DNA Base Excision Repair: Mutation Induction and Novel Functions

by

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Abstract

DNA is susceptible to chemical modifications corrupting its cellular information processing function, necessitating correction of such modifications. Cells are formed mostly by water – giving rise to hydrolytic reactions – and aerobic metabolism is a source of reactive oxygen species (ROS) – causing oxidation. Deamination of cytosine to uracil is an example of the former and oxidation of thymine to 5-formyluracil (f5U) is an example of the latter.

In order to avoid the incorporation of wrong nucleosides into DNA, f5U must be eliminated and substituted by the correct base. The main mechanism for the repair of f5U is the base excision repair (BER) pathway, in which specific glycosylases recognize the damage and excise it from its ribose residue. Several glycosylases have been found to be involved in the repair of f5U in *Escherichia coli*, where 3-methyladenine DNA glycosylase II (AlkA) may be the most important. However, here we present evidence to indicate that the nucleotide excision repair protein UvrA is also involved in the repair of f5U, although the mechanism has yet to be elucidated.

Interestingly, we have found that the AlkA glycosylase, in addition to alleviating is also able to promote mutation induction by 5-formyldeoxyuridine in *E. coli*. Extrapolated to the mammalian system this observation suggests that DNA repair genes may act as oncogenes under certain cellular conditions.

Uracil lesions in DNA are repaired by the BER pathway initiated by a monofunctional uracil-DNA glycosylase (UDG), and the family 1 UDGs are the most extensively studied glycosylases. We have found that UDG-mediated repair initiation by *E. coli* Ung and hUNG also involves DNA strand incision generating a 3’-α,β-unsaturated aldehyde (UIP) and a 5’-phosphate, which demonstrates that they are indeed bi-functional enzymes. However, while the
cleavage of the N-glycosyl bond by β-elimination occurs through a covalent Schiff base intermediate between a reactive active site lysine and the deoxyribose moiety in other bi-functional glycosylases, the uracil–deoxyribose bond is suggested to be cleaved by an activated water molecule carrying out the elimination reaction.
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“In the end, we only regret the chances we didn’t take”

Nicholas Sparks, ‘The Guardian’
Contents

Abstract .............................................................................................................................. i
Acknowledgements .................................................................................................... iii
Contents ....................................................................................................................... v
List of papers ........................................................................................................... vii
List of figures .............................................................................................................. ix
Glossary ....................................................................................................................... xi
1 Introduction ............................................................................................................. 1
   1.1 DNA damage formation ............................................................................... 2
      1.1.1 Hydrolytic damage .................................................................... 4
      Uracil in DNA ................................................................................... 4
      1.1.2 Oxidative damage .................................................................... 6
      5-Formyluracil ............................................................................... 7
   1.2 DNA repair and response mechanisms .................................................... 9
      1.2.1 Base excision repair ............................................................... 10
      Uracil-DNA glycosylases ............................................................ 13
      DNA glycosylases for lesions induced by oxidation .......................... 13
      5-Formyluracil-DNA glycosylases .............................................. 14
      1.2.2 Nucleotide excision repair ..................................................... 16
      The SOS response ......................................................................... 18
      Nucleotide excision repair of 5-formyluracil .................................. 19
2 Aim of the studies

3 Overview of results

3.1 DNA uracil excision includes strand incision by hUNG (Paper 1)

3.2 Severe repair-deficiency for oxidized DNA bases causes primarily GC to AT transitions in E. coli (Paper 2)

3.3 Damage-specific mutation induction promoted by repair (Paper 3)

3.4 Alleviation and promotion of damage-specific mutation induction in E. coli are highly dependent on the uvrA gene (Paper 4)

4 Discussion

4.1 Family 1 glycosylases are most probably bi-functional enzymes

4.2 Oxidative DNA damage may initiate tumorigenesis due to C to T transitions

4.3 Repair of 5-formyluracil in DNA by base excision repair can either alleviate or promote mutagenesis in E. coli

4.4 Nucleotide excision repair is involved in the repair of oxidative base lesions

5 Future perspectives

6 References

Appendix
List of papers

Paper 1
Marina Alexeeva, Marivi N. Moen, Xiang Ming Xu, Kristin Grøsvik, Izaskun Muruzábal-Lecumberri, Kristine M. Olsen, Ingar Leiros, Finn Kirpekar, Arne Klungland and Svein Bjelland: DNA uracil excision includes strand incision by hUNG. Manuscript.

Paper 2

Paper 3

Paper 4
List of figures

1.1 Chemical composition of ssDNA and RNA molecules……………… 1
1.2 Mechanism of mutagenesis for one base substitution mutation….. 2
1.3 Chemical DNA base modifications………………………………… 3
1.4 Products formed from deamination of bases in DNA………………. 5
1.5 Some important DNA base damages produced by ROS…………… 6
1.6 Chemistry of f5U in DNA………………………………………….. 8
1.7 Base-pairing abilities of f5U in DNA……………………………….. 8
1.8 Diagram of general BER……………………………………………. 11
1.9 Base-damage repair mechanism by mono- and bi-functional DNA glycosylases………………………………………………….. 12
1.10 UvrABC system of nucleotide excision repair (NER). Mechanism in E. coli…………………………………………………………… 17
1.11 Regulation of SOS response in E. coli gene of the UmuD protein…………………………………………………………………… 18
<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
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<td>hSMUG</td>
<td>single-strand selective monofunctional uracil-DNA glycosylase</td>
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1 Introduction

Genetic information in all living cells is contained in their DNA, at first assumed to be a highly stable molecule. This contrasts with many viruses, which use RNA as genetic material, which leads to extremely increased mutation rates. Presently it is believed that DNA replaced RNA during early cellular evolution (Lindahl, 1993). Although the only chemical difference between DNA and RNA is an H rather than an OH group at the 3’C position of the sugar moiety, respectively (Figure 1.1), this causes a highly increased stability of the sugar-phosphate backbone (Friedberg et al., 2005). However, the price paid is a more labile N-glycosyl bond in DNA compared to RNA (Figure 1.1). Due to hydrolysis of this N-glycosyl bond, apurinic/apyrimidinic (AP) sites are the most common DNA damage in all cells. Although being

![Figure 1.1 Chemical composition of ssDNA and RNA molecules. Phosphodiester bonds are marked in purple and N-glycosyl bond, in red.](image-url)
mainly cytotoxic due to inhibition of replication or transcription, AP sites are believed to contribute significantly to mutagenesis when they are bypassed by certain translesion DNA polymerases, where adenine is the preferred (mis)incorporated base (the “A-rule”) (Pagès et al., 2008; Strauss, 2002). Because of this and because AP sites are the most common DNA lesion, cell health and survival is dependent on its almost total removal. This also applies to other lesions (Kim and Wilson, 2012).

When lesions arise in DNA bases and are not properly repaired, mutations may appear after replication due to misincorporation of a wrong base opposite the lesion. An example of C to T transition is shown in Figure 1.2.

![Figure 1.2 Mechanism of mutagenesis for one base substitution mutation. Hydrolytic deamination of C to U. After the first round of replication (1 Pol), U misincorporates A instead of G. In the second round of replication (2 Pol), A incorporates T. The result is a transition from GC to AT.](image)

### 1.1 DNA damage formation

All cells are constantly suffering damage to their DNA caused by physical agents and chemical species coming from the outside environment or formed by the cell itself. DNA lesions can affect single nucleotides or result in changes of the helix structure. UV-B (and UV-C) light is a major producer of thymine dimers and 6–4 photoproducts (6–4PP), which both disturb the helical structure of the DNA, while ionizing radiation is particularly disruptive due to the formation of double-strand breaks (DSB). Certain aromatic compounds present in oil and tar (e.g. tobacco smoke) attach to DNA as bulky DNA adducts disturbing its helical structure (Henkler et al., 2012). The cellular methyl donor S-adenosylmethionine (SAM) (Bauerle et al., 2014) contains a reactive methyl group – like methyl methanesulfonate (MMS) (Figure 1.3) – that methylates DNA erroneously at a certain frequency. SAM is, in addition to other alkylating agents present in the environment, an important source of DNA damage. Such
damage can arise via two different chemical reactions which are called nucleophilic substitutions 1 and 2 (S_N1 and S_N2) and methylate oxygen and nitrogen residues in both single- and double-stranded DNA (ss- and dsDNA) (Sedgwick, 2004).

However, the quantitatively most important lesions formed endogenously in DNA are due to the water component of all cells and the generation of reactive oxygen species in aerobic cells described below (Friedberg et al., 2005; Kim and Wilson, 2012).

Figure 1.3 Chemical DNA base modifications. Formation of bulky adducts (A), methylation (B) and oxidation (C). Deamination is shown in Figure 1.4.
1.1.1 Hydrolytic damage

Since water is a major component of all cells, hydrolytic reactions are a major threat to the chemical integrity of their DNA and, as already mentioned, generate AP sites as the most abundant lesion. Purines are liberated from DNA at similar rates, while pyrimidines are liberated at 5% of the rate of the purines (Lindahl, 1993) due to their higher N-glycosyl bond stability (Friedberg et al., 2005). The difference in depurination between ss- and dsDNA is only four-fold, suggesting a poor protection by the double helical structure (Lindahl, 1993). The measured depurination rate for duplex DNA in vivo translates to loss of approximately one purine per generation per *Escherichia coli* chromosome; given a doubling time of one hour. For mammalian cells, with a larger genome and a longer cell cycle, this rate is equivalent to loss of 9 000–10 000 purines during each 24 hours period (Kim and Wilson, 2012; Lindahl, 1979). In addition to being responsible for the formation of AP sites, hydrolytic deamination of cytosine, adenine, guanine and 5-methylcytosine (m^5C) resulting in uracil, hypoxanthine, xanthine and thymine, respectively (Figure 1.4), occurs extensively in a pH- and temperature-dependent manner (Friedberg et al., 2005). Uracil arising from cytosine is the second most common hydrolytic DNA damage and, if not repaired prior to replication, C to T transition mutations are formed (Lindahl, 1993) (Figure 1.2).

*Uracil in DNA*

Although uracil is the adenine partner base in RNA it appears erroneously in the DNA as a consequence of cytosine deamination (Figure 1.3) or misincorporated from dUTP. The latter common source of genomic uracil is the principal source of abasic sites in yeast (Guillet and Boiteux, 2003) and is especially relevant in cycling cells. dUMP is a normal intermediate in the biosynthesis of dTMP and dTTP but its levels are kept very low by dUTPase (Ladner and Caradonna, 1997). Genomic uracil by deamination results in mutagenic U·G mispairs and non-mutagenic but may be genotoxic U·A pairs (Kavli et al., 2007).

However, deamination of cytosine plays an important biological role in immunoglobulins diversification (Durandy and Honjo, 2001) and protection against retroviral infection (Pham et al., 2005) and so it has been observed targeted cytosine deamination by specialized enzymes like APOBEC
1 INTRODUCTION

(apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like) and AID (activation-induced cytidine) deaminases (Kavli et al., 2007).

Spontaneous cytosine deamination has been estimated to take place at a rate of 60–500 events per human genome per day (Barnes and Lindahl, 2004; Krokan et al., 2002). However, based on different studies on ss- and dsDNA and estimating the percentage of ssDNA in the total DNA of a cell as approximately 0.1%, this first estimation would change to 70–200 deaminations per cell per day (Kavli et al., 2007).

Figure 1.4 Products formed from deamination of bases in DNA. The amine group (-NH₂, circled in green) is hydrolyzed and replaced by the oxygen from the water molecule. Adapted from Friedberg et al., 2005.
1.1.2 Oxidative damage

Due to reactive oxygen species (ROS) formed as byproducts in the electron transport chain of bacterial plasma membranes as well as mitochondria, chloroplasts and some other organelles of eukaryotes, oxidative damage to DNA is substantial in all aerobic cells. ROS are also formed by photosensitization reactions involving both ultraviolet and visible light. The most reactive ROS is the hydroxyl radical (-OH), which is formed from hydrogen peroxide (H₂O₂) and Fe²⁺ in the Fenton reaction as well as by ionizing and UVB and UVC radiations. Another important reactive but non-radical form of oxygen is singlet oxygen (Gutteridge and Halliwell, 1989).

ROS cause oxidative damage to DNA including base modifications (Figure 1.5) and strand breaks, which is believed to be of similar importance as hydrolytic damage. The most studied ROS-induced base modification is the mutagenic oxoG, which mispairs with adenine. Indeed, oxoG is easier oxidized than guanine itself giving rise to secondary oxidation products in DNA (Klungland and Bjelland, 2007). Many oxidized bases – e.g. the thymine lesions 5-hydroxy-5,6-dihydrothymine (Th₅), 6-hydroxy-5,6-dihydrothymine (Th₆) and 5-hydroxy-5-methylhydantoin (hmh) – are cytotoxic rather than mutagenic by inflicting an effective block to DNA replication and/or transcription (Bjelland and Seeberg, 2003).

![Figure 1.5 Some important DNA base damages produced by ROS](image)

oxoG incorporates an oxygen in position 8 of a guanine (so it is also known as 8-oxoguanine). Thymine can incorporate hydroxyl groups in cis (as shown in the figure) or trans form, resulting in cTg or tTg. hm5U and f5U (Figure 1.3) have a common precursor in 5-hydroperoxymethyluracil (hpm5U).
5-Formyluracil

5-Formyluracil (f5U) is formed by oxidation of the 5-methyl group of thymine (Figure 1.3C) and seems to be present in DNA at similar concentrations as oxo8G (Bjelland et al., 2001). It was reported to be formed in DNA 25 years ago (Kasai et al., 1990).

The most common form of f5U is the one with a keto group in the 5th position. The enol form arises when the keto group is reduced to hydroxyl. Moreover, at basic pH the keto group adopts the anionic form. These f5U forms (Figure 1.6) have different base-pairing abilities. The keto form (f5U\textsuperscript{C=O}) does not obliterate the Watson-Crick pairing ability, so pairing with adenine is preferred. But the formyl group alters the charge distribution of the pyrimidine ring as well as it may directly participate in hydrogen bond formation causing e.g. mispairing with cytosine (Figure 1.7). The abundance of the anionic form (f5U\textsuperscript{\neg}) as opposed to the preferred keto form increases with pH. The amount of f5U\textsuperscript{\neg} and thus the f5U\textsuperscript{\neg}\cdot G mispair is significant at neutral pH. The f5U\textsuperscript{C=O}\cdot A and f5U\textsuperscript{C=O}\cdot G pairs have been demonstrated by X-ray analyses, in contrast to the f5U\textsuperscript{C=O}\cdot C mispair which is tentative (Kamiya et al., 2002).

The f5U deoxynucleoside (5-formyl-2'-deoxyuridine, f5dU) can be used to induce mutations in cells (Kamiya et al., 2002; Kasai et al., 1990; Yoshida et al., 1997). f5dU added to culture medium of cells is converted \textit{in vivo} to its 5'-triphosphate derivate (f5dUTP) which is used by cellular polymerases to incorporate fdUMP into the DNA. It has been observed in recent studies that the most common mutations induced by f5dU in \textit{E. coli} (Ånensen et al., 2001) are different from the ones arising in mammalian cells (Kamiya et al., 2002).
**INTRODUCTION**

**Figure 1.6 Chemistry of f^5^U in DNA.** Oxidative attack on the 5-methyl group of thymine converts it to a formyl group (circled in purple). Other forms of modifications in the 4th position are circled in orange. Adapted from Knævelsrud et al., 2009.

**Figure 1.7 Base-pairing abilities of f^5^U in DNA.** The different forms of f^5^U have different base-pairing abilities: cognate or Watson-Crick hydrogen bonding pattern is shown in blue dotted lines; non-cognate patterns of mispairs are shown in red dotted lines. Adapted from Kamiya et al., 2002; Knævelsrud et al., 2009.
1.2 DNA repair and response mechanisms

When DNA suffers a damage, this can be repaired or tolerated, or start an apoptotic response in the worst-case scenario for the cell. There are several biochemical mechanisms for DNA repair, and the enzymes involved may take part in more than one.

Sometimes the damage itself can just be removed, what restores the DNA; this is called direct reversal. When this is not possible, the damage is removed together with the damaged base/nucleotide or with a whole fragment of DNA. The cell may also show tolerance to the damage by employing specialized translesion bypass polymerases that insert a correct or an incorrect base across the lesion. Thus, cell death may be avoided at the expense of some mutation induction.

Table 1.1 lists the known biological responses to DNA damage. The main ones are explained in detail later in this text.

Table 1.1 Biological responses to DNA damage (Table 1-1 from Friedberg et al., 2005)

<table>
<thead>
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<th>Reversal of base damage</th>
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<tr>
<td>Excision of damaged, mispaired or incorrect bases</td>
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<tr>
<td>Base excision repair (BER)</td>
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<td>Nucleotide excision repair (NER)</td>
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<td>Transcription-coupled nucleotide excision repair (TC-NER)</td>
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<td>Mismatch repair (MMR)</td>
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<td>Strand break repair</td>
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<td>Single-strand break repair (SSBR)</td>
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<td>Double-strand break repair (DSBR)</td>
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<td>Tolerance of base damage</td>
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<td>Translesion DNA synthesis (TLS)</td>
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<td>Postreplicative gap filling</td>
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<td>Replication fork progression</td>
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<td>Cell cycle checkpoint activation</td>
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<td>Apoptosis</td>
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The majority of the DNA damages in cells are repaired by the excision repair mechanisms: base excision repair (BER) and nucleotide excision repair (NER). These two pathways share most of the enzymes involved in insertion and ligation of the correct nucleotide, but they differ in the way they eliminate – excise – the damaged or incorrect base (Figures 1.8 and 1.10). The BER pathway employs somewhat specialized glycosylases for the excision of aberrant bases. Uracil-DNA glycosylases are known to recognize uracil in
INTRODUCTION

DNA and excise it, starting a chain of downstream reactions able to convert the damaged strand to its original state. There are several enzymes reported until now as U-glycosylases (Liu et al., 2003), making BER the most suitable mechanism for the repair of U and U-like damages, among other base lesions. The NER pathway is an important mechanism in the repair of “bigger” damages that distort the conformation of the DNA double helix making difficult or impossible normal copying and transcription to mRNA (Petit and Sancar, 1999). However, in vitro studies (Kino et al., 2004) suggest the implication of NER in the repair of f5U in DNA.

1.2.1 Base excision repair

Spontaneously arisen DNA base damages are primarily repaired by BER. Both prokaryotes and eukaryotes show similar pathways with slight differences (Figure 1.8). The mechanism starts with the recognition of the damaged base by a DNA glycosylase followed by cleavage of the N-glycosyl bond (Figure 1.1) between the base and the deoxyribose, leaving an AP site. There exist glycosylases specialized in many types of base damages or mispairings. These relatively small enzymes (~30–50 kDa) are monomeric and do not require cofactors. They seem to act by flipping-out the base 180° followed by N-glycosyl bond cleavage (Slupphaug et al., 1996). In the case of bi-functional glycosylases, they exhibit in addition an AP lyase activity (O’Connor and Laval, 1989) which cleaves the phosphodiester backbone at the 3’-end of the AP site by a β- or β/δ-elimination reaction (Figure 1.9), leaving behind the AP site remnant as a (saturated/unsaturated) 3’-dRP or 3’-phosphate, respectively. The latter can be removed by a polynucleotide kinase phosphatase (PNKP) (Friedberg et al., 2005).

In the short-patch BER, the DNA backbone is cleaved either by an AP endonuclease at the 5’-side or by an AP lyase – by bi-functional glycosylases – at the 3’-end of the AP site. The AP site remnant is removed afterwards by a 5’-deoxyribose phosphate (5’-dRP) lyase or a 3’-phosphodiesterase, respectively. The gap is filled by a polymerase with the correct nucleotide followed by a ligase, which seals the nick and completes the process. In the long-patch BER, the free 3’-OH group formed by incision of the AP site by an AP endonuclease is targeted by a DNA polymerase, which extends a displaced DNA strand (a flap) that is removed by a flap endonuclease (Figure 1.8). Although the molecular basis for the selection of one or the other path
Figure 1.8 Diagram of general BER. The BER pathway follows the same steps in prokaryotes and eukaryotes, with different enzymes and cofactors involved. Adapted from Moen et al., 2011; Xu et al., 2008.
remains unclear, the presence or lack of some polymerases and cofactors seems to influence the final decision (Sander and Wilson, 2001).

Because of its abundance, the AP site is the most common substrate for BER (Kim and Wilson, 2012). In *E. coli*, the AP sites are incised by AP endonucleases such exonuclease III (Xth) and endonuclease IV (Nfo) (Hang *et al.*, 1998). In humans, this is a role for hAPE1 (human apurinic/apyrimidinic endonuclease 1) (Friedberg *et al.*, 2005).

**Figure 1.9 Base-damage repair mechanism by mono- and bi-functional DNA glycosylases.**
A) Mono-functional: a water molecule (blue) in the active site (red) is deprotonated by carboxylic acid side chain of the enzyme and positioned for nucleophilic attack at the anomeric position. The product of the reaction is an abasic site. B) Bi-functional: the nucleophile in this case is an amine group in a lysine residue of the enzyme (blue). The product of glycosidic bond cleavage is rearranged (Schiff base intermediate). A β-elimination leads to incision of the phosphodiester bond between the abasic site and the phosphate in 3’ (Figure 1.1). Some enzymes also have δ-elimination function, incising the phosphate in 5’ (5'-dRP lyase). Adapted from Drohat and Maiti, 2014; Schärer and Jiricny, 2001.
1 INTRODUCTION

Uracil-DNA glycosylases

Uracil-DNA glycosylase (UDG) was the first DNA glycosylase activity to be reported 40 years ago (Lindahl, 1974). Since then, UDGs have been grouped into several families distinguished by primary structure and all members of this UDG superfamily have exclusively been described as evolutionary conserved (Schormann et al., 2014) mono-functional enzymes. The most important UDGs in E. coli and human cells are displayed in Table 1.2.

Human uracil-DNA glycosylase (hUNG) is the human homologue for E. coli uracil-DNA glycosylase (EcUng) and has been the most studied UDG. It occurs in alternative sliced forms from the same gene – ung – designated UNG1 for distribution in the nuclei or UNG2 in mitochondria (Krokan et al., 2001). The hUNG active site consists of a deep uracil-binding pocket with an overlying groove that binds one DNA strand, but the entire DNA-binding surface on hUNG extends considerably beyond the immediate active pocket. The non-specific DNA-binding surfaces may aid local uracil search, contribute to binding abasic DNA product and help present the DNA product to hAPE1 (Roberts et al., 2012).

DNA glycosylases for lesions induced by oxidation

A multitude of different nucleic acid base modifications are induced in DNA by ROS (Bjelland and Seeberg, 2003). Many of these, including ring-saturated and ring-fragmented pyrimidines, are believed to be mainly cytotoxic lesions contributing only slightly to mutation induction. In contrast, oxo8G is considered a principal lesion responsible for the mutagenic events induced by ROS, where several repair proteins counteracting its damaging effects have been extensively characterized (Friedberg et al., 2005; Lindahl, 1993). Also the cytosine oxidation products 5-hydroxycytosine (h5C), h5U and uracil glycol (Ug) have been shown to cause mispairing contributing to mutation induction (Bjelland and Seeberg, 2003).

In E. coli, most oxidized base damages are removed by three enzymes: formamidopyrimidine-DNA glycosylase (Fpg) – the main enzyme for the repair of oxo8G residues (Serre et al., 2002) – endonuclease III (Nth) and endonuclease VIII (Nei). They exhibit somewhat overlapping specificities (Prakash et al., 2012). However, the oxidized thymine residues hm3U and F5U are most efficiently repaired by the 3-methyladenine-DNA glycosylase II
INTRODUCTION 1

(AlkA) (Bjelland et al., 1994). Most of these glycosylases have analogues in mammalian cells (Table 1.2). The former enzymes are bi-functional and were described initially by their AP lyase activity, but they are able to recognize different oxidized substrates and, after nicking the N-glycosyl bond, they cleave the ribose by β-elimination rather than hydrolysis (Bailly and Verly, 1987).

5-Formyluracil-DNA glycosylases

f5U has a potential mutagenic or cytotoxic effect in bacteria and may be even more mutagenic in mammalian cells (Klungland et al., 2001). Several studies, both in vitro and in vivo, have demonstrated f5U-mediated mutation induction (Ånensen et al., 2001; Fujikawa et al., 1998; Terato et al., 1999; Yoshida et al., 1997; Zhang et al., 1997). It has been observed that f5U in DNA is removed efficiently by the AlkA DNA glycosylase of E. coli as well as by DNA glycosylase activities present in human, murine and rat cell-free extracts (Bjelland et al., 1994; Bjelland et al., 1995; Zhang et al., 1995). Efficient removal of f5U from DNA was demonstrated and confirmed in vitro (Masaoka et al., 1999).

The AlkA enzyme is a 31-kDa polypeptide containing 282 amino acids (Clarke et al., 1984; Nakabeppu et al., 1984). It has a broad substrate specificity, being able to recognize and catalyze the excision of several methylated bases (Table 1.2). AlkA takes part in the adaptive response to alkylation damage, since adaptation to survival in E. coli is associated with the induction of AlkA synthesis. The adaptive response to alkylation is regulated by the 6-oxoalkylguanine transferase I (Ada) protein. Mutations in the ada gene prevent the induction of AlkA (Friedberg et al., 2005). However, there is no evidence of alkA induction by ROS exposure.

3-Methyladenine-DNA glycosylase (MPG) is the enzyme responsible for initiating repair of alkylated bases in human cells, however, exhibits no activity for f5U. The human 8-oxoguanine-DNA N-glycosylase 1 (hOGG1) – one of the major mammalian glycosylases for oxidized bases – do not seem to recognize f5U (Masaoka et al., 1999). This contrasts with the human endonuclease III (hNTH1), which shows f5U activity in vitro (Miyabe et al., 2002). However, its physiological importance in f5U repair has been questioned since it needs large amount of substrate and does not seem kinetically efficient (Matsubara et al., 2003). The main mammalian f5U-DNA glycosylase activity is believed to be a function of the single-strand selective mono-functional uracil-DNA
<table>
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<th>Protein</th>
<th>Gene</th>
<th>Common name</th>
<th>Substrates</th>
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<td>No</td>
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<td>No</td>
<td>MPO</td>
<td>unm</td>
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<td>No</td>
</tr>
<tr>
<td>Nei</td>
<td>nei</td>
<td>Endonuclease VIII</td>
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<td>Yes</td>
<td>NEIL1/</td>
<td>unm</td>
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<td>Tg, hU-A, hC-G, o xoA-G, FpyG-C</td>
<td>Yes</td>
</tr>
</tbody>
</table>

Table 1.2 DNA glycosylases for uracil and lesions induced by oxidation. UDGs in E. coli and their equivalents in human cells for the lesions mentioned in this thesis. Adapted from Bjelland and Sørensen, 2003; Friedberg et al., 2005; Kim and Wilson, 2012; Krokan et al., 1997; Masaoka et al., 2003; Serre et al., 2002; Takeo et al., 2002; Wallace, 2013.
INTRODUCTION 1

glycosylase (hSMUG1) (Masaoka et al., 2003; Matsubara et al., 2003), which is a backup enzyme for hUNG and the primary repair enzyme for a subset of oxidized pyrimidines such as hm5U and h5U in addition to f5U (Masaoka et al., 2003).

1.2.2 Nucleotide excision repair

Some DNA lesions distort the double-helix structure significantly, which is the qualification for being a substrate for NER. Because of this, common NER substrates are cross-linked base residues caused by UV radiation, like cyclobutane pyrimidine dimers (CPDs) – including thymine dimers (Figure 1.3A) – and 6–4PPs, as well as bulky chemical species, originating from e.g. tar covalently attached to bases which often are guanine. An extensive review of NER substrates is described in Table 1 from Truglio et al. (2006).

In *E. coli* and other prokaryotes, NER is carried out by the UvrABC system, which is composed of the three proteins UvrA, UvrB and UvrC. The genes for two of these proteins – *uvrA* and *uvrB* – are controlled by the SOS response (Figure 1.11) (Janion, 2001). The *uvrC* gene, however, is not such regulated (Van Houten, 1990). The NER pathway starts with the formation of a complex consistent in one UvrB protein and two UvrA (UvrA$_2$B complex), which docks the DNA some distance from the damage. This complex scans the DNA using its helicase activity. When the damage is encountered, UvrA$_2$B forms an unstable complex with the DNA. Using ATP, UvrB forms a stable complex with the damaged strand and UvrA dissociates. UvrC binds to the UvrB-DNA complex and activates UvrB, which makes an incision in the damaged strand four nucleotides 3’ from the lesion. UvrC is then activated and incises the damaged strand seven nucleotides 5’ a few seconds after. DNA helicase II (UvrD) releases the incised fragment and UvrC. UvrB remains bound to the gapped DNA and is released prior to the new DNA synthesis performed by polymerase I. DNA ligation completes the repair (Figure 1.10) (Friedberg et al., 2005; Petit and Sancar, 1999).

The endonuclease mode characteristic for NER is well conserved through evolution from prokaryotes to human cells. An important difference is, however, that the mammalian system is much more complex. Only three proteins are employed by *E. coli* in contrast with 16/17 polypeptides by human cells, among other differences (Petit and Sancar, 1999).
1 INTRODUCTION

Figure 1.10 UvrABC system of nucleotide excision repair (NER). Mechanism in *E. coli*. The two molecules of UvrA bind one of UvrB in solution using ATP. ATP is also required for the stable binding between UvrB and the damaged DNA strand. UvrD (helicase II) is needed to remove UvrC and the incised fragment. Downstream enzymes – polymerase and ligase – repair the damage in the same way as in BER. Adapted from Friedberg *et al.*, 2005; Petit and Sancar, 1999.
The $E. \text{coli}$ SOS response is a global response to DNA damage in which the cell cycle is arrested and DNA repair and translesion synthesis/mutagenesis are induced. It was described for the first time in 1975 (Radman, 1975). The repressor protein LexA binds to the operator regions of SOS genes – the SOS box – and negatively regulates their transcription. Treatment of the cells with DNA-damaging agents causes increased inhibition of DNA synthesis and thus increased ssDNA regions where RecA protein (Rad51 in eukaryotes) binds, which induces LexA to cleave itself resulting in the expression of the SOS genes (Figure 1.11). Under high load of oxidative stress, genes with a weak SOS box are fully induced, and those include $uvrA$, $uvrB$ and $uvrD$ (Crowley and Hanawalt, 1998), activating the NER repair pathway. The increased rate of mutation during the SOS response is caused by three low-fidelity DNA polymerases with stronger SOS boxes, induced when NER does not suffice: Pol II, Pol IV and Pol V (Saha et al., 2007). Pol IV is involved in bypass replication across DNA damages.

**Figure 1.11 Regulation of SOS response in $E. \text{coli}$ gene of the UmuD protein.** DNA damage activates RecA protein, which induces LexA to degrade at its Ala84–Gly85 bond. The operon (SOS box) is released and the $umuD$ gene expresses into the UmuD protein, a subunit of the DNA polymerase V, low-fidelity polymerase that repairs the damaged DNA introducing replication errors. Adapted from Friedberg et al., 2005.
of damaged bases generated by ROS, and seems to play an important role in misincorporation of dCTP opposite 1,2-dihydro-2-oxoadenine (oxo2A), leading to G·C → A·T mutations. Pol IV prefers to insert dATP opposite f5dU, not leading to any mispairing as know until know (Hori et al., 2010).

Nucleotide excision repair of 5-formyluracil

As previously mentioned, f5U is repaired by the BER pathway, which in E. coli is initiated by damaged base removal by the AlkA, Fpg, Nth or Nei DNA glycosylases. In addition, the mammalian NER system has been reported to exhibit activity for repair of f5U in vitro – through the XPC-HR23B complex (Kino et al., 2004) – while no such evidence exists for bacterial NER (Van Houten et al., 2005). Transcription coupled NER (TC-NER) do not require XPC (Le May et al., 2010) indicating that f5U seems to be interacting with the global genome NER (GG-NER).

Previous studies in E. coli have shown that 5-fluorouracil (F5U) – antimetabolite employed clinically to manage solid tumors (Wyatt and Wilson, 2009) – induces the SOS response via expression of the umuC gene in the wild-type and uvrA strains, but not in lexA and recA strains (Oda, 1987). Moreover, the same glycosylases seem to be involved in the repair of F5U and f5U, both in E. coli and humans (Liu et al., 2003; Wyatt and Wilson, 2009).
2 Aim of the studies

During enzymatic characterization of UDGs using 5'-labelled oligonucleotides with uracil incorporated at a specific site, we surprisingly discovered that hUNG and hSMUG1 glycosylases might incise the DNA strand at the uracil site following base removal. Thus, the task was to verify this activity and characterize the incision products chemically (Paper 1).

The main BER glycosylases involved in the removal of virtually all the base damages formed by ROS are AlkA, Fpg, Nth and Nei. Since, to our knowledge, the consequences on mutagenesis if knocking out these enzymes have yet to be investigated, we decided to study the spontaneously arisen base substitutions in the quadruple mutant alkA fpg nth nei (Paper 2).

Our research group has previously shown that f^5U is efficiently removed from DNA in vitro by the E. coli AlkA DNA glycosylase, which later has been confirmed by others. To possibly provide in vivo evidence for this enzyme function, experiments using different concentrations of f^5dU were performed on E. coli wild-type and bacteria deficient in the alkA gene (Paper 3).

Since in vitro experiments suggest a role for mammalian NER in f^5U repair (Kino et al., 2004), we decided to investigate whether this also was the case in E. coli, a much simpler system to compare the possible roles or relationships between BER and NER in f^5U repair. First, we wanted to possibly establish an in vivo effect regarding mutagenesis using different concentrations of f^5dU in E. coli by monitoring the consequences of knocking out each of the three genes coding for the UvrABC proteins system. The study was initiated by determining
AIM OF THE STUDIES 2

the mutation rate and base substitutions induced by f'dU in the uvrA mutant (Paper 4).
3 Overview of results

3.1 DNA uracil excision includes strand incision by hUNG (Paper 1)

During the performance of DNA incision assays with different uracil-DNA glycosylases (UDGs), we observed that the major UDGs in human cells (hUNG) and in *E. coli* (EcUng) – both family 1 UDGs – were able to incise the lesion site after base excision. hUNG was not able to recognize and incise AP-containing DNA, showing that it needed uracil for damage recognition.

Gel migration analyses using certain DNA glycosylases/AP lyases and AP endonucleases to define different 3’-end products indicated that the 3’-end was identical to a 3’-α,β-unsaturated aldehyde, which we decided to designate uracil-DNA incision product (UIP). The chemical nature of this new UDG-endonuclease activity was also investigated using MALDI-TOF-MS (matrix-assisted laser desorption/ionization time of flight mass spectrometry) for product analysis, confirming that the incision resulted in a 3’-α,β-unsaturated aldehyde as well as a 5’-phosphate (Paper 1, Fig. 3a). Enzyme reactions were performed in the presence H₂¹⁶O and H₂¹⁸O followed by subsequent sample preparation confirmed the unsaturated nature of the 3’-remnant by showing post-enzymatic quantitative addition of either water or ammonia to the double bond of the 3’-α,β-unsaturated aldehyde. These experiments were consistent with both a β-elimination and a hydrolytic reaction mechanism. However, when the UDG–DNA reaction intermediate could not be trapped as a stable covalent complex it points to the latter mechanism. hUNG also lacks a lysine residue in the active site to carry out a β/δ-elimination reaction, which indicates a hydrolytic mechanism. Thus, we proposed that a catalytic or activated H₂O molecule initiates a β-elimination reaction at the 2’C–3’C bond by acting as a general base and attracting a proton from the deoxyribose 2’C. The electron-
OVERVIEW OF RESULTS 3

withdrawing power of the deoxyribose 1'C formyl group should greatly facilitate such a reaction, which in addition might be stabilized by His268 in the hUNG active site (Paper 1, Fig. 3a). This scheme accords well with crystal structures of hUNG in complex with damaged DNA, showing both accessibility of water molecules, formyl group stabilization (Paper 1, Fig. 3c) and reactive distance between a reactive H2O and the 2'C atom of the flipped-out abasic nucleoside (Paper 1, Fig. 3c).

3.2 Severe repair-deficiency for oxidized DNA bases causes primarily GC to AT transitions in E. coli (Paper 2)

Inactivation of the main BER glycosylase genes (alkA, fpg, nth and nei) involved in repairing lesions induced by ROS in E. coli caused a ten-fold reduction in the bacterial growth. The mutation rate was three times higher as measured by scoring for rifampicin resistance (rifR) (Paper 2, Table 1). This agrees with the described cytotoxicity of some ROS-induced lesions (Bjelland and Seeberg, 2003).

The abundance of the six types of base substitutions detected by sequencing the rifR region of mutants obtained from wild-type and alkA fpg nth nei varied significantly between the former and latter. In wild-type, the most common mutation was the G·C → A·T transition accounting for almost 40% whilst the G·C → C·G transversion as the most infrequent one accounted for roughly 4%, of the total base substitutions recorded. Interestingly, in the quadruple alkA fpg nth nei mutant more than 90% of the mutations were G·C → A·T transitions while the rest were A·T → G·C transitions (Paper 2, Table 1).

Two thirds of the G·C → A·T transitions recorded in alkA fpg nth nei occurred in two hot spots (1576 and 1592) shared with the wild-type; most of the rest occurred in two other hot spots (1546 and 1586). Virtually all A·T → G·C transitions recorded in alkA fpg nth nei occurred at the site 1547, which also was a common site for such mutations in wild-type (Paper 2, Figure 2). Taken together with known mispairing abilities of oxidized G and C (Bjelland and Seeberg, 2003), our results suggest that cytosine oxidation seems more important than oxidation of other bases including guanine in spontaneous mutagenesis.
3.3 Damage-specific mutation induction promoted by repair (Paper 3)

When f5dU at different concentrations (0.1–0.4 mM) was added to exponentially growing *E. coli* – wild-type, *alkA* and *xth nfo* – the relative growth decreased only moderately and never fell below 50%. This agrees with the character of f5U as a mutagenic rather than cytotoxic lesion (Bjelland and Seeberg, 2003). However, since *alkA* seems slightly more affected than wild-type, a phenotypic difference may exist.

The spontaneous mutation rates (scored using the rif\(^\text{R}\) system as above) were similar for wild-type, *alkA* and *xth nfo*, although slightly lower for *xth nfo*. The addition of 0.1 mM f5dU increased the mutation rate of all three to roughly the double, demonstrating the mutagenic capacity of f5dU. However, higher concentrations of f5dU (0.2 and 0.4 mM) decreased the mutation rates. In wild-type this decrease was moderate while the decrease in mutation rate for *alkA* was >50% when the f5dU concentration added was increased from 0.2 to 0.4 mM, which fell even below the spontaneous level (Paper 3, Table I). Thus, the presence of AlkA (in wild-type) seems to prevent an abrupt decrease in f5dU-induced mutagenesis at the highest concentration of f5dU studied.

The A·T → G·C transition was clearly the most predominant base substitution formed in repair-proficient *E. coli* at the lowest concentration of f5dU (0.1 mM). In addition to its large increase in abundance by f5dU supplementation (Paper 3, Figure 3A), its connection with the f5dU damage per se was indicated by its lower formation in *xth nfo* (Paper 3, Figure 3C), arguing against un-specific formation via AP sites. The increased A·T → G·C formation in *alkA* at 0.2 mM f5dU (Paper 3, Figure 3B), which theoretically should be connected to poorer f5U repair (Bjelland et al., 1994), supports this notion. The A·T → G·C transition is challenged by G·C → A·T at higher (0.2 and 0.4 mM) f5dU concentrations (Paper 3, Figure 3A). Evidence that the latter is induced by f5dU is indicated by its higher rate in *alkA* than in wild-type at 0.1 mM f5dU (Paper 3, Figure 3B). The G·C → A·T transition is a typical A-rule mutation that can be formed via AP sites, which is underscored by its consistent higher rates in *xth nfo* (Paper 3, Figure 3C). The formation of G·C → T·A transversions is favored at higher f5dU concentrations, and evidence that it is formed as a consequence of the A-rule at the higher rate in *xth nfo* compared to wild-type and *alkA* at 0.2 mM f5dU (Paper 3, Figure 3A,C). However, the
similar rate of G·C→T·A at 0.1 mM f5dU under the different repair conditions (Paper 3, Figure 3) may indicate that translesion of AP sites is not the only mechanism for G·C→T·A formation. Thus, Ånensen et al. (2001) reports all three mutations induced in E. coli by f5dU using another mutation detection system.

Based on the above results we can conclude that the presence or absence of AlkA does not seem to be straightforward, and may have different and even opposite consequences on the f5dU-mediated induction of the most prominent substitutions at different mutagen concentrations (Paper 3, Figure 4). The most striking result applies to the G·C→A·T transition, which rate decreases notably in the wild-type compared to alkA at 0.1 mM f5dU but opposite increases dramatically at 0.2 and 0.4 mM in the former compared to the latter (Paper 3, Figure 4B). This indicates mutation alleviation by AlkA at low f5dU concentrations but a pronounced promotion at the highest concentrations measured.

Base substitutions were detected at 27 different sites in the rifR region, 24 of them described previously by Garibyan et al. (2003). Eight sites were identified as “hot spots”, harboring 8% or more of the total mutations detected in the wild-type and alkA strains (Paper 3, Figure 2). The majority of all A·T→G·C transitions were localized at only two sites, both exhibiting the sequence context 5’-GTC-3’. Mutation initiation requires a T to be replaced by f5U, which would be alleviated or promoted by AlkA.

The excision of f5U from DNA opposite A and G by AlkA at increasing enzyme concentrations was analyzed. Kinetic analysis showed that the f5U·G substrate was cleaved much more efficiently than the f5U·A substrate (Paper 3, Figure 5B,C). The same results have been previously reported for the human glycosylase hSMUG1 (Knævelsrud et al., 2009), and accord with the flipping-out mechanism for base excision, where the enzyme must disrupt the DNA base-pairing and base-stacking interactions of the targeted base to be accommodated in the active site pocket, what determines the reaction rate (McCullough et al., 1999; Wibley et al., 2003).

Automated docking simulations were carried out with f5U-DNA into the substrate-binding pocket of AlkA. While the keto form of f5U fits well into the active site being stabilized by a hydrogen bond from the 5-formyl group to Arg22, the ionized f5U® form is excluded from the AlkA active site pocket (Paper 3, Figure 6A). That can be seen by the much stronger decline in
3 OVERVIEW OF RESULTS

glycosylase activity for f5U compared to the primary AlkA substrate m7A at high pH when f5U is abundant (Paper 3, Figure 6B).

3.4 Alleviation and promotion of damage-specific mutation induction in E. coli are highly dependent on the uvrA gene (Paper 4)

The growth of the uvrA mutant was only modestly affected by the addition of f5dU to the culture medium (Paper 4, Figure 1), which we, as mentioned above, also observed for wild-type, alkA and xth nfo (Paper 3, Figure 1). Therefore, the results confirm the notion that f5U is a mutagenic rather than cytotoxic lesion (Bjelland and Seeberg, 2003).

The spontaneous mutation (scored using the rifR system as previously) rate was slightly lower in uvrA than in wild-type and alkA. The distribution of the spontaneously arisen base substitutions in E. coli uvrA deviated significantly from the wild-type (Paper 4, Table 2), but they were in both cases detected mostly at the same hot spots. Most spontaneous mutagenesis in uvrA causes G·C → A·T and G·C → T·A mutations – in contrast with a more equal distribution of the six base substitutions in wild-type (Paper 4, Figure 2) –, thus being closer to alkA in its behavior (Paper 4, Table 2).

Interestingly, supplement with 0.1 mM f5dU doubled the mutation rates in wild-type and alkA but only increased it slightly in uvrA (Paper 4, Table 1) indicating that the UvrA protein or the UvrABC complex seems necessary for promoting mutations induced by f5dU (Paper 4, Table 1). However, the abundance of A·T → G·C transitions increased ~6 times and the abundance of A·T → T·A transversions increased ~3 times above the spontaneous level (Paper 4, Table 2). It should be noticed that no increase in the A·T → T·A transversion was found by addition of f5dU to wild-type, alkA and xth nfo (Paper 3, Figure 3). In conclusion, although the presence of the UvrA protein only affects the spontaneous mutagenesis slightly, it almost doubles the f5dU-induced mutagenesis, which is mostly due to promotion of A·T → G·C transitions.
4 Discussion

4.1 Family 1 glycosylases are most probably bi-functional enzymes

Uracil formed by hydrolytic deamination of cytosine must be replaced in DNA prior to replication to avoid generation of C to T transition mutations (Friedberg et al., 2005; Lindahl, 1993), which are the most common type of spontaneous mutation arising in cells and are frequently found in human tumors (Alexandrov et al., 2013). DNA glycosylases of the BER system involved in uracil excision have been described until now as mono-functional enzymes, requiring downstream proteins to complete the BER pathway and repair the damage (Figure 1.8). The studies performed show that UDG-mediated repair initiation by EcUng and hUNG also involves DNA strand incision generating a 3'-α,β-unsaturated aldehyde (UIP) and a 5'-phosphate. UIP can be removed by hAPE1 to prime for replication whereas the 5'-end is ready for ligation, demonstrating that the U-DNA incision products are appropriate for complete repair.

Hitherto, all known bi-functional glycosylases are lyases, which incise an AP site, either formed by the enzyme itself or another enzyme, by a β- or β/δ-elimination reaction (Friedberg et al., 2005). This contrasts largely with the U-DNA incision activity exhibited by the family 1 UDGs, which carries out the reaction by an activated water molecule rather than by a Schiff base intermediate and is not able to recognize and incise AP-DNA, only U-DNA, thus showing strong dependency on uracil.

The rationale for this novel enzyme activity is obscure. It may be argued that it is a consequence of the inaccuracy of the hydrolytic uracil excision activity rather than being evolved to serve a cellular function. However, U-DNA incision generates a 3’ block to the initiation of repair replication, which under certain cellular stress conditions could be beneficial to the cell. Another
DISCUSSION 4

argument is that if U-DNA incision has been detected in vitro it is also probably takes place to a certain extent in vivo, and consequently must play a role, although minor, in cell function.

4.2 Oxidative DNA damage may initiate tumorigenesis due to C to T transitions

Since guanine is the DNA base most susceptible to oxidation, and its major oxidation product – oxo\(^8\)G – has a high capacity for mispairing with adenine during replication, oxo\(^8\)G has been regarded as the most important oxidized base generated during aerobic respiration (Fortini et al., 2003; Markkanen et al., 2013), leading to G·C → T·A transversions. This is indicated by an increased level of such mutations in *E. coli* when Fpg, the major enzyme for oxo\(^8\)G removal, and MutY, the enzyme removing mispaired A opposite oxo\(^8\)G, are knocked out (Garibyan et al., 2003). In contrast, our study on the spontaneous mutagenesis in the *alkA fpg nth nei* mutant, where virtually all glycosylase activity for oxidized bases are knocked out, shows that ~90% of the base substitutions were G·C → A·T and the rest A·T → G·C. Specifically, only one (<1%) G·C → T·A transversion was detected. Consequently, our study on the BER deficient quadruple mutant supports previous studies on repair proficient *E. coli* concluding that the most common base substitution, G·C → A·T arising from oxidative damage to DNA, is caused by mispairing of oxidized cytosines, where presented evidence pointed to h\(^5\)U and Ug as important mutagenic lesions (Kreutzer and Essigmann, 1998).

However, G·C → A·T transitions also originate by deamination of cytosine to uracil, but since the *alkA fpg nth nei* mutant has complete capacity for uracil removal, virtually all the transitions found are expected to having arisen because of mispairing by unrepaired and thus persistent oxidized bases. The present and previous results underscore the importance of oxidized cytosines in the generation of C to T transitions which are frequently formed in human tumors.
4.3 Repair of 5-formyluracil in DNA by base excision repair can either alleviate or promote mutagenesis in *E. coli*

More than 20 years ago, AlkA was found to have significant activity for removal of 5FU from DNA *in vitro* (Bjelland *et al.*, 1994). At that time this was unexpected since the enzyme was known to be induced in the adaptive response to alkylation (Evensen and Seeberg, 1982; Karran *et al.*, 1982). Since then, no evidence of its role in the repair of oxidized bases *in vivo* has been demonstrated. Thus, this is the first study showing a role of AlkA in the repair of an oxidized base *in vivo*. We found that the presence of AlkA influences the distribution of base substitutions induced by 5FU and, more intriguingly, that AlkA, in addition to alleviating, promotes mutagenesis dependent on the concentration of mutagen. We have suggested a working hypothesis to explain both mutation alleviation and promotion by AlkA. This model takes into account whether 5FU removal takes place before or after replicative mispairing and considers that the ionic form of 5FU (5FU\(^{+}\)) is excluded from the AlkA active site which makes it accessible for mispairing (Paper 3, Figure 7).

It is also important to take into account that several back-up activities for 5FU removal like Fpg, Nth and Nei (Zhang *et al.*, 2000) are present in the absence of AlkA.

4.4 Nucleotide excision repair is involved in the repair of oxidative base lesions

Our results show that the distribution of spontaneously arisen base substitutions deviates significantly more between *E. coli* uvrA and the wild-type than between alkA and the wild-type (Paper 4, Table 2), which is not surprising since the UvrA protein is necessary for a functional NER pathway which is involved in the repair of several DNA lesions counteracting mutation induction. However, it was intriguing the observation that *uvrA* exhibits a more different distribution of 5dU-induced base substitutions compared with the wild-type than *alkA* (Paper 4, Table 2), considering the believed importance of AlkA in 5U repair. Indeed, the results indicate that the presence of the UvrA protein is necessary for mutation alleviation as well as induction (Paper 4, Figure 3B). Whether this is due to repair activity for 5U exhibited by the UvrABC complex
DISCUSSION 4

per se, which has been shown for human NER in vitro (Kino et al., 2004), or might be a consequence of UvrA or UvrABC collaborating with BER e.g. in the recognition of the f5U lesion, we cannot yet conclude.
5 Future perspectives

After showing a novel bi-functionality of EcUng and hUNG suggested being able to incise U-DNA by a water-mediated elimination reaction, similar studies on hSMUG1 has been initiated, and should be extended to other mono-functional glycosylases like E. coli Tag and Mug, hMPG and hTDG. This might tell us whether the novel U-DNA incision activity is a specific function of some few enzymes or is coupled to the involvement of activated water in catalysis.

The spontaneous mutagenesis due to damage by ROS was measured in E. coli by comparing wild-type with a quadruple mutant (alkA fpg nth nei) deficient in the genes for the main glycosylases involved in the repair of oxidized base residues. A future goal would be to extent the comparison of the spontaneous mutagenesis using the same system for mutation detection to alkylation repair-deficient (involving the genes tag, alkA, alkB, ada and ogt) and deamination repair-deficient mutants (involving the genes ung, mug and alkA). This might tell us something about the importance of these different chemical processes in mutagenesis.

DNA repair involves many different pathways and enzymes establishing a complex network in the cell cycle. Defects in the DNA repair system have been demonstrated to have a connection with cancer development, e.g. deficiency in NER genes related to xeroderma pigmetosum and skin cancer (Pesz et al., 2014). Mutations in the OGG1 gene have been reported to be related to several types of renal cancers (Audebert et al., 2000) and UNG-deficient mice have shown an increased morbidity and higher than normal incidence of B-cell lymphomas (Nilsen et al., 2003). However, correlation of BER with tumorigenesis needs to be further investigated. Since the results presented in this thesis show that repair glycosylases both alleviate and promote mutagenesis in E. coli, BER might behave in the same way in human cells and thus be linked to cancer initiation and progression.
Since $f^5U$ causes a minimal perturbation in the DNA structure (Bjelland and Seeberg, 2003), it is tempting to speculate whether the large effect on $f^5dU$ mutagenesis exhibited by the deficiency in the UvrA protein is due to collaboration with BER glycosylases rather than due to NER activity \textit{per se}. To answer this interesting question, we have started similar mutagenesis experiments with \textit{E. coli} \textit{uvrB} and \textit{uvrC}, from which results we hope can encourage further experiments to reach a conclusion. Molecular interaction and repair studies involving $f^5U$-DNA, BER glycosylases and the UvrA, UvrB and UvrC proteins should be appropriate.
6 References


REFERENCES


36


