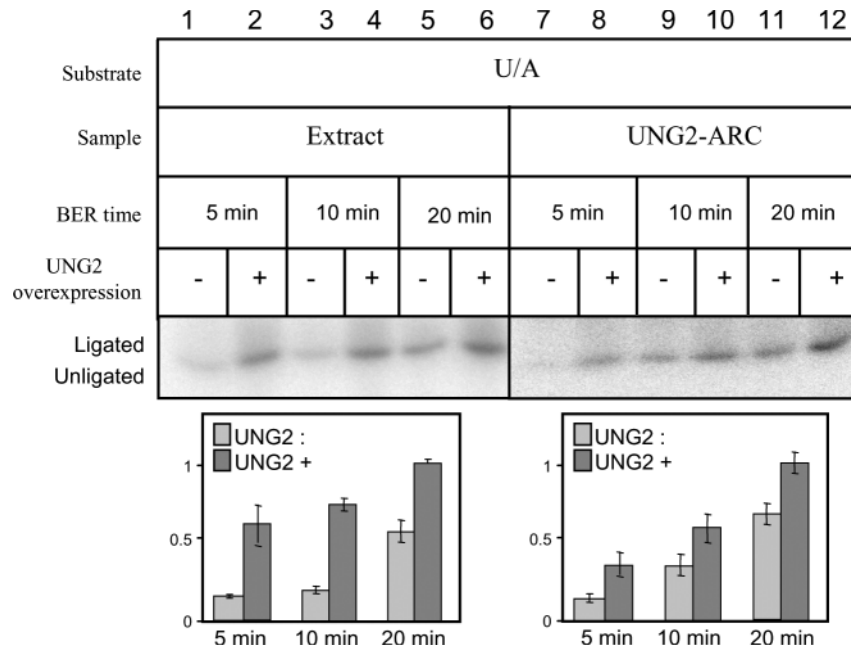


Figure 10. Increased cellular UNG2 protein enhances BER activity in whole cell extract and UNG2-ARC. Exponentially growing HeLa cells carrying a UNG2 expressing construct were either induced with 2 $\mu\text{g/ml}$ of doxycycline for 48 h (+) or mock-induced (-) as control. UNG2-ARC was prepared by incubating 40 μl of PU1 sub-linked beads with 100 μg of whole cell extract for each reaction. BER assays with 500 ng of U/A DNA substrate and [α - ^{32}P]dTTP as isotope were carried out using whole cell extracts (40 μg) (lanes 1–6) or UNG2-ARC (lanes 7–18) for the indicated incubation times. The panels below show the results calculated from two independent experiments.

and the panel below). The data from crude extracts indicate that UNG2 may be a rate-limiting factor, but since the BER rate increases 4-fold when UNG2 increases 15-fold, it does not seem to be the sole rate-limiting factor. The results of BER analysis of UNG2-ARC indicate that formation of repair complexes could be independent of the presence of damage in DNA.

DISCUSSION

Our work demonstrates the existence of a BER complex that is proficient in repair of uracil-containing DNA and western analysis confirmed that the factors known to be required for the process were present in the complex. Furthermore, the buildup of the complex is not dependent on the presence of uracil-damaged DNA. This indicates that at least one form of BER for uracil-containing DNA takes place in a preassembled multi-protein complex. Alternative models assuming sequential recruitment of enzymes to the site of repair have been suggested (45). Several observations, particularly the pattern of protein-protein interactions, support this model in which an incoming protein is recruited by one or more proteins present at the site of repair. After release of the base in the initial step, the DNA glycosylase may remain bound to the product DNA (AP-site) for which it has high affinity, and may shield it from damage (45). In some cases, DNA glycosylases have virtually no turnover (TDG), or low turnover (SMUG1), when present alone, but the turnover is enhanced several-fold by AP-endonuclease (7,41). The structure of APE1 bound to abasic DNA showed that APE1 uses a rigid, pre-formed surface to bend the DNA helix by $\sim 35^\circ$ and retains its kinked DNA product

efficiently (46). The kinked APE1-DNA complex is proposed to recruit POL β to sites of damage (46,47), where it binds to the nicked DNA, and induces a further bend to $\sim 90^\circ$ (47). DNA kinking could also provide an effective means of promoting the directional and sequential exchange of factors at each step of the BER pathway (46). This recruitment model does not require the formation of a more stable BER complex, but is not incompatible with the preassembled BER complex model, where the substrate may be handed from one component of the complex to the other. One possibility is that these mechanisms operate in different contexts. Some components may be recruited as single proteins and others as small complexes, forming larger complexes with limited lifetime. Another possibility, in agreement with our results, is that the biochemistry of the individual steps operates just as suggested for the sequential recruitment model, but that this process simply takes place in a complex.

In a previous paper, a multi-protein BER complex from bovine testis containing POL β , DNA ligase I and uracil-DNA glycosylase was isolated by a POL β affinity method, and if proteins were present in equimolar concentrations, the complex had a cumulative mass of 196 kDa (20). This complex was captured in the absence of DNA by POL β -affinity chromatography. This argues against damage recognition by the glycosylase being a trigger for complex assembly. The fact that BER complexes can be isolated by two different methods supports the existence of such complexes in the cell. However, our work indicates that PCNA, POL δ , XRCC1, a DNA ligase and APE1 are also part of a BER complex, which must then be significantly larger than the complex reported previously. Possibly, these differences may be explained by the fact that we used lower concentrations of monovalent salt during isolation

and washing of the complex. Since we did not detach the BER complex(es) from the beads, and did not examine them as complexes in solution, it is possible that UNG2 is present in complexes with different compositions. In NER, at least some repair factors are recruited sequentially to the site of damage after localized subnuclear exposure to ultraviolet light. Here the damage recognition complex XPC-hHR23B appears to be essential for the recruitment of all subsequent NER factors (48). Thus, at best a fraction of NER proteins is present in preassembled repair complexes.

Based on experiments using isolated nuclei (22) and UNG-deficient mice (49), a role for UNG2 in removal of misincorporated uracil in U/A pairs has been established. However, the roles of UNG2, SMUG1 or other enzymes, in removal of uracil from U/G mispairs have remained unsettled (7,49). We find that UNG2-ARC and whole cell extracts are at least as effective in repair of U/G mispairs, as with U/A pairs. Previously, we defined kinetic properties of homogenous recombinant SMUG1 and UNG2 using U/A or U/G substrates in oligonucleotide cleavage assays. We concluded that these properties were consistent with a major role for UNG2 in repair of U/G mispairs, as well as U/A pairs. Complete BER in crude extracts of HeLa cells supported this view, in agreement with results presented here (7). However, a different paper concluded that murine SMUG1 has the major uracil-DNA glycosylase activity initiating repair on U/G mispairs, with UNG2 as a back-up (50). In these studies, the glycosylase step was examined using nuclear extracts in oligonucleotide cleavage studies, and low substrate concentrations were used to mimic the *in vivo* situation. The different conclusions reached in the latter study and the present study are not due to differences in the concentration of DNA-uracil, which are fairly equal in the two studies. It could, however, possibly be due to a much higher total DNA concentration but much lower relative lesion *density* used in our study as compared with study on murine extracts (50). The different results are, however, more probably explained by the use of different methods for preparation of extract, and by different kinetics due to assay conditions. It should also be noted that activities and kinetic properties of human UNG2 (7) and human and mouse SMUG1 (7,49) are dependent on concentrations of monovalent salts and Mg^{2+} , as well as the presence or absence of APE1. Contrary to the kinetic properties originally reported for *Xenopus* SMUG1, and the erroneous entries of kinetic parameters for hUNG in this paper (51), kinetic parameters for human SMUG1 and UNG2 would be consistent with a major role for UNG2 and a backup function for SMUG1 in removal of uracil from DNA both in U/G mispairs and U/A pairs (7,52). Thus, the K_m values of SMUG1 and UNG2 are 1.3 μM and 0.4 μM , respectively, whereas K_{cat} for UNG2 is 100-fold higher than that of SMUG1 and K_{cat}/K_m some 300-fold higher than that of SMUG1 (7). Even if different assay conditions may modify the picture several-fold, the properties of SMUG1 does not make it a likely primary candidate in performing the important first step in BER of uracil-containing DNA. Our present data, both with BER complexes and whole cell extracts, supports this view. Furthermore, murine (53) and human UNG2 (36) have been demonstrated to be required for removal of uracil in U/G pairs resulting from deamination by AID in somatic hypermutation and class switch recombination in B-cells. The major role of SMUG1 is more likely to be in

repair of oxidative base lesions, such as 5-hydroxymethyluracil (7,52,54) 5-hydroxyuracil and 5-formyluracil (52), where alternative repair enzymes are not identified, or are less efficient. Thus, we conclude that UNG2 is the major enzyme initiating BER of uracil-containing DNA both in a U/G and a U/A context. UNG2-ARC also proficiently repair abasic sites, but whether this type of complex represents the major type, completing repair initiated by glycosylases other than UNG2 remains to be examined.

Components of short- and long-patch BER have been identified and their roles studied both *in vitro* and *in vivo* as well as in reconstitution systems. However, mechanisms that determine the branching of BER pathway into short-patch and long-patch repair are not completely understood. The type of DNA damage was found to determine the mode of pathway (55). Thus, repair of hypoxanthine (HX) and 1, N^6 -ethenoadenine (ϵA) by the monofunctional alkyl- N -purine glycosylase (ANPG/MPG) was via both short- and long-patch BER, whereas repair of 8-oxo-G by the bifunctional hOOG1 protein was mainly via short-patch pathway (55). However, in a recent study on repair of 8-oxo-G, 55–80% repair patches were >1 nt in an *in vivo* system (56). However, the biochemistry of the repair pathway was not analyzed in this study, nor was possible impact of cell cycle status on the mode of repair. Modified 5'-dRp residues are refractory to the dRPase activity of POL β (57). Thus, repair DNA synthesis of such lesions could be initiated by POL β (58), but further removal of the 5'-dRp and completion of repair is thought to require a 'switchover' to a PCNA and POL δ/ϵ dependent long-patch pathway (59). Although single nucleotide insertion is a dominant function of POL β , this DNA polymerase is able to replace several nucleotides containing a modified 5'-dRp, resulting in the generation of a flap oligonucleotide structure (11,60). The flap is cleaved by FEN1 (11), resulting in a nick in DNA which is subsequently sealed by a DNA ligase. A direct involvement of PCNA in BER outside sites of replication has not so far been demonstrated. However, a PCNA-independent long-patch BER outside sites of replication consisting of a core of four proteins; APE1, POL β , FEN1 and DNA ligase III may occur. Such long-patch repair might be the main mode of BER of modified AP-sites in resting cells. This model may be consistent with previous reports (11,42) as well as our data. The rate-limiting step in BER has previously been studied and suggested to be the removal of 2-deoxyribose 5-phosphate (dRp) when BER is initiated by a monofunctional glycosylase (e.g. UNG) (61), or the removal of 3'-blocking deoxyribose residue when a base damage is removed by a bifunctional DNA glycosylase (e.g. hOOG1) (62). Moreover, in a separate study, the joining of repair ends by a DNA ligase was suggested to be a rate-limiting step in BER (63,64). Although the rate of excision of uracil from DNA by UNG-proteins is highly sequence dependent (35), UNG2 and other UNG-proteins have very high catalytic turnover numbers (7,65). This suggests that glycosylase step is not the most likely rate-limiting step in UNG2-ARC and probably not in extracts. Previously, the excision of certain base lesions was also found to be a rate-limiting step in BER when a DNA glycosylase with low turnover activity removed the lesions (66). Apparently, the type and the position of a damaged base are additional factors in the kinetics of BER. In fact, we found that placing uracil opposite adenine at the neighboring nucleotide at the 5'-side of the U/A

substrate used in our experiments (Figure 1) resulted in a significant reduction of rate of repair (data not shown).

In conclusion, we immunoprecipitated BER complexes that carry out short-patch and long-patch BER for uracil-containing DNA, using antibodies specific for the N-terminal non-catalytic part of UNG2. Short-patch repair is the predominant mode both in extracts and in BER complexes. Furthermore, UNG2 appears to be the major glycosylase responsible for repair of U/G mispairs and possibly sole enzyme for repair of U/A pairs in human cells. On the basis of the results presented here, we suggest that at least a significant fraction of BER proteins for repair of uracil-containing DNA, and possibly for other BER substrates as well, are present in preassembled repairosome multiprotein BER complexes. These may be dynamic of nature.

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Paper IV

Different organization of Base Excision Repair of uracil in DNA in Nuclei and Mitochondria

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ABSTRACT

Nuclei and mitochondria efficiently carry out base excision repair (BER) of uracil in DNA. To examine the overall organization of these processes, we constructed cell lines expressing mitochondrial UNG1 or nuclear uracil-DNA glycosylase UNG2, both fused to enhanced yellow fluorescent protein (EYFP) placed C-terminally. UNG1 and UNG2 have identical catalytic domains, but different N-terminal regions. Complete BER of uracil in crude mitochondrial or nuclear extracts required the respective UNG-proteins. Although APE1 is highly enriched in nuclei relative to mitochondria, it was apparently the major AP-endonuclease required for BER in both organelles. APE2 is enriched in mitochondria, but its possible role remains uncertain. UNG1-EYFP and UNG2-EYFP from mitochondrial or nuclear extracts had essentially similar uracil-DNA glycosylase activities. Immunoprecipitation experiments demonstrated that a fraction of UNG2-EYFP was present as a multiprotein-complex that carried out complete BER. In contrast, UNG1-EYFP and mitochondrial proteins were not physically associated in a complex competent in BER. However, APE1 was apparently localized in proximity of UNG1-EYFP, as demonstrated by formaldehyde-crosslinking of these proteins in intact mitochondria. These results demonstrate that nuclear and mitochondrial BER processes are differently organized, with nuclear BER proteins in part being present in a complex, while mitochondrial BER proteins do not form stable complexes.

Keywords: Base excision repair; uracil-DNA glycosylase, repair complexes, mitochondria

INTRODUCTION

Base excision repair (BER) of DNA is the major mechanism for repair of minor base lesions that do not cause helix distortions (1). A DNA glycosylase initiates BER by releasing the damaged base, leaving an abasic site (AP-site) that is cleaved by an AP-endonuclease. In nuclei the subsequent steps follow two different paths; either short patch repair in which a single nucleotide gap is filled and ligated, or long patch repair in which a gap of 2-8 nucleotides is filled and sealed (2). In mitochondria short patch is the dominant BER pathway (3). Nuclei and mitochondria are both competent in BER, but use largely different proteins (4,5). However, nuclear and mitochondrial isoforms of DNA glycosylases are frequently encoded by the same gene and have common catalytic domains, but different N-terminal or C-terminal regions that may be involved in subcellular sorting and macromolecular interactions (6-9). Uracil in DNA results from misincorporation of dUMP during replication, chemical deamination of cytosine, and in B-cells deamination by activation induced deaminase (10). Nuclear and mitochondrial forms of human uracil-DNA glycosylase (UNG) are encoded by the *UNG*-gene and have a common catalytic domain (6,8). These forms are generated by alternative splicing and use of different promoters (6). The mitochondrial preform UNG1 contains 35 unique N-terminal amino acids, approximately 29 of which are removed upon mitochondrial import to give the mature UNG1 (11). Nuclear UNG2 has 44 unique N-terminal amino acids, all of which are retained after nuclear import (12).

Mitochondrial DNA (mtDNA) is associated with the inner membrane (13). In several organisms mtDNA binds to distinct proteins, forming so-called nucleoids probably helping to anchor DNA to the inner membrane. Mitochondrial transcription factor A (mtTFA) was among proteins in nucleoids (14,15). The mitochondrial inner membrane has high protein content. Among others, subunits of oxidative phosphorylation are anchored there, forming large protein complexes (16). Recently, several mitochondrial BER proteins were reported to associate with the inner membrane (17). Direct interaction between several mammalian nuclear BER proteins has been reported for a large number of proteins (18). A fraction of BER proteins apparently forms relatively stable complexes that can be isolated by biochemical fractionation or immunoprecipitation (19,20). Thus, antibodies to the non-catalytic part of UNG2 allowed isolation of functional uracil-BER complexes carrying out complete repair (19).

Here, we present results indicating different organization of BER in nuclei and mitochondria. We used organelle extracts of HeLa cells stably expressing UNG2-EYFP or UNG1-EYFP and anti-EYFP-antibodies linked to paramagnetic beads to isolate the glycosylases and proteins associated with them. A fraction of UNG2-

EYFP was present in a complex competent in complete repair of uracil-containing DNA, supporting previous results using a different method (19). However, we could not detect significant BER activity associated with immunoprecipitated UNG1-EYFP from mitochondrial extracts, although crude extracts efficiently repaired uracil-containing DNA. UNG1 may interact functionally with downstream BER proteins, but such interactions do not result in the buildup of stable repair complexes.

MATERIALS AND METHODS

Chemicals and antibodies

Synthetic oligonucleotides were purchased from MedProbe (Oslo, Norway). [α - 32 P] dTTP (3000 Ci/mmol), and [γ - 33 P] ATP (3000 Ci/mmol) were from Amersham Biosciences. Primary antibodies against APE1 (mouse monoclonal, ab194), APE2 (rabbit polyclonal, ab13691), and serum anti-GFP antibody (rabbit polyclonal, ab290), were from Abcam Ltd, UK. The GFP antibody recognizes and binds to EYFP and is hereafter referred to as anti-EYFP-Ab. IgG from non-immunized rabbits was from Santa Cruz Biotechnology, Inc. USA. Anti-UNG-antibody (PU101) against the catalytic domain has been described previously (21). The ProteosilverTM silver stain kit was from Sigma-Aldrich, USA.

Cell culture and cell lines

We prepared UNG1-EYFP and UNG2-EYFP fusion proteins under control of a cytomegalovirus (CMV) promoter by transfecting HeLa cells with pUNG1-EYFP and pUNG2-EYFP (6). The cells were then cultured under selection with 0.4 mg/ml genetecin (G418) in Dulbecco's modified Eagle's medium with 10% fetal calf serum, 0.03% glutamine, and 0.1 mg/ml gentamicin in 5% CO₂. The UNG1 / UNG2-EYFP positive cells were sorted out from G418 resistant cells by flowcytometry. The levels of expression of UNG1-EYFP and UNG2-EYFP were lower than the levels of endogenous UNG1 and UNG2 (Figure 1D).

Confocal microscopy

A Zeiss LSM 510 Meta laser scanning microscope equipped with a Plan-Apochromate 63x/1.4 oil immersion objective was used to examine images of 1 μ m thick slices of cycling living cells. EYFP was detected with excitation at $\lambda = 514$ detection at $\lambda > 530$ nm.

Isolation of mitochondria

We harvested the cells by trypsination and washed the cells once with cold PBS and once with an isotonic buffer (20 mM HEPES-KOH pH 7.8, 5 mM MgCl₂, 5 mM DTT, and 0.25 M sucrose). The cells were resuspended in a hypotonic buffer (20 mM HEPES-KOH pH 7.8, 5 mM MgCl₂, 5 mM DTT) and incubated on ice for 15 min before disruption of the cells by a Dounce homogenizer (15-20 strokes). We immediately added (1:1 v/v) 2× MSH buffer (20 mM HEPES-KOH pH 7.8, 2 mM EDTA, 2 mM EGTA, 0.42 M manitol, 0.14 M sucrose, 0.3 mM spermine, and 1.5 mM spermidine) to the mixture as described previously (22). We then centrifuged the cell lysate at 600 g for 12 min to separate cell debris and nuclei (the pellet) from mitochondria (supernatant), and pelleted the crude mitochondria at 10,000 g for 20 min. The pellet was then resuspended in 1 ml 1× MSH/50% Percoll (Sigma), the suspension loaded on top of a 1× MSH/50% Percoll gradient (9 ml) and centrifuged at 50,000 g for 1h at 4°C. The mitochondria were removed from the gradient and washed with 1× MSH buffer and resuspended in 2 ml buffer B (10 mM HEPES-KOH pH 7.4, 0.25 M sucrose, 1 mM EDTA, 1 mM EGTA, and 1 mM DTT). We divided the mixture in two parts and treated one part with 0.04 mg trypsin at room temperature for 20 min, while keeping the other half on ice. We inactivated trypsin by adding 0.120 ml Complete[®] protease inhibitor (1 tablet in 1 ml water) to the mixture, and washed the mitochondria three times in 2 ml buffer B containing protease inhibitor.

Isolation of nuclei

We cultured and harvested stably expressing UNG2-EYFP HeLa cells as above, except omission of MSH buffer to the mixture after disruption of the cells with the Dounce homogenizer. The cell homogenate was centrifuged at 600 g for 12 min and the pellet used for preparation of nuclear extract.

Preparation of nuclear and mitochondrial extract

We used a modification of the procedure described before (23). Briefly, mitochondria or nuclei were resuspended at 1× packed cell volume (PCV) in buffer I (10 mM Tris-HCl pH 8.0, and 100 mM KCl) and 1× PCV of buffer II (10 mM Tris-HCl pH 8.0, 100 mM KCl, 2 mM EDTA, 2 mM DTT, 40% glycerol, 1% Nonidet P-40, Complete[®] protease inhibitor, and Phosphatase Inhibitor cocktails). The mixtures were then rotated at 4°C for 1 h, nuclear or mitochondrial debris were pelleted at 22,000 g at 4°C for 15 min, and the supernatants recovered.

Immunoprecipitation

We covalently linked 0.01 ml anti-EYFP-Ab, or non-immunized rabbit IgG to 0.1 ml paramagnetic Dynabeads[®] protein A according to the manufacturer's instructions (DynaL, Norway). For immunoprecipitation we incubated mitochondrial or nuclear extracts with the antibody coated beads (anti-EYFP-Ab-beads or IgG-Ab-beads as control) under gentle rotation for 4 h at 4°C. We washed the beads 4 times with 0.2 ml washing buffer (10 mM Tris-HCl, pH 7.5, 100 mM KCl) and used the beads directly in BER assay or uracil-DNA glycosylase (UDG) assay, or resuspended the beads in SDS buffer, heated at 80°C (unless otherwise indicated) and separated proteins by SDS-polyacrylamide gel electrophoresis.

Formaldehyde crosslinking of mitochondrial proteins

We resuspended intact mitochondria (after trypsin treatment) in PBS with 0.5% formaldehyde and incubated the mixture at 37°C for 20 min. We stopped the reaction by adding 0.125 M glycine and further incubation at room temperature for 5 min. Mitochondria were washed twice with PBS and resuspended in buffer I and II as above and sonicated. The mitochondrial debris was pelleted at 22,000 g for 15 min at 4°C, and the supernatant recovered.

DNA substrate, BER assay, and uracil-DNA glycosylase (UDG) activity assay

We prepared DNA substrates containing uracil at a single position (Figure 2B) as described before (19,24). BER assays using nuclear or mitochondrial extracts, and their respective immunoprecipitates were carried out as described before (19). UDG activity assay was performed as described earlier (12).

Western analysis

We separated proteins in 10% denaturing SDS-polyacrylamide gels, and transferred them to PVDF membranes (Immobilon[™], Millipore). We incubated the membranes with the primary antibodies for 1 h at room temperature, followed by incubation for 1 h with either peroxidase-labeled Polyclonal Rabbit Anti-mouse IgG/HRP or peroxidase-labeled Polyclonal Swine Anti-Rabbit IgG (DakoCytomation, Denmark). We treated the membranes with chemiluminescence reagent (SuperSignal[®] West Femto Maximum, PIERCE, USA), and visualized the bands in Image Station 2000R (Eastmann Kodak Company, USA).

RESULTS

In the present study, we wanted to compare the overall organization of nuclear and mitochondrial BER. For this purpose we constructed cell lines expressing either UNG1 or UNG2 with a fluorescent tag (enhanced yellow fluorescent protein, EYFP). This allowed immunoprecipitation of the glycosylases from each of the subcellular compartments using anti-EYFP antibodies. This made possible studies on how these DNA glycosylases interact with other proteins in nuclei and mitochondria. The HeLa cell lines used expressed either UNG2-EYFP or UNG1-EYFP from a CMV promoter, which is usually considered a strong promoter. However, as shown below the expression of the fusion proteins was significantly lower than that of the endogenous proteins, thus a general perturbation of the balance of the repair proteins was unlikely.

Subcellular localization of UNG-EYFP fusion proteins

We found that UNG2-EYFP proteins expressed in the UNG2-EYFP HeLa cell line were exclusively localized in nuclei (Figure 1A), similar to UNG2-EYFP under the regulation of the UNG2 promoter (P_A) (12). In the UNG1-EYFP HeLa cell line UNG1-EYFP co-localized with mitochondria, and was entirely absent from nuclei, in agreement with previous results (6) (Figure 1B). To ensure that UNG1-EYFP molecules (Figure 1B) were not merely attached to the outer membrane of mitochondria, we treated intact mitochondria with trypsin before lysis of the mitochondria (25). This treatment cleared contaminating nuclear UNG2 from the mitochondrial extract, whereas UNG1 and UNG1-EYFP fusion proteins remained unaffected (Figure 1C) and was routinely used for preparation of mitochondrial extracts. It is noticeable that also APE1 is strongly reduced, indicating that it is mainly, but not exclusively, a nuclear protein. In contrast, APE2 was not significantly reduced by trypsin-treatment, indicating that it is a genuine mitochondrial protein (Figure 1C). Figure 1D shows western analysis of nuclear and mitochondrial extracts from UNG2-EYFP and UNG1-EYFP expressing HeLa cells, respectively. The EYFP-tagged UNG proteins and the endogenous forms were all detected using the same antibodies (PU101), and the endogenous forms of UNG were significantly more abundant than the tagged forms.

UNG2-EYFP, but not UNG1-EYFP, is present in uracil-BER competent complexes

Recently, we used antibodies against the non-catalytic N-terminal domain of UNG2 and isolated BER complexes (UNG2-ARC) that repaired uracil in DNA (19). In this study, we used nuclear and mitochondrial extracts from UNG2-EYFP and UNG1-EYFP expressing cells, respectively, for immunoprecipitations with anti-EYFP-antibody-beads. Figure 2A shows western analysis of immunoprecipitates from UNG2-EYFP (lane 1) and

UNG1-EYFP (lane 2) cell extracts. We detected UNG2-EYFP (lane 1, upper panel) and UNG1-EYFP (lane 2, upper panel) proteins, but not their respective endogenous proteins (lower panel), indicating that only the fusion proteins were immunoprecipitated. Next, we used a uracil-containing circular plasmid to test BER activity of the immunoprecipitates (Figure 2B). We succeeded in isolating BER-competent complexes from nuclear extracts using this approach (Figure 2C, lane 3), consistent with our recent report where we used a different procedure to isolate complexes (19). However, we did not find detectable BER activity associated with the immunoprecipitated material from mitochondrial UNG1-EYFP extract (Figure 2C, lane 4). Importantly, equal amounts of protein (0.5 mg) were used for preparing immunoprecipitates from nuclear and mitochondrial extracts, excluding trivial explanations for the differences observed. This indicates that no, or insignificant amounts of uracil-BER protein complexes were present in the mitochondrial extract. However, the extracts as such repaired uracil in DNA in the same BER assay (Figure 2C, lanes 5-10). We did not find detectable differences between BER activity of pre-immunoprecipitated and post-immunoprecipitated UNG2-EYFP (Figure 2C, lanes 5 and 7) indicating that a small fraction of uracil-BER proteins in the nuclear extract associated with UNG2-EYFP. To test if immunoprecipitated UNG1-EYFP protein had UDG activity comparable to the UNG2-EYFP immunoprecipitates, we incubated the immunoprecipitates from nuclear and mitochondrial extracts with 5'-end labeled uracil-containing double-stranded oligonucleotides. We found that UNG2-EYFP and UNG1-EYFP immunoprecipitates had roughly similar UDG activities (Figure 3A, lanes 3, 4, 12-mer bands), and this activity was completely inhibited by neutralizing anti-UNG-antibodies (PU101) (Figure 3A, lanes 7, 8). In conclusion; when equal amounts of protein from either nuclear or mitochondrial extracts are immunoprecipitated using EYFP-antibodies, similar amounts of UDG-activity are detected, but only UNG2-EYFP is present in a complex that carries out complete BER of uracil in DNA.

Supplementing UNG1-depleted mitochondrial extract with recombinant UNG protein restores uracil-BER in the extract

Next, we tested if our failure to immunoprecipitate putative UNG1-EYFP BER-complexes was due to preferential interaction of BER factors with endogenous UNG1. For this analysis, we immuno-depleted (IP-depleted) UNG proteins (both UNG1-EYFP and UNG1) from the UNG1-EYFP mitochondrial extract using PU101 antibodies attached to beads, and used the depleted extract in the BER assay. Repair of uracil was strongly reduced after the immuno-depletion (Figure 3B, lane 2). However, the level of the repair was restored to the level of the untreated extract (lane 3, control) when the reaction mixture was supplemented with recombinant

UNG protein (21) (rec.UNG) (lane 4). This demonstrates that sufficient amounts of free BER proteins acting downstream to UNG1 were present in the extract, and supports the conclusion that BER proteins do not interact stably with UNG1.

APE1 is the major AP endonuclease in mitochondrial uracil-BER in HeLa cells

To test the role of APE1 in the nuclear and mitochondrial uracil-BER, we incubated nuclear UNG2-EYFP as well as mitochondrial UNG1-EYFP extracts on ice with or without anti-APE1-antibodies. Then uracil-DNA substrate was added to the extracts and the samples were incubated at 32°C for 60 min. Recently, we showed that these antibodies inhibited uracil-BER in immunoprecipitated UNG2-BER complexes (19). We found that anti-APE1-antibodies moderately inhibited nuclear uracil-BER (Figure 4, lane2). However, uracil-BER of the mitochondrial extract was strongly inhibited by these antibodies (Figure 4, lane 4), indicating that APE1 is the major AP-endonuclease in HeLa cell mitochondria.

The inner membrane of mitochondria harbors a large number of proteins including mitochondrial DNA glycosylases (17). Therefore, it would seem possible that UNG1 may be located in close vicinity to other BER proteins in the inner membrane, thus facilitating functional interactions, although without forming stable complexes. To test this, we carried out formaldehyde crosslinking of intact mitochondria. Formaldehyde is useful for the detection of protein-protein interactions. The formaldehyde crosslinking requires a short distance (~2Å) between the molecules, such that proteins must be in close physical proximity at least for a limited time period (26). Moreover, the crosslinks are reversible, enabling subsequent identification the proteins. We incubated extract prepared from crosslinked mitochondria with anti-EYFP-Ab-beads in the presence of DNase. After washing, we resuspended the beads in SDS buffer and heated at 65°C for 15 min to release proteins from the beads. Then one half of the eluted fraction was heated at 95°C for 30 min to reverse crosslinking reactions, and proteins in the both eluted fractions were separated on an SDS gel, and visualized by silver-staining (Figure 5A, lanes 2, 3). We used normal IgG as control (Figure 5A, lanes 4, 5). We detected several bands and smeared protein in the gel after reversing the crosslinking reactions as compared with not reversed (Figure 5A, lanes 2, 3), suggesting that UNG1 is located close enough to other proteins for crosslinking to occur. We also carried out western analysis of the eluted proteins after reversing crosslinking reactions at 95°C for 30 min (Figure 5B, lanes 1, 2). We detected UNG1-EYFP, and APE1, but not APE2 in the eluted material (Figure 5B, lanes 1, 2). Furthermore, using western analysis we detected APE1 in the immunoprecipitates from the UNG2-EYFP nuclear extract (Figure 5B, lane 3), but not in the immunoprecipitates from the non-crosslinked UNG1-EYFP

mitochondrial extract (Figure 5B, lane 4). These results suggest that UNG1 and the downstream BER protein APE1 may be transiently located in close vicinity. However, despite the presence of DNase during the immunoprecipitation, we cannot rule out the possibility that this interaction may take place through a common mtDNA substrate, as DNA may theoretically be protected from digestion by proteins crosslinked to DNA.

Recently, several mitochondrial DNA glycosylases as well as DNA polymerase and DNA ligase, but not AP endonuclease, were demonstrated to tightly associate with the inner membrane (17). Therefore, it was possible that all uracil-BER enzymes except for AP endonuclease were present in the UNG1-EYFP immunoprecipitates, resulting in the isolation of inactive complexes. To test this we carried out BER assay analysis of UNG1-EYFP immunoprecipitates supplemented with recombinant APE1. We could not reconstitute detectable repair activity by adding APE1 (not shown), indicating that the inability of UNG1-EYFP immunoprecipitates to repair uracil in DNA (Figure 2, lane 4) was not solely due to the lack of AP endonuclease in the immunoprecipitates.

DISCUSSION

Nuclear UNG2 and the mitochondrial preform UNG1 differ in the N-terminal sequences required for targeting (6) (Figure 1A and B). The entire UNG2 protein is imported to nuclei, but a large fraction of the N-terminal region of UNG1 is proteolytically removed upon mitochondrial import (Figure 1B) (11). The 6 amino acids remaining in UNG1 are all different from the 44 amino acids in the N-terminal region of UNG2. The successful isolation of UNG2-specific uracil-BER complexes by two different methods (19) (and Figure 2C, lane 3), suggests that the presence of UNG2-BER complexes in nuclei may be biologically relevant. We found no evidence for formation of similar BER complexes in mitochondrial extract.

In mitochondria, most BER proteins including UNG1 are not freely soluble in the matrix, but associate tightly with the inner membrane independently of mtDNA (17). However, such sub-organelle localizations apparently do not mediate formation of stable BER complexes, since we were unable to isolate sufficient amounts of uracil-BER complexes for detectable BER using our assay system (Figure 2C, lane 4). In the more complex nuclear environment the presence of the essential BER proteins in complexes may aid binding of different repair proteins to the relevant repair intermediate. Our results are compatible with a model in which the BER proteins are not stably interacting with each other, but close enough (17) to “pass the baton” (27), thus avoiding exposure of damage-sensitive repair intermediates such as highly reactive abasic sites.

As shown in Figure 1C, treatment of intact mitochondria with trypsin removed a considerable fraction of contaminating nuclear APE1, while APE2 remained unchanged. However, our formaldehyde crosslinking experiments suggest that the small amounts of APE1 remaining in mitochondria may be located in the proximity of UNG1, while we were unable to detect APE2 in the eluted immunoprecipitate from crosslinked material. Moreover, mitochondrial uracil-BER was completely inhibited in the presence of anti-APE1-antibodies. These results suggest that APE1, rather than APE2, may be the main AP endonuclease in mitochondrial uracil-BER in HeLa cells. APE2 was reported to display very weak AP endonuclease activity compared with APE1 (28,29). Previously, a fraction of APE2 was shown to localize in mitochondria (30). Our results (Figure 1C) support the view that APE2 is a genuine mitochondrial protein, but its role remains elusive.

In recent years, identification and analysis of BER protein complexes have attracted attention (19,31-33). The differences between mitochondrial and nuclear uracil-BER may reflect the higher complexity of nuclear organization and consequently a higher requirement for formation of macromolecular compartments. Repair foci and replication foci are examples of one level of subnuclear compartmentalization. Complexes of repair proteins

within such foci would represent yet another level of organization, and like replication and repair foci they are likely to be highly dynamic.

Generation of suitable antibodies for immunoprecipitation of the many proteins is usually a time consuming process requiring careful characterization of each new antibody. The use of fusion proteins where EYFP or similar proteins form a separate domain may be a useful alternative for isolation of putative BER protein complexes for biochemical and structural analysis.

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LEGENDS TO FIGURES

Figure 1. UNG2- and UNG1-EYFP fusion proteins. (A) Left; schematic illustration of nuclear UNG2 and, right; confocal image showing HeLa cells stably expressing UNG2-EYFP. (B) Left; schematic illustration of mitochondrial UNG1 preform and the processed, mature form of mitochondrial UNG1 and, right; confocal image of UNG1-EYFP. The UNG1 preform is thought to be processed by mitochondrial processing peptidase (MPP). Both fusion proteins were expressed from the CMV promoter. The unique N-terminal ends are required for subcellular sorting, but not for uracil-DNA glycosylase (UDG) activity. The catalytic domain alone is a fully active UDG enzyme. (C) Western analysis of mitochondrial extract before trypsin treatment (lane 1) and after trypsin treatment of intact mitochondria prior to lysis of the mitochondria (lane 2). (D) Western analysis of nuclear extract (lane 1), and mitochondrial extract (lane 2) from UNG2-EYFP and UNG1-EYFP expressing HeLa cells, respectively, showing the fusion proteins (upper panel) and the corresponding endogenous proteins (lower panel). Anti-UNG-antibody (PU101) was used to detect all UNG proteins. Broken line (---) indicates that a part of the gel has been deleted to shorten the image.

Figure 2. Functional analysis of the immunoprecipitates and extracts. (A) Western analysis of immunoprecipitated UNG2-EYFP and UNG1-EYFP proteins. We prepared immunoprecipitates from 0.25 mg of each extract using 0.01 ml anti-EYFP-Ab-beads (lanes 1, 2) or IgG-Ab-beads for control (lanes 3, 4) and separated the eluted proteins in a SDS gel as described in materials and methods and subjected to western analysis with anti-UNG-antibody (PU101). Symbol × shows IgG contamination. Broken line (---) indicates that a part of the gel has been deleted to shorten the image. (B) Schematic illustration of plasmid in BER assay with a single U at defined position. (C) BER assay of different immunoprecipitates or crude extracts. We prepared immunoprecipitates by incubating 0.05 ml anti-EYFP-Ab-beads with 0.5 mg UNG2-EYFP nuclear extract (lane 3) or UNG2-EYFP mitochondrial extract (lane 4) as well as 0.05 ml IgG-Ab-beads with the same amounts of each extract for control (lanes 1, 2), and used the beads directly in the BER assay. For BER assay of the extracts

we used 0.05 mg of each extract in each reaction. DNA substrate containing T instead of U was used as control in the BER assay (lanes 11-12). The repair reactions were carried out at 32°C for 60 min. High molecular weight bands (HMW) represent repair products longer than ten nucleotides, as well as unspecific repair products at other parts of the DNA substrate, as demonstrated in experiments not shown here.

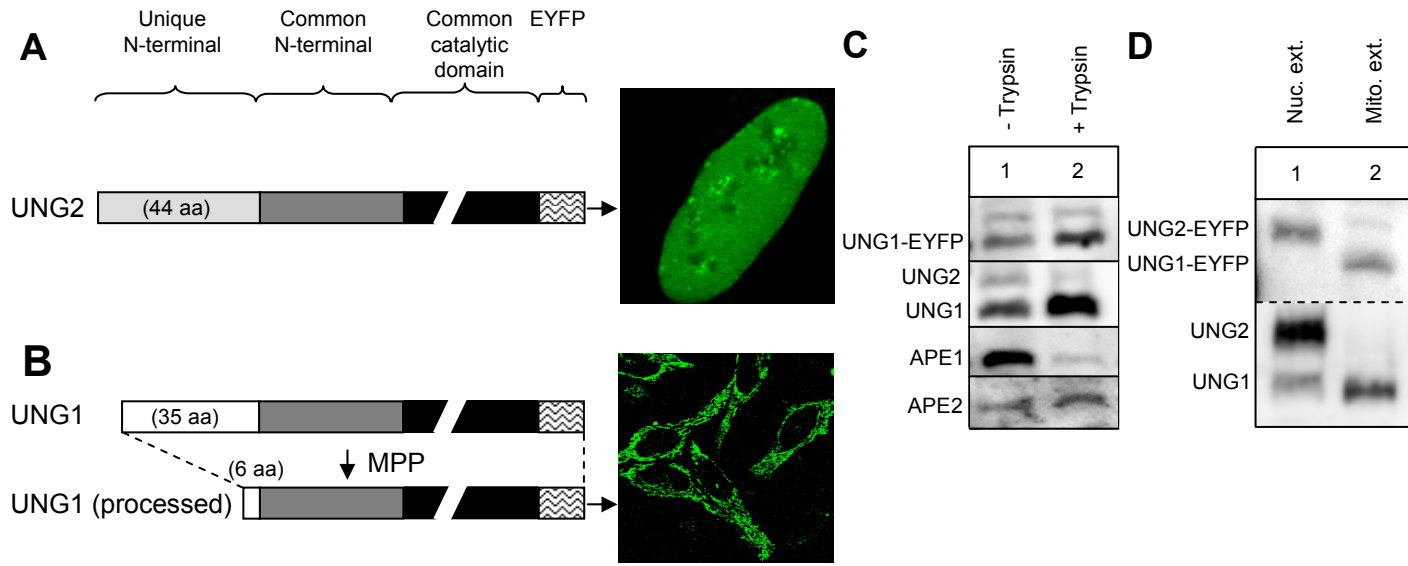
Figure 3. UDG-activity of UNG2-EYFP and UNG1-EYFP immunoprecipitates and BER assay analysis of reconstituted mitochondrial extract. **(A)** We tested UDG activity of the immunoprecipitates by oligonucleotide cleavage assay. We prepared immunoprecipitates from 0.25 mg nuclear extract of UNG2-EYFP expressing cells (Nuc. ext.) and from 0.25 mg mitochondrial extracts (Mito. ext.) from UNG1-EYFP expressing cells, using 0.01 ml anti-EYFP-Ab-beads, or normal IgG-Ab-beads as control. Immunoprecipitates were incubated for 30 min at 32°C with a uracil-containing 22-mer oligonucleotide, labelled at the 5'-end with ³³P, and cleaved products separated (12-mer) from the intact 22-mer in a denaturing polyacrylamide gel as described before (12). Lane 1, untreated oligonucleotide; lane 2, oligonucleotide incubated with recombinant UNG; lane 3, oligonucleotide incubated with immunoprecipitate from nuclear extracts prepared with anti-EYFP-Ab-beads; lane 4, oligonucleotide incubated with immunoprecipitate from mitochondrial extract prepared with anti-EYFP-Ab-beads; lanes 5-6, oligonucleotide after “mock”-immunoprecipitation of extracts with control IgG-Ab-beads; lanes 7-8 oligonucleotide after incubation in the presence of neutralizing anti-UNG-antibody (PU101) of immunoprecipitates prepared with anti-EYFP-Ab beads. **(B)** BER assay of reconstituted mitochondrial extract. We immuno-depleted mitochondrial extract (IP-depleted) for UNG1-EYFP and endogenous UNG1 using beads carrying anti-UNG-Ab (PU101). We then used the depleted extract for BER assays. Lane 1, BER of mitochondrial extract before IP-depletion; lane 2, immunodepleted mitochondrial extract; lanes 3 and 4, similar to lanes 1 and 2, but reconstituted with recombinant UNG (21).

Figure 4. Role of APE1 in uracil-BER of mitochondrial and nuclear extracts. We incubated UNG2-EYFP nuclear extract (lanes 1, 2) and UNG1-EYFP mitochondrial extract (lanes 3, 4), with or without anti-APE1-antibodies as indicated, on ice for 30 min. The repair reactions were initiated by adding DNA substrate and were carried out at 32°C for 60 min.

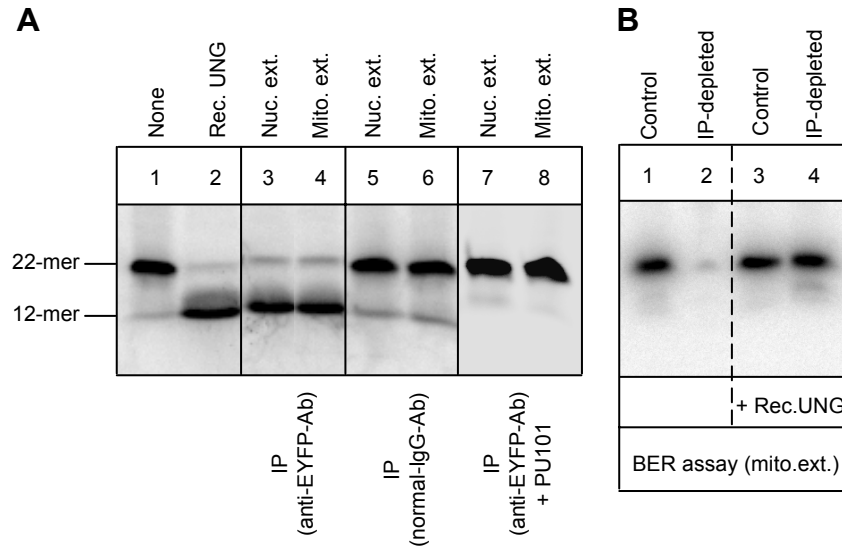
Figure 5. Silver-staining and western analysis of immunoprecipitates after formaldehyde crosslinking and reversal. **(A)** We prepared immunoprecipitates from 0.5% formaldehyde-treated mitochondria, using anti-EYFP-

Ab-beads. For control we used IgG-Ab-beads. The immunoprecipitates were eluted at 65°C (elute, 65°C), and one half of the eluates were heated at 95°C to reverse crosslinking (elute, 95°C). The two fractions were analyzed in SDS gel combined with silver-staining (lanes 2-5). Arrows show the bands seen after reversal of crosslinking. Symbol × shows IgG contamination. **(B)** We prepared immunoprecipitates from 0.5% formaldehyde-treated mitochondria, using anti-EYFP-Ab-beads. For control we used IgG-Ab-beads. We heated the immunoprecipitates at 95°C for 30 min to elute proteins and to reverse crosslinking reactions and carried out western analysis of eluates for UNG, APE1, and APE2 (lanes 1, 2). Lanes 3 and 4 show western analysis of immunoprecipitates from nuclear UNG2-EYFP extract and mitochondrial UNG1-EYFP extract (not crosslinked) respectively, for APE1 and APE2.

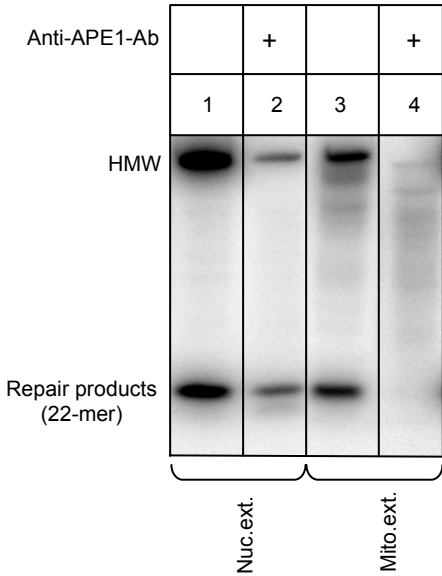
Akbari et al. Fig. 1



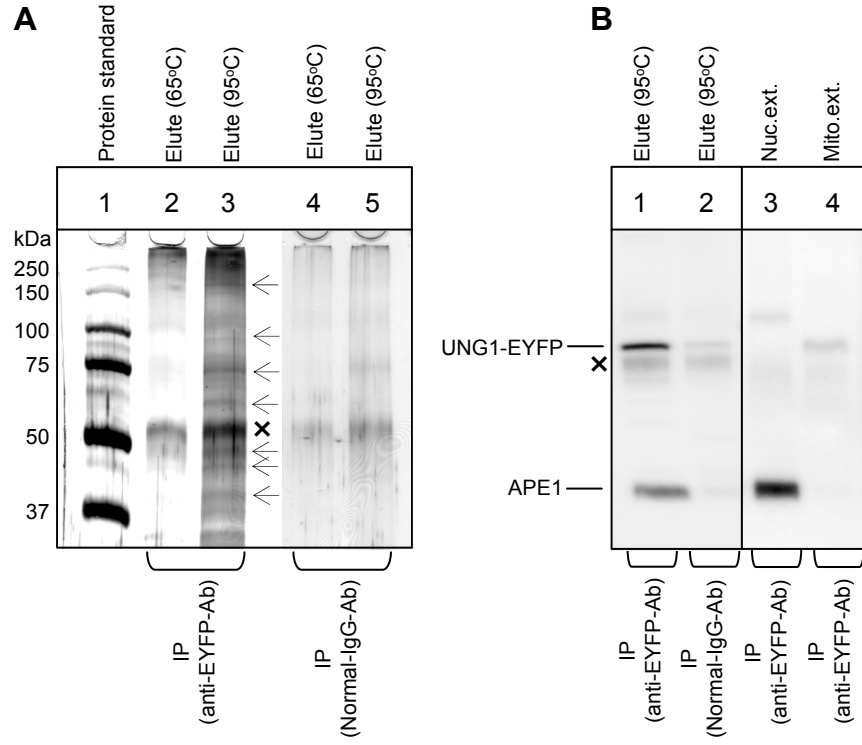
Akbari et al. Fig. 3



Akbari et al. Fig. 4



Akbari et al. Fig. 5



Paper V

Sequence variation in the human uracil-DNA glycosylase (*UNG*) gene

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Abstract

Spontaneous deamination of cytosine results in a premutagenic G:U mismatch that may result in a GC → AT transition during replication. The human *UNG*-gene encodes the major uracil-DNA glycosylase (UDG or UNG) which releases uracil from DNA, thus, initiating base excision repair to restore the correct DNA sequence. Bacterial and yeast mutants lacking the homologous UDG exhibit elevated spontaneous mutation frequencies. Hence, mutations in the human *UNG* gene could presumably result in a mutator phenotype. We screened all seven exons including exon–intron boundaries, both promoters, and one intron of the *UNG* gene and identified considerable sequence variation in cell lines derived from normal fibroblasts and tumour tissue. None of the sequence variants was accompanied by significantly reduced UDG activity. In the *UNG* gene from 62 sources, we identified 12 different variant alleles, with allele frequencies ranging from 0.01 to 0.23. We identified one variant allele per 3.8 kb in non-coding regions, but none in the coding region of the gene. In promoter B we identified four different variants. A substitution within an AP2 element was observed in tumour cell lines only and had an allele frequency of 0.10. Introduction of this substitution into chimaeric promoter–luciferase constructs affected transcription from the promoter. UDG-activity varied little in fibroblasts, but widely between tumour cell lines. This variation did not however correlate with the presence of any of the variant alleles. In conclusion, mutations affecting the function of human *UNG* gene are seemingly infrequent in human tumour cell lines. © 2001 Elsevier Science B.V. All rights reserved.

Keywords: Human uracil-DNA glycosylase; Genetic variation; Cancer cell lines

1. Introduction

Spontaneous base-loss, deamination and oxidation of DNA bases are frequent events and the resulting lesions are repaired predominantly by BER. It has

been estimated that at least 10 000 lesions are formed spontaneously in every human cell every day [1]. BER is therefore considered quantitatively as the most important means of DNA repair of spontaneously arising damage and impaired repair capacity towards these lesions could add substantially to the mutation load. Damage specific DNA glycosylases hydrolyse the *N*-glycosylic bond between the target base and deoxyribose, releasing the damaged base and leaving an apurinic/aprimidinic (AP) site in DNA (reviewed in [2]). Uracil-DNA glycosylase (UDG) initiates the

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BER pathway for the removal of uracil. Uracil in DNA results from incorporation of dUMP instead of dTMP during replication [3–5], or from cytosine deamination [6] that gives rise to G:U mismatches resulting in transition mutations (G:C → A:T) during replication. U:A pairs are not mutagenic but may alter binding of transcription factors to their response elements [7], thus influencing gene-expression. The major UDG from prokaryotes, eukaryotes and eukaryote viruses are highly conserved [2] and UDG-deficient yeast [8,9] and *Escherichia coli* [10,11] exhibit several-fold increases in spontaneous mutation frequencies due to C → T transitions. In addition to loss of BER activity, altered expression levels of BER proteins have been shown to cause mutator phenotypes in yeast [12], in *E. coli* [13] and in human cells [14].

The human *UNG* gene spans 13.8 kb and comprises two promoters and seven exons [15–17]. The gene encodes both nuclear (UNG2) and mitochondrial (UNG1) forms that differ in their N-terminal amino acid sequences. They are generated by transcription from two different promoters, promoter A (P_A) and promoter B (P_B), respectively, and the use of alternative splicing [17]. The level of UDG activity varies between organs [18] and between individuals [19,20]. However, this variation has not been correlated to mutations in the *UNG* gene.

Germline mutations in genes thought to maintain the integrity of the genome, caretaker genes, may cause inherited predisposition to cancer [21]. Rare germline mutations in nucleotide-excision repair genes result in recessive human syndromes like xeroderma pigmentosum where severely affected individuals have 1000-fold increased risk for UV-induced skin cancer [22]. Interestingly, sequence polymorphisms resulting in reduced repair capacity exist in the human population [23,24]. Mutations in mismatch-repair genes are found in at least 70% of patients with the cancer-prone syndrome HNPCC (hereditary non-polyposis colorectal cancer) [21]. In contrast, no human cancer prone syndrome is clearly known to originate from BER deficiency. Mutations in caretaker genes are thought to result in earlier presentation of cancer. We set out to screen the *UNG* gene for mutations in mismatch repair proficient colon cancer cell lines derived from relatively young patients. Furthermore, since genetic instability has been observed in the region of the *UNG* locus (12q24.1) in gastric cancers [25] we included

gastric cancer cell lines in the analysis in addition to a selection of other cancer cell lines. By sequencing regions of the *UNG* gene from 62 sources, we identified 12 different substitution variants in non-coding regions of the gene whereas no sequence variation was found in coding regions. Four sequence variants were identified in promoter B, one of which affected transcription from chimaeric promoter–luciferase constructs. However, the variations in measured UDG activity could not be correlated to the presence of sequence variants.

2. Materials and methods

2.1. Samples

Cell lines are listed in Table 1. All cell lines, except HaCaT 72 PS were purchased from American Type Culture Collection (ATCC). HaCaT 72 PS was kindly provided by Dr. Norbert E. Fusenig (German Cancer Research Centre, Heidelberg, Germany). Cell lines derived from human foreskin fibroblasts with an initial passage number of 1–9 are denoted normal cell lines. DNA from healthy, normal individuals was isolated from blood with the QiaAmp Blood kit (Qiagen). DNA from Noonan syndrome patients was kindly provided by Dr. Anette Bakken (Ullevål hospital, Department of Medical Genetics, Oslo, Norway). P1-DNA was isolated from a P1-phage clone containing the *UNG* gene (Genome Systems). Jx fibroblasts were normal cells taken from a male individual suffering from multiple basal cell carcinomas of unknown aetiology. High molecular weight DNA from cell lines was prepared by phenol/chloroform extraction and isopropanol precipitation using a model 340A Nucleic Acid Extractor (PE Applied Biosystems) or by using the High Pure PCR Template Preparation Kit (Boehringer Mannheim). DNA from the P1-clone and plasmid constructs for in vitro mutagenesis was isolated using the Qiagen Plasmid Midi Kit (Qiagen) with additional phenol/chloroform to remove any endotoxin from the plasmid constructs before transfection.

2.2. PCR amplification

PCR primers directed to intronic or non-coding sequences (Table 2) were designed using the Oligo

Table 1
Cell lines and their UDG-activity

Type of cell line	Source	Name	UDG-activity (U/mg total protein) ^a
Normal	Human foreskin fibroblast	CCD 32	0.07
		CCD 34	0.09
		CCD 39	0.10
		CCD 43	0.07
		CCD 1064	0.07
		CCD 1070	0.07
		CCD 1072	0.08
		CCD 1077	0.09
		CCD 1079	0.06
Various cancer	Glioblastoma	A172	0.42
	Epidermoid carcinoma	A253	0.62
	Lung adenocarcinoma	A427	0.39
	Lung carcinoma	A549	0.53
	Spontaneous transformed keratinocyte	HaCaT 72 PS	0.70
	Cervical adenocarcinoma	HeLa	0.21
	Blooms syndrome lymphoblast	Hg1525	0.26
	Retinoblastoma	RBWERI	0.41
	Retinoblastoma	RBV79	0.28
	Glioblastoma	U87	0.68
	SV40 transformed fibroblast	WI 38-VA13	0.46
	Lung adenocarcinoma	SkLu1	0.24
	Colon cancer	Colon adenocarcinoma, female 44 years	CX-1
Colon adenocarcinoma, female 55 years		Colo320 DN	0.43
Ileocecal adenocarcinoma, male 67 years		HCT-8	0.28
Colon adenocarcinoma		Hs 587. Int	0.08
Colon adenocarcinoma, female 44 years		HT-29	0.23
Colon adenocarcinoma, male 56 years		LOVO	0.10
Colorectal carcinoma, male 32 years		LS411N	0.33
Colorectal carcinoma, male 63 years		LS513	0.26
Colon adenocarcinoma, 58 years		LS 180	0.23
Colon adenocarcinoma, male 51 years		SW 480	0.29
Colon adenocarcinoma, male 51 years		SW 620	0.19
Colorectal adenocarcinoma, male 53 years		SW 837	0.26
Testis cancer	Embryonal carcinoma, testis, male 22 years	NTERA-2 c1.DM	0.26
	Embryonal carcinoma, testis, male 34 years	CATES-1B	0.03
	Testis cancer, male 15 years	HS444(B)T	0.04
Gastric cancer	Gastric adenocarcinoma, female 54 years	AGS	0.07
	Gastric carcinoma, male 55 years	KATOIII	0.16
	Gastric adenocarcinoma, male 62 years	RF-1	0.30
	Gastric adenocarcinoma, male 62 years	RF-48	0.30
	Gastric carcinoma, male 74 years	Hs 746T	0.08
	Gastric carcinoma, male	NCI-N87	0.30
	Gastric carcinoma, male 44 years	SNU-1	0.09
	Gastric carcinoma, female 33 years	SNU-5	0.10
	Gastric carcinoma, female 54 years	SNU-16	0.25
	Gastric adenocarcinoma, male 72 years	23132/87	0.16
Breast cancer	Breast, non-tumorigenic, female 27 years	HBL-100	nd ^b
	Breast carcinoma, female 54 years	T-47D	0.33
	Breast carcinoma, female 69 years	MCF-7	0.28
	Breast adenocarcinoma, female 51 years	MDA-MB-231	0.08
	Breast carcinoma, female 63 years	ZR-75-1	0.17

^a 1 unit of UDG is defined as the amount of enzyme that releases 1 nmol uracil from standard UDG-substrate per minute at 30°C. UDG activity was measured in triplicates and presented as arithmetic mean.

^b Not determined.

Table 2
PCR and sequencing primers

Region	PCR primers (5'–3')	Sequencing primers (5'–3')
ProA/exon IA (411–905) ^a	kudgproA+: GAC CAG TCT TCT CTT CTT GC kudgproA–: GAT CCT GCA CGC CCT CCG	ProA428+seq: AAG AGC CTG TCC AAA GAG ProA640+seq: CAC AGC CAC AGC CAG GG ProA866–seq: TCC CCC TTC ACC CCC TA
ProB/exon IB (838–1717)	kudgproB+: CTG AGG AAA GCG GAG ATG C kudgPB–: CTA CAC TAA CAA GAC AAA AGG G	ProB926+seq: GCC TCT GAC TCG GTA AAC ProB1268+seq: CGC TCC AGT TTA GAA CCT ProB975–seq: GCG GCG GCT ATT TTG AAC ProB1315–seq: GGA CAG TAA GAG CCC AG ProB1697–seq: GCA GCC TCC ACA TTT ACC
Exon II (2022–2428)	hung 2+: GAC CCA GCC CAG AGA AAG AG hung 2–: TCA GCC AAA CCT CGG GAT TTC	X2+seq: TTT CCA ATG AGC AAT CTG AT X2–seq: ATA CTA TTT GAC TCC TCC TC
Exon III (4703–5091)	kudg.ex3+: CAG CCT GAG CAA CAC AGC AAA C kudg.ex3–: GTG AAC AGT GCC CCA GAT AGT C	ex3+seq: GGG TCT GTG CTG CTT AC Exon3–seq: CCC AGA TAG TCC CAC TTG
Exon IV (5778–6064)	hung 4+: AGG GCT GGC TGT AAC TTC TAA C hung 4–: TGA GAA TCG CCT GAA CCT GGG	X4+seq: GTT TTT TGT TTT TCT TGT GGC X4–seq: AAA AAT CTA GCA GTC GCT GG
Intron IV (5911–6577)	hum 4+: GTC TAC AGA CAT AGA GGA TTT TG hum 5–: GCC CCA GAG CAA GAA AAC AAG	sh4.1+: ATT TTT AGT AGA GAC AGG G sh5.1–: TGA GGA CAG CGT TGA GAA G sh4.1–: GCA CTT TGG GAA GCC GAG
Exon V (6347–6848)	kudg.ex5+: CAC TGG ATC CCA TCA AGC ATT G kudg.ex5–: GTC AGA AGT TGG CCA GTT GCT T	ex5+seq: CTG ATT TGC CTG AGC CTA X5–ny: AAA ACA AAA CAT ACC CTA TC
Exon VI (12650–13392)	kudg.ex6+: ATG TTC TAA TGC AAA TGC TGA ATG kudg.ex6–: CAA AGG GTA GCC TTT GTT GTT TGT	X6+seq: AAA AGT CCC AAA TCT GCC Exon6–seq: AGT GGA ACT TCG TAA CTG
Exon VI NTR ^b (13116–13932)	Exon 6.1+: GTA CTA CAG ACG GCT CAT CC Yac 2–: GGA GAA AAT CAC CCC ACC CC	sh6.2+: ATC TCC CTT GCC TTT ATG sh6.2–: AAA ACC AAG GAG CAG CAG sh6.1+: CAA GAA GCC CAT TGA CTG sh6.1–: CTT TTC TCC TCT TCC TTG

^a Amplified regions (excluding primer sequences). Positions referring to the cloned *UNG* gene (GenBank Accession No. X89398).

^b Non translated region.

Primer analysis software, version 5.0. PCR amplification of genomic DNA by denaturation for 2 min at 96°C followed by 35 amplification cycles with 30 s denaturation at 96°C, annealing for 30 s at 60–65°C and 1 min extension at 72°C were carried out using a MiniCycler™ (MJ Research Inc.). Annealing temperatures were optimised for each primer pair. Reactions contained 100 ng high molecular weight genomic template DNA, 0.2 µM of each primer, 250 µM dNTP, 1.7–2.5 mM Mg²⁺ and 1.7 U Taq polymerase in a total volume of 50 µl. The reactions were set up according to the protocol provided for Expand™ High Fidelity PCR System (Boehringer Mannheim). PCR products were purified using the Qiagen QIAquick-spin PCR purification kit (Qiagen). Purity and yields of PCR products before and after purification were estimated performing agarose gel electrophoresis.

2.3. DNA sequencing

Internal primers for sequencing (Table 2) of both strands of the PCR products were designed as described above. Purified PCR products were sequenced using an Applied Biosystems 373 or 377 DNA sequencer (PE Applied Biosystems) with ABI Prism BigDye Terminator Cycle Sequencing Ready reaction kit as instructed by the manufacturer. Conditions for sequencing of PCR products were 30 cycles with 30 s at 96°C, 15 s at 50–54°C (optimised for each primer) and 4 min at 60°C. Residual dideoxy terminators were removed by ethanol precipitation largely according to the manufacturer (PE Applied Biosystems).

2.4. DNA sequence analysis

Initial analysis of sequences were performed using ABI Prism DNA sequence analysis software. Ambiguously called bases in the chromatograms were identified using the ABI Prism Factura feature identification software. Sequences were accepted if less than 3% ambiguities were observed in the clear data range. Sequences from both strands of the PCR products were compared with the wild type sequence by visual inspection using the ABI Prism Auto Assembler software.

2.5. UDG-assay

All cell lines were grown according to recommendations and harvested by trypsination for isolation of DNA and activity measurements. Cell-free extracts for activity measurements were prepared by resuspending cell pellets in 800 µl assay buffer (20 mM Tris-HCl pH 7.5, 60 mM NaCl, 1 mM EDTA pH 8, 1 mM DTT) prior to sonication for 3 min on ice (output control 2.5, 30% duty cycle). Supernatants for determination of enzyme activity were collected after centrifugation at 14 000 × g for 10 min at 4°C. UDG activity was assayed from appropriate dilutions using [³H]dUMP-containing DNA as described previously [26]. Protein concentration was determined by the Bio-Rad protein assay.

2.6. Site-directed mutagenesis

The plasmid constructs containing promoter B in the pGL2-Basic vector (Promega) are described elsewhere [27]. The pGL2-Basic vector carries the coding region for firefly (*Photinus pyralis*) luciferase. Site-directed mutagenesis was performed using the QuickChange™ Site-directed Mutagenesis kit (Stratagene). Mutagenesis primers (altered base in bold italic) were for variant number 1: ProB (998 C → T): 5'-GCC GCT GTC **C**T**C** CAT GGG CCG-3'. Variants 2 and 3 were introduced consecutively into the same construct using ProB (1034 A → G): 5'-GGC CAG CCA ATG **G**G**G** ACG CGT CTC GGG GCC-3' and ProB (1082 T → A): 5'-GCC GCA GGC CCT **C**C**A** GGC TCG GTG CGC TG-3'. All mutations were confirmed by sequencing.

2.7. Transfection assay

Transfection of HeLa cells was performed using 3 µl FuGENE 6 (Boehringer Mannheim) per transfection reaction according to recommendations from the manufacturer. DNA (1 µg) of each reporter construct was used per 2.5 × 10⁵ cells plated in 60 mm culture dishes 24 h prior to transfection. Aliquots of 150 ng pRL-TK vector encoding Renilla luciferase were co-transfected as an internal control. Activities expressed from the firefly luciferase reporter gene

and the Renilla luciferase control in transfected cells were detected using the Dual-Luciferase™ Reporter Assay system (Promega) as recommended by the manufacturer in a Turner Design luminometer. pGL2-Basic was used as negative control in each transfection experiment.

3. Results

UDG-activity was measured in nine normal and 41 cancerous cell lines. Activity measurements (U/mg protein) are shown in Table 1, right column. UDG activities for individual cell lines within groups are shown in Fig. 1. From this, we see that the UDG-activity in normal fibroblasts is low and consistent within the group. UDG activities in the cancer cell lines are highly variable. With exception of the testis cancer cells CATES-1B and Hs444(B)T, cancer cell lines exhibited as high as or higher activity than normal cells. Lowest activity (0.03 U/mg protein) was found in the testis cancer cell line CATES-1B. Highest activity was found in the spontaneously transformed keratinocyt HaCaT 72 PS (0.70 U/mg protein). The group called various cancer cell lines in Table 1 is included in Fig. 1 even though it comprises a non-uniform group since neither the mean UDG ac-

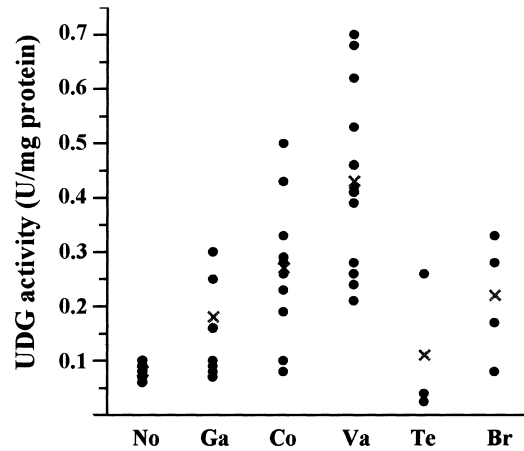


Fig. 1. UDG-activity in normal fibroblast and cancer cell lines based on data in Table 1. Groups of cell lines are abbreviated as follows: No: normal, Ga: gastric, Co: colon, Va: various, Te: testis, Br: breast. Average activity for each cell line (●); average activity for each group of cell lines representing a common type (×).

tivity or the variance are significantly different from the other uniform groups.

A sequencing strategy was set up to reveal variant alleles within the *UNG* gene. All seven exons were amplified by PCR using primers upstream and downstream of exon–intron boundaries. Both promoters (P_A and P_B), intron IV (IntIV) and the untranslated part of

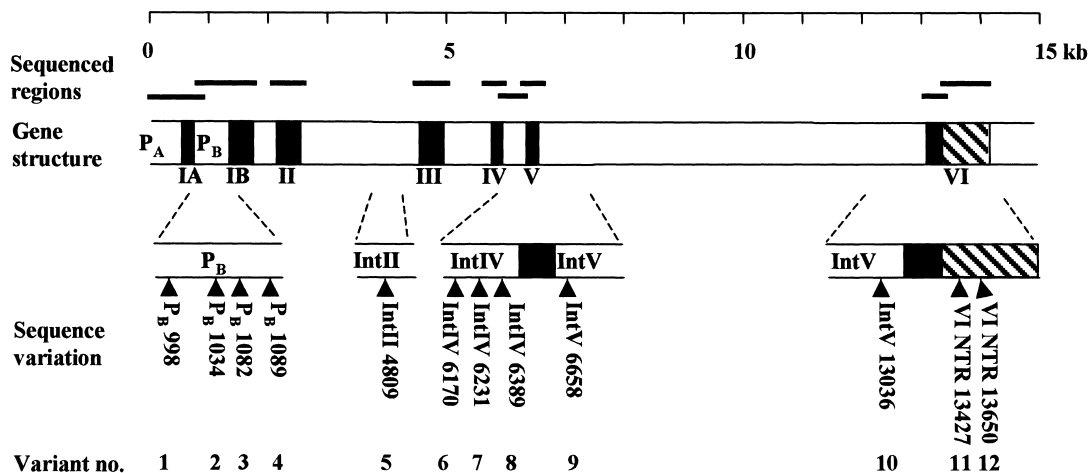


Fig. 2. Schematic organisation of the human *UNG* gene. Exons are marked with roman numbers. Translated exons are shown as black boxes and the non-translated region of exon VI is shown as a hatched box. Sequenced regions are indicated above the gene structure and positions of nucleotide substitutions are indicated underneath. The substitutions are for simplicity called 1–12. The position and nature of substitutions are in accordance with the sequence submitted to GenBank under Accession No. X89398.

Table 3
Allele frequencies of the various substitutions in the *UNG* gene

Localisation	Position	Specifications	Allele frequency									
			Number ^a	No	Ga	Co	Te	Br	Va	Noo	N/Noo	Cancer
ProB	998	C → T transition, within AP2 element	1	–	0.15	0.04	–	–	0.17	–	–	0.10
	1034	A → G transition, downstream of CCAT box	2	0.28	0.30	0.25	–	0.20	0.17	0.10	0.18	0.21
	1082	T → A transversion, within a Yi element	3	0.28	0.30	0.25	–	0.20	0.17	0.10	0.18	0.21
	1089	G → A transition, downstream of Yi element	4	–	–	0.04	–	–	–	–	–	0.01
Intron II	4809	T → C transition, 119 bp upstream of exon III	5	–	–	–	–	0.20	–	–	–	0.02
	6170	G → A transition, 286 bp upstream of exon V	6	–	0.05	–	–	–	–	–	–	0.01
Intron IV	6231	G → A transition, 225 bp upstream of exon V	7	–	0.05	–	–	0.10	–	–	–	0.02
	6389	C → T transition, 67 bp upstream of exon V	8	0.11	0.05	0.17	0.50	–	–	0.15	0.15	0.10
Intron V	6658	T → C transition, 23 bp downstream of exon V	9	–	0.10	0.04	–	–	–	0.05	0.03	0.04
	13036	A → T transversion, 49 bp upstream of exon VI (substitution is at the 3' end of a polyA tract)	10	0.33	0.35	0.25	–	–	0.13	0.15	0.23	0.19
Exon I	13427	T → C transition, 205 bp downstream of coding exon VI	11	–	–	0.04	–	–	–	–	–	0.01
NTR	13650	C → T transition, 428 bp downstream of coding exon VI	12	0.11	–	–	–	–	–	–	0.05	–

^a Numbering of substitutions 5' from the gene. Abbreviation as in Fig. 1 and Noo: Noonan syndrome. The allelic frequencies were calculated assuming homozygous alleles when only one PCR variant was achieved in the sequence analyses. Allelic frequencies for the normal (incl. Jx)/Noonan (N/Noo) and the cancers (two left columns) were based on number of samples defined in the two groups separately.

Table 4
Nucleotide substitutions in the *UNG* gene

Type	Sample	1	2	3	4	5	6	7	8	9	10	11	12
Normal	<i>UNG</i> ^a								+				
	CCD32												
	CCD34		+	+							+		+(h) ^c
	CCD39		+(h)	+(h)							+(h)		
	CCD43		+	+							+		
	CCD1064								+				
	CCD1070												
	CCD1072			+	+							+	
	CCD1077											+	
	CCD1079												
Noonan ^e	Jx ^d								+				
	Noonan 1								+				
	Noonan 2										+		
	Noonan 3												
	Noonan 4												
	Noonan 5												
	Noonan 6								+				
	Noonan 7			+	+					+	+		
	Noonan 8			+	+							+	
	Noonan 9												
Gastric	Noonan 10								+				
	AGS								+				
	KATOIII	+(h)											
	RF-1												
	RF-48												
	Hs746T			+(h)	+(h)							+(h)	
	Nci-N87	+					+	+					
	Snu-1		+	+								+	
	Snu-5											+	
	Snu-16		+	+								+	
Colon	23132/87		+(h)	+(h)						+(h)	+(h)		
	CX1		+	+					+		+		
	Colo320												
	HCT-8		+	+	+				+		+		
	Hs587		+	+					+	+	+		
	Ht-29		+	+					+		+		
	LOVO												
	LS411N		+	+							+		
	LS513	+											
	LS180		+	+							+		+
Testis	SW480												
	SW620												
	SW837												
Breast	NTERA-2										+		
	CATES-1B										+		
	HS444(B)T										+		
Breast	HBL100												
	MCF-7												
	MDA-MB-231		+	+									
	T47-D		+	+		+(h)							
Breast	ZR-75-1							+					

Table 4 (Continued)

Type	Sample	1	2	3	4	5	6	7	8	9	10	11	12
Various	A172		+	+							+		
	A253												
	A427		+	+									
	A549												
	HaCaT												
	HeLa	+(h)											
	Hg1525			+	+								+
	RBWERI			+	+								
	RBV79												+
	U87	+(h)											
	SKLU1												
	WI38												

^a P1-clone from a human genomic library containing the whole *UNG* gene.

^b + indicate presence of nucleotide substitution.

^c (h) denotes homozygous state.

^d Jx-patient, see M & M.

^e These samples comprise five normal samples and five from patients with Noonan syndrome.

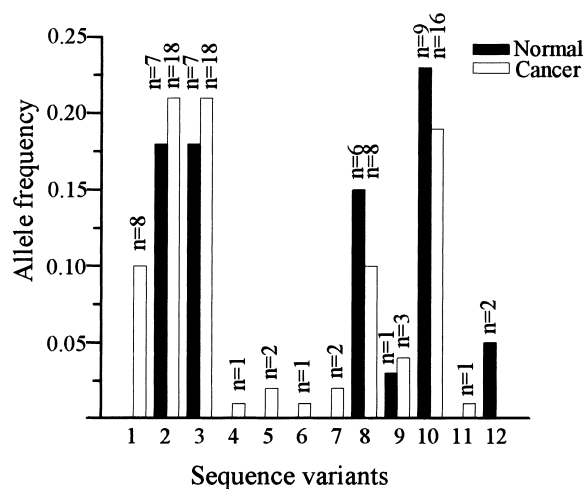


Fig. 3. Allelic frequencies of nucleotide substitutions in the *UNG* gene. Numbering of substitutions are the same as in Fig. 2. The presence of substitution number 1 is analysed in 42 cancer cell lines and 40 non-cancerous sources. The other frequencies are based on screening of 42 cancer cell lines and 20 non-cancerous sources. Numbers of substitutions retrieved n , are indicated above the bars.

exon VI (VI NTR) were included in the analysis to get information on sequence variation within non-coding regions. The amplified regions are illustrated in Fig. 2. In addition to DNA from cell lines listed in Table 1, we included DNA from 10 individuals of whom five are diagnosed with Noonan syndrome and one patient

sample, Jx. The *UNG* gene was screened for sequence variation from a total of 62 sources.

We revealed 12 different nucleotide substitutions (for simplicity called 1–12 in Fig. 2) in the sequence compared to the sequence from cloned *UNG* gene (GenBank Accession No. X89398). The individual sequence variants are described in Table 3. All cell lines and which sequence variants they contain are shown in Table 4. Nucleotide substitutions were found in 67% of the cell lines. As can be inferred from Fig. 2, no nucleotide substitutions were found in any of the translated exons. Four base pair substitutions were located in promoter B. A transition (C → T) was found in a putative binding site for transcription factor AP2 in position 998 (number 1). Sequence variants in position 1034 (A → G, number 2) downstream of a CCAAT-box and within a Yi element in position 1082 (T → A, number 3) always appeared together suggesting they are genetically linked. The last variant identified in promoter B resides downstream of a Yi element in position 1089 (G → A, number 4). Interestingly, variants 1 and 4 were only identified in cancer cell lines with allele frequencies of 0.1 and 0.01, respectively (Table 3). Variants 2 and 3 were common both in normal and cancer cell lines with allele frequencies 0.18 and 0.21, respectively. For verification of P_B variants 1, and 2 and 3 together restriction enzyme digests with *BpmI* and *BsmFI* were used, respectively (data not shown). The other variant alleles were

all identified in introns (numbers 5–10), and two variants (11 and 12) were identified in the non-translated part of exon VI (Table 3).

Of the 12 nucleotide substitutions identified, 10 were transitions and two were transversions. These were mostly found in a heterozygous state, although in three of the samples, one normal fibroblast (CCD39) and two gastric cancer cell lines (Hs746T and 23132/87), all the observed substitutions appeared in homozygous state (Table 4). Assuming homozygosity when only one allele was found, the allelic frequencies of the various nucleotide substitutions within the cell line types are summarised in Table 3. Allele frequencies for the different sequence variants from normal and cancerous samples are shown in Fig. 3. As seen in the figure, several of the substitutions had similar frequencies in normal and cancerous cell lines. Moreover, none of the allelic variants can be correlated with UDG activity measurements in extreme low or extreme high range.

We were intrigued by the existence of many variant alleles in promoter B compared to other regions of the gene. To analyse whether any of the variants could affect transcription from the promoter we prepared and transfected chimaeric promoter–luciferase constructs harbouring substitutions 1 and 2 + 3 into HeLa cells. Previous work performed in our laboratory indicated functional interaction between the two promoters [27]. We therefore analysed the effects of the substitutions in constructs harbouring promoter B only (pGL2- P_B) and in constructs comprising both promoters and the intervening exon IA (pGL2- P_{AB}). As can be seen from Table 5, variants 2 and 3 together did not change transcription from either promoter construct. Variant 1 altered expression of luciferase significantly although the effect differed in the two constructs. Transcription was up-regulated approximately 1.5-fold from promoter B alone (pGL2- P_{Bm1}), but down-regulated 1.4-fold in pGL2- P_{ABm1} , harbouring both promoters and the intervening exon IA. Substitution number 1 was present only in cancerous samples and with high frequency (allele frequency of 0.1). To ensure this was not an artefact from a difference in sample sizes between normal and cancer cells, we screened 20 additional DNA samples isolated from blood taken from normal individuals for this particular sequence variant without detecting it.

4. Discussion

In our material derived from 62 human sources, we identified 12 different base pair substitutions in the *UNG* gene. In total 110 variant alleles were identified in 422 kb of non-coding DNA (3.4 kb non-coding DNA sequenced \times 2 alleles \times 62 samples), corresponding to one substitution per 3.8 kb of non-coding sequence of the *UNG* gene. In contrast, no sequence variation was found in the coding regions which represented 129 kb in total (1.04 kb coding DNA sequenced \times 2 \times 62). Thus, the allelic variation in *UNG* in these samples is considerably lower than the expected values of 0.5–2 variants per kb [28]. The sequence variation within nucleotide excision repair genes was recently found to be one variant allele in every 2.3 kb in coding and 2.1 kb in non-coding regions [23]. Thus, there is apparently less variation in the *UNG* gene than in genes for nucleotide excision repair. Of the 12 substitution variants observed, 10 (83%) were transitions and two (17%) transversions. This is in good agreement with previous reports showing 75 and 70% transitions [23,29]. Only one sequence variant (number 7 in intron IV) was a G \rightarrow A transition at a CpG dinucleotide. This is lower than expected since CpG dinucleotides are frequently mutated in human cells [30]. This discrepancy could be a consequence of genomic hypomethylation seen in human cancer cells [31]. Substitution variants in promoter B correspond to one substitution every 0.95 kb (59 variants/(0.5 \times 2 \times 62) kb), thus, conforming with the expected occurrence of allelic variants [28]. Surprisingly, no sequence variation was observed in promoter A, which represents 37 kb of sequence (0.3 \times 2 \times 62). Several substitution variants were found to be present in cancer cell lines, but absent in normal cell lines. The frequencies of most of these substitutions were low (1–2%), thus, the far lower number of normal compared to cancerous cell lines included in the study could account for this difference. Taken together, the number of nucleotide substitutions in normal cell lines was one substitution per 2.1 kb whereas in cancer cell lines, one variant was found per 3.5 kb. The opposite was observed in promoter B where one substitution per 1.4 and 1 kb was found in normal and cancerous samples, respectively.

In some cell lines, all identified sequence variants appeared to be homozygous (Table 4). Substitutions

Table 5
Relative activities of chimaeric promoter–luciferase constructs in HeLa cells^a

Promoter constructs	Mutated element	Luciferase activity (%)
pGL2- <i>P_B</i>	<u>CCCATGGG</u> (AP2: 998–1005) <u>CCAATGGGAA</u> (CCAAT: 1026–1035) <u>CCCTCCTGGCT</u> (Yi: 1076–1086)	100 ± 6
pGL2- <i>P_Bm1</i>	<u>TCCATGGG</u> (AP2: 998–1005)	146 ± 6
PGL2- <i>P_Bm2 + 3</i>	<u>CCAATGGGGA</u> (CCAAT: 1026–1035) <u>CCCTCCAGGCT</u> (Yi: 1076–1086)	90 ± 6
PGL2- <i>P_{AB}</i>	<u>CCCATGGG</u> (AP2: 998–1005) <u>CCAATGGGAA</u> (CCAAT: 1026–1035) <u>CCCTCCGGGCT</u> (Yi: 1076–1086)	100 ± 6
pGL2- <i>P_{AB}m1</i>	<u>TCCATGGG</u> (AP2: 998–1005)	71 ± 2
PGL2- <i>P_{AB}m2 + 3</i>	<u>CCAATGGGGA</u> (CCAAT: 1026–1035) <u>CCCTCCAGGCT</u> (Yi: 1076–1086)	93 ± 7

^a HeLa cells were transiently transfected with promoter–luciferase constructs (1 µg/2.5 × 10⁵ cells/60 mm dish). Luciferase activity was measured after 24 h. Results are given as percent of luciferase activity expressed from PGL2-*P_B* and PGL2-*P_{AB}* alone. Transcription factor binding motifs are underlined and position of sequence shown. Mutations are indicated in bold italic. Data are presented from two separate experiments as the mean ± S.E.M., each carried out in triplicate.

2 + 3 in promoter B are present with allelic frequencies of 0.18 and 0.21 in normal and cancer cells, respectively. Three cell lines showed homozygosity for these substitutions (CCD39, Hs746T and 23132/87) whereas 19 were heterozygous and 40 were wild type. Thus, these variants might exist as polymorphisms and identification of homozygous variants is not unexpected. Variant 10 has allelic frequency of 0.23 and 0.19 in normal and cancer cell, respectively, and this variant might similarly to 2 + 3 exist as a polymorphism. However, all homozygous 2 + 3 variants are found in concert with homozygosity for substitution number 10. This might be indicative of loss of heterozygosity (LOH) in these cell lines. The gastric cancer cell lines were analysed for LOH (data not shown). Hs746T, which appeared homozygous for variants 2 + 3 and 10 was homozygous for highly polymorphic markers in exon II and intron V. This cell line was also homozygous for markers centromeric (D12S1583) and telomeric (D12S1605) [32] of the *UNG* gene. Thus, the apparent homozygosity of variants in this cell line is probably a result of LOH. Similarly, the gastric cancer cell line 23132/87 that appeared to be homozygous for substitutions 2 + 3, 9 and 10, exhibited only one form of all *UNG* markers and could therefore have undergone LOH. The

normal fibroblast cell line CCD39, homozygous for 2 + 3 and 10 was heterozygous for the centromeric marker and homozygous for the telomeric and intron V markers (data not shown). Thus, some rearrangements have occurred in the locus but since no marker in the *UNG* promoter gene has been evaluated, we cannot conclude LOH in this cell line. Two apparently homozygous substitutions, numbers 12 and 5, were identified in CCD34 and T47D, respectively. These variants were not identified in other cell lines. Identification of a homozygous variant without identifying any heterozygous variants is unlikely, but both cell lines were heterozygous for other sequence variants (see Table 4) and CCD34 was heterozygous for all polymorphic markers. Another puzzling observation is the appearance of substitution 1 in homozygous form in three cell lines (KATOIII, HeLa and U87) and in heterozygous form in only two cell lines (LS513 and Nci-N87). HeLa and U87 were heterozygous for the centromeric and intron V markers and KATOIII and Nci-N87 are heterozygous for the intron V marker, suggesting that both *UNG* alleles are at least partly, intact in these cell lines. The existence of local deletions in one allele might in principle, explain these findings. However, since only one fragment length of the PCR products were identified on

agarose gels, the deletions must include binding sites for at least one of the PCR primers used to amplify the region.

In the present study, UDG activity was measured in nine normal fibroblast and 41 cancer cell lines of various types. No correlation could be found between the measured UDG activity and presence of promoter variants even if one mutation in an AP2 element in promoter B affected transcription from the promoter. We cannot conclude whether mutations in promoter B have effect on UNG1 transcription specifically since the activity assay does not discriminate between UNG1 and UNG2. To measure UNG1 activity specifically we would need to measure UDG activity in mitochondrial fractions of all cell lines. This is laborious and beyond the scope of the present study. It has previously been demonstrated that UDG activity [33,34] and transcription from both promoters were cell cycle regulated showing induction in late G1 to early S-phase [27]. Moreover, UNG2 associates preferentially with replicating SV40 DNA [35], is present in replication foci [36] and is highly expressed in replicating tissue [27]. Measures were not taken to relate UDG activity to growth rate of the cell lines. This hampers interpretation of correlation between UDG activity and sequence variation in the promoter. The main finding remains however, that mutations abolishing UDG activity were not identified in these cell lines.

From our data, it appears that the *UNG* locus is highly conserved also within humans as it is between species [2,37]. If mutations in *UNG* result in a slight reduction of fitness the mutants would experience negative selection and never proliferate to dominate a rapidly dividing culture. This scenario could provide an explanation for the absence of human cancer-predisposition syndromes caused by BER deficiency. Mutated MMR (mismatch repair) genes might in contrast provide a selective advantage to the growing tumour cell to prevent it from being eliminated. Indeed, it has been proposed that certain MMR deficient cells escape elimination by the immune system [38], which might explain why these genes are frequently found to be mutated in tumours whereas BER genes are not. Variant alleles have been identified in tumour tissue for other BER proteins including hOGG1 DNA glycosylases [39,40] and DNA polymerase β [41,42]. There is no evidence linking

those variant alleles to tumour initiation. However, subtle mutations in single enzymes might have effect when combined and a natural follow-up of the present study would be to measure BER capacity from a uracil-containing substrate.

5. Conclusion

The *UNG* gene appears to contain less than expected sequence variation both in coding and non-coding regions. However, we have identified considerable sequence variation in non-coding regions of the *UNG* gene in cell lines derived from normal fibroblasts and tumour tissue. None of the sequence variants was accompanied by significantly reduced UDG activity. In conclusion, mutations affecting the function of human *UNG* gene are seemingly infrequent in human tumour cell lines.

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