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Mechanistic study of the effects of 8-oxo-7,8-dihydroguanine (8-oxoG) on reporter gene expression in mammalian cells

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CONTENTS

ABSTRACT

DNA damage causes replication errors, leading to genetic instability or cell death. Besides that, many types of DNA base modifications have been shown to interfere with transcriptional elongation if they are located in the transcribed DNA strand of active genes, acting as roadblocks for RNA polymerases. It is widely assumed that transcription blockage by endogenous DNA damage is responsible for the early cell senescence in organs and accelerated ageing observed in individuals with compromised nucleotide excision repair.

The aims of this work were to design new experimental systems for testing transcription blocking potentials of DNA base modifications in an individual gene and to apply these test systems to the investigation of the effects of a frequent endogenously generated base modification, namely 8-oxo-7,8-hydroxyguanine (8-oxoG), on the gene transcription in cells. Several experimental strategies were employed for this purpose. First, I constructed an episomal vector encoding for a short-lived EGFP-ODC fusion protein and measured expression of the reporter gene in permanently transfected clonal cell lines exposed to DNA damaging agents. Second, the expression of plasmid-borne EGFP gene damaged with photosensitisers to obtain one or several oxidative purine modifications per plasmid molecule was determined in transiently transfected human and mouse host cells in an approach known as “host cell reactivation”. As a prerequisite for these experiments, a robust method of precise quantitative measurement of the EGFP gene expression in transiently transfected cells by flow cytometry was developed and validated. Third, I elaborated a very efficient procedure for insertion of synthetic oligonucleotides carrying 8-oxoG into plasmid DNA, avoiding any unwanted base damage and strand breaks. The consequences of 8-oxoG placed in defined positions in opposing DNA strands of the EGFP gene for transcription were measured by host cell reactivation in cells with functional 8-oxoguanine DNA glycosylase (OGG1) gene and in OGG1 null cells.

The results obtained in *Ogg1*$^{-/-}$ cells demonstrated that unrepaired 8-oxoG, even if situated in the transcribed DNA strand, does not have any negative effect on the reporter gene transcription. On the other hand, as few as one 8-oxoG was sufficient to cause a significant decrease of the gene expression in OGG1-proficient cell lines, i.e. in the presence of base excision repair. For two analysed positions of 8-oxoG in the plasmid DNA, the inhibition of gene transcription by the base modification correlated with the efficiency of its excision by purified OGG1 protein under cell-free conditions. Based on these findings, it has to be concluded that the observed decrease of transcription is mediated by excision of the base modification by OGG1 and probably caused by the repair-induced single-strand breaks. The mechanism of transcription inhibition by 8-oxoG is therefore clearly distinct from stalling of elongating RNA polymerase II complexes at the modified base.

ZUSAMMENFASSUNG

DNA-Schäden führen zu Fehlern bei der DNA-Replikation und damit zu genetischer Instabilität bzw. Zelltod. Außerdem beeinflussen viele Arten von DNA-Schäden auch die Transkription, insbesondere wenn sie im transkribierten Strang der DNA lokalisiert sind, indem sie als "Wegsperre" für die RNA-Polymerasen wirken. Es wird angenommen, dass die Blockade der Transkription durch endogene gebildete DNA-Schäden dafür verantwortlich ist, dass Defekte der Nukleotidexzisionsreparatur zur frühen zellulären Seneszenz und beschleunigten Alterungsprozessen führen.

Ziel dieser Arbeit war es, eine geeignete Methode zur Untersuchung des Ausmaßes der Transkriptionsblockade durch eine DNA-Basenmodifikation in einer definierten Position eines Gen zu entwickeln und diese dann anzuwenden, um die Folgen eine häufigen endogen gebildeten Basenmodifikation, nämlich 8-Oxo-7,8-dihydroguanine (8-oxoG), für die Gentranskription zu ermitteln. Dazu wurden verschiedenen Strategien eingesetzt. Erstens wurde ein episomaler Vektor konstruiert, der für ein kurzlebiges EGFP-ODC-Fusionsprotein kodiert. Die Expression dieses Reporterproteins wurde sodann in permanent transfizierten klonalen Zelllinien untersucht, die mit verschiedenen DNA-schädigenden Agentien behandelt wurden. Zweitens wurde die Expression eines Plasmid-kodierten EGFP Gens nach transienter Transfektion in humane und murine Wirtszellen untersucht, nachdem in den Plasmiden zuvor durch Behandlung mit einem Photosensibilisator eine oder mehrere oxidative Purinmodifikationen pro Plasmidmolekül induziert worden waren ("host cell reactivation"). Als Vorraussetzung für diese Experimente wurde eine verlässliche Methode zur genauen Quantifizierung der EGFP Genexpression in transient transfizierten Zellen mittels Durchflusszytometrie entwickelt und validiert. Drittens wurde eine sehr effiziente Methode ausgearbeitet, um synthetische Oligonukleotide, die eine Basenmodifikation wie 8-oxoG enthalten, in Plasmide einzubauen, ohne unerwünschte zusätzliche DNA-Schäden oder Strangbrüche zu induzieren. Die Auswirkungen von 8-oxoG in definierten und sich gegenüber liegenden Positionen beider Stränge des EGFP Gens auf die Transkription wurde sodann in Zellen mit und ohne Defekt in der 8-Oxoguanine-DNA-Glykosylase (OGG1) gemessen.

Die Ergebnisse mit *Ogg1*$^{-/-}$-Zellen zeigten, dass nicht repariertes 8-oxoG keine negativen Auswirkungen auf die Transkription des EGFP-Gens hat, selbst wenn es im transkribierten Strang der DNA positioniert ist. Andererseits führte in OGG1-profizienten Zellen (mit funktionsfähiger Basenexzisionsreparatur) schon ein einzelnes 8-oxoG in der DNA zu einer signifikanten Abnahme der Genexpression. Für zwei untersuchte Positionen von 8-oxoG korrelierte dabei das Ausmaß der Inhibition der Genexpression mit der Effizienz der Exzision

der Modifikation durch reines OGG1 unter zellfreien Bedingungen. Auf Grund dieser Ergebnisse muss angenommen werden, dass die beobachtete Expressionsminderung durch die Exzision der Basenmodifikation durch OGG1 entsteht und wahrscheinlich auf die durch die bei der Reparatur gebildeten DNA-Strangbrüche zurückzuführen ist. Der Mechanismus dieser Transkriptionshemmung ist daher grundsätzlich verschieden von einer Arretierung des transskribierenden RNA-Polymerase II-Komplexes an einer modifizierten Base.

1. INTRODUCTION

1.1 8-Oxo-7,8-dihydroguanine (8-oxoG) as the major oxidation product of guanine in DNA

8-Oxo-7,8-dihydroguanine (8-oxoG) is a most common oxidative base modification that arise from the damage of DNA by reactive oxidative species (ROS). ROS (such as superoxide anions, hydroxyl radicals and hydrogen peroxide) are formed in cells either by normal cellular metabolism or under the influence of environmental agents. Guanine is particularly susceptible to oxidation mediated by •OH radicals, singlet oxygen and one electron oxidants, because it has the lowest ionization potential among the four canonical nucleobases present in DNA (Cadet et al, 1999), (Steenken, 1997). The 8-oxoG lesion is formed by oxidation of guanine at position 8 (C8) (**Figure 1.1**).

8-OxoG is a pre-mutagenic DNA lesion, which has been implicated in carcinogenesis (Xie et al, 2004). Many organisms have development the efficient DNA repair mechanisms that remove 8-oxoG from the genome and thus prevent genomic instability The steady-state level of 8-oxoG in the nuclear genome of a human cell has been estimated to be one to several modified bases per 10^6 guanines (Ohno et al, 2006), (Pflaum et al, 1997). This stationary level is assumed to reflect the balance between the continuous damage generation by endogenously formed ROS and the removal of the modified bases by DNA repair (Epe, 2002). In confirmation of this thesis, accumulation of 8-oxoG in the organs of repair-deficient animals during lifetime has been demonstrated (Klungland et al, 1999), (Osterod et al, 2001).

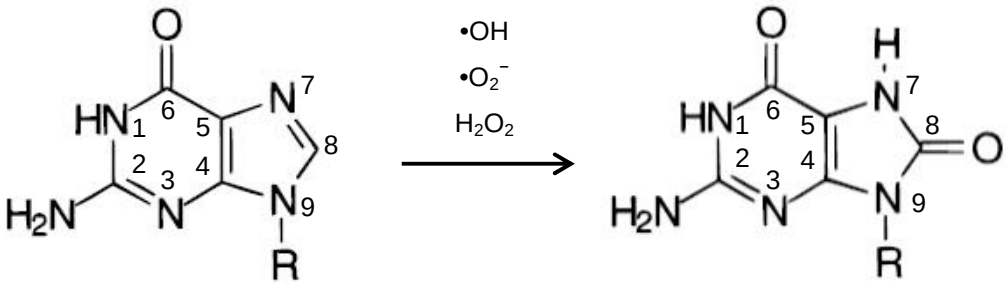

Figure 1.1 Generation of 8-oxo-7,8-dihydroguanine (8-oxoG). Numeration of atoms is shown according to Burrows et al. (Burrows & Muller, 1998)

1.2 Mutagenicity of 8-oxoG and its avoidance

8-oxoG in DNA can be involved in two types of base paring interactions. Similarly to the unmodified guanine, it forms a Watson-Crick base pair with cytosine in the complementary DNA strand. Such an 8-oxoG:C base pair contains 8-oxoG in *anti* conformation (**Figure 1.2**). Consequently, DNA synthesis on the template containing 8-oxoG results in a fraction of daughter DNA molecules containing cytosine in the newly synthesized DNA strand. However, another large fraction of newly synthesized DNA was found to contain an atypical 8-oxoG:A base pair (Shibutani et al, 1991), which is formed by 8-oxoG rotated into a *syn* conformation (**Figure 1.2**). This Hoogsteen type base pair is remarkably stable and – what is of critical biological importance – escapes the intrinsic proofreading activity of the replicating DNA polymerases (Hsu et al, 2004), (Maga et al, 2007). In consequence, an 8-oxoG mispaired with adenine can result in a G to T transversion mutations, if it remains unrepaired till the next cycle of DNA replication (Friedberg, 2006). This mechanism of mutagenicity is of huge biological importance, resulting in the accumulation of spontaneous mutations which can lead to cancer and aging. It is worth to underline here that C:G to A:T transversions have been shown to be the most predominant type of somatic mutation in many cancers (Greenman et al, 2007).

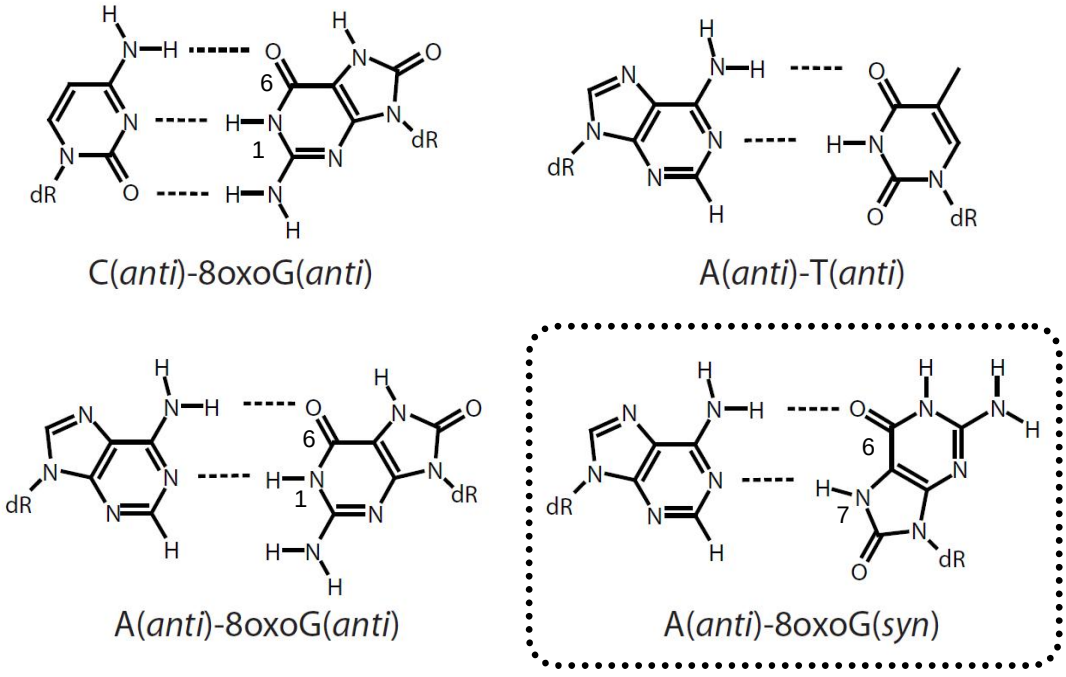

Figure 1.2 Base paring properties of 8-oxoG, according to Damsma et al (Damsma & Cramer, 2009). A stable Hoogsteen base pare of 8-oxoG with adenine in DNA (frame) escapes the proofreading activity of the replicative DNA polymerase leading to incorporation of wrong bases. The same type of base pairing occurs in the active site of RNA polymerases during transcription of damaged DNA, leading to transcription errors.

The replicational mutagenicity of 8-oxoG is prevented by three independent enzymatic mechanisms which are conserved throughout the life kingdoms from bacteria to humans. The human homologs of the *E. coli* MutT protein (MTH1, MTH2 and NUDT5) are 8-oxo-dGTP hydrolases which convert the 8-oxo-dGTP nucleotide formed by ROS into the monophosphate (8-oxo-dGMP), so that the modified base cannot be incorporated into DNA any longer (Friedberg, 2006), (Hori et al, 2010). The Human MUTYH DNA glycosylase (a homolog of the bacterial MutY enzyme) functions in a post replicative long-patch BER mechanism to selectively recognise and remove adenines inappropriately paired with 8-oxoG (Parlanti et al, 2002). The mechanism initiated by MUTYH thus corrects the replication errors of DNA polymerases reconstituting the 8-oxoG:C base pair. A complementary mechanism for excision of 8-oxoG incorporated during replication is provided by the AP endonuclease Apn1 in yeast (Ishchenko et al, 2005). However, the relevance of this mechanism for humans is not clarified. In contrast, mutations in the MUTYH gene are associated with high frequency of colorectal cancer (reviewed in (David et al, 2007)), thus underlining the importance of this protein. As MUTYH does not remove 8-oxoG from DNA, this does not provide a repair mechanism for 8-oxoG. This is performed by still another enzyme called 8-oxoguanine DNA glycosylase (OGG1). An analogous activity in bacteria is represented by the fapy-guanine DNA glycosylase (Fpg) also known as MutM. The mechanisms of repair of 8-oxoG are discussed in the subsequent sections.

In the same way as replication of the 8-oxoG containing DNA can lead to incorporation of adenine into the daughter DNA strand, bypass of 8-oxoG by the transcription machineries can lead to incorporation of incorrect nucleotides (preferentially adenosine) opposing to the damaged base and production of mutant transcripts. This process can lead to synthesis of mutant proteins and is called transcriptional mutagenesis (TM) (Saxowsky et al, 2008). Transfections of the OGG1-null MEFs cell lines deficient in repair of 8-oxoG with replication-incompetent 8-oxoG plasmid constructs have demonstrated that 8-oxoG causes transcriptional mutagenesis. Transcriptional mutagenesis was absent in repair proficient cells lines indicating the specificity of the observed effect. An independent work of Brégeon et al. demonstrated an efficient, but error-prone transcriptional bypass of 8-oxoG in human cells by analyses of the luciferase reporter gene expression from two DNA templates containing a single 8-oxoG (paired with either thymine or cytosine) in the transcribed strand of the gene (Bregeon et al, 2009). The authors proposed that 8-oxoG mediated transcriptional mutagenesis may play an important role in the pathogenesis of human neurodegenerative diseases.

1.3 Repair of 8-oxoG in mammalian cells

The major mechanism that removes 8-oxoG from mammalian chromosomal DNA is base excision repair (BER) initiated by the 8-oxoguanine DNA glycosylase (OGG1) (Radicella et al, 1997), (Rosenquist et al, 1997), (Klungland et al, 1999), (Osterod et al, 2001). Within this DNA repair pathway, OGG1 recognises and removes 8-oxoG bases paired with cytosines. It is a bifunctional DNA glycosylase that has a relatively weak apurinic/apyrimidinic (AP) lyase activity, which can lead to cleavage of the sugar-phosphate backbone on the 3′ side of the abasic site by a β-lyase mechanism after excision of the damaged base (Dherin et al, 1999), (Friedberg, 2006). Although most of the AP-sites are probably cleaved by this mechanism, it appears that some of them cannot be directly hydrolysed by pure OGG1 (Parsons et al, 2005). The efficiency of 8-oxoG excision by OGG1 can be modulated by other BER proteins. The most important of them is the major human AP endonuclease APE1 which stimulates the turnover rate of hOGG1 by the bypass of the AP lyase step (Vidal et al, 2001). Another important molecular partner of OGG1 is the DNA glycosylase NEIL1. Due to its relatively high AP site affinity and a stronger AP lyase activity, NEIL1 also can stimulate turnover of OGG1 in a similar manner as APE1 does (Mokkapati et al, 2004). Besides these BER enzymes, the X-ray repair complementing defective repair in Chinese hamster cells 1 (XRCC1) protein was shown to interact with OGG1. Although lacking own enzymatic activity in the DNA damage processing, XRCC1 was shown to stimulate the DNA glycosylase activity of OGG1 up to 3-fold (Marsin et al, 2003).

The dual enzymatic activities of OGG1 enable several possible scenarios for the subsequent steps of repair. In case if hydrolysis of the sugar-phosphate backbone on the 3′ side of the AP site by the β-lyase activity of OGG1, the generated DNA single-strand break (SSB) will contain the rest of the sugar in the form of an unsaturated aldehyde (3′-*trans*-4-hydroxy-2-pentenal-5-phosphate) attached to the 3′ DNA end. This is subsequently hydrolysed by APE1 to generate a 1-nt gap with a 3′ OH. Alternatively, APE1 can directly excise the AP site, generating a 3′ OH and a deoxyribose phosphate (dRP) attached to the 5′ DNA end (**Figure 1.3**). Both SSB structures generated by alternative processing of 8-oxoG in the course of BER are recognised by the DNA polymerase β (pol β), which incorporates G into the 1-nucleotide gap created by APE1 (Frosina et al, 1996), (Pascucci et al, 2002). Removal of the 5′-dRP residue may occur concomitantly with the DNA synthesis step, thanks to the pronounced intrinsic lyase activity of pol β (Sobol et al, 2000), (Pascucci et al, 2002). DNA ends produced by pol β can thus be directly ligated. This is accomplished by DNA ligase I or DNA ligase III/XRCC1 complex. The whole pathway is referred to as “single-nucleotide” or “short-patch” BER, because only one nucleotide is substituted in the process of repair. The mentioned proteins are essential and sufficient to reconstruct the whole base excision repair

process under cell free conditions (Pascucci et al, 2002), and it is likely that a great part of 8-oxoG repair in living cells also occurs by this mechanism. The steps of BER of commonly occurring DNA base modifications are summarised in **Figure 1.3**.

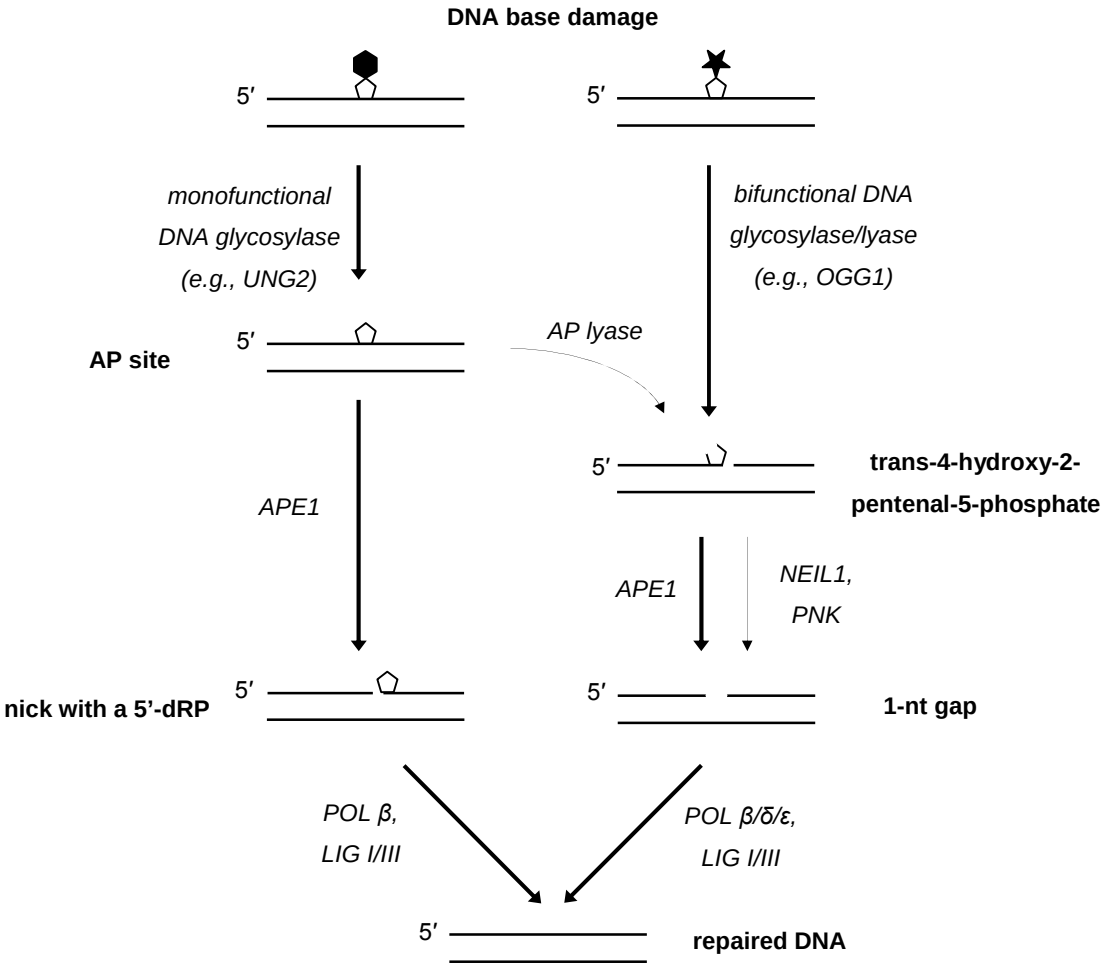

Figure 1.3 Processing of common DNA base modifications by BER initiated by monofunctional (left) and bifunctional (right) DNA glycosylases. The major participating enzymes and structural repair intermediates are shown. DNA polymerisation mechanism can occur by a single-nucleotide or a long-patch mechanism (not shown, see discussion in text).

When the excision of the dRP residue by pol β is blocked for some reason, the downstream repair steps can switch to the “long-patch” BER mechanism. In this mechanism the DNA polymerase β and δ accomplish the incorporation of several (2 to 7) nucleotides at the damage site (Matsumoto et al, 1999). The DNA strand displacement during the synthesis generates a 5′-flap with the sugar-phosphate residue at the end which subsequently undergoes cleavage by the specific flap endonuclease FEN1, providing a necessary 5′-

phosphate for the DNA ligase I. The “long-patch” BER is stimulated by human proliferating cell nuclear antigen (PCNA) (Klungland & Lindahl, 1997). Besides PCNA and the proteins that accomplish the single-nucleotide BER, the “long-patch” BER additionally requires the replication factor C and DNA ligase I (Pascucci et al, 1999).

The exact nature of the molecular switch between the short-patch versus long-patch pathways is unknown. Some researchers believe that this can be modulated by the Poly [ADP-ribose] polymerase 1 (PARP1) protein (Barnes & Lindahl, 2004). On the other hand, it is well possible that real BER in cells is assisted and regulated by at least several additional enzymes and scaffold proteins (Hegde et al, 2008). For instance, the XRCC1 protein (which is known to interact with DNA ligase III, OGG1, pol β and PARP) was proposed to play an orchestrating role from the base excision step to the resealing of the repaired DNA strand through its multiple protein-protein interactions (Marsin et al, 2003).

The single-nucleotide BER was proposed to be the preferential mechanism for base excision repair of 8-oxoG paired with C, because the repair rates were significantly decreased in cells obtained from the pol β null mouse compared to the wild type cells (Fortini et al, 1999). Furthermore, strand displacement DNA synthesis at the incised 8-oxoG occurred in the reconstituted in vitro repair system only in the absence of DNA ligase and additionally required FEN1 or high concentrations of pol β. In the presence of DNA ligase I, the DNA repair synthesis was restricted to the replacement of 1 nt, thus proving the short-patch BER mechanism (Pascucci et al, 2002).

The homozygous *Ogg1* null mice accumulate significant amounts of 8-oxoG in organs (Klungland et al, 1999), (Osterod et al, 2001). Moreover, repair of the oxidative purine modifications (including 8-oxoG) exogenously induced in the chromosomal DNA is greatly retarded in cell lines derived from the *Ogg1* null mice and therefore deficient in the OGG1 protein (Osterod et al, 2001). These findings clearly demonstrate the major contribution of OGG1 to overall repair of 8-oxoG. On the other hand, a very mild phenotype of *Ogg1* null mice and the presence of measurable residual repair activity in the *Ogg1*$^{-/-}$ cells (>50% of the exogenously induced oxidative purine modifications repaired within 16 hours) clearly indicate the existence of some additional back-up pathway for repair of this kind of DNA damage in cells (Osterod et al, 2001).

The putative candidates to initiate BER of 8-oxoG in an OGG1-independent manner have been identified within the family of the NEIL (Nei-like) oxidized base-specific DNA glycosylases. NEIL1 and NEIL2 are bifunctional DNA glycosylases that have associated β,δ-AP-lyase activities. The cleavage of DNA strand by NEILs produces a 3′-phosphate terminus. For this reason, a polynucleotide kinase (PNK) is essential to produce a hydroxylated 3′ end suitable for the Pol β gap-filling reaction, thus allowing the subsequent steps of repair to take

place. The principal substrates for NEIL1 activity are oxidized pyrimidines (thymine glycol, urea) and formamidopyrimidine derivatives of A and G (FapyA and FapyG). However, NEIL1 demonstrated activity against 8-oxoG (in an 8-oxoG:C pair) as well, being able to excise 8-oxoguanine from oligonucleotides (Hazra et al, 2002), (Parsons et al, 2005), (Dobbs et al, 2008), (Morland et al, 2002). Furthermore, this enzyme was identified as major DNA glycosylase that excises oxidative base damage (8-oxoG) located near the 3′ end of a DNA single-strand break, whereas the activity of OGG1 on this type of cluster lesion was compromised (Parsons et al, 2005). The S phase-specific activation of NEIL1 in cells and its interaction with PCNA support the putative role of this enzyme in a preferential repair of oxidized bases in DNA prior to replication (Dou et al, 2008). Another member of the NEILs family (NEIL2) showed a unique preference for excising 8-oxoG lesions present in a DNA bubble structure (Dou et al, 2003). Altogether, it seams that NEILs could take part in BER of some particularly positioned 8-oxoG bases, which cannot be efficiently excised by OGG1. However, the physiological relevance of such repair mechanism is not sufficiently clarified and still awaiting for the compelling in vivo data. Considering the lack of the 8-oxoG excision activity in the OGG1-free cell extracts (Klungland et al, 1999), it appears unlikely that other glycosylase could significantly contribute to BER of this DNA base modification.

There is a persistent belief in the DNA repair community that at least some types of oxidative base modifications could be repaired by a nucleotide excision repair (NER) mechanism (Hanawalt & Spivak, 2008). This opinion is largely based on the severe clinical features of the patients with various defects of NER. These diseases (xeroderma pigmentosum, trichodistrophy, Cockayne syndrome and the intermediate conditions) are manifested as neurological abnormalities and early ageing, which have been ascribed to inability to repair some unknown kind of the endogenously generated DNA damage. There is no direct evidence that 8-oxoG is involved in pathogenesis of any of these diseases. However, the versatility of NER in detection and repair of a great variety of structurally unrelated DNA lesions makes it a reasonable candidate backup mechanism for repair of 8-oxoG.

NER is a multistep process by which wide spectrum of bulky helix-distorting DNA lesions are enzymatically excised from genome as oligonucleotides (Friedberg, 2006). Two sub-pathways of NER that use different mechanisms for recognition of DNA damage are characterised. These are global genome repair (GGR), removing the lesions from the overall genome, and transcription-coupled repair (TCR), which is only active in the transcribed genes, in particular, in the DNA strand undergoing transcription by a RNA polymerase (strand selective repair). The GGR is initiated by recognition of a distorted DNA helical structure by the XPC protein in complex with hHR23B protein and centrin-2 (CEN2). Recognition of some DNA lesions (such a cyclobutane pyrimidine dimer) additionally requires the DDB (DNA damage binding) protein complex. Then the pre-incision complex is formed

around the lesion by coordinated recruitment of the transcription factor IIH (TFIIH), XPA, RPA and XPG. The two subunits of the TFIIH complex, ATP dependent helicases XPB and XPD catalyse local separation (melting) of DNA strands at a site of DNA damage, forming a bubble. Such open structure enables the incision of the DNA strand containing the base modification by structure-specific endonucleases. The first incision is done by the XPF-ERCC1 (excision repair cross complementing-1) at the 5′ side of the damage, several nucleotides away from the damaged base. The second incision is done by the xeroderma pigmentosum G (XPG) endonuclease cutting at the 3′ side of the lesion (O'Donovan et al, 1994). The order of the two excision reactions is strictly regulated in NER, as it has been shown that full catalytic activity of XPG is only revealed once ERCC1-XPF has made the 5′ incision, and that the repair DNA synthesis begins before the 3′ incision is produced by XPG (Staresincic et al, 2009). Following the first incision, a protein named replication factor C (RFC) binds to the 3′ end of the cut DNA to catalyse the loading of the proliferating cell nuclear antigen (PCNA) on DNA. PCNA provides a sliding clamp which stabilises interaction of DNA polymerase δ (DNA polδ) with template DNA thus increasing the processivity of the enzyme. The non-damaged DNA strand remains protected from degradation by the replication protein A (RPA) until the DNA repair synthesis takes place. The repair patch synthesis is accomplished by DNA polymerase δ that uses the DNA strand complimentary to damage as template (reviewed in (Hanawalt & Spivak, 2008) and (Friedberg, 2003)), after which the ligation takes place.

The second sub-pathway of NER is called transcription-coupled repair (TCR), because it selectively removes the lesions from the transcribed DNA strand of actively transcribed genes. This pathway was first characterised in mammalian cells by faster repair of UV-induced cyclobutane pyrimidine dimers (CPDs) in the active dihydrofolate reductase (DHFR) gene than in genome overall (Bohr et al, 1985) and in the transcribed strand than in the rest of the same gene (Mellon et al, 1987). The mechanism of TCR is overall similar to that of the GGR, but differs in the damage recognition mechanism. It appears that transcription coupled repair is initiated by arrest or stalling of RNA polymerase II on a bulky lesion (CPD, 6-4 photoproduct, acetaminofluorene adduct, etc.) during transcription. Initiation of repair by the stalled transcription complexes is assisted by the RNA pol II elongation factor (TFIIS) and the Cockayne syndrome (CS) proteins CSA (also known as ERCC8) and CSB (ERCC6) (Fousteri & Mullenders, 2008), (Hanawalt & Spivak, 2008). TFIIS stimulates the intrinsic transcript cleavage activity of the RNA pol II improving the access of the repair machineries to damaged bases (Fish & Kane, 2002), (Kettenberger et al, 2003), while the precise molecular functions of the CS proteins remain puzzling.

The ATP-dependent chromatin remodelling factor CSB possesses a DNA binding activity and interacts with the stalled RNA pol II complex, playing essential role for the assembly of

NER proteins, which altogether constitute the TCR complex (Laine & Egly, 2006a), (Fousteri et al, 2006) and could be important for the chromatin modification at the damage site by the recruitment of the histone acetyltransferase (HAT) p300 (Fousteri et al, 2006), (Fousteri & Mullenders, 2008). The recruitment of CSB to the lesion-stalled transcription complexes is an ATP-dependent process which involves a gross conformational change of CSB (Lake et al, 2010). In spite of its strong homology with the SNF2 family DNA helicases, CSB does not display helicase activity (Fousteri & Mullenders, 2008) but rather catalyses the re-annealing of the separated DNA strands (Muftuoglu et al, 2006).

CSA is a WD-repeat protein which has been implicated in the E3 ubiquitin-protein ligase activity, which drives ubiquitylation and degradation of CSB during the TCR (Groisman et al, 2006). It also promotes recruitment of the nucleosomal binding protein HMGN1, XAB2, and TFIIS (Fousteri et al, 2006). There is evidence that even in the absence of damage, CSA associates with the hypophosphorylated forms of the RNA polymerase and, possibly in conjunction with CSB, grants for the polymerase the ability to perform TCR at any time. To allow subsequent repair of the DNA lesion by the NER machinery, the associated E3 ligase activity of CSA has to be inactivated (Groisman et al, 2003).

In summary, the CS proteins play key roles in the pre-incision step of TCR, being required for recognition of the distorted transcription bubble containing the RNA Pol II stalled at the damage site. CSA and CSB are involved in the protein interactions and required for the molecular transitions, which are essential for recruitment of core NER factors to the stalled RNA Pol II. The downstream events in TCR (excision of the DNA lesion and the subsequent repair steps) are the same as in GG-NER.

It is important to consider that both NER subpathways lead to the excision of an oligonucleotide containing the DNA modification, but not of the modified base alone. Therefore, virtually any structural DNA modification can potentially be repaired by either TCR or GG-NER, provided that it is recognised by the appropriate DNA damage-sensing proteins. So it can be that BER and NER pathway have overlapping specificities in repair of oxidative damage as it was shown in in vivo experiments in yeast (Swanson et al, 1999), (Doetsch et al, 2001). In GG-NER the damage sensory function relies on XPC which recognise the DNA helix distortion. Base modifications that do not lead to a structural change in the helical shape of DNA (like 8-oxoG) are thus unlikely to be straight substrates for GG-NER. However, the XPC-HR23B complex apparently can co-operate with BER by improving the excision efficiency of 8-oxoG by the specific OGG1 DNA glycosylase (D'Errico et al, 2006).

TCR may be a more plausible candidate backup repair pathway for the 8-oxoG lesions, as its specific components, particularly the CSB protein, have been implicated in repair in multiple experimental systems. Several mechanisms have been proposed for the role of CSB in

repair of 8-oxoG. First, the CSB deficiency was shown to cause down-regulation of the OGG1 gene expression in cell lines derived from the Cockayne syndrome patients, which led to a reduced 8-oxoG incision activity in the cell extracts (Dianov et al, 1999). Second, CSB protein accelerated the global repair of Fpg-sensitive oxidative base lesions (most of which are believed to be 8-oxoG) in the cell lines expressing OGG1 (Sunesen et al, 2002), (Osterod et al, 2002) and in MEFs from the OGG1-null mice (Osterod et al, 2002). Finally, the removal of 8-oxoG from a plasmid gene in the *Ogg1*$^{-/-}$ MEFs could occur only in the presence of transcription (Le Page et al, 2000), thus feeding the hypothesis about a transcription-coupled repair of 8-oxoG. However, TCR of 8-oxoG has not been demonstrated experimentally till now, with the exception of several papers that have been subsequently retracted. By contrast, other authors did not provide any evidence that repair of 8-oxoG in chromosomal DNA is confined to the transcribed DNA sequences, rather proposing a role of CSB in the whole genome repair (Sunesen et al, 2002), (Osterod et al, 2002). Altogether, in spite of intense investigation during the last decade, the question about the contribution of TCR to the repair of 8-oxoG remains open.

1.4 The effects of 8-oxoG on elongating RNA polymerases in the reconstituted transcription reactions

The mechanistic insights from the reconstituted TC-NER reactions confirm the idea that blocking of the elongating RNA polymerase II at the damage site is a necessary and sufficient signal for initiation of repair, provided that the whole set of necessary protein factors is available (Laine & Egly, 2006a). The principal role of RNA polymerase as a damage recognition factor in this repair pathway is further supported by massive data demonstrating the correlation of the efficiency of TC-NER with the capacity of a DNA modification to block the progression of the RNA polymerases in the reconstituted transcription systems (reviewed in (Tornaletti & Hanawalt, 1999), (Tornaletti, 2009)). In summary, current understanding of the transcription-coupled repair mechanism implies that virtually every transcription-blocking DNA modification can be repaired by the TC-NER and that only transcription blocking DNA damage can be repaired by TC-NER. Applying this paradigm to 8-oxoG, the researchers investigated the potential of 8-oxoG to block the progression of the RNA polymerase II (Pol II). The existence of a transcription block in an in vitro reconstituted transcription system would predict that 8-oxoG could be a TCR substrate.

Kuraoka and co-workers (Kuraoka et al, 2003) showed that a significant fraction of the human Pol II stalled at a single 8-oxoG located on the transcribed strand in an in vitro transcription elongation assay with a oligo dC-tailed template containing a single DNA base lesion. The fraction of Pol II that stalled at 8-oxoG lesions did so after inserting the correct

base, while the rest bypassed the site using either correct or incorrect base insertions. In contrast to human Pol II, the T7 RNA Pol did not stall at the lesion on this template. Other lesions, such as AP site and uracil, were efficiently bypassed by Pol II without blocking transcriptional elongation. These authors suggested that partial stalling of Pol II caused by 8-oxoG in the transcribed strand in principle could be sufficient to trigger TCR. On the other hand, transcriptional bypass of 8-oxoG was also reported in the same study, leading to mutations in a fraction of the transcripts (Kuraoka et al, 2003). In a later paper, the same group reported that transcription elongation factor TFIIS (SII) enabled RNAPII to bypass 8-oxoG. The authors hypothesised that transcriptional bypass of the oxidative DNA damage prevents cellular death (Kuraoka et al, 2007).

The group of Hanawalt (Tornaletti et al, 2004) investigated the effect of 8-oxoG on transcription elongation by both T7 RNA Polymerase and mammalian Pol II (from rat liver) using a pair of DNA substrates containing single 8-oxoG in the transcribed or the non-transcribed strand. The 8-oxoG in non-transcribed strand had no detectable effect on transcription elongation by either polymerase. Both polymerases demonstrated either a weak block (≈5 % for the T7 RNA Polymerase) or only transient pausing (mammalian Pol II) at an 8-oxoG located in the transcribed strand. In contrast, a CPD (a known substrate for TCR) located in the transcribed strand of template DNA in different sequence contexts was an absolute block to transcription elongation by mammalian Pol II (Donahue et al, 1994), (Tornaletti et al, 1997). Similar to CPD, cisplatin-induced lesions in the transcribed DNA strand constitute a strong in vitro block for both T7 RNA polymerase and mammalian Pol II (Tornaletti et al, 2003). Tornaletti and co-workers suggested that, because of the weak blocking potential of 8-oxoG for RNA polymerase elongation, the initiation of TCR at this lesion may require additional factors to ensure the Pol II arrest. Such factors could be missing from the purified protein preparations used. Therefore, transcription reactions performed with the whole nuclear extracts could better predict the physiologically relevant elongation-blocking potentials of the DNA base modifications than reactions reconstituted with the purified proteins.

Contrary to expectations of the researchers advocating the transcription-blocking potential of 8-oxoG, this modification (positioned in the transcribed DNA strand) did not block the RNA Pol II transcription in reactions performed with nuclear extracts of HeLa cells. In contrast, a site-specific single-stranded break in the template strand and a site-specific cyclobutane pyrimidine dimer did strongly block the Pol II transcription in this experimental system as well. The DNA-helix distorting oxidative pyrimidine lesion thymine glycol (Tg) in the transcribed strand initially blocked transcription or caused the RNA transcription complex to pause, but permitted transcription to go on short time afterwards. The authors explained the eventual bypass of the Tg lesion (or at least some of it) by the capacity of the cell extracts to

repair the lesion. In addition the authors proposed that oxidative base damage that does not block transcriptional elongation directly can nevertheless block transcription after incision by a repair DNA glycosylase (Kathe et al, 2004).

A weak block of RNA pol II at an 8-oxoG positioned in the transcribed DNA strand was also observed by Larsen et al. (Larsen et al, 2004). In run-off transcription assays with purified transcription factors, approximately 95% of the transcripts demonstrated bypass of 8-oxoG. The control DNA substrate containing a single cisplatin lesion in the transcribed strand caused a solid block for Pol II in the same transcription system. Transcription activity was also measured by using HeLa nuclear extract on the same substrates as described before with very similar results. The observed weak blockage (5%) could be due to single-strand breaks generated at 8-oxoG during the incubation with nuclear cell extracts for run-off transcription (Larsen et al, 2004).

The Egly group (Charlet-Berguerand et al, 2006) used a reconstituted transcription system (RTS) using recombinant basal transcription factors TBP, TFIIB, TFIIE and TFIIF or RNA pol II and TFIIH purified from HeLa cell nuclear extract. These researchers found conditions under which 8-oxoG in transcribed strand could slightly block elongation by RNA pol II (20%-40%). Importantly, this block was efficiently counteracted by addition of the elongation factors, such as elongin, CSB and TFIIS which all enhanced bypass of an 8-oxoG lesion by Pol II, contributing to transcriptional mutagenesis. Interestingly, recent biochemical and structural analyses of the yeast RNA Pol II accomplishing RNA synthesis on an 8-oxoG containing DNA template revealed that neither incorporation of C nor misincorporation of A into DNA structurally interfered with the arrangement of the Pol II catalytic subunit or impeded further elongation (Damsma & Cramer, 2009).

In summary, many authors who worked with the in vitro reconstituted transcription systems with DNA templates containing 8-oxoG concluded that this oxidative DNA base modification can be relatively easily bypassed by RNA polymerases (**Table 1.1**). The weak block of RNA Pol II at 8-oxoG observed in some studies most probably cannot provide a sufficient signal for initiation of TCR. However, the base modifications could be converted to SSBs by incision by the DNA glycosylases and consequently block transcription elongation (Charlet-Berguerand et al, 2006), (Kathe et al, 2004). An abasic site (AP-site), which is another structural intermediate of BER of 8-oxoG, can also arrest transcription when located in the transcribed strand of the template DNA (Tornaletti et al, 2006).

Table 1.1 Effects of 8-oxoG located in transcribed DNA strand (TS) on transcription elongation reported in different reconstituted transcriptions systems.

RNA Polymerase	Reported results	Possible physiological outcome	References
Pol II	significant fraction of Pol II is **stalled**, while the rest can **bypass** the lesion, inserting either correct or incorrect base	transcriptional mutagenesis; possibly, TCR	(Kuraoka et al, 2003)
Pol II	**transiently paused transcription** (substrates containing 8-oxoG in the TS and purified Pol II)	additional factors may be required to ensure Pol II arrest and initiate TCR	(Tornaletti et al, 2004)
Pol II	**no block** of transcriptional elongation (run-off transcription assays with HeLa cell nuclear extracts)	SSB that remained after incision of 8-oxoG could prevent forward movement of the transcription complex	(Kathe et al, 2004)
Pol II	**slightly blocked** elongation **(20%-40%)**, that can be overcome by elongin, CSB and TFIIS (purified Pol II and proteins)	transcriptional mutagenesis	(Charlet-Berguerand et al, 2006), (Kuraoka et al, 2007)
Pol II	**weak block (5%)** at 8-oxoG, possibly due to single-strand breaks generated by OGG1, present in nuclear extract	no indication for a definite block for Pol II at the lesion	(Larsen et al, 2004)
T7 RNA pol	**weak block** (read-through frequency of up to 95% with the purified protein)	slightly affected transcription	(Tornaletti et al, 2004)

1.5 The effects of 8-oxoG on transcription in cells

Even though 8-oxoG most likely does not block elongation by RNA polymerases in reconstituted transcription systems, evidence has been provided that this lesion has an inhibitory effect on transcription in mammalian cells. As there is no way to specifically generate high levels of 8-oxoG in the chromosomal DNA (because of the high reactivity of oxidants with non-DNA cellular compartments and related cytotoxicity), a useful tool to study the effects of 8-oxoG on transcription is transfection of plasmids containing this DNA modification. This technique is referred to as “host cell reactivation assay”, because it was

originally applied to measure the repair capacity of cells as a degree of reactivation of damaged exogenous genes transfected to the host cell.

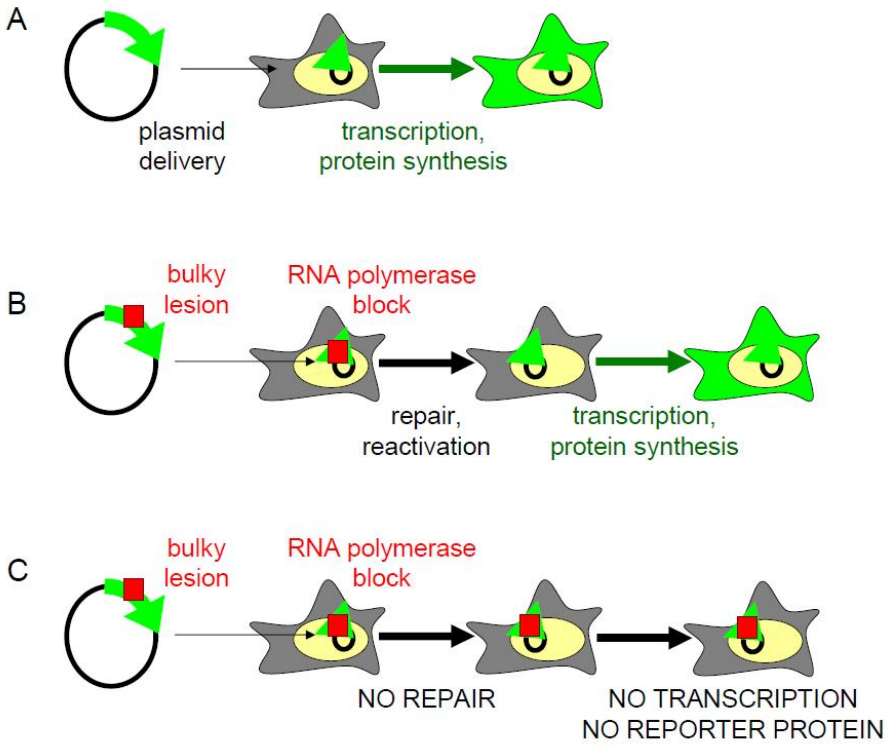

Figure 1.4 The principle of a plasmid based host-cell reactivation assay (HCR). (A) Transfection of a plasmid DNA encoding for a reporter protein results in a detectable signal (e.g., EGFP fluorescence). (B) The presence of a transcription-blocking damage (red square) in the template DNA strand of the reporter gene prevents transcription. Transcription blocks can be removed by the host-cell repair mechanisms, resulting in gene reactivation and expression of detectable levels of the reporter protein. (C) Reactivation of the damaged reporter gene cannot occur in repair deficient host cells.

Host cell reactivation (HCR) is a sensitive assay to measure the effect of DNA damage on the expression of a reporter gene. HCR methods are based on the capacity of host cells to repair DNA damage present in a transiently transfected plasmid DNA (or in the genome of a virus or a bacteriophage which cannot perform the repair by their own). If the damaged foreign DNA encodes for a reporter gene, its expression is inactivated by the DNA damage. However, repair of the plasmid or viral DNA by the repair machineries of the host cell leads to reactivation of the gene expression (**Figure 1.4**). The relative level of expression of the reporter gene is thus assumed to be an indicator of the repair capacity of the host (Ganesan et al, 1999). Indeed, transient transfections of reporter vectors containing known type of

transcription-blocking DNA lesions (such as UV-induced pyrimidine adducts) into cell lines with different genetic backgrounds have shown that the cells deficient in TCR are characterised by the lowest expression level of the damaged DNA (Spivak & Hanawalt, 2006). This feature has proven helpful in identifying and cloning the genes responsible for DNA repair-deficient syndromes, such as the human CSA gene (Henning et al, 1995). The reporter genes commonly used in HCR studies are those enconing for β-galactosidase (Spivak & Hanawalt, 2006), (Kassam & Rainbow, 2007), luciferase (Carreau et al, 1995) and fluorescent proteins, such as EGFP (Slebos & Taylor, 2001), (Khobta et al, 2009).

Transfections of reporter plasmids encoding for β-galactosidase that contained various numbers of UV-induced photoproducts into wild type and TCR-deficient CSB cells demonstrated a correlation between proficiency of TCR and efficiency of the reactivation of a damaged reporter gene. The β-galactosidase activity was decreased proportionally to the UV dose applied to the plasmid DNA, and the decrease was more pronounced in CS-B and $UV^{S}S$ cells (both lacking a functional CSB gene) than in the wild type (Spivak & Hanawalt, 2006). Similar dose-dependent decrease of the reporter gene expression was observed by oxidative DNA base modifications, such as thymine glycol (induced by treatment with osmium tetroxide) and 8-oxoG (induced by photosensitisation in the presence of methylene blue). Intriguingly, the oxidative base modifications were more powerful in inhibiting the reporter gene expression than equal amounts of the UV-induced lesions in all investigated cell lines. The HCR of the pCMVβ vector which contained 8-oxoG randomly generated by treatment with methylene blue and visible light was significantly less efficient ($p < 0.0001$) in CS-A and CS-B cells than in wild type or $UV^{S}S$ cells These results suggest that oxidative base damage can be extremely harmful for transcription and that CS proteins are important for transcription of plasmid DNA containing 8-oxoG (Spivak & Hanawalt, 2006). These findings were subsequently supported by an independent study of HCR of the recombinant adenovirus damaged with methylene blue plus light (Leach & Rainbow, 2011).

It is important to underline that DNA treatment with methylene blue plus light induced not only 8-oxoG, but probably other oxidative base modifications, single-strand breaks (Schneider et al, 1990) and AP sites (Epe et al, 1993), that also could interfere with expression of the reporter plasmids (Spivak & Hanawalt, 2006). Moreover, DNA damage generated by treatment of reporter plasmid DNA with methylene blue plus light is randomly distributed in the plasmid and thus does not allow to distinguish between the effects of the damage in the reporter gene and in the non-genic DNA. Neither allows it to address the site- and DNA strand-specific effects of 8-oxoG on transcription. To overcome this, Larsen and co-workers (Larsen et al, 2004) constructed plasmid DNA substrates containing a single 8-oxoG in the transcribed strand of a plasmid-borne luciferase gene and transfected them into MEF cell lines with different genetic backgrounds relevant for the repair of 8-oxoG (wild type,

Csb$^{-/-}$, *Ogg1*$^{-/-}$ and *Ogg1*$^{-/-}$*Csb*$^{-/-}$). No significant effect of 8-oxoG on the plasmid expression was observed in wild-type cells, as well as in cells carrying targeted mutations of the genes required for removal of 8-oxoG. The authors conclude that 8-oxoG does not significantly obstruct transcription in vivo, even in double knockout *Ogg1*$^{-/-}$*Csb*$^{-/-}$ cell line, in which global repair of 8-oxoG is virtually absent (Osterod et al, 2002). The authors hypothesise that sequence context at 8-oxoG sites could play a role in elongation through this lesion (Larsen et al, 2004).

An independent study of HCR of the plasmid gene carrying a single 8-oxoG in the same cell model reported completely different results (Pastoriza-Gallego et al, 2007). The luciferase reporter gene containing the lesions located at different sites on the transcribed strand was transfected into DNA repair-deficient MEF cells (isolated from the *Ogg1*$^{-/-}$, *Csb*$^{-/-}$ and the respective double knockout mice). These authors demonstrated that both proteins (OGG1 and CSB) modulate the expression of the reporter gene. The strongest effect of 8-oxoG on the luciferase protein expression was observed when both repair proteins were missing (in *Csb*$^{-/-}$*/Ogg1*$^{-/-}$ cells). It was further found that the transcriptional effect of the 8-oxoG lesion was quantitatively different, depending on the promoter driving the gene transcription (Pastoriza-Gallego et al, 2007). To explain the quantitative differences between the effects of 8-oxoG in different positions in the gene and to reconcile the striking discrepancy of these results with the previous work of the Klungland group (Larsen et al, 2004), the authors proposed that the sequence context surrounding the lesion and/or the distance from the promoter can additionally modulate the efficiency of the damage bypass by the RNA Pol II in cells. However, a closer analysis of the published works indicates the possibility that the discordant results could originate from suboptimal technical factors in the experiments. The most critical problem is the lack of a reliable method for preparation of plasmid DNA embedding a single defined DNA base modification. This made only very low amounts (just 10 ng!) of DNA available for transfections in one of the studies (Pastoriza-Gallego et al, 2007). Under such conditions, any chemical or topological impurities in the final plasmid preparation could influence the result in an unpredictable manner. The second problem was obviously the absence of a fine quantification method for gene expression, which would be required to allow a valid correction for different transfection efficiencies between the cell lines.

1.6 Scope of this work

The aim of this investigation was a precise quantitative measurement of the effects of single 8-oxoG residues on gene transcription in cells, depending on the DNA strand (transcribed versus non-transcribed) and the status of the genes relevant for repair. This required

development of highly productive techniques for incorporation of single 8-oxoG into the double-stranded circular DNA and invention of a robust method for quantitative gene expression analyses in the transiently transfected cells. This was based on the enhanced green fluorescent protein, which provides a bright and stable fluorophore directly detectable in the individual cells by the flow cytometry or microscopy methods (Cormack et al, 1996). We thus hoped to produce a sufficient methodological breakthrough to be able to resolve the conflicts in the published data and answer the question about the transcription-blocking potential of 8-oxoG.

2 MATERIALS AND METHODS

2.1 Materials

2.1.1 Instruments

Biowave S2100 Spectrophotometer	WPA; Cambridge, UK
ClustalW2 Software	EBI, Cambridge, UK
Eppendorf Centrifuge 5415D	Eppendorf, Hamburg
FACScalibur FACS System and CellQuest™ Pro Software	Becton Dickinson GmbH, Heidelberg
Gel Doc 1000 Geldokumentationssystem and Molecular Analyst® Software	Bio-Rad; Hercules, USA
Lightcycler® 1.5 Real Time PCR System and Lightcycler® Software Version 3.5.3	Roche Diagnostics GmbH; Mannheim
Primer Designer 4 V4.1	Scientific & Educational Software; Cary NC, USA
Biometra® TGradient Thermocycler	Biometra GmbH; Göttingen
Universal 320R Centrifuge	Hettich Zentrifugen, Tuttlingen
Z2™ Coulter Counter®	Beckman Coulter Inc.; Fullerton, USA
Dosimeter Luxmeter 110	PRC Krochmann GmbH, Berlin, Germany)

2.1.2 E. coli strains and genotypes

SCS-8	*recA1 endA1 mcrA* Δ(*mcrBC-hsdRMS-mrr*) Δ(*argF-lac*)*U169* φ80*dlacZ*Δ*M15* Tn*10* (Tetr)
SCS-110	*rpsL* (Strr) *thr leu endA thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB*) [F´ *traD36 proAB lacI*q*Z*Δ*M15*]
JM110	*rpsL* (Strr) *thr leu thi-1 lacY galK galT ara tonA tsx dam dcm supE44* Δ(*lac-proAB)* [F' *traD36 proAB lacIqZ*Δ*M15*]
JM105	F' *traD36 proA*$^+$*B*$^+$ *lacI*^{q} Δ*(lacZ)M15* / Δ*(lac-pro) thi-1 endA1 sbcB15 rpsL* (Str^r) *hsdR4* ($r_k^-m_k^+$)
JM109	F$^-$ e14$^-$ (McrA$^-$) Δ*(lac-pro) endA1 gyrA96* (Nal^r) *thi-1 hsdR17* ($r_k^-m_k^+$) *glnV44 relA1 recA1*

2.1.3 Mammalian cell lines

HeLa

The human epithelial cervical cancer HeLa cell line was received from R.J. Wiesner (Institut für Vegetative Physiologie, Universität Köln). Doubling time is circa 17h.

Spontaneously immortalized mouse embryonic fibroblasts (MEFs)

Wild-type (*F11.1*)

Spontaneously immortalized mouse embryonic fibroblasts (MEFs) were kindly provided by Deborah Barnes (Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire, UK). Doubling time is circa 20h.

$Ogg1^{-/-}$ (1.2)

Spontaneously immortalized mouse embryonic fibroblasts from homozygous OGG1-null mice were kindly provided by Deborah Barnes (Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire, UK). Doubling time is circa 20 h.

$Csb^{m/m}$ (1.3)

Spontaneously immortalised mouse embryonic fibroblasts (MEFs) derived from CSB-mutated *$Csb^{-/-}$* mice were kindly provided by A. Klungland (Department of Molecular Biology, The National Hospital, University of Oslo, Oslo, Norway). Doubling time is circa 18h.

$Csb^{m/m}Ogg1^{-/-}$ (2.3)

Spontaneously immortalized mouse embryonic fibroblast from *$Ogg1^{-/-}$/$Csb^{-/-}$* double knockout mice were kindly provided by Deborah Barnes (Imperial Cancer Research Fund, Clare Hall Laboratories, South Mimms, Hertfordshire, UK).Doubling time is circa 22 h

Parp-$1^{-/-}$ $Ogg1^{-/-}$

Spontaneously immortalized mouse embryonic fibroblast from *$Parp1^{-/-}Ogg1^{-/-}$* double knockout mice were kindly provided by A. Klungland (Department of Molecular Biology, The National Hospital, University of Oslo, Oslo, Norway). Doubling time is circa 22 h

The MEF cell lines were propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM sodium pyruvate, 100 U/mL penicillin, 100 mg/L streptomycin, and 15% foetal calf serum (all reagents from PAA Laboratories GmbH, Pasching, Austria). Identities of the MEF cell lines were routinely controlled by genotyping of the knockout and wild-type alleles. MEFs were taken for transfections at passage numbers between 15 and 20. The HeLa cervical carcinoma cells were cultivated at 10% foetal calf serum.

2.1.4 Plasmid vectors

plasmid	description	source
pMARS	episomal expression vector	(Jenke et al, 2004)
pEGFP-mODC-ZA	modified pEGFP-C3 expression vector with inverted mouse *Odc* gene exon 10, coding for stable EGFP	A. Khobta, University of Mainz
pDsRed-Monomer-N1	expression vector encoding for pDsRed-Monomer	Clontech (France)

2.1.5 Synthetic oligonucleotides used for construction of the modified plasmid substrates containing 8-oxoG

DNA strand, modification	Sequence (5′ → 3′)
Transcribed strand (TS, bottom),	
no modification	TCAGGGCGGACTGGGTGC
8-oxo-7,8-dihydroguanine (8-oxoG)	TCAGGGC**[8-oxoG]**GACTGGGTGC
Non-transcribed strand (NTS, top)	
no modification	TGAGCACCCAGTCCGCCC
8-oxo-7,8-dihydroguanine (8-oxoG)	TGAGCACCCAGTCC**[8-oxoG]**CCC

2.1.6 Stock solutions and buffers

BEH buffer (modification of BE1)	10 mM Hepes pH 7.5, 200 mM NaCl, 1 mM EDTA
Buffered 2% formaldehyde solution	Freshly prepared by mixing 1 ml 37% HCOH with 17.5 ml PBS CMF (stable for several days at 4°C)
Bovine Serum Albumin (BSA) ,100 µg/ml	New England Biolabs GmbH, Frankfurt an Main, Germany
Cycloheximide, 20 mg/ml in EtOH	20 mg of CHX powder (Sigma)

	1 ml of EtOH, ultrapure
Ethidium bromide solution	stock solution 10 g/L in H_2O, Sigma-Aldrich Co, Seelze, Germany
TE buffer, pH 8.0	10 mM Tris HCl (pH 8.0), 1 mM EDTA (pH 8.0)
G418, Geneticin®	50 mg/ml, stock solution in water, used concentration 800 mg/L in DMEM medium.
Proteinase K stock solution	20 mM Tris HCl (pH 7.4), 1 mM CaCl2, 50% Glycerol
RNAse A (stored frozen)	1 mg/ml in water
RNAse A-supplemented buffer P1	Qiagen GmbH, Hilden, Germany
Phenol solution equilibrated with 10 mM Tris HCl, pH 8.0, 1 mM EDTA,	Sigma-Aldrich Co, Seelze, Germany BioReagent, for molecular biology
TE-saturated phenol:chloroform:isoamyl alcohol (25:24:1)	Sigma-Aldrich Co, Seelze, Germany BioReagent, for molecular biology

2.1.7 Enzymes

Antarctic Phosphatase, (5 U/µl)	New England Biolabs GmbH, Frankfurt am Main, Germany
T4 DNA ligase(30 U/µl), supplied with T4 DNA Ligase buffer (10x)	Fermentas, St. Leon-Rot, Germany
T4 DNA polymerase (5 U/µl)	Fermentas, St. Leon-Rot, Germany
T4 Polynucleotide Kinase (10 U/µl)	New England Biolabs GmbH, Frankfurt am Main, Germany
Nb.Bpu10I/Nt.Bpu10I (5 U/µl each)	Fermentas, St. Leon-Rot, Germany
R buffer (10x)	Fermentas, St. Leon-Rot, Germany
Nb.BsrDI (10 U/µl)	New England Biolabs GmbH, Frankfurt am Main, Germany

DNase I, RNase-free (1 U/µl)	Fermentas, St. Leon-Rot, Germany
Afl III (5 U/µl)	New England Biolabs GmbH, Frankfurt am Main, Germany
Bgl II (10 U/µl)	New England Biolabs GmbH, Frankfurt am Main, Germany
DpnI,(10 U/µl) supplied with 10x Buffer Tango™	Fermentas, St. Leon-Rot, Germany
EcoR I (10 U/µl)	New England Biolabs GmbH, Frankfurt am Main, Germany
ScaI (10 U/µl)	New England Biolabs GmbH, Frankfurt am Main, Germany
Exonuclease III (200 U/µl)	Fermentas, St. Leon-Rot, Germany
PfuTurbo® DNA Polymerase	Stratagene (now Agilent Technologies), Waldbronn, Germany
Recombinant Fpg protein	kind gift from Serge Boiteux

2.1.8 Transfection reagents and equipment

Effectene® Transfection Reagent	Qiagen GmbH, Hilden
Matra-A Reagent	PromoCell GmbH, Heidelberg
Magnetic plate	IBA GmbH, Göttingen

2.2 Methods

2.2.1 Basic DNA and RNA techniques

2.2.1.1 Phenol-chloroform extraction of DNA

Equal volumes of buffer-saturated phenol were added to the DNA solutions in disposable tubes (2 ml Eppendorf® Safe-Lock® tubes or 50 ml Greiner-type polypropylene centrifuge tubes). Samples were vortexed for 1 min and centrifuged for 3 min (3000–5000 × g). Aqueous phase was carefully (to avoid the interface) removed to a new tube. Steps were repeated until any material was no longer visible in the interface. Then samples were

extracted once with equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and once with chloroform to remove the traces of phenol. Aqueous phase was mixed with 0.1 volume 3 M NaOAc, 20 µg glycogen, and 2.5 volumes of ice-cold 95% EtOH. DNA was then precipitated by centrifugation (16000 × g, 30 min, 4°C). Supernatant was carefully removed and pellet washed with ice-cold 70% ethanol. Supernatant was discarded and pellet air-dried 5–10 min (RT). Pellet was dissolved in sterile water or 10 mM Tris-HCl buffer (pH 8.0), as appropriate.

2.2.1.2 DNA precipitation with isopropanol

DNA solution was mixed with 0.1 volume sodium acetate (3 M, pH 5.2), and equal volume of isopropanol was added. The mix was centrifuged 30–45 min at 4°C (10000–15000 × g). The pellets were washed by ice-cold 70% ethanol and centrifuged 15–25 min at 4°C (10000–15000 × g). The pellets were air-dried 5–20 min (depending on the size of the pellet) and dissolved in appropriate buffer (TE or BEH).

2.2.1.3 Agarose gel electroforesis

For analyses of DNA we used 0.8–2% agarose gels, which were prepared and run in TAE electrophoresis buffer. Samples were mixed with 6× loading dye (with 0.1% SDS or without SDS). Hind digested lambda DNA or GeneRuler™ 1 kb DNA ladder (Fermentas, St. Leon-Rot, Germany) were used as molecular size markers. DNA electrophoresis was performed for 46–60 min at 80–100 V. To visualize DNA under UV light the gels were stained with 0.5 mg/L ethidium bromide in TAE buffer for 30 min and rinsed briefly in deionized water. Images of the gels were taken with a Gel Doc 1000 Molecular Imager and analysed with Molecular Analyst Software. For quantitative analyses care was taken that the fluorescence signal in single bands does not reach the detection saturation.

2.2.1.4 Alkaline agarose gel electrophoresis

Electrophoresis of separate DNA strands under alkaline conditions was applied for analyses of the components of the second strand synthesis reactions. Alkaline electrophoresis was performed in 0.8% agarose gels. Agarose was melted in water and polymerised gels equilibrated in the alkaline electrophoresis buffer (50 mM NaOH, 1 mM EDTA). Sample loading buffer (10×) contained 0.3 M NaOH, 2 mM EDTA, 10% glycerol, 0.25% bromcresol green. Gels were run for 120–150 minutes at a relatively low voltage (2 V/cm). The gels pH was neutralised 45 min in 0.1 M Tris-HCl (pH 8.0), followed by staining with ethidium bromide, as described above.

2.2.1.5 RNA denaturing agarose gel electrophoresis

The isolated RNA samples (4 µl of the 25 µl column eluates) were mixed in safe-lock Eppendorf tubes with 4 µl of 2× RNA loading dye (Fermentas, catalog # R0641) and denatured at 65–70°C for 5 minutes (longer time up to 15 minutes can result in better denaturation). Then RNA samples were run in 1% TAE gel (prepared with DEPC water) 1 hour at 100 V in the Bio-Rad 15×10 cm chamber. Marker was prepared by mixing 8 µl GeneRuler™ 1 kb DNA ladder (Fermentas) with 8 µl RNA loading dye (without heating).

2.2.1.6 Purification of DNA fragments with the QIAEX II system

PCR products were run in 1.8% agarose gel, the bands cut with a razor blade, and DNA extracted with QIAEX II gel extraction kit following the Qiagen standard protocol. Eluted DNA was precipitated with EtOH and stored at -20°C.

2.2.2 Construction of stably replicating episomal vectors for analyses of gene expression in the presence of DNA

2.2.2.1 Preparative PCR of a fragment of mODC exon 10

The mODC exon 10 fragment for cloning was amplified by the preparative PCR (6 reactions) with PfuTurbo® DNA Polymerase in 50 µl reaction volume in 0.2 ml thin-wall PCR-tubes. Reactions were set by mixing 48 µl master mix (**Table 2.1**) and 2 µl template DNA (20 pg pAZ plasmid with a cloned mODC fragment). Forward primer (5′-TCTCATGAAGCAGATCCAGA) contained an extra T on its 5′ end to reconstitute the restriction site for endonucleases BglII at 5′ end of the ODC-coding DNA sequence. The restriction site for the BglII endonuclease on the ODC 3′ side was converted to a ScaI restriction site by PCR with the reverse primer 5′-**TAG**TACTCAT**CTA**CACATTGATCCTAG (nucleotide sequence corresponding to the mouse ODC gene is underlined). Stop codons (bold typeset) for the two possible insert orientations were included in the reverse primer. Cycling program is reported in **Table 2.2**. PCR products from six reactions were pooled together, and the DNA band of specific size (152 bp) was purified from an agarose gel with the QIAEX II gel extraction kit and quantified for subsequent ligation into the pMARs vector.

Table 2.1 PCR reaction components for cloning of the mODC gene fragment.

Reagent	End concentration
buffer 10× (Mg-)	1×
dNTPs mix (10mM each)	0.8 mM each
ddH_2O	
primer (forward) , 2µM	0.4 µM
primer (reverse) , 2µM	0.4 µM
Pfu-TURBO DNA pol, 2.5U/µl	0.05 U

Table 2.2 PCR program to amplify the mODC exon 10 fragment.

Step	Temperature	Time		Functions
	4°C	∞		
1	95°C	2 min		Initial denaturation
2	95°C	30 s		Denaturation
3	50°C	45 s		Annealing
4	72°C	45 s	to 2, 32×	Primer extension
5	72°C	10 min		Final extension
7	4°C	∞		Final hold

2.2.2.2 Digestion of pMARs vector with the restriction endonuclease Bgl II

5 µg of pMARS vector was digested with 30 units of BglII in 1× NEBbuffer 3 in a reaction volume of 150 µl. The reaction was incubated in a TGradient Thermocycler at 37°C for 2 hours. After digestion plasmid DNA was extracted by the phenol/chloroform method, as described above (2.2.1.1).

2.2.2.3 Blunting the ends of linearized pMARS by filling-in the overhangs

The linearized pMARs was blunted by filling-in the 5′ overhangs produced by BglII with T4 DNA Polymerase in 35 µl volume of the following reaction (**Table 2.3**). Reaction was incubated 15 minutes at 12°C and was stopped by adding EDTA to a final concentration of 10 mM and heating to 75°C for 20 minutes

Table 2.3 Fill-in reaction components.

Component	(µl) / sample
linearized pMARS (100 ng/µ)l	25
NEB buffer 2 (10×)	3.5
BSA *(*100 µg/ml)	0.5
dNTPs (1 mM of each)	3.5
T4 DNA Polymerase, 3 units/µl	1
ddH_20	2.5

2.2.2.4 Removal of 5′ phosphate groups from blunted vector with Antarctic Phosphatase

To prevent self-ligation, the vector was dephosphorylated with Antarctic phosphatase (AP) in 50 µl of the following reaction (**Table 2.4**). Dephosphorylation proceeded at 37°C for 1 hour and followed by heat inactivation of the enzyme 20 minutes at 65°C

Table 2.4 The components of vector dephosphorylation reaction.

Component	(µl) /sample
Antarctic phosphatase (5 units/µl)	2.5
Antarctic phosphatase buffer (10X)	5
pMARS, linearized and blunted (100ng/µl)	25
NEB buffer 3 (10×)	5
ddH_20	12.5

2.2.2.5 Ligation of the 5′-phosphorilated insert into the dephosphorylated blunted vector

Agarose gel-purified PCR products of the fragment of the mODC exon 10 were ligated into the linearised blunted pMARs vector in random orientations. Two different molar ratios of insert to vector (1:1 and 3:1) were used in parallel with a “no insert” control reaction (**Table 2.5**). Ligations were performed at 16°C for 17 hours in a TGradient Thermocycler.

Table 2.5 Ligation conditions for cloning of the ODC gene fragment.

molar [insert] :[vector] ratio	0 (no insert)	1	3
linearised dephosphorylated vector (50 ng/µl), µl	8	8	8
Insert (4 ng/µl), µl	-	3.5	10
10× ligase buffer, µl	4	4	4
ddH_2O, µl	26	22.5	16
T4 DNA ligase, µl	2	2	2
final volume, µl	40	40	40

2.2.2.6 Three-primer PCR screening of transformants to determine the insert orientation

Transformed bacteria (15 clones) were screened for the presence and orientation of mODC insert by three-primer PCR with primers 5′-GACCACTACCAGCAGAACAC (GFP), 5′-TCTCCTGGGCACAAGACAT (AZ-ODC), and 5′-GCCTGTGCTTCTGCTAGGAT (ZA-ODC). Clones obtained from ligation with molar insert to vector ratios 1:1 (7 clones) and 3:1 (8 clones) were named 1.1−1.7 and 3.1−3.8, respectively. PCR reactions were performed in volume of 25 µl with 2 µl of boiled *E. coli* suspension from each clone as a template. pEGFP-ODC_AZ and pEGFP-ODC_ZA plasmids (2 ng) were used as PCR positive controls. PCR was performed for 30 cycles with annealing temperature of 60°C. Other conditions were as described above (**Table 2.2**). PCR products were analysed by electrophoresis in 1.5 % agarose gel.

Table 2.6 Three-primer PCR reaction components.

Reagent	End concentration
buffer 10× (Mg-)	1×
$MgCl_2$ 50 mM	1.5 mM
dNTPs mix (10 mM each)	0.8 mM each
Primer 1, 2 µM	0.2 µM
Primer 2, 2 µM	0.2 µM
primer 3, 2 µM	0.2 µM
DNA pol, 5 U/µl	0.05 U

2.2.2.7 Screening of pMZA and pMAZ plasmids by restriction analyses with BglII and ScaI

Plasmids from 6 selected clones obtained by ligation and confirmed by three-primer PCR for the presence and orientation of mODC insert were isolated by GenElute Plasmid Miniprep Kit (Sigma Aldrich) from 5 ml overnight cultures of recombinant *E. coli* as specified by the kit manufacturer. Samples were eluted in 75 µl of TE buffer (pH 8.0). Isolated plasmids from six clones were screened by restriction analyses with BglII and ScaI restriction enzymes to check for possible degradation of DNA ends at the ligation sites. In the first step plasmids were digested with BglII restriction enzyme (as described in 2.2.2.2) in a reaction volume of 50 µl. The pMARs plasmid was used as a control of digestion. After the reaction, 12 µl of samples (prepared by combining 50 µl reaction volumes with 10 µl loading buffer containing 1% SDS) were checked for complete digestion in 1% agarose gel. Remained sample volumes were extracted with phenol:chlorophorm:isoamil alcohol and DNA precipitated with ethanol. Dry plasmids pellets were resuspended in 34 µl ddH_2O. 1 µl BSA (100 µg/ml) and 4 µl 10× NEB Buffer 3 were then added to samples. Then every sample was split in two equal portions, one of which was further digested with ScaI (according to the protocol suggested by the manufacturer).

2.2.2.8 Sequencing of plasmid DNA

The plasmid DNA from several selected positive clones (each “AZ” and “ZA”) was isolated using the miniprep kit from Sigma and eluted in 75 µl TE (pH 8.0). Then 3 µl of plasmid DNA from each clone was mixed with 3 µl of 0.2 µM solution of the “pEGFP-C3_1153_1” sequencing primer (5′-GACCACTACCAGCAGAACAC) and sent for sequencing to GENterprise GmbH (University Campus Mainz). The obtained sequence data was manually

edited using the Staden Package Trev 1.9 Software and aligned by ClustalW2 with the reference sequence.

2.2.2.9 Transient transfection of HeLa cells with the help of Effectene®

Exponentially growing cells were taken for transfections. 500000−700000 HeLa cells were plated 16−24 hours before transfection per well of the 6-well plates (Nunc, Wiesbaden, Germany). Directly before transfection the medium was substituted with a fresh one (1.6 ml per well). Cells were transfected with 800 ng supercoiled plasmid DNA with the help of Effectene® (Qiagen, Hilden, Germany). Effectene® transfection mix was prepared at room temperature as indicated in **Table 2.7**. Different concentrations of vector DNA could be used as well, however I recommend to transfected at least 400 ng of plasmid DNA per well for the optimal transfection efficiency. The transfection medium containing plasmid DNA and transfection reagent was replaced with the fresh medium after 4 hours. Transfected cells were analysed by flow cytometry at 24 hours after the transfection start. Alternatively, the cells were monitored under the direct Nikon Eclipse 400 fluorescence microscope (blue excitation) equipped with a 5× objective, sufficient to observe the transfected cells as fluorescent dots. An inverted fluorescence microscope would be required to observe the cells under higher magnification.

Table 2.7 Pipetting chart for transfections with Effectene®.

Wells to transfect (6-well plate) / Reagent	1	4
Supercoiled Vector DNA (50−100 ng/ul in BEH or TE)	8	32
EC buffer (Qiagen), µl	100	400
Enhancer (Qiagen), µl	3.2	12.8
	>>Vortex briefly, leave 2−5 minutes, RT<<	
EFFECTENE reagent (Qiagen), µl	10	40
	>>Vortex 10 s, leave 5−10 minutes, RT<<	
Full medium (with FCS), µl	600	2400
Final transfection mix volume, µl	721.2	2884.8
	>>Add dropwise to culture (gently swirling)	
Volume to add per well, µl	700	700

2.2.3 Generation and analyses of stably transfected cell lines expressing stable or short-lived green fluorescent protein (GFP)

2.2.3.1 Permanent transfection of HeLa cells with pMAZ (clone 3.6) and pMZA (clone 3.7) plasmids

Confluent HeLa cells were plated 24 hours before transfection at 500000 cells per well in 6-well plates. The cells were transfected with 800 ng supercoiled pMAZ-ODC or pMZA-ODC plasmids with the help of Effectene® as described in 2.2.2.8. 24 hours after transfection the medium was removed and the cells were washed with PBS buffer, trypsinised and collected in PBS supplemented with 1% FCS. The cells expressing EGFP were separated by fluorescence-activated cell sorting (FACS) using a BD FACSVantage™ cell sorter (Becton Dickinson, GmbH, Heidelberg, Germany). The sorted cells were plated in the 145×20 mm Cellstar tissue culture dishes (Greiner Bio-One, Frickenhausen, Germany) with the selection medium containing 800 mg/L G418. Single fluorescent clones were identified under a microscope, picked, and expanded under the selection pressure for 4 weeks. Corning® cloning cylinders (Corning, Fogostraat, Netherlands) were used to pick single clones (as described in manufacturer's protocol).

2.2.3.2 Fixation of cells with formaldehyde

Cells were formaldehyde-fixed prior to FACS analyses to avoid any uncontrolled GFP degradation during the sample processing or storage. To this end, cells were detached with 0.45 ml trypsin, resuspended by repeated pipetting after addition of 0.55 ml of cold medium, and collected in the 2 ml safe-lock disposable tubes. Cells were pelleted in a benchtop microcentrifuge 40 s and washed once with ice-cold phosphate-buffered saline (PBS). Wet pellets were resuspended in 0.45 ml ice-cold PBS (calculating per one well of a 6-well plate) by mild vortexing (≤70% power setting, 15 seconds) in order to obtain a single-cell suspension, instantly mixed with 0.5 ml ice-cold 2% formaldehyde solution prepared in PBS, and allowed to fix 12–24 h in the refrigerator. All centrifugation steps were at 250 × g. On the next day, the fixed cells were washed with PBS, resuspended in suitable volumes of PBS (≥0.4 ml) and analysed by flow cytometry. The samples were kept ice-cold at all times till analysed. The use of the original Eppendorf tubes is advisable in order to avoid the loss of cells during the centrifugation steps. The described fixation procedure had no detectable effect on the EGFP fluorescence.

2.2.3.3 Detection of expression of the short-lived EGFP-ODC protein in the permanently transfected HeLa cell clones by flow cytometry

GFP fluorescence in cells was measured by flow cytometry. The FACSCalibur^{TM} instrument was calibrated by the non-transfected HeLa cells. The fluorescence threshold (FL1-H) was arbitrarily chosen to cut off 99.9% of non-transfected cells. Cells stably transfected with pEGFP-ODC-MAZ plasmid were analysed under these settings, thus defining a fraction of the cells expressing significant EGFP levels. The effect of DNA damaging agents on the EGFP transgene expression was determined as change in a fraction of the EGFP-positive cells, relative to the undamaged control.

2.2.3.4 DNA damage induction in the permanently transfected HeLa-MAZ and HeLa-MZA cells

Cells were plated 16–20 hours before the experiments at (3–3.2) $\times 10^5$ per well of the 6-well plates. UV-C damage was generated by the 254 nm single band lamp covered with a metal grid in order to minimise the intensity. The medium was fully removed immediately before irradiation and plates irradiated against black background at the fluency of 0.3125 J/(m²×s) , which corresponds to 60 nA dosimeter Luxmeter 110 reading. Following the addition of the fresh medium, the plates were incubated under standard conditions, the cells fixed after the specified time intervals as described in 2.2.3.2, and the EGFP expression analysed by flow cytometry.

Oxidative damage was induced by visible light from the halogen lamp (Osram SLG 1000W, Osram, München) from the distance of 38 cm in the presence of Ro19-8022 as a photosensitiser. The cells were rinsed with ice-cold PBS and irradiated on ice after addition of 2 ml (per well of a 6-well plate) PBS containing desired concentrations of Ro18-8022. Controls were irradiated in PBS without the photosensitiser. Further procedures were as described for UV damage.

2.2.4 Induction of base modifications in plasmid DNA by photosensitisation and quantitative analyses of expression of reporter genes transfected into mammalian host cells

2.2.4.1 Generation of 8-oxo-7,8-dihydroguanine in plasmid DNA by photosensitisation in the presence of methylene blue

Covalently closed plasmids were damaged with light from a halogen lamp (Philips PF811 or Osram SLG 1000W) from the distance of 90–100 cm in the presence of 0.8 µM methylene blue (Sigma-Aldrich, Taufkirchen, Germany). Average numbers of strand breaks (SSB) were calculated by the Poisson formula from the ratios of the amounts of covalently-closed and

nicked plasmid molecules determined after separation of the two forms by gel electrophoresis. Oxidised guanines were quantified after full conversion into SSB by treatment with an excess of specific DNA repair endonuclease Fpg as previously described (Epe et al, 1993; Will et al, 1999), (Speckmann, 2006). The sites of base loss were detected in an analogous approach using endonuclease IV as a probe.

2.2.4.2 Generation of cyclobutane pyrimidine dimers in plasmid DNA by UV

UV photoproducts, mostly cyclobutane pyrimidine dimers (CPD) and some 6-4 photoproducts, were generated by UVB as previously described (Flohr et al, 2003). The average numbers of pyrimidine photoproducts per plasmid molecule were determined by the plasmid relaxation assay with T4 endonuclease V as a probe. Up to 3 CPD per plasmid molecule were quantified directly from the proportion of the residual plasmid DNA in the covalently closed form. Higher numbers of T4 endonuclease V-sensitive lesions per plasmid molecule were generated by proportionally increasing the UV dose, assuming a linear dose-effect relationship (Speckmann, 2006).

2.2.4.3 Transfections of MEF cell lines

MEF cell lines were subjected to magnet-assisted transfection with MATra-A system (IBA, Göttingen, Germany). 16−24 hours before transfection 180000−210000 cells were plated in 2 ml medium per well in 6-well plates. Co-transfections were done with equal amounts (1.5 µg) of the specified GFP-encoding plasmids (non-damaged or carrying the indicated numbers of DNA lesions) and non-damaged reference plasmid encoding for DsRed-Monomer. The plasmids DNA volumes were adjusted (in a Greiner Tube) with a supplement-free DMEM to 200 µl per each well. Then MATra-A reagent was added to the DNA solution (1 µl MATra-A per 1 µg DNA). The samples were vortexed or mixed by pipetting and left for incubation for 20 minutes at room temperature. Full DMEM (2 ml per well) was added to the transfection mix. Cell conditioning medium was then substituted with the transfection mix. The cells were transfected for 20 minutes at the magnet plate. After transfection the medium was immediately replaced with pre-warmed fresh full medium. At 24 hours after transfections (unless otherwise specified) cells were fixed as described in 2.2.3.2. Green fluorescent protein (GFP) fluorescence was visualised by fluorescence microscopy and quantitatively measured by flow cytometry. For simultaneous mRNA and protein expression analyses, the cells were split 4 hours post transfection, and each portion was processed accordingly.

2.2.4.4 Quantitative EGFP expression analyses by flow cytometry

Cells were fixed with formaldehyde prior to FACS analyses as previously described (2.2.3.2). Non-transfected cells were used for setting up the photomultiplier tube voltages. GFP expression was measured in the fluorescence detection channel FL1 compensated from the

FL2, and DsRed-Monomer expression at the channel FL2 compensated from FL1. Fluorescence compensation settings were calibrated with the cells transfected separately with each plasmid and were the same in all experiments. For the quantitative gene expression analyses, EGFP fluorescence was only measured in cells that expressed high levels of DsRed-Monomer protein and therefore exhibited red fluorescence (FL2) above a threshold level of 100. This approach allowed exclusion of non-transfected cells from analyses, thus minimising the interexperimental variability resulting from the fluctuating transfection efficiencies. Average GFP expression per transfected cell was quantified as mean FL1 fluorescence.

2.2.4.5 Quantification of the EGFP mRNA in MEFs after transfections with plasmids damaged by photosensitisation

Total cellular RNA was isolated with the help of TRIZOL® Reagent (Invitrogen, Karlsruhe, Germany) and treated with DNase I (Fermentas, St. Leon-Rot, Germany). DNaseI was removed by phenol extraction, RNA precipitated with isopropanol, and its integrity was confirmed by denaturing agarose gel electrophoresis. First strand of the complementary DNA (cDNA) was produced by Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas, St. Leon-Rot, Germany). Efficiency of reverse transcription was confirmed with a control 1.1 kb mRNA. Oligo$(dT)_{18}$ primer and the control RNA supplied by the kit manufacturer. cDNA samples were diluted fivefold and analysed by real-time quantitative PCR. In parallel, the samples that were not subjected to reverse transcription ("no-RT" control) were analysed in order to detect potential contamination with plasmid DNA. Real-time PCR was performed for 30 cycles using LightCycler® 1.5 system and a LightCycler® FastStart DNA MasterPLUS SYBR Green I kit (Roche Diagnostics, Mannheim, Germany). Primers specific for EGFP-mODC-ZA cDNA were: 5′-GACCACTACCAGCAGAACAC and 5′-GCCTGTGCTTCTGCTAGGAT. Primers for DsRed-Monomer were: 5′-CCTCCACCGAGAAGCTGTA and 5′-TCCACCACGGTGTAGTCCT. Cycling program is reported in **Table 2.8**. Standard curves were generated by at least four points obtained from amplification of known amounts of plasmid DNA (serial dilutions). The specificity of PCR products was confirmed by the melting curve analyses (performed accordingly to the manufacturer's instruction). Relative EGFP-mODC-ZA gene expression from the damaged plasmid was calculated for every cell line as the ratio of mean EGFP-mODC-ZA cDNA levels between the cells transfected with the damaged and non-damaged plasmids. These ratios were corrected for the levels of DsRed-Monomer cDNA in corresponding samples. The levels of DsRed-Monomer cDNA thus served as internal reference for the efficiencies of transfection, mRNA preparation and reverse transcription in the samples.

Table 2.8 Real-time PCR cycling program.

Step	Temperature	Time	option
1	95°C	10 min	
2	95°C	10 s	
3	65°C	10 s	
4	72°C	15 s	
5	88°C	5 s	to 2, 30 times*
6	65°⟶95°C	10 s	
7	40°C	30s	20°C/sec

*fluorescence acquired at the end of every cycle.

2.2.5 Experimental procedures for construction of vectors suitable for incorporation of single modified bases into different strands of the EGFP gene

2.2.5.1 Inversion of the f1 replication origin of the pEGFP-mODC-ZA plasmid

To allow directional insert ligation into vector, the polarities of the two available AflII sites were inverted as following. The pEGFP-mODC-ZA vector was amplified by PCR with the Pfu TURBO proofreading DNA polymerase (Stratagen), using the primers, containing the inverted AflIII sites on their 5′ ends: ZAss-minus-vect-A1 (5′-CTTAACATGTAAATTGTAAGCGTTAAT) and ZASS_minus-vect-A2 (5′-AGAACGCGTAAAGGCCAGGAACCGTAAA). Reactions were prepared in 0.2 ml PCR tubes on ice with sample volume of 25 µl, using 20 pg linearised pEGFP-mODC-ZA plasmid as a template. PCR master mix was prepared for 20 samples, calculating the final concentrations of the components as specified in **Table 2.9**. Primers annealing temperature was 60.2°C and extension/elongation steps were repeated 30 times. Other conditions were as described above (**Table 2.2**).

After preparative PCR reaction products were purified by the MinElute Reaction Cleanup Kit following the standard protocol of the manufacturer and eluted in 40 µl of 10 mM Tris HCl (pH 8). Then 30 µl of eluted PCR products were incubated with 10 units of restriction endonuclease AflIII (restriction sites 5′-A^CATGT and 5′-A^CGCGT) in total reaction volume of 50 µl at 37°C for 1 hour to produce the sticky ends for ligation. The AflIII-digested 3033 bp

PCR product was purified from the agarose gel, combined with the 1849 bp. DNA fragment obtained by digestion of the pEGFP-mODC-ZA plasmid with AflIII, and ligated at different temperatures (25°C and 16°C). A three-fold molar excess of the fragment containing the EGFP gene was used for ligation (25°C, 10 min).

Table 2.9 PCR reaction components for the pEGFP-mODC-ZA vector amplification.

Reagent	End concentration
buffer 10× (Mg-)	1×
dNTPs mix (10 mM each)	0.8 mM
primers (2 µM each)	0.4 µM
Pfu-TURBO DNA pol, 2.5 U/µl	0.05 U/µl

2.2.5.2 PCR screening of the pEGFP-mODC-ZA clones containing the f1 origin of replication in “minus” and “plus” orientations

Competent *E. coli* SCS-8 cells were transformed with the ligation reaction products. Colonies obtained were expanded in 700 µl LB medium with 30 µg/ml kanamycin and allowed to grow for 6 h or overnight. 50 µl of bacterial suspensions were spin down, pellets resuspended in 200 µl water and lysed by boiling 10 min. The obtained lysates of bacterial clones (2 µl) were directly used as templates for PCR screening with two primer pairs. 2 ng of pure pEGFP-mODC-ZA plasmid was used as a positive control for the “plus” orientation of the EGFP gene with respect to the f1 replication origin. Primer combinations used and the PCR product sizes for both “minus” and “plus” orientations are listed in **Table 2.10**.

Table 2.10 PCR primers for screening of the pZA(-) and pEGFP-mODC-ZA bacterial clones.

Primers name	Primers sequence 5′ to 3′	expected size of PCR product, bp.
pZA-PLUS-A1	TTCGCCACCTCTGACTTGA	**173**
pZA_PLUS-MIN-A2	GGCGGTAATACGGTTATCCAC	“plus orientation”
pZA-MINUS-A1	GACGTTGGAGTCCACGTTCTT	**238**
pZA_PLUS-MIN-A2	GGCGGTAATACGGTTATCCAC	“minus orientation”

PCR master mixes were prepared for each pair of primers, as specified in **Table 2.11**. PCR was performed in a TGradient Thermocycler. Primers annealing temperature was 60°C and extension/elongation steps were repeat 32 times. Other conditions were as described above (**Table 2.2**). PCR products were analysed by electrophoresis in 2.2 % agarose gel.

Table 2.11 PCR reaction components for the insert orientation screening.

Reagent	End concentration
buffer 10× (Mg-)	1×
$MgCl_2$ 50 mM	1.5 mM
dNTPs mix (10 mM each)	0.8 mM each
Primer 1, 2 µM	0.2 µM
Primer 2, 2 µM	0.2 µM
Taq "Invitrogen" DNA pol, 5U/µl	0.05 U

2.2.5.3 Characterisation of the pZA(-) plasmid by digestion with AflIII and transfection into the HeLa cells

To check for possible degradation of DNA ends at the ligation sites the newly generated pZA(-) plasmid (also referred to as pZASS in the working protocols) was digested with AflIII restriction endonuclease in a following reaction. Plasmid DNA (200 ng) was incubated in 1× NEB buffer 3 supplemented with 100 µg/ml BSA and AflIII enzyme (2.5 Units) at 37°C for 2 hours in a reaction volume of 25 µl. The enzyme was killed by heat inactivation (80°C, 20 min).

To verify that the EGFP gene in pZA(-) plasmid can be efficiently expressed, HeLa cells were transfected with the plasmid. Transfections were done in parallel with 400 ng/well of pZA(-) or control pEGFP-mODC-ZA plasmid DNA in a 6 well plate. 24 hours after transfections cells were fixed as described (2.2.3.2) and analysed by flow cytometry.

2.2.6 Annealing and extension of synthetic oligonucleotides on a single-stranded circular DNA template as a method for incorporation of 8-oxoG into plasmid DNA

2.2.6.1 Procedure for isolation of circular ssDNA containing the coding strand of the EGFP gene from the M13 helper phage

The overnight culture of *E. coli* transformed with pZA(-) vector (also referred to as pZASS, "ss" standing for "single-stranded") was grown from an individual kanamycin-resistant colonies picked from a fresh plate. 2 ml of LB broth containing 30 mg/L kanamycin was inoculated and shaken at 37°C overnight. The next day 400 ml of LB broth containing 30 mg/L kanamycin was inoculated with 200 µl of the overnight culture and shaken vigorously (180 rpm) with good aeration at 37°C for 3 hours in a 2000 ml flask. The bacterial culture was infected with 400 µl helper phage M13K07 (10^{11} pfu/ml, NEB Frankfurt am Main, Germany), shaken vigorously (200 rpm) for good aeration at 37°C. Followed the 2-hour incubation, kanamycin concentration was adjusted to 85 µg/ml (733 µl of 30 mg/ml kanamycin solution added to the infected bacterial culture). After 18−24 hours vigorous agitation supernatant was harvested by pelleting the cells (7500 × g, 20 minutes, 4°C) and filtered through a 0.2 µm filter (Nalgene® Labware). Phage particles were precipitated by adding 0.25 volume of phage precipitation solution (3.75 M ammonium acetate pH 7.5 and 20% PEG 8000) and incubation on ice for ≥1 hour. Then solution was centrifuged (9000 rpm in a Beckman Ti-60 rotor for 20 minutes) .The supernatant was drained and pellets resuspended in 3.8 ml TE buffer (pH 8.0) supplemented with 80 µl RNAse A (1 mg/ml), followed by incubation 20 minutes at 37°C. Then 80 µl SDS (10%) and 40 µl Proteinase K (20 mg/ml) were added. The samples were further incubated 2 hours at 50°C followed by phenol/chloroform extractions according to a standard procedure (2.2.1.1). Aqueous phase was mixed with 0.5 volume 7.5 M ammonium acetate (pH 7.5) and 2.5 volumes of ice-cold 95% EtOH and left at -20°C for 30 min. The phagemid DNA was precipitated by centrifugation (20000 × g, 20 min, 4°C). Pellets were resuspended in ddH_2O, and concentration and purity of ssDNA were estimated by spectrophotometry.

2.2.6.2 Treatment of ssDNA isolated from phage with EcoRI

To avoid the carry-over of minor quantities of double-stranded plasmid DNA of bacterial origin, which were shown to contaminate the ssDNA isolated from M13 helper phage, the ssDNA preparations were treated with the EcoRI restriction endonuclease, which has one recognition site 5′-GAATTC in pZAss plasmid. 20 units of EcoRI (NEB) were incubated with 1 µg of total DNA in 1× EcoRI buffer for 2 hours at 37°C (40-fold overdigestion). The enzyme was heat inactivated 20 min at 65°C. DNA was extracted with phenol:chloroform:isoamyl

alcohol (25:24:1) and precipitated by addition of 0.5 volumes 7.5 M ammonium acetate and 2.5 volumes of ice-cold 95% EtOH.

2.2.6.3 Generation of DNA strand-specific nicks with endonuclease Nb.BsrDI (NEB)

Both pZA and pZAss plasmids contain unique recognition sequences for the BsrDI restriction endonuclease downstream from the EGFP gene. However, the orientations of the DNA sequence motif recognised by BsrDI (5′-CATTGC) are different in the two plasmids with respect to the EGFP gene direction. Consequently, different DNA strands with respect to the EGFP gene are cut in the two plasmids by an available Nb.BsrDI nicking mutant of the restriction nuclease, which specifically cuts one DNA strand 5′ from the 5′-CATTGC sequence (**Table 2.12**).

Table 2.12 Orientations of the nicking sites with respect to direction of the EGFP gene transcription.

plasmid	EGFP strand nicked
pZA	template (-)
pZAss	non-template (+)

Reaction conditions for nicking of pZA and pZAss plasmids with Nb.BsrDI are shown in **Table 2.13**. Plasmid DNA was incubated with Nb.BsrDI enzyme (NEB, Frankfurt am Main) 2 hours at 65°C. Reactions (**Table 2.13**) were prepared in ice and stopped by heat inactivation 20 min at 80°C. The complete relaxation of plasmid DNA was controlled by agarose gel electrophoresis (0.8% agarose in TAE buffer). Only plasmid DNA that demonstrates complete conversion of the supercoiled form into the open circular form was taken for subsequent degradation by Exonuclease III (**Figure 2.1**).

Table 2.13 Nicking conditions of plasmid DNA with Nb.BsrDI.

reaction component	concentration	volume
Plasmid DNA in BEH buffer	100 ng/µl	1000 µl
NEB buffer 2	10×	113 µl
Nb.BsrDI	10,000 units/ml	10 µl

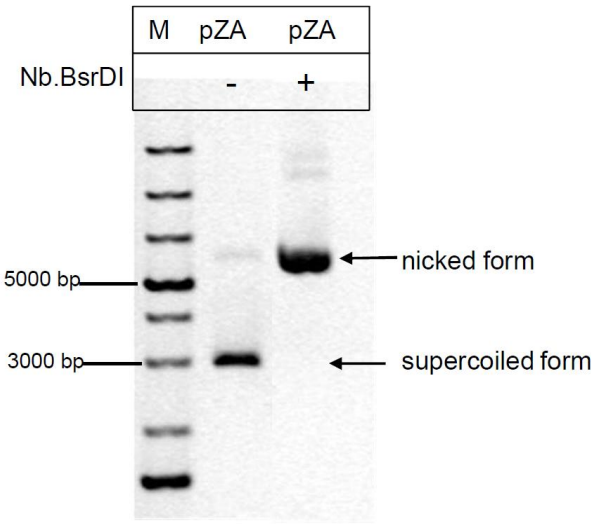

Figure 2.1 Nicking of pZA plasmid DNA with Nb.BsrDI nicking endonuclease.

2.2.6.4 Procedure for production of circular ssDNA by degradation of the nicked plasmid strand by Exonuclease III

Plasmid DNA nicked by Nb.BsrDI was mixed on the ice with exonuclease III (5000 units for 100 µg nicked plasmid). No buffer adjustment was required after heat inactivation of the Nb.BsrDI digestion reactions. Nicked DNA was incubated with exonuclease III 16 hours at 37.9°C. Enzyme was killed by heat inactivation 20 min at 70°C. Obtained ssDNA was extracted with phenol/chloroform and precipitated with isopropanol (described in 2.2.1.1 and 2.2.1.2). DNA pellet was dissolved in nuclease-free water and quantified by spectrophotometer. The homogeneity of ssDNA was controlled by agarose gel electrophoresis (0.8% agarose in TAE buffer).

2.2.6.5 Procedure for production of covalently closed plasmids by primer extension around a circular single-stranded DNA template and ligation

Template ssDNA was isolated from M13 Helper phage (Section 2.2.6.1) or obtained by combined treatment of plasmid DNA with BsrDI and exonuclease III (Section 2.2.6.4) .Synthetic oligonucleotides containing no base modifications or 8-oxoG were used as primers in the strand extension reactions catalysed by T4 DNA polymerase (Fermentas). The list of synthetic oligonucleotides used for construction of the modified plasmid substrates is shown in **Table 2.14**.

Table 2.14 The list of synthetic oligonucleotides used for annealing and extension on the circular ssDNA templates.

Primer	Sequence
pNK1_synth915-t_G906	5′- [Phos]GAAGAAGATGGTGCGCTCCTGGAC
PNK1_SYNTH915-T_8OG906	5′- [Phos]GAAGAAGAT[8oxoG]GTGCGCTCCTGGAC
PNK1_SYNTH771-T_G762	5′- [Phos]CTTGCCGGTGGTGCAGATGAACTT
PNK1_SYNTH771-T_8OG762	5′- [Phos]CTTGCCGGT[8oxoG]GTGCAGATGAACTT

The oligonucleotides used in the early stages of the work were 5′-phosphorylated (**Table 2.14**). However, it was later found out that the product yield was improved by addition of polynucleotide kinase to the reactions. Non-phosphorylated primers were equally efficient under these conditions. The reactions were performed in two steps. First, a primer was annealed together with the circular ssDNA template. Then the enzymes (T4 DNA polymerase, T4 polynucleotide kinase, and T4 DNA ligase) were added, and primer extension and strand ligation reactions were performed in a single step.

Annealing reactions (**Table 2.15**) were prepared on ice in 0.2 ml in PCR tubes in a 100 µl reaction volume. The temperature conditions of annealing are specified in **Table 2.16**. Following the completion of annealing, 1 µl of T4 polynucleotide kinase (10 U/µl), 1 µl of T4 DNA ligase (30 U/µl) and 2 µl of T4 DNA polymerase (5 U/µl) were added to the reactions. The second strand polymerization and ligation of plasmid DNA were then performed in TGradient Thermocycler (10 minutes at 12°C followed by 15−20 hours at 37°C). Reactions were stopped by heat inactivation (65°C, 10 minutes).

Table 2.15 Reaction set-up for annealing of the synthetic oligonucleotides on the circular ssDNA templates.

Reagent	final concentration
ssDNA in H_2O	100 µg/ml
dNTP	1 mM
ATP	1 mM
Ligase buffer	1×
primer	800 nM

Table 2.16 Standard temperature mode for annealing of synthetic oligonucleotides on the circular ssDNA templates.

Step	Target temperature	Incubation time	Function
1	4°C	∞	
2	90°C	1 min	melting
3	50°C	1 min	annealing 1
4	70°C	5 min	melting of false-primed
5	30°C (ΔT=-0.02°C/min)	1 min	slow accurate annealing
6	4°C	∞	

Products of the primer extension/strand ligation reactions were isolated by extraction with phenol-chloroform (described 2.2.1.1) and isopropanol precipitation (described 2.2.1.2). Alternatively, the ligation products were conveniently purified with a Qiagen MinElute Reaction Cleanup Kit following standard protocol from manufacturer and eluted in 40−80 µl TE.

2.2.6.6 Separation of covalently closed circular plasmid DNA in agarose gels

The band of covalently closed circular plasmid DNA migrates in ethidium bromide-containing agarose gel as supercoiled form and thus can be separated from plasmid DNA in linear and open circular forms. Products of the extension/strand ligation reactions (≥95% of the total reaction product) were loaded in a wide well (two or three joined wells) of a 0.8% agarose gel containing EtBr, along with a small aliquot of the same reaction (loaded in the next well) and with supercoiled plasmid DNA isolated from bacteria. DNA was separated at 5−8 V/cm for 90−120 min. The piece of the gel with a wide well containing the most of the reaction product (“Part 1”) was cut with a scalpel and separated from the rest of the gel in order to avoid the DNA exposure to UV light of the gel-imager transilluminator lamp. The rest of the gel (“Part 2”) containing the control aliquot of the reaction product, supercoiled plasmid DNA, and a molecular size marker was photographed under UV-light along with a transparent plastic ruler to make a note of the migration distance of the band of interest from the well. After excision of the appropriate position in the gel, Part 1 was aligned with the rest of the gel to verify the excision of the band corresponding to the covalently closed plasmid DNA, as shown in **Figure 2.2**.

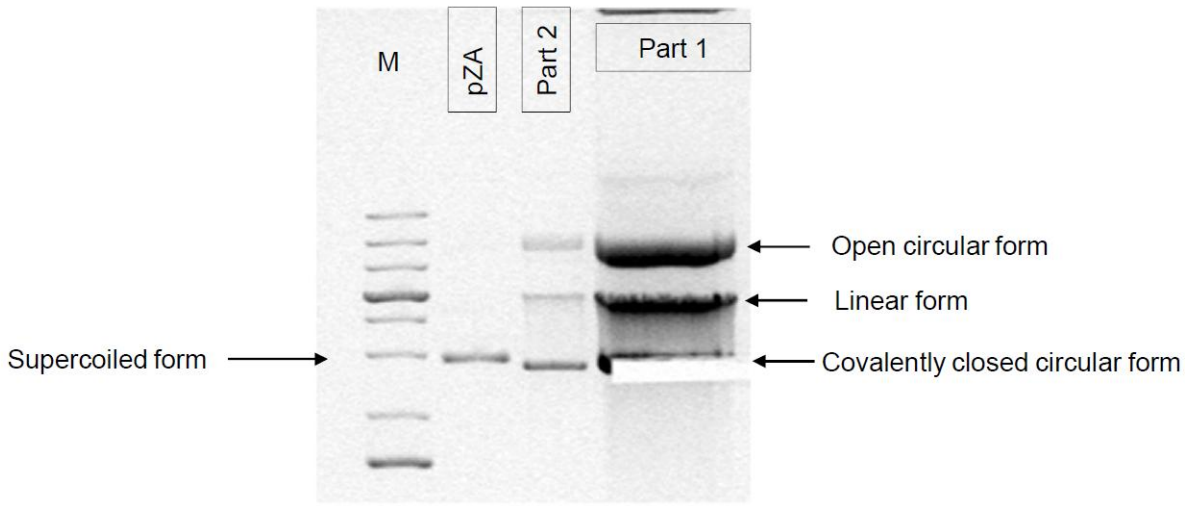

Figure 2.2 Excision of the band of covalently closed circular plasmid DNA from agarose gel.

2.2.6.7 Electro elution of DNA from EtBr agarose gel using D-Tube Dialyzers

Maxi D-Tube™ Dialyzer was prepared to use by incubating with 2–3 ml of water for at least 5 min. The excised agarose slide, containing the band of covalently closed circular DNA was placed in a D-Tube™ device parallel to the cellulose membrane. The remaining volume was filled with 1× TAE buffer. The D-Tube™ Dialyzer was positioned in an electrophoresis chamber in a way that two membranes were perpendicular to the electric field, thus permitting the electric current to pass through the tube, as described in the manufacturer's protocol (Novagen).

Electro elution was performed in horizontal electrophoresis units (length 12 cm) at 80–100 V for 60–120 min. The current polarity was reversed for 2 min. Then the agarose slide was removed from the D-Tube™ Dialyzer device, and membrane rinsed many times with elution buffer. Dialyzed DNA was extracted with phenol/chloroform, supplemented with 0.1 volume 3 M sodium acetate (pH 5.2) and precipitated with ethanol. DNA was resuspended in BEH buffer (pH 7.5) and stored frozen at -20°C.

2.2.6.8 Extraction of DNA from EtBr agarose gel with a Fermentas kit

The DNA Extraction Fermentas Kit utilizes ability of DNA to bind to specially prepared glass particles in the presence of chaotropic salts. To extract covalently closed circular DNA from slice of agarose gel, the original manufacturer's protocol was followed. The volume of gel slice was determined by weight (1 g corresponds to ~1 ml). The slice was placed in a 2 ml Safe-Lock Eppendorf® tube. Then 3 volumes of binding solution (6 M NaI, pH 5.0) were added to 1 volume of gel and incubated 5 min at 55°C to dissolve the agarose with occasional agitation (time can be extended until complete dissolving). Resuspended silica

powder suspension was added to solutions (5 µl of silica to every 2.5 µg of DNA) and incubated 5 min at 55°C, inverting the tube every 2 min. Silica powder/DNA complex was spun down and washed 3 times with ice cold washing buffer (provided by the kit manufacturer). Supernatant was removed, pellet air-dried, and DNA eluted in BEH buffer by incubation at 55°C, 5 min. This step was repeated to increase DNA yield.

2.2.6.9 Extraction of DNA from EtBr agarose gel by ultracentrifugation

A very simple and low cost method for isolating DNA fragments from agarose gels by ultracentrifugation was described by Z. Wang and all (Wang & Rossman, 1994). The protocol was adjusted towards the optimal recovery and quality of the covalently closed pZA plasmid as follows. The 0.8% agarose gel slices containing ccc plasmid DNA were ultracentrifuged 25−35 min (45000 rpm, Beckman TI-60 rotor). Agarose pellets were extracted with 500 µl TAE buffer per ultracentrifugation tube and centrifugation repeated. Supernatants were joined, extracted with phenol/chloroform, adjusted to 0.3 M sodium acetate and ethanol-precipitated. Eluted DNA was dissolved in BEH buffer (pH 7.5) and stored frozen at -20°C. Freezing of gel slices by incubation at -20°C or in liquid nitrogen prior to ultracentrifugation has been advised (Koenen, 1989). However, in my hands this did not significantly improve the DNA yields and was therefore omitted.

2.2.6.10 Measurement of DNA concentration using built-in fluorimeter of LightCycler 1.5 (SYBRGreen master mix)

Small concentrations of DNA in the samples recovered from agarose gels were measured by fluorescence in the presence of SYBR Green. Serial dilutions of standard plasmid DNA and plasmid DNA of unknown concentrations (2 µl of DNA solution) were mixed in the LightCycler capillaries with 8 µl of 1.25× concentrated master mix prepared in water from 10× DNA Master SYBR Green (Roche Applied Science). Fluorescence was measured using a LightCycler 1.5 (Roche Applied Science) in the real-time fluorimeter mode at 30°C. Calibration curves were built with known concentrations of pure plasmid DNA (pZA). Fluorimeter settings were calibrated by the DNA sample with highest concentration.

2.2.6.11 Detection of 8-oxoG within the covalently closed plasmid DNA

The presence of 8-oxoG in the plasmid DNA was verified by using purified DNA repair enzyme Fpg (formamidopyrimidine [fapy]-DNA glycosylase) from *E. coli* (Boiteux et al, 1987), (Boiteux et al, 1990) as a probe. Plasmids DNA (150 ng) was incubated with 90 µg of purified Fpg 2 hours, 37°C. After incubation with Fpg, plasmids were run in agarose gels in the presence of 0.5 mg/L ethidium bromide. Relative intensities of DNA bands were determined with a GelDoc 1000 and the Molecular Analyst 2.1 software (Bio-Rad Laboratories,

München, Germany. Recombinant Fpg protein was a kind gift of Serge Boiteux, CEA Fontenay-aux-Roses, France.

2.2.7 Procedures for construction and analyses of plasmids containing 8-oxoG by annealing and ligation of synthetic oligonucleotides between two nicks in the DNA strand of choice

2.2.7.1 Nicking of the pZA plasmid DNA with nicking endonucleases Nt.Bpu10I or Nb.Bpu10

Supercoiled pZA plasmid isolated from bacteria was incubated with the mutants of Bpu10I restriction endonuclease Nt.Bpu10I and Nb.Bpu10I to introduce nicks in the non-transcribed ("top") and transcribed ("bottom") strands of the EGFP gene, respectively. The 250 µl reactions containing 50 µg plasmid DNA in reaction buffer R (Fermentas, catalog # BR5) and 50 units of Nb.Bpu10I or 55 units of Nt.Bpu10I nicking endonucleases were incubated 2 hours at 37°C. Enzymes were killed by heat inactivation 20 minutes at 80°C.

2.2.7.2 Analytical ligation in the presence of excess of a non-phosphorylated competitor oligonucleotide

To verify that both Bpu10I sites were nicked, the cut DNA strand was melted and re-annealed in the presence of a non-phosphorylated competitor oligonucleotide (18-mer with the sequence identical to the excised native oligonucleotide) (**Table 2.17**). Exchange of the excised fragment with the synthetic oligonucleotide prevents the strand ligation by the T4 DNA ligase, because of the lack of the 5′-phosphate. Therefore, no covalently closed circular DNA can be formed in the case of a complete exchange.

Nicked pZA plasmid (0.4 µg DNA digested with either Nb.Bpu10I or Nt.Bpu10I) was incubated in the presence of 0.023 nmol synthetic oligonucleotide (184-fold molar excess) in the T4 DNA ligase buffer in a TGradient Thermocycler under slow annealing conditions (**Table 2.17**).

Table 2.17 The program for melting and re-annealing of nicked plasmid DNA in the presence of a non-phosphorylated competitor oligonucleotide.

Step	Temperature	Time
1	4°C	∞
2	80°C	10 min
3	50°C	1 min, rate 0.02°C/s
4	4°C	∞

T4 DNA ligase (2 units/reaction) was added to the cooled reactions and further incubated 1 hour at 22°C, after which the enzyme was killed by heat inactivation (65°C, 15 min). A sample incubated without the competitor oligonucleotide was used as a control for the T4 ligase activity. The reaction products were analysed in 0.8% agarose gels in TAE buffer containing ethidium bromide.

2.2.7.3 Optimised protocol for incorporation of single 8-oxoG in the DNA strand of choice

Plasmids containing two nicks generated by a respective strand-specific nicking enzyme in the same strand of the EGFP gene (transcribed or non-transcribed) were used as starting material. 20 µg nicked plasmids were supplemented with the T4 DNA ligase buffer (Fermentas) and 5.7 µl of 200 µM synthetic competitor oligonucleotides (180-fold molar excess), incubated with T4 polynucleotide kinase (Fermentas) at 37°C 30 minutes in a reaction volume of 750 µl. The 8-oxoG-containing and unmodified oligonucleotides used for construction of the hybrid plasmid molecules are listed in (2.1.5). Native 18-mers were melted out at 80°C for 10 minutes, followed by cooling to 50°C at a rate of 0.02°C/s, as described above (**Table 2.17**). Reactions were transferred to ice, and T4 DNA ligase (100 units) was added in 250 µl T4 DNA ligase buffer. Ligation was conducted 1 hour at 22°C, following by heat-inactivation of the enzyme 15 minutes at 65°C. Hybrid plasmids molecules containing the built-in synthetic 18-mers were extracted with phenol:chlorophorm and precipitated with ethanol, as described previously (2.2.1.1). Modified plasmids were resuspended in 150−200 µl TE buffer (pH 8.0), and DNA concentration was determined by measuring A_{260} in an aliquot using a UV-spectrophotometer and ultra-micro sized UV-Cuvettes (BrandTech Scientific). The yield of the modified plasmid DNA obtained with this method approaches 90−100%. The A_{260}/A_{280} ratio of the final DNA preparation is typically 1.75±0.05.

2.2.7.4 Detection of 8-oxoG within the covalently closed plasmid DNA

The presence of 8-oxoG in the plasmid DNA was verified by incubation with Fpg from *E. coli*, as described above (2.2.6.11).

2.2.7.5 Excision of 8-oxoG within the covalently closed plasmid DNA by purified OGG1

Purified human OGG1 protein (hOGG1) was a kind gift of Pablo Radicella, CEA Fontenay-aux-Roses, France. 100 ng of plasmid DNA containing synthetic 8-oxoG were incubated (1 hour, 37°C) with a range of concentrations of purified hOGG1 enzyme (56.7 ng; 18.9 ng; 5.67 ng; 1.89 ng; 0.56 ng) in the presence of 1 U Endonuclease IV (NEB, Frankfurt am Main). Following the incubation with enzymes, the samples were run in agarose gels in the presence of 0.5 mg/L ethidium bromide. Gels were briefly rinsed in deionised water, and relative intensities of bands corresponding to covalently closed and open circular DNA were

determined with a GelDoc XP+ imager and the Image Lab 3.0 software (Bio-Rad Laboratories, Munich, Germany).

2.2.7.6 Protein expression analyses of plasmid substrates containing synthetic 8-oxoG by flow cytometry

MATra-A system (PromoKine, Heidelberg, Germany) was employed for transfection of MEFs as described in 2.2.4.3. Effectene (Qiagen, Hilden, Germany) was used for transfection of HeLa cells. The EGFP encoding hybrid plasmids, produced by insertion of the synthetic oligonucleotides containing the specified bases or base modifications, were transfected together with equal amounts of the pDsRed-Monomer-N1 plasmid encoding for the DsRed-Monomer. Transfections were done in duplicates for each individual plasmid preparation in 6-well plates (Nunc, Wiesbaden, Germany) with 800 ng (for the Effectene transfections) or 3 mg (for the transfections with MATra-A) of total plasmid DNA. Cells were harvested and fixed with 1% formaldehyde (2.2.3.2) at indicated times after transfections and analysed by FACSCalibur™ flow cytometer and CellQuestTM Pro software (Beckton Dickinson, GmbH, Heidelberg, Germany), as described in the previous sections (2.2.4.4) EGFP expression was determined as a median FL1 fluorescence among the transfected cells, defined by a significant content of DsRed-Monomer (FL2 >30).

2.2.7.7 Procedure for determination of mRNA expression levels and plasmid survival in HeLa cells

Cells were split in three equal parts (for DNA, RNA and protein expression analyses) 8 hours after transfections and incubated in 6-well plates for additional 16 hours. For isolation of DNA, cells were collected in 0.5% sodium dodecyl sulphate, supplemented with 100 mg/L Proteinase K, and incubated 3 hours at 50°C. Total cellular DNA was isolated by standard phenol–chlorophorm extraction procedures and ethanol precipitation (2.2.1.1). The plasmid copy numbers in DNA samples were determined by real-time quantitative PCR with a LightCycler 1.5 (Roche Applied Science).

RNA was isolated with a ZR RNA MiniPrep™ Kit (HiSS Diagnostics, Freiburg, Germany) according to the supplier's instructions, including in-column treatment with DNase I (Fermentas) 30 min at room temperature. RNA integrity was verified by denaturing agarose gel electrophoresis (2.2.1.5). Reverse transcription (RT) reactions were performed following standard protocol from manufacturer with Revert Aid^{TM} First Strand cDNA Synthesis Kit (Fermentas) using random hexamer primers (see following **Table 2.18**) cDNA samples were diluted 1:200 and quantified by real-time quantitative PCR relative to serial dilutions of DNA isolated in parallel from the same transfection samples. The absence of contaminating plasmid DNA was confirmed by PCR of aliquots removed prior to RT.

One primer pair was designed for amplification of the DsRed-Monomer sequence in the reference plasmid and two primer pairs were designed for the EGFP cDNA, one of which encompassed the 8-oxoG sites in the positions 1230 (transcribed strand) and 1231 (non-transcribed strand), as summarized in **Table 2.18**. Positions of the primers in the plasmid DNA refer to the sequence reported by the supplier (Clontech). The transcription start sites correspond in both vectors to the position 583.

Table 2.18 Oligonucleotides used as PCR primers in the real-time quantitative PCR:

Primer pair	Template	Position and strand	Sequence (5′ to 3′)	Product length (bp)
DsRed	pDsRed-Monomer-N1	701 (+) 939 (–)	TCAAGGAGTTCATGCAGTTC GAAGGACAGCTTCATGTAGT	239
EGFP1	pEGFP-mODC-ZA	739 (+) 938 (–)	CTGACCCTGAAGTTCATCTG GTCTTGTAGTTGCCGTCGTC	200
EGFP2	pEGFP-mODC- ZA	1153 (+) 1368 (–)	GACCACTACCAGCAGAACAC GCCTGTGCTTCTGCTAGGAT	216

2.2.8 Strategy and procedure for analysis of replication of pEGFP-mODC-ZA plasmid DNA in HeLa cells

To detect replication of dam-methylated plasmids in mammalian hosts, we adapted the previously described techniques based on the resistance of the replicated plasmids to combined digestion with DpnI and exonuclease III (Burns et al, 2010; Taylor & Morgan, 2003). DpnI is a restriction endonuclease, which specifically cuts GATC sequences containing methylated adenine. Dam-methylated plasmid DNA is efficiently cut by DpnI, while DNA derived from the dam- bacteria and DNA replicated in mammalian cells are not because of the lack of adenine methylation at GATC sequences. This peculiarity allows experimental detection of replication of plasmid DNA in mammalian hosts as a loss of sensitivity to DpnI digestion. HeLa and MRC-5 V1 cells were co-transfected in parallel with equal amounts (400 ng each) of the pEGFP-mODC-ZA and pDsRed-Monomer plasmids, one of which was isolated from a dam- strain of *E. coli* (SCS-110) and another from the dam+ strain (SCS-8). Episomal DNA was recovered from cells 48 hours after transfections with a plasmid miniprep kit (Sigma-Aldrich) and quantified by real-time qPCR.

The same amounts of plasmid DNA re-isolated from mammalian cells (equivalent to 100 pg of pEGFP-mODC-ZA) were supplemented with 500 ng heterologous DNA (phage lambda DNA, Fermentas), 1× Tango™ buffer (Fermentas) and incubated with 5 units DpnI (Fermentas) or without the enzyme 1 hour at 37°C followed by heat-inactivation 20 minutes at 80°C. Reactions (20 µl) contained 100 mM NaCl to inhibit the cleavage of hemimethylated GATC sites (Sanchez et al, 1992). One fifth of the reaction was removed, and the uniform digestion verified by gel electrophoresis. The rest of the samples were treated with 200 units exonuclease III (Fermentas) 1 hour at 45°C, killed 20 min at 80°C, in order to degrade the linearised DNA but not the DpnI-resistant circular plasmid DNA. Finally, remaining amounts of plasmid DNA were measured by real-time qPCR with primers listed in **Table 2.19** to determine the fraction of DpnI-resistant DNA molecules as an estimation of the fraction of plasmid DNA synthesized post-transfection in the mammalian host cells.

Table 2.19 PCR primers for analyses of DpnI- and exonuclease III-digested plasmid DNA recovered from cells:

Primer pair	Amplified template	Position and strand	Sequence (5′ to 3′)	Product length (bp)	DpnI sites
DsRed	pDsRed-Monomer-N1	701 (+) 1394 (–)	TCAAGGAGTTCATGCAGTTC TGTGGTATGGCTGATTATGA	694	3
EGFP2	pEGFP-mODC- ZA	1153 (+) 1584 (–)	GACCACTACCAGCAGAACAC TGTGGTATGGCTGATTATGA	432	8

3 RESULTS

3.1 The effects of DNA damage induced in cells by exogenous agents on transcription of reporter transgenes

3.1.1 Construction of stably replicating episomal vectors for analyses of gene expression in the presence of DNA damage

Many of the DNA damaging agents (for instance, UV radiation) cause an overall decrease of RNA synthesis in cells (Mayne & Lehmann, 1982). This effect has been attributed to stalling of the elongating RNA polymerase at the bulky DNA modifications (Tornaletti et al, 2003). In accordance with this model, recovery of RNA synthesis after UV irradiation correlates with repair of UV- induced DNA lesions (cyclobutane pyrimidine dimers and 6-4 photoproducts) (Hanawalt, 1994). The influence of the UV–induced DNA damage on transcription is well studied on the genome level, but not so much on the level of single genes. On the other hand, the effects of oxidative DNA damage are altogether poorly studied both at the level of global transcription and of expression of single genes, mainly because the exogenous oxidants fail to induce significant amounts of DNA damage at non-cytotoxic conditions of exposure.

The effect of DNA damage on transcription of single genes can be studied by measuring expression of reporter transgenes. Among the available reporters, the enhanced green fluorescent protein (EGFP) gene seems well suitable for such gene expression studies, particularly because the EGFP protein expression level can be directly measured in individual cells by fluorescence detecting techniques, such as fluorescence-activated flow cytometry (Cormack et al, 1996), (Soboleski et al, 2005). However, the relatively long protein lifetime of EGFP (about 26 hours) makes detection of short lasting perturbations of transcription problematic, because the average repair times of many types of DNA damage, including the oxidative base modifications, are much shorter under physiological conditions. To detect inhibition of RNA polymerase II transcription following DNA damage, our strategy was to modify the EGFP gene by addition of specific degradation signals to the messenger RNA and the protein in order to achieve a more rapid protein turnover.

The protein lifetimes can be considerably reduced by fusion of heterologous natural protein degradation signals, for instance the one of the ornithine decarboxylase (ODC) protein, which has a very short (between 10 and 40 min) intracellular half-life in mammalian cells. The PEST (proline(P)-glutamate(E)-serine(S)-threonine(T)-rich) sequence in carboxyl-terminal domain of mODC protein has been shown to be responsible for the rapid intracellular degradation of murine ODC (Ghoda et al, 1989). Several groups successfully used the PEST-containing sequences from mouse ODC gene fused to GFP, which resulted

in shortening of the protein half-life to 5.5 hours in permanent transfection experiments (Corish & Tyler-Smith, 1999) and even 2 hours in transiently transfected cells (Li et al, 1998). A mammalian expression vector (pEGFP_mODC-AZ) containing a reporter gene encoding for short-lived GFP protein was previously constructed in our group by A. Khobta. In this vector, a fragment of mouse ornithine decarboxylase gene (mODC) exon 10 was inserted in-frame with enhanced green fluorescent protein (EGFP). In addition, the same (mODC) fragment has been inserted in opposite direction, preceded by a STOP codon to generate a control construct encoding for stable EGFP (pEGFP_mODC-ZA). Unfortunately, these vectors had some inconveniences for use as reporters for studies of the effects of DNA damage on transcription. Permanent transfections with these vectors require the integration into chromosomal DNA by recombination. Since the genomic site of integration cannot be controlled, this could lead to variable gene expression because of either epigenetic down regulation or even partial destruction of the construct. As a result, the transfected HeLa cells showed intrinsically unstable gene expression levels with some of the selected or sorted clones losing the EGFP fluorescence already after few cell divisions (data not shown).

To improve the stability of gene expression, we chose a backbone of an episomally replicating non-viral vector pMARS for cloning of the short-lived EGFP reporter protein. This vector harbours the synthetically designed minimal matrix attachment region (MAR), repeated four times in the 3′ untranslated region (UTR) of the EGFP gene. Thanks to this motif, the plasmid is expected to replicate as an episome and maintain stable number of copies in host cells (Jenke et al, 2004). We hoped that this vector would provide more stable gene expression levels in the permanently transfected host cells, because integration into chromosomal DNA is not required for episomal vectors. In addition, we identified two classes of AU-rich elements (AREs) within the MAR elements in the 3′ UTR (Kitsera et al, 2007). AREs are known to promote a rapid decay of variety of human messenger RNAs (Shaw & Kamen, 1986), (Bakheet et al, 2006), thus signalling for a rapid mRNA turnover. For the above listed reasons we decided to construct a pair of episomal reporter vectors each encoding for destabilized (pMAZ-ODC) or stable (pMZA-ODC) EGFP. This was achieved by cloning the mouse ODC gene fragment in two directions into the pMARS plasmid.

The mODC exon 10 fragment was amplified by PCR using the previously constructed pEGFP_mODC-AZ plasmid as a template and the 5′-phosphorylated primers. The PCR product was blunt end ligated into the pMARS vector linearised with BglII after filling the 5′-overhangs with the T4 DNA polymerase and dephosphorylation of the vector (Methods section 2.2.2). The colony counts after transformation of *E. coli* SCS-8 with the ligation products are shown in Table 3.1. All 15 clones were screened by three-primer PCR for the presence and orientations of mODC insert (Methods section 2.2.2.6). Two of the primers (“B” and “C”) anneal in the opposing directions to the mODC fragment (**Figure 3.2**). Thus, only

the clones containing the insert can generate the PCR product. The expected product lengths were 215 bp for the AZ orientation (corresponding to the EGFP-ODC fusion protein) and 270 bp for the ZA orientation (corresponding to the unmodified EGFP).

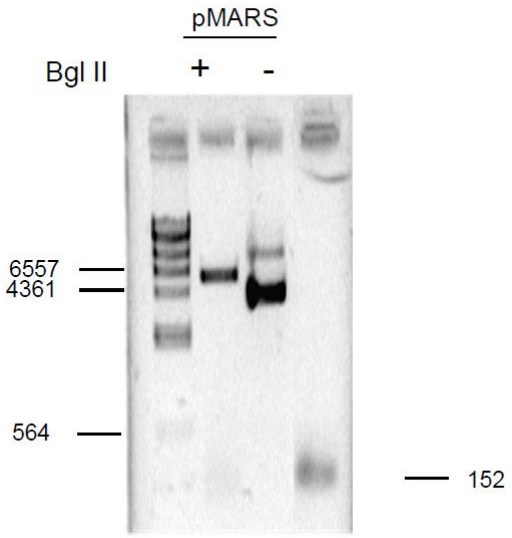

Figure 3.1 Agarose TAE gel electrophoresis of the linearised vector and insert prior to ligation. The acceptor pMARS vector (band of 5585 bp) was linearised by Bgl II, blunted and dephosphorylated. The mODC exon 10 insert (specific band of 152 bp) was obtained by PCR and purified from gel.

Table 3.1 Colony counts after transformation of the *E. coli* SCS-8 strain with ligation products of pMARs vector and mODC exon 10 insert.

DNA used to transformation	Number of colonies
no insert (negative control)	1
1/1 insert / vector	7
1/3 insert / vector	8
supercoiled pZA plasmid (positive control)	≥200

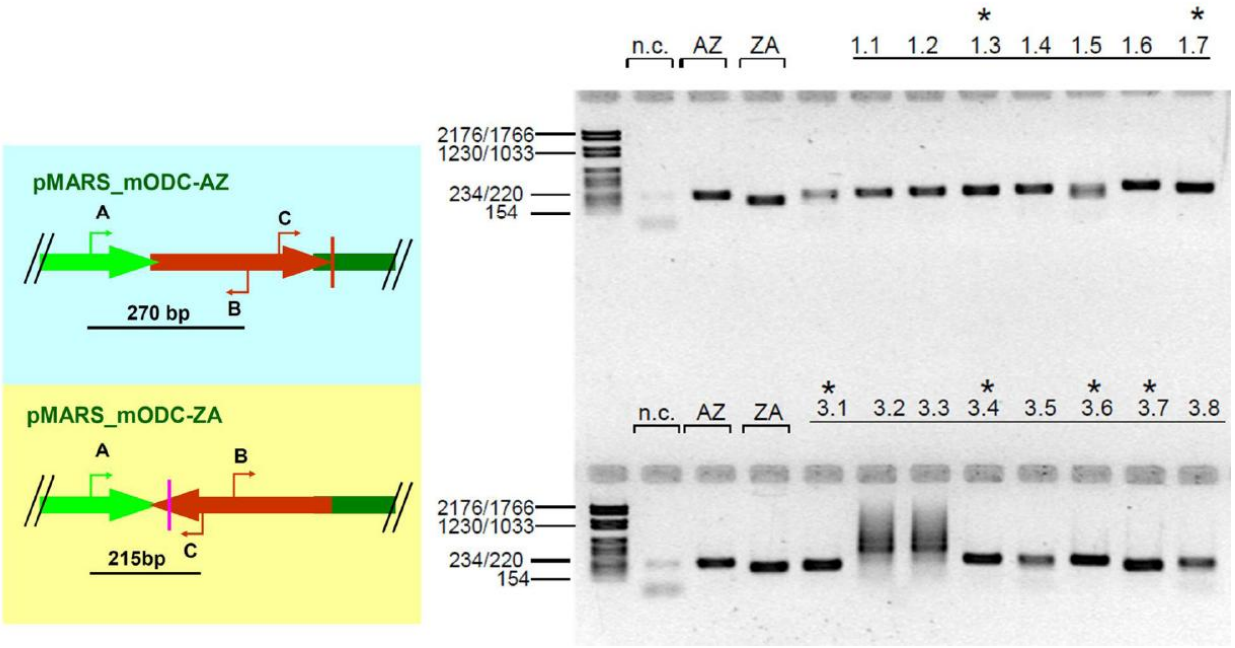

Figure 3.2 Agarose TAE gel electrophoresis of the products of three-primer PCR screenings for possible orientation of ligated mODC exon 10 insert in pMARS vector. Left is a scheme of three-primer PCR for both pMARS-mODC-AZ and pMARS-mODC-ZA constructs with different insert orientations. A, B, and C are PCR primers. Vertical bar shows a STOP codon.

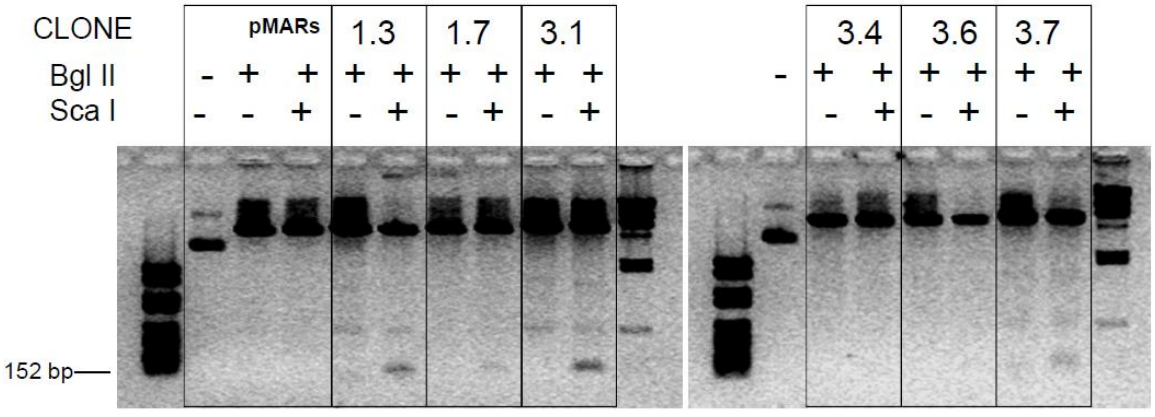

Figure 3.3 Screening of selected clones by digestion with BglII and ScaI restriction endonucleases to check possible degradation of DNA ends at ligation sites.

Plasmids from six selected clones (marked with asterisks in **Figure 3.2**), namely 1.3; 1.7; 3.1; 3.4; 3.6; 3.7 were isolated by by GenElute Plasmid Miniprep Kit and screened by restriction analyses with Bgl II and Sca I restriction enzyme to check for possible degradation of DNA ends at the ligation sites. The specific band of 152 bp was released after Bgl II + Sca I digestion in all chosen clones (**Figure 3.3**). In the clones that produced somewhat lower DNA yields (3.4 and 3.6) this band was hardly detectable, because it contains only about 3% of total DNA in the lane.

The clones 1.3; 3.4; 3.6; 3.7 were sequenced. One clone (1.3) contained a mutation and was therefore discarded. We named the plasmid isolated from a mutation-free clone 3.6 coding for the EGFP-mOFC fusion protein "pMAZ-ODC". This plasmid was later assigned a Genbank accession number EU421131. The counterpart plasmid (clone 3.7) coding for unmodified EGFP and containing the inverted ODC fragment, preceded by the PCR-introduced translation stop-codon, was named "pMZA-ODC" (Genbank accession number EU421132). The pMAZ-ODC and pMZA-ODC vectors have the same size of 5742 bp and have the same size, direction, and base content of the transcribed regions, as well as the entire 3′ portion of the of GFP transcripts; the ODC insert orientation is the only difference between these plasmids at the molecular level.

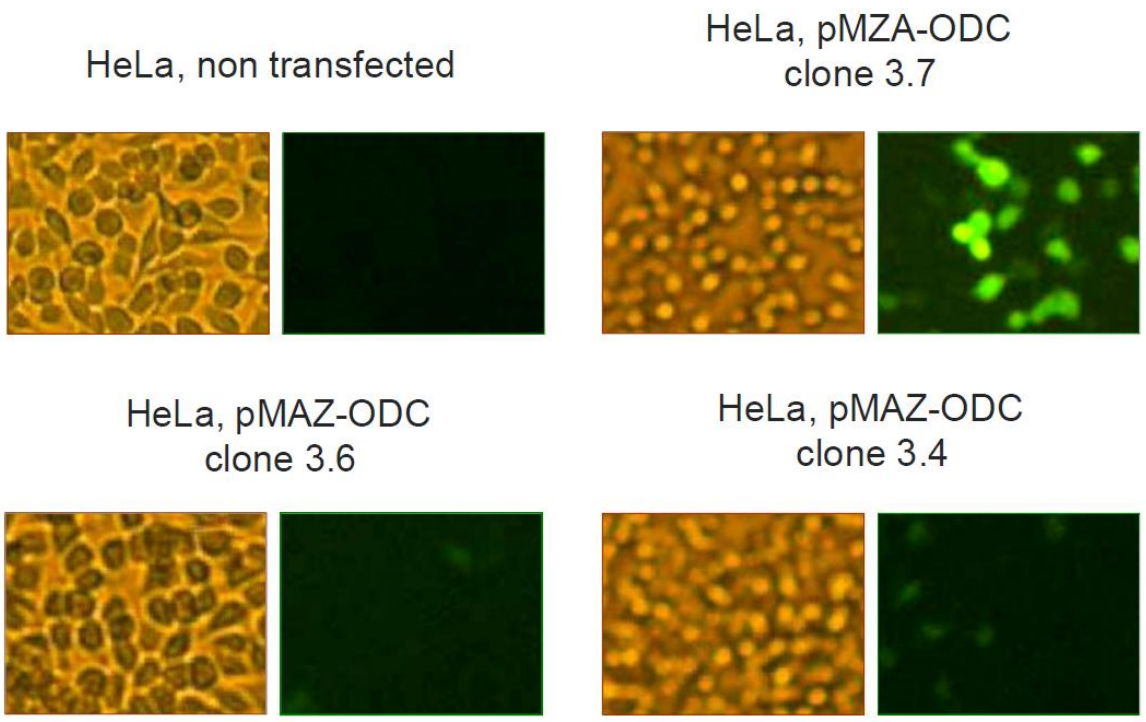

Figure 3.4 Fluorescent microscopy images of HeLa cells 24 hours after transfection with pMAZ-ODC and pMZA-ODC plasmids.

The EGFP expression levels of the newly-produced vectors were next checked by transient transfection into HeLa cells. The detectable levels of EGFP expression were observed under the fluorescent microscopy in case of transfection with each plasmid. The cells, transfected with the pMZA-ODC vector (clone 3.7) encoding the stable protein demonstrated high level of the GFP fluorescence. The cells transfected with the pMAZ-ODC vector encoding for destabilized EGFP (clones 3.4 and 3.6) demonstrated much weaker fluorescence signals, in agreement with the presumed decreased lifetime of the protein **Figure 3.4**.
In summary two vectors were obtained for subsequent use for permanent transfection of mammalian cells with the aim to study the effects of exogenously induced DNA damage on transcription in cells.

3.1.2 Generation of stably transfected cell lines expressing stable or short-lived enhanced green fluorescent protein (EGFP)

To study the effect of DNA damage on transcription in living cells I generated HeLa cell lines permanently transfected with pMAZ-ODC coding for the destabilized EGFP-ODC fusion protein or with pMZA-ODC encoding the unmodified EGFP. Exponentially growing HeLa cells were transfected with 800 ng supercoiled pMZA-ODC (clone 3.7) or pMAZ-ODC (clone 3.6) plasmids. Following cultivation for 24–48 hours, the cells expressing EGFP were sorted by FACS, diluted in fresh medium and plated in the presence of geneticin (G418). Single clones were examined by fluorescence microscopy for the EGFP expression. Fluorescent clones of 100–200 cells were picked and further expanded under the selection pressure (G418, 800 mg/L) for 4 weeks as described (Methods section 2.2.3.1). The expanded clones were analysed by FACS for expression of EGFP. The analysed HeLa clones expressing the stable GFP protein from the pMZA-ODC vector had green fluorescence similar to each other (97.3-99.7% of fluorescent cells), and clearly distinguishable from the non-transfected cells (**Figure 3.5**). The clones obtained after transfection with pMAZ-ODC vector generally demonstrated much weaker fluorescence. Of 25 analysed clones that were obtained, only three showed distinct fluorescence levels when compared with non-transfected cells. Not more that 20% of cells demonstrated a significant EGFP fluorescence signal even in the clone with the highest EGFP expression level (**Figure 3.5**).

The low levels of the EGFP fluorescence in the HeLa-MAZ cells were attributed to rapid degradation of GFP-ODC fusion protein in the proteasome, since the EGFP fluorescence could be saved by treatment with proteasome inhibitor MG115. Moreover, the treatments with MG115 showed that the EGFP gene was expressed in all cells (**Figure 3.6**). Permanence of the EGFP expression in the selected clones was verified by prolonged incubation without the selection pressure. The green fluorescence was stably maintained

without major quantitative changes for at least 45 rounds of cell division after the removal of G418 (data not shown). Longer times were not tested.

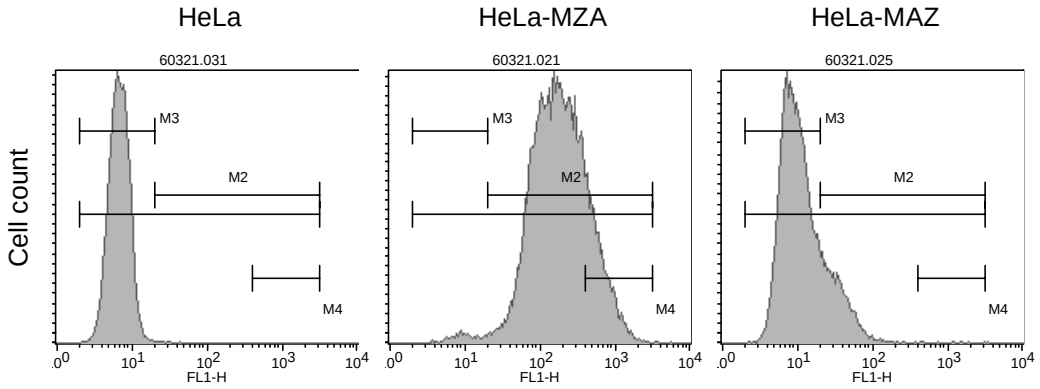

Figure 3.5 Flow cytometry analyses of the EGFP fluorescence in non-transfected HeLa cells (left panel) and in two selected permanently transfected clones.

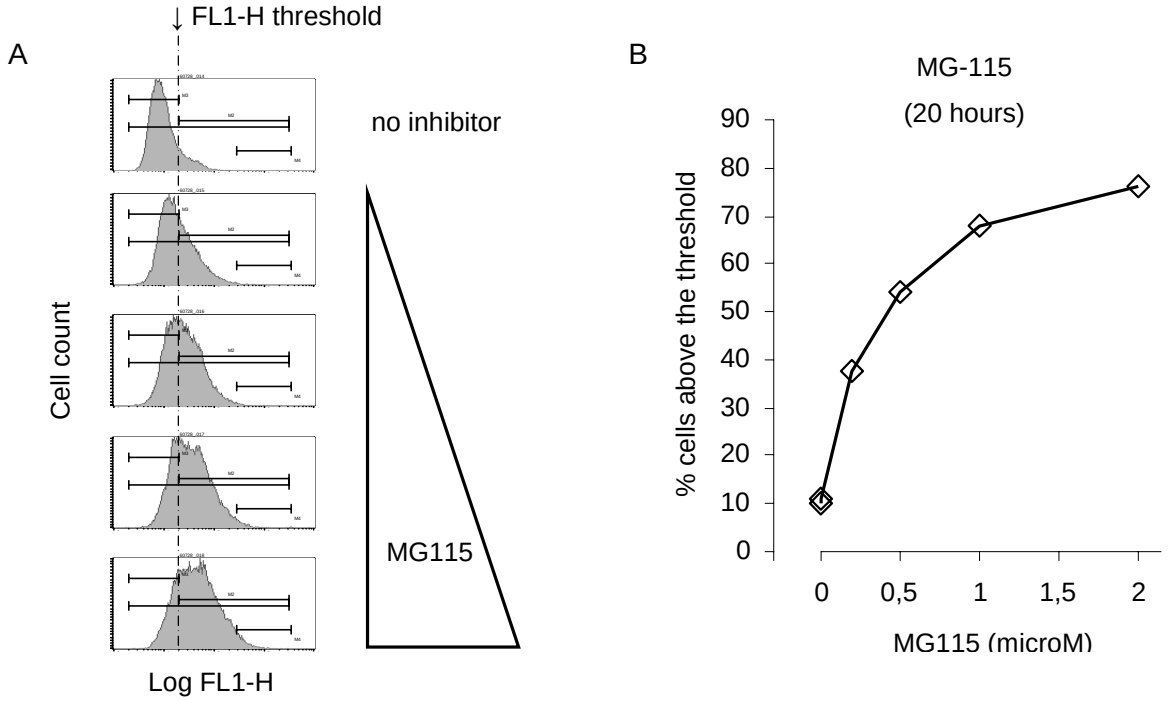

Figure 3.6 Effect of the proteasome inhibitor MG115 on the EGFP fluorescence in a selected permanently transfected clone (s06) of HeLa-MAZ cells. (A) Flow cytometry analyses of cells treated for 20 hours with the increasing concentrations of the inhibitor. The fluorescence threshold was arbitrarily selected to cut off 99.9% non-transfected cells. (B) Quantification of the fractions of EGFP-positive cells after treatments with MG 115 shown in A.

To measure the average lifetimes of the EGFP-ODC fusion protein in cells, the single clones of the permanently transfected cells were incubated with the inhibitor of protein synthesis cycloheximide. In case of the cells expressing the unmodified EGFP (HeLa-MZA), a 3-hour incubation with the inhibitor caused only a slight decrease of the fraction of the cells expressing the high levels of the EGFP fluorescence. Strikingly, a virtually complete loss of the specific fluorescence signal was observed in the cells expressing the EGFP-ODC fusion protein (HeLa-MAZ) under the same experimental conditions (**Figure 3.7.A**). We next tested four separate HeLa-MAZ clones, which had different steady-state EGFP expression levels under the normal conditions. The decrease of the EGFP-specific fluorescence signal was very rapid in four clones of the HeLa-MAZ cells tested, independently of the initial EGFP expression levels. The fraction of the EGFP-positive cells halved within approximately 30 minutes from the addition of cycloheximide in all clones analysed, thus providing an indication of a very rapid protein turnover (**Figure 3.7.B**).

To test if the stationary levels of the short-lived EGFP-ODC protein are sensitive to perturbations of the gene transcription, we next treated the HeLa-MAZ cells with the inhibitors of transcription α-amanitin and 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB), both of which specifically affect the RNA polymerase II transcription. The clone s06 of HeLa-MAZ cells was chosen for these experiments based on its relatively high EGFP expression under the normal conditions. The effect of α-amanitin could not be detected during the first six hours of treatment, probably because of a slow uptake by the cells. However, at the later times, the fraction of EGFP expressing HeLa-MAZ cells decreased significantly in comparison with the non-treated cells. After 24-hour incubation in the presence of α-amanitin, EGFP fluorescence disappeared almost completely in this cell line. In contrast, only a modest quantitative decrease of the EGFP expression was documented in the HeLa-MZA cells under the same conditions of incubation (**Figure 3.8**).

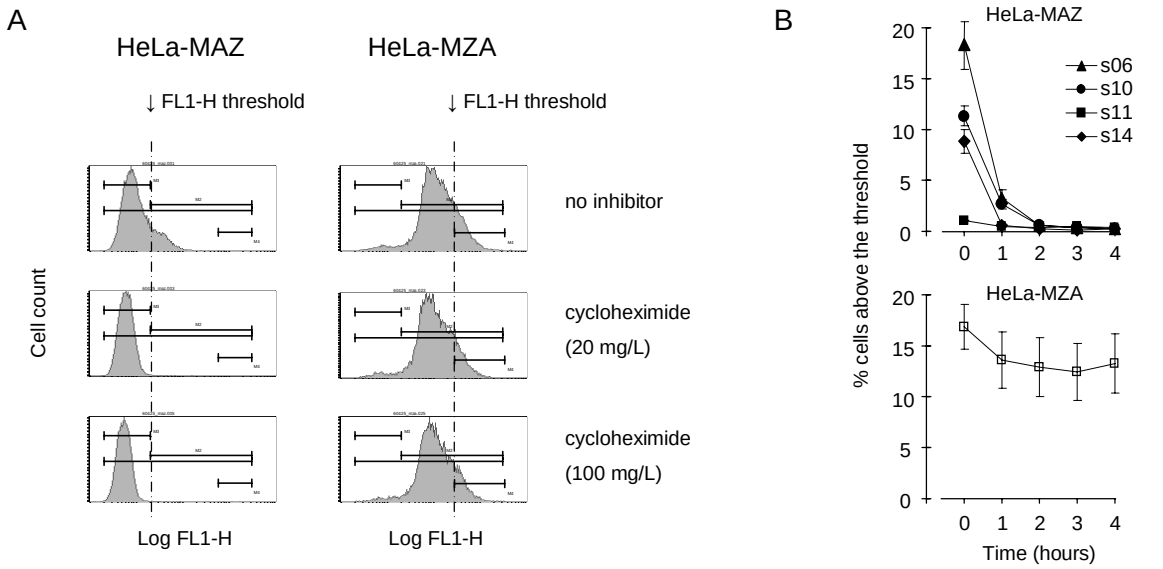

Figure 3.7 Effect of the inhibitor of protein synthesis cycloheximide on the EGFP fluorescence in the permanently transfected HeLa-MAZ (clone s06) and HeLa-MZA (clone 01) cell lines. (A) Flow cytometry analyses of cells treated for 3 hours with with 20 or 100 mg/L cycloheximide. (B) Time-dependent decrease of the fractions of highly fluorescent cells (defined by the FL1 threshold) during the incubations in the presence of 100 mg/L cycloheximide. Four independent HeLa-MAZ clones were analysed.

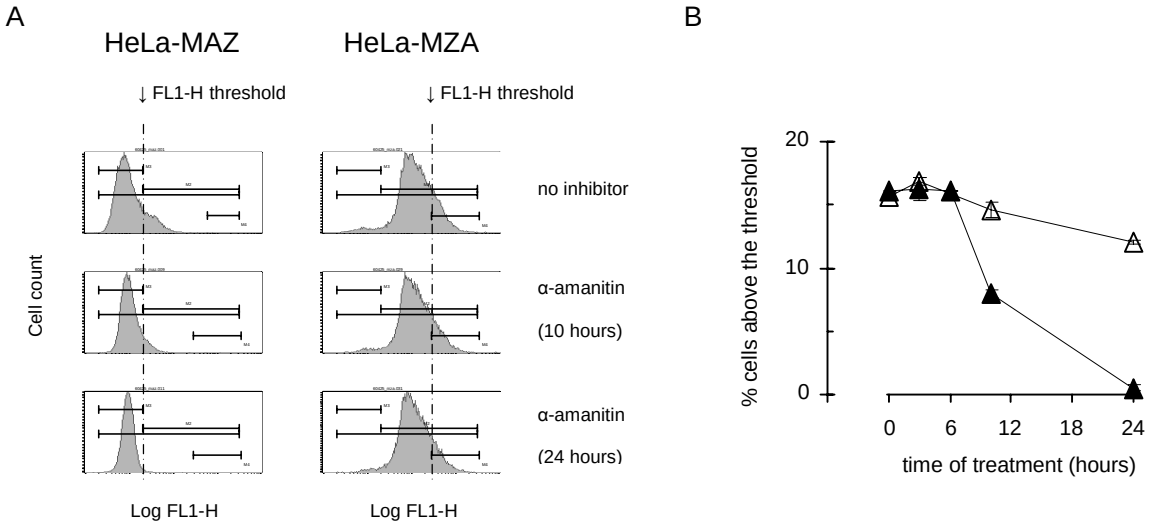

Figure 3.8 Effect of α-amanitin (10 mM) on the EGFP fluorescence in the permanently transfected HeLa-MAZ (clone s06) and HeLa-MZA (clone 01) cell lines. (A) Fluorescence distribution plots obtained by flow cytometry. (B) Time-dependent decrease of the fractions of highly fluorescent cells (defined by the FL1 threshold). Treatments with α-amanitin were done in duplicates.

Inhibition of transcription by DRB caused a slightly different dynamics of the EGFP expression response. The expression of the short-lived EGFP (HeLa-MAZ cells) was affected by the inhibitor much earlier (3 hours) than in the case of treatments with α-amanitin. The protein expression continued to decrease within the first 10 hours of incubation with DRB in this cell line (**Figure 3.9**). As no measurement was done between 10 and 24 hours of incubation with DRB, it is possible that the minimum of protein expression was achieved around 10 hours after addition of DRB or maybe somewhat later. Interestingly, this was followed by partial recovery of the EGFP expression at 24 hours, reflecting either instability of DRB or some indirect response to the inhibitor. At all tested time points, DRB did not cause any notable decrease of the specific fluorescence in HeLa-MZA cell line expressing the non-modified EGFP (**Figure 3.9**). Altogether, it can be concluded that the use of destabilised GFP markedly improved the response of the protein level in the cell (measured as direct fluorescence) to the perturbations of the gene transcriptional activity.

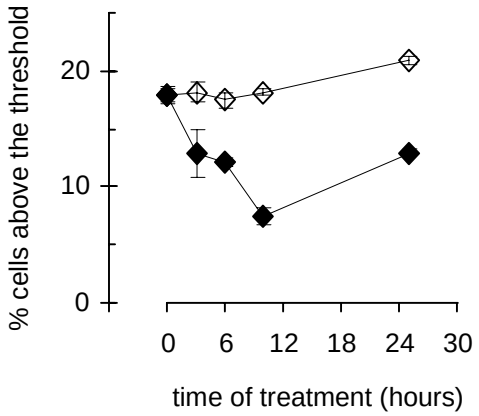

Figure 3.9 Effect of 5,6-dichloro-1-ß-D-ribofuranosylbenzimidazole (DRB) (20 mM) on the EGFP fluorescence in the permanently transfected HeLa-MAZ clone s06 (black symbols) and HeLa-MZA clone 01 (open symbols) cells. Treatments done in duplicates.

3.1.3 Effects of DNA damaging agents on expression of episomal GFP gene in HeLa cells

Previous experiments have shown that levels of the short-lived EGFP-ODC protein in cells provide enough fluorescence for detection of the gene expression by flow cytometry. At the same time, the protein turnover is fast enough to allow detection of the decrease of transcription caused by the specific inhibitors (α-amanitin and DRB) with a delay of several hours. We therefore hoped that the effects of transcription-blocking DNA damage could be detected in the HeLa-MAZ cells stably expressing the episomal EGFP-ODC transgene. UV-C irradiation specifically modifies DNA, without damaging other biomolecules. The spectrum of DNA base modifications induced at the chosen damage conditions consists almost exclusively of cyclobutane pyrimidine dimers and 6-4 photoproducts. Both of these bulky DNA adducts are very efficient in blocking the elongation phase of transcription (Donahue et al, 1994), (Brueckner et al, 2007). Accordingly, UV irradiation is well known to induce an immediate halt of global transcription in the exposed cells, making the subsequent recovery of transcription possible only after removal of the transcription-blocking DNA damage by nucleotide excision repair (NER). We exposed the monolayers of cultivated cells to different doses of UV-C (254 nm wavelength) and analysed the EGFP fluorescence in cells after 150 minutes by flow cytometry. The results demonstrated that the percentage of the fluorescent cells decreased gradually in a dose-dependent manner, starting from the UV-C dose of 75 J/m² (**Figure 3.10**). However, no detectable decrease of the EGFP fluorescence was observed at lower UV-C doses. In summary, EGFP expression in permanently transfected HeLa-MAZ cells showed an overall inverse correlation with UV-C dose and therefore with the probability of damage generation in the transcribed region of the gene (Kitsera et al, 2007).

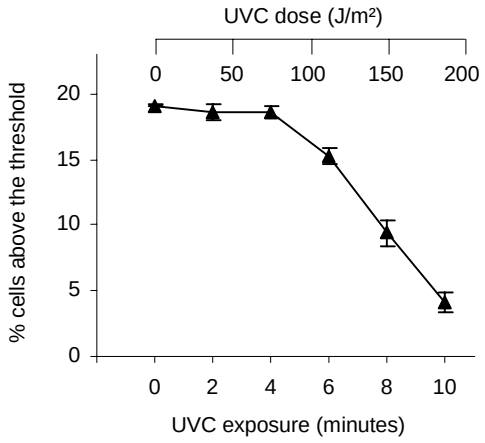

Figure 3.10 Percentages of HeLa-MAZ (clone "s06") cells, expressing significant amounts of EGFP fluorescence (FL1-H detected by flow cytometry), depending on the applied UV-C dose. Cells were irradiated in duplicates 150 minutes prior to analyses.

Decrease of the EGFP expression in UV-C treated HeLa-MAZ cells is consistent with interruption of transcriptional elongation at the sites of DNA damage, which is a generally accepted mechanism of transcription inhibition by UV irradiation. According to this model, ongoing DNA repair in cells chased after irradiation should lead to recovery of transcription, thus restoring the EGFP expression levels. To test this, the cells were allowed to repair for various time intervals following the UV-C dose of 150 J/m². Considering the average CPD yield of 1.7×10^{-5} $m^2/J\times bp$ (previously determined by Epe and co-workers), this dose is expected to induce on average $1.7\times10^{-5}\times150\times0.5\times1870=2.38$ CPDs per transcribed strand of the EGFP-ODC gene (the 1870 bp-long transcribed region).

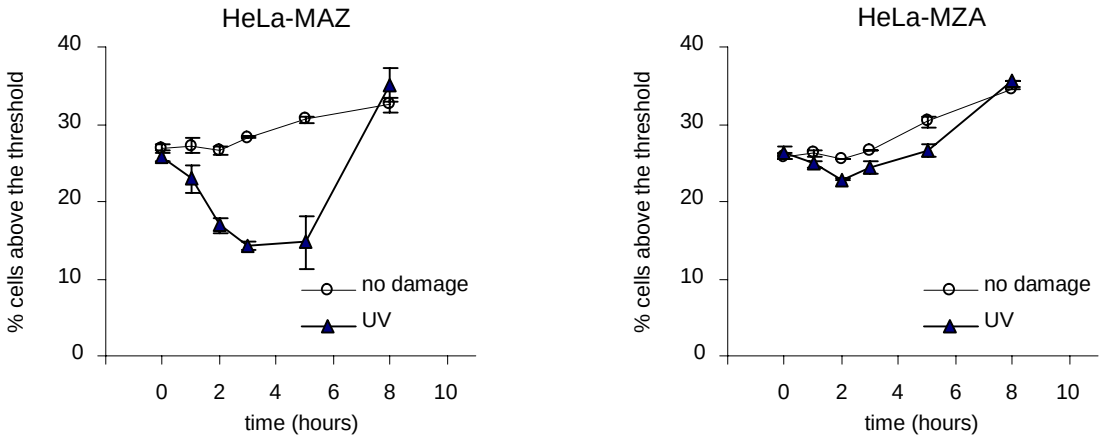

Figure 3.11 EGFP expression in HeLa-MAZ (clone "s06") and HeLa-MZA (clone "01") cells released after single exposure to 150 J/m² UV-C for the specified time intervals (duplicates). Error bars indicate data range.

In HeLa-MZA cells expressing the short-lived EGFP-ODC protein UV irradiation resulted in a rapid decrease of the specific fluorescence after the damage induction (**Figure 3.11**). The dynamics of this process was thus very similar to the effects caused by the specific inhibitors of RNA polymerase II (discussed in 3.1.2), in agreement with the transcription blocking potential of the induced DNA damage. As the UV-induced decrease of the gene expression was barely detectable in HeLa-MZA cells, which express conventional EGFP, the protein fluorophore was not significantly bleached or otherwise affected by UV, once again supporting the idea that the changes detected with the destabilised EGFP reflect the decrease of transcription. Remarkably, the decrease of gene expression slowed down gradually and ceased completely between 3 and 5 hours after the damage induction, being

followed by a rapid recovery between 5 and 8 hours. Subsequent experiments showed that decreasing the UV-C dose resulted in even more rapid fluorescence recovery (**Figure 3.12**). It is noteworthy that the beginning of recovery of the EGFP expression in these experiments approximately coincides in time with the repair time for pyrimidine dimers by TCR reported in the literature (Spivak et al, 2002), (Ljungman, 1999).

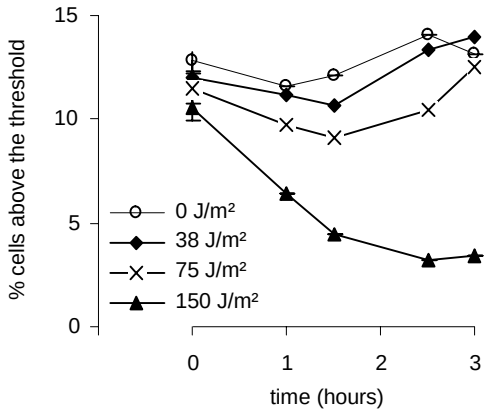

Figure 3.12 The effect of the UV-C dose on the EGFP expression in HeLa-MAZ (clone "s06") cells at different time intervals after irradiation.

As expression analysis of the short-lived EGFP-ODC protein in the UV damaged cells is in good agreement with current understanding of the mode of interaction of the UV-induced DNA damage with transcription, we interpret these results as an indication that this system is suitable for detection of DNA damage (decrease of the specific fluorescence), as well as repair (recovery of the fluorescence). We hoped that the effects of oxidative DNA base damage on the gene expression and repair of the oxidative DNA base modifications could be deduced in a similar approach from the EGFP fluorescence in HeLa-MAZ cells. We have chosen visible light in combination with a well-characterised photosentisizer Ro19-8022 (Will et al, 1999) as a damaging agent, because it induces with a high specificity oxidative purine modifications (primarily 8-oxoG) and because previous results from our lab (Epe and co-workers, unpublished) and of other group (Rybanska et al) indicated that this mode of damage causes a significant overall inhibition of RNA synthesis.

Damage in the presence of Ro19-8022 in the concentration range of 50–200 nM did not cause any detectable effect on the specific EGFP fluorescence in HeLa MAZ cells (data not

shown). Damage in the presence of 400–600 nM Ro19-8022 caused no significant decrease of the EGFP-specific fluorescence, but rather some increase at late times (between 4 and 6 hours) after the damage induction (**Figure 3.13** and data not shown). It is only starting from 800 nM concentration when the decrease of EGFP fluorescence was clearly detectable (**Figure 3.13**). However, no sign of transcription recovery was observed during 6 hours of incubation. It has to be noted that the analyses were complicated by the toxic effects of the concentrations ≥400 nM, which caused visible changes of cell morphology and death or loss of most of cells within several hours of incubation. It is thus hard to be sure that the observed decrease of the EGFP fluorescence level occurred specifically in consequence of a transcription block. The reported yields of Fpg-sensitive DNA modifications induced by 50 nM Ro19-8022 in AS52 cells at the same light exposure conditions averaged ~5×10^{-4} /kbp (Will et al, 1999). Extrapolating this value to my experimental conditions, the same dose is expected to induce approximately $5\times10^{-4}\times1.9\approx9.5\times10^{-4}$ Fpg-sensitive DNA modifications in the EGFP-ODC gene (≈1.9 kb). Consequently, the probability of damage DNA induction in the EGFP-ODC gene even at the highly cytotoxic dose of 1 µM Ro19-8022 is estimated to be less than 0.02. Therefore, the observed effect on the EGFP fluorescence was most likely indirect.

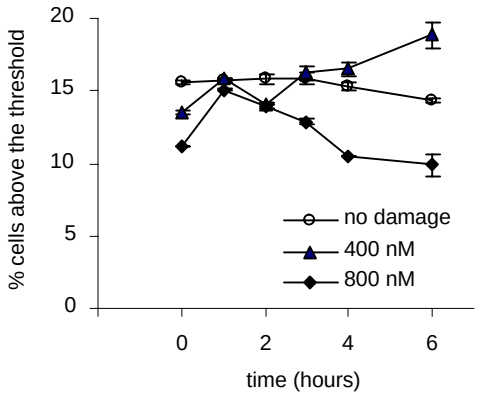

Figure 3.13 The effect of oxidative damage induced by photosensitisation in the presence of indicated concentrations of Ro-19-8022 on the EGFP expression in HeLa-MAZ (clone “s06”) cells.

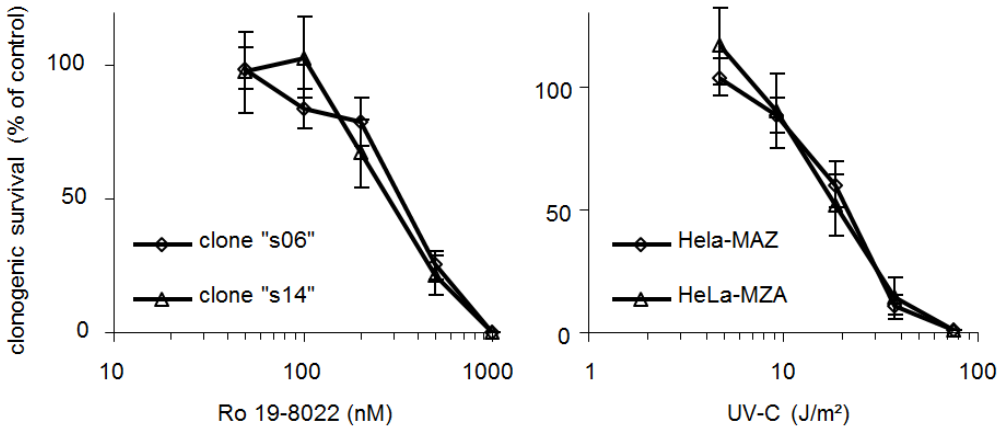

Figure 3.14 Clonogenic survival of HeLa-MAZ (two different clones) and HeLa-MZA (clone “01”) cells following damage induction with Ro19-8022 plus light (left) or UV-C (right).

In summary, application of the newly-constructed EGFP-ODC reporter protein allows detection of transcriptional changes in a single gene following DNA damage and in course of repair. The effects of DNA damage (induced by UV-C and by Ro19-8022 as a photosensitiser) confirm that both agents cause a significant inhibition of transcription, which can be detected from the changed levels of the short-lived EGFP protein in the stably transfected cells. The decrease of the gene expression is probably caused by direct transcription block in case of damage induced by UV-C, but most likely indirect in case of the oxidative damage induced by photosensitisation. It has to be stated however that in both cases inhibition of transcription required very high loads of damage (**Figures 3.10–3.13**). Clonogenic survival testing showed that such damage levels are highly cytotoxic (**Figure 3.14**). Unfortunately, the high levels of stress in cells under such damaging conditions make the application of the established cell lines problematic for studies of physiological transcriptional responses to DNA damage, especially if the effects of oxidative damage are to be addressed. For this reason I further concentrated on the development of complementary experimental approaches, which would allow investigation of the effects of 8-oxoG on transcription at a single gene level.

3.2 Effect of oxidative DNA damage induced in plasmid DNA by photosensitisation on expression of reporter genes transfected into mammalian host cells

3.2.1 Expression of oxidatively damaged plasmids in HeLa cells

To study the effect of oxidative DNA base damage on transcription in cells, the plasmids encoding for reporter proteins (EGFP and DsRed-Monomer) were damaged by exposure to visible light in the presence of methylene blue as a photosensitiser and transfected into HeLa cells. Exposure to visible light in the presence of methylene blue induces in double-stranded DNA 8-oxoG as a predominant base modification. Other types of DNA damage, such as oxidised pyrimidines, sites of base loss and single-strand breaks (SSB), are induced at many fold lower frequencies at these conditions (Epe et al, 1993), (Khobta et al, 2009). Firstly, I studied the effects of various doses of oxidative DNA damage on the expression of reporter plasmids in host cells. The pEGFP-mODC-ZA (encoding for EGFP) or pDsRed (encoding for DsRed-Monomer) plasmids were exposed to light in the presence of methylene blue for different times and then co-transfected together with a tracer plasmid into HeLa cells. The tracer plasmid was not damaged and always encoded for a different protein than the damaged plasmid. Thus, the non-damaged plasmid coding for DsRed-Monomer was co-transfected with the EGFP-encoding damaged plasmids and vice versa. Fluorescence signals of both proteins in cells expressing the fluorescent reporter proteins were detected by flow cytometry. Importantly, the combination of DsRed-Monomer and EGFP allows simultaneous detection of both proteins in the same cells without mutual interference between the specific fluorescent signals. So the cells expressing only DsRed-Monomer localised in the upper left quadrant of the fluorescence scatter plots, while the cells expressing only EGFP localize mostly in the lower right quadrant (**Figure 3.15**, panel A). The cells co-transfected with pZA and pDsRed plasmids localised in the upper right quadrant of the fluorescence scatter plots. If pEGFP-mODC-ZA was damaged and pDsRed-Monomer was used as a non-damaged tracer, EGFP expression in the cells decreased in dependence on the light exposure time of the plasmid. This could be detected as redistribution of population of the transfected cells from the upper right to the upper left quadrant in the fluorescence scatter plots. (**Figure 3.15**, upper row in panel A). Conversely, when pDsRed-Monomer was damaged and pZA used as the tracer plasmid, with increasing of irradiation time the population of transfected cells moved from the upper right to the lower right quadrant, thus reflecting the decreased DsRed-Monomer expression (**Figure 3.15**, lower row in panel A). In both cases the data clearly demonstrate dose-dependent decrease of protein expression in host cells because of the oxidative DNA base damage generated in the vector DNA by the photosensitiser methylene blue (**Figure 3.15**, panel B).

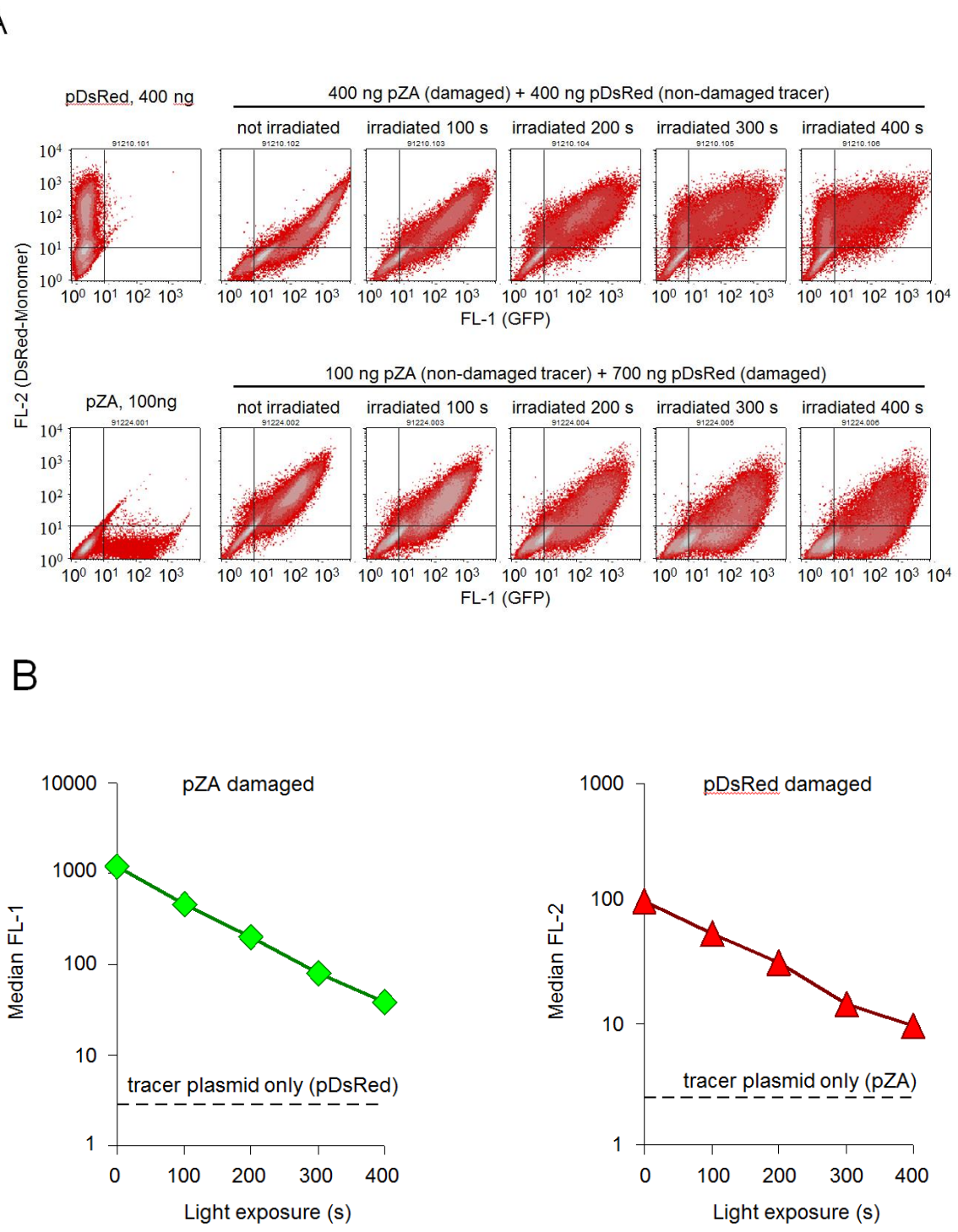

Figure 3.15 Effects of various doses of oxidative DNA base damage generated by methylene blue plus light on the expression of plasmid DNA in HeLa cells. (A) Dot density plots obtained by flow-cytometry of the transfected cells fixed 24 hours after transfections. (B) Expression of the damaged genes (calculated as median specific fluorescence in cells expressing the reference non damaged reporter gene). Dotted lines show the background fluorescence levels in cells transfected with reference plasmids only.

The influence of oxidative DNA damage (oxidised guanines) on plasmid expression were next analysed at various times after delivery into the host cells. HeLa cells are repair proficient. Therefore, if the oxidative base damage present in the plasmid DNA was capable of blocking the elongating RNA polymerase II complexes, this effect was expected to decrease and finally disappear after the damage is repaired in cells. We and others observed this previously during the host cell reactivation of UV-damaged plasmids (Khobta et al, 2009). In contrast, the EGFP-encoding plasmid containing on average three randomly distributed oxidised guanines (oG=3) generated by light in presence of photosensitiser demonstrated a progressing decrease of EGFP expression in time dependent manner (**Figure 3.16 A**). At early time point of 8 hours, damage had only a small effect on the EGFP gene expression, but a strong decrease of median GFP fluorescence was detected at subsequent time points. A time course of DsRed-Monomer expression in the cells transfected with oxidatively damaged pDsRed plasmid also showed a gradual and time-dependent decrease in expression of the damaged gene (**Figure 3.16 B**).

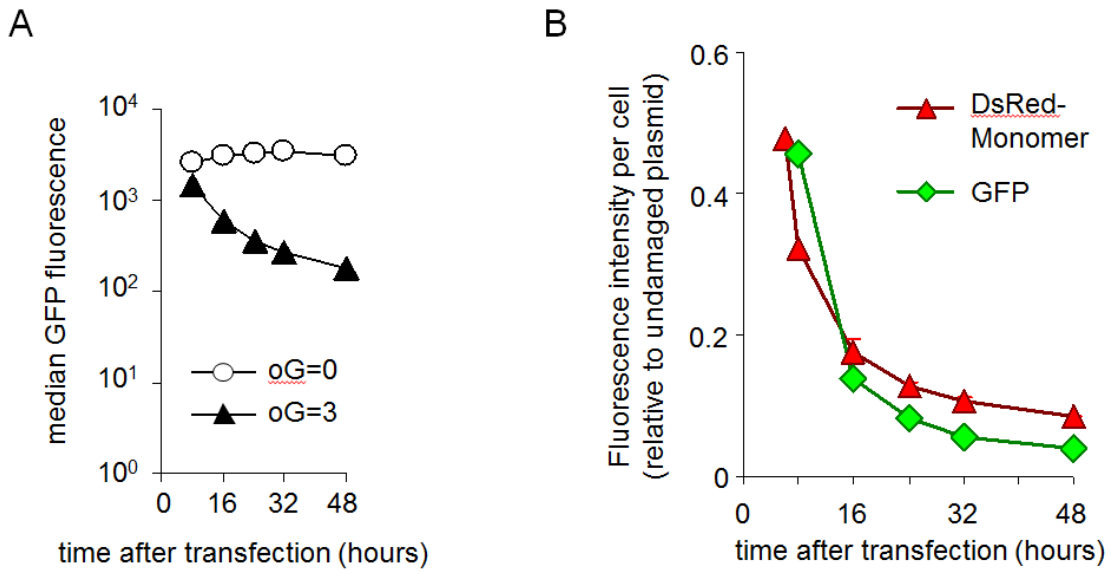

Figure 3.16 Gene inactivation by oxidative guanine lesions. A: median EGFP fluorescence of the HeLa cells transfected either with not damaged plasmid (oG=0) or the plasmid containing on average 3 oxidised guanines (oG=3). (B) Quantification of expression of the damaged pDsRed plasmid relative to the non-damaged pDsRed. The expression of the pZA plasmid exposed to the same damaging conditions relative to the non-damaged pZA is plotted for comparison.

In summary, the results indicate that, in contrast to the UV-damaged plasmid genes, which undergo repair-dependent reactivation in the host cells, the plasmid-borne genes containing the oxidative purine modifications do not undergo reactivation in the repair proficient host cells, but rather demonstrate decline of the gene transcription over time (Khobta et al, 2010a). This indicates that the effect of oxidised guanines (which are the predominant DNA base modifications induced by photosensitization in the presence of methylene blue) on gene expression could be different from the direct blockage of the elongating RNA polymerases at the damage site. To investigate the mechanism of transcription inhibition by these oxidative base modifications, we decided next to analyse their influence on gene expression in the host cells, which are deficient in repair of these lesions, such as Ogg1-null mouse embryonic fibroblasts (Klungland et al, 1999). In addition we tested the effect of deficiency in the Cockayne syndrome B protein (CSB), which is essential for the transcription recovery after various kinds of DNA damage (Colella et al, 1999), (Proietti-De-Santis et al, 2006). Importantly, CSB was also implicated in the bypass of 8-oxoG by RNA polymerase II in vitro (Charlet-Berguerand et al, 2006) and shown to participate in the repair of 8-oxoG (Osterod et al, 2002). These considerations stimulated us to extend the gene expression studies to the CSB-deficient cells as well.

3.2.2 Effects of CSB and OGG1 on expression of the oxidatively damaged plasmid gene in normal and repair-deficient MEFs

In Section 3.2.1 it was demonstrated that oxidative DNA base damage strongly decreased the expression of plasmid genes in repair proficient human cells. Preliminary results previously obtained in our laboratory by transfection of oxidatively damaged plasmid DNA to cells deficient in the repair of 8-oxoG provided indication that quantitative differences in gene expression were present between the repair-deficient (Ogg1-null) and repair-proficient cells. It has been suggested that oxidative damage inhibits transcription to a stronger extent in the absence of repair, although this result was not observed in all cell lines used in the study (Speckmann, 2006).

To address more systematically the role of repair protein in transcription inhibition by oxidative base modification we co-transfected non-damaged plasmid encoding for DsRed-Monomer together with equal amounts of non-damaged (OG=0) or damaged pEGFP-mODC-ZA plasmids in wild-type, *Ogg1*$^{-/-}$, *Csb*$^{m/m,}$ and *Csb*$^{m/m}$*/Ogg1*$^{-/-}$ cells. The levels of GFP expression were measured at 24 hours after transfection and calculated relative to non-damaged plasmid for every cell line. The plasmids were damaged by light in presence of the photosensitiser methylene blue prior to transfection and contained on average 1 (OG=1) and 3 (OG=3) oxidised guanines per plasmids molecule (Section 2.2.4.1). Oxidised guanines in

plasmid DNA had effect on expression levels of the reporter protein in all four mouse embryo fibroblast (MEF) cell lines. Interestingly, transfection of the same amounts of the same preparations of the damaged plasmids into different MEF cell lines showed that the absence of OGG1 protein did not cause significant decrease on the expression levels of oxidatively damaged plasmid DNA in cells. The inhibition of reporter protein expression in OGG1-null mouse embryonic fibroblasts was similar as in in wild-type MEFs (**Figure 3.17**). The strongest effect of damage on EGFP expression was observed in cells without functional Cockayne syndrome group B protein (CSB) but containing the wild-type *Ogg1* alleles (**Figure 3.17**). In Csb-deficient mouse cells even a single oxidised guanine per plasmid molecule caused a two-fold decrease in expression of the EGFP gene, while three oxidised guanines reduced the gene expression almost 5-fold, compared to the non-damaged plasmid (**Figure 3.17**). Deficiency in both OGG1 and CSB proteins resulted not in additive effects, but rather caused in a significantly improved expression of the damaged plasmids (2-fold reduction of gene expression against the 5-fold in the $Csb^{-/-}Ogg1^{wt/wt}$ MEFs in the presence of the same amounts of DNA damage) (**Figure 3.17**). The result thus show that CSB protein has an essential function for transcription of oxidatively damaged DNA, while OGG1 can cause decrease of gene expression of oxidatively damaged DNA when CSB protein is absent.

In the presence of CSB, the effect of the *Ogg1* gene status on expression of the damaged plasmid gene was not significant. On average, expression was reduced to a slightly bigger extent in OGG1-null cells, in comparison with wild-type. However this difference was not observed in all experiments. A plausible explanation of this result could be that CSB activity is sufficient to promote transcription of the damaged gene, regardless of functional base excision repair.

Besides the EGFP fluorescence levels, the amounts of GFP mRNA produced from the oxidatively damaged and non-damaged plasmids, as well as pDsRed-Monomer mRNA expressed from the co-transfected non-damaged reference plasmid were quantified in parallel in the same transfection experiment by reverse-transcription and real-time quantitative PCR, as described in Section 2.2.4.5. As shown in **Figure 3.18** the EGFP fluorescence measured after transfections with the damaged plasmid containing on average three oxidised guanines correlate well with the mRNA expression levels. The data show that oxidative DNA damage induced by photosensitisation in the presence of methylene blue resulted in decreased mRNA levels, especially in the MEF cell lines lacking the CSB protein, confirming the essential role of CSB protein in transcription of oxidatively damaged plasmid. The difference between $Csb^{m/m}$ and $Csb^{m/m}$ $Ogg1^{-/-}$ MEFs was also detectable at the level of mRNA, thus indicating that inhibition of transcription by oxidative damage is enhanced in the presence of the OGG1 protein.

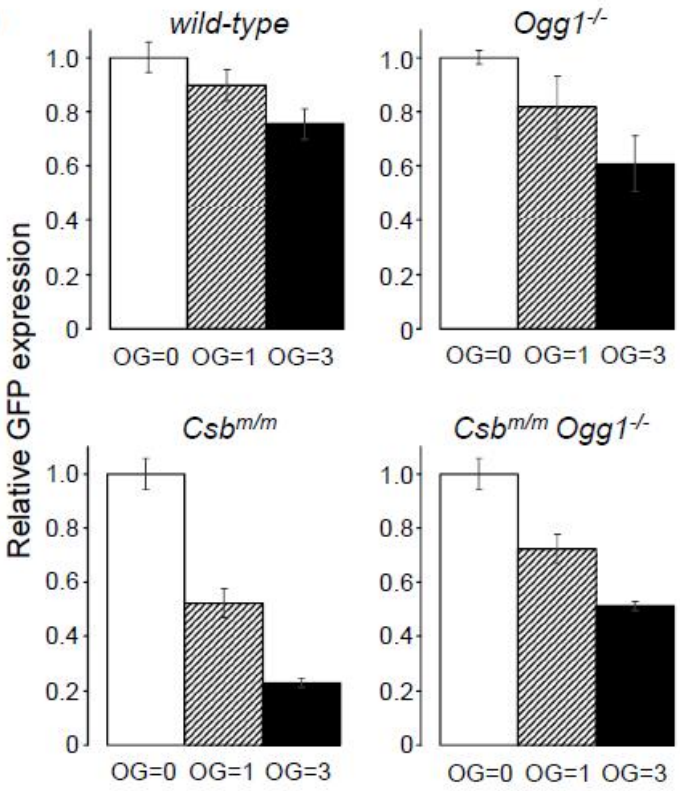

Figure 3.17 Effects of Cockayne syndrome group B protein (Csb) and 8-oxoguanine DNA glycosylase (OGG1) on plasmid inactivation by oxidative guanine lesions. Columns indicate means of four to six transfection experiments ±S.D.

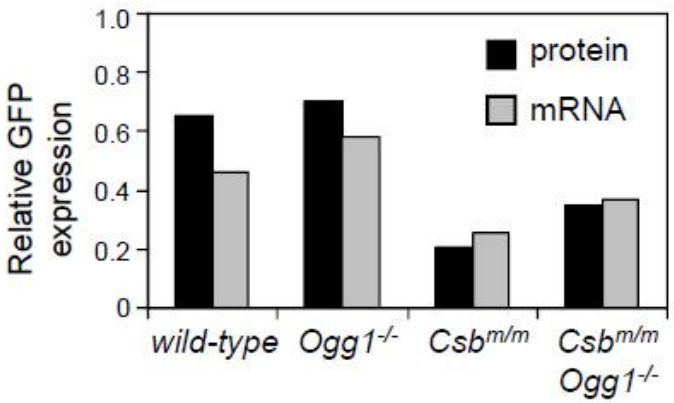

Figure 3.18 Comparison of EGFP protein (FL-1 fluorescence) and mRNA levels (rtq-PCR) measured 24 hours after transfections with the plasmid containing 3 oxidised guanines (generated by light in the presence of methylene blue), relative to the corresponding dark-incubated plasmid DNA.

Altogether these results put on evidence a pronounced negative effect of oxidative damage induced by photosensitisation on gene expression. The inhibition of transcription reported here was probably caused by 8-oxoG, which was the predominant base modifications under our experimental conditions. To verify this, plasmid substrates containing single 8-oxoG in the transcribed region of the EGFP gene had to be constructed.

3.3 Production of plasmid DNA containing single synthetic 8-oxoG in defined positions within the reporter EGFP gene

3.3.1 Incorporation of synthetic 8-oxoG residues by annealing and extension of the modified oligonucleotides on a single-stranded circular DNA template

To incorporate synthetic 8-oxoG into various positions in the reporter plasmid encoding for EGFP, it was initially planned to perform annealing and extension of the modified oligonucleotides on single-stranded circular DNA template as a variation of the method proposed by Bregeon and Doetsch for targeted incorporation of 8-oxoG into the luciferase gene sequence (Bregeon & Doetsch, 2004). The original procedure had to be modified to meet the requirements of the present study, particularly, the need for precise quantitative measurement of the reporter gene expression. Therefore, the EGFP gene was chosen as a reporter compatible with flow cytometric analyses, and the experimental protocol was adjusted towards minimisation of the fraction of plasmid DNA containing single-strand breaks.

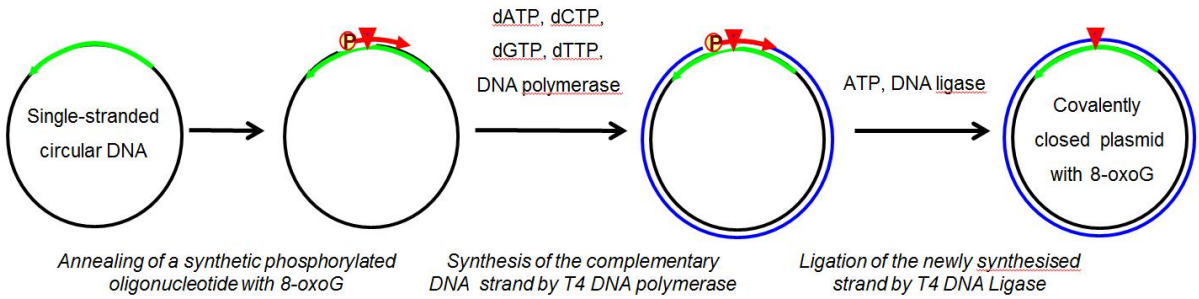

Figure 3.19 A strategy for the production of plasmid DNA, containing synthetic 8-oxoG in defined position in the reporter EGFP gene, from the single stranded circular DNA. Green arrow indicates the EGFP coding sequence

The outline of the procedure is shown in **Figure 3.19**. The DNA strand corresponding to the coding strand of the EGFP gene was obtained from the filamentous phage M13. This single-stranded circular DNA was then used as a template for annealing of synthetic deoxyribo-oligonucleotides containing G or 8-oxoG, which served as primers for synthesis of the second strand of the plasmid DNA with the help of the T4 DNA polymerase. Covalently closed plasmid DNA harbouring the synthetic oligonucleotides was obtained by intramolecular ligation in the presence of the T4 DNA ligase. To pursue this strategy, the pair

of vectors suitable for production of each of the DNA strands of EGFP gene in the M13 phage had to be produced. Subsequently, each step of the procedure of incorporation of the synthetic 8-oxoG was optimised, starting from the production of the single-stranded circular template DNA.

3.3.1.1 Construction of vectors suitable for incorporation of single modified bases into different strands of the EGFP gene

As a backbone for the vector with defined positioning of synthetic lesions, I used the pEGFP-mODC-ZA (clone C2) phagemid vector previously constructed in our lab by A. Khobta (Khobta et al, 2009). The experimental procedure for the introduction of a modified base into the transcribed ("minus") strand of EGFP gene starts from packaging of the coding ("plus") strand of EGFP into the M13 phage particles. A complementary phosphorylated oligonucleotide with the desired base modification is then annealed to the single stranded circular DNA, isolated from the phage, and extended with DNA polymerase to produce the second DNA strand. Finally, covalently closed double-stranded circular DNA is obtained by intramolecular ligation. This strategy can be used to introduce single modified bases in various positions and also to insert "tandem lesions" (several base modifications, present within a short distance in the same DNA strand) with a single synthetic oligonucleotide.

Upon infections of the *Escherichia coli* host with the M13KO7 helper phage, the strand of the phagemid DNA containing the phage origin of replication (f1 ori) undergoes processive single-stranded replication, which results in production of recombinant virions, containing the circular form of this single DNA strand. In the pEGFP-mODC-ZA plasmid the EGFP coding sequence and the phage origin of replication are localised in different DNA strands. This will be referred to as "plus" orientation, because it allows replication of the transcribed strand of the EGFP gene by the helper phage, while the synthesis of the non-transcribed strand of the gene will be primed in the subsequent step by a complementary synthetic oligonucleotide, thus leaving the modified oligonucleotide incorporated in the non-transcribed ("plus") strand of the resulting double-stranded plasmid DNA. Conversely, to insert a modified base into the transcribed strand of EGFP gene, a plasmid containing the f1 ori and the EGFP coding sequence in the same DNA strand ("minus" orientation) was required.

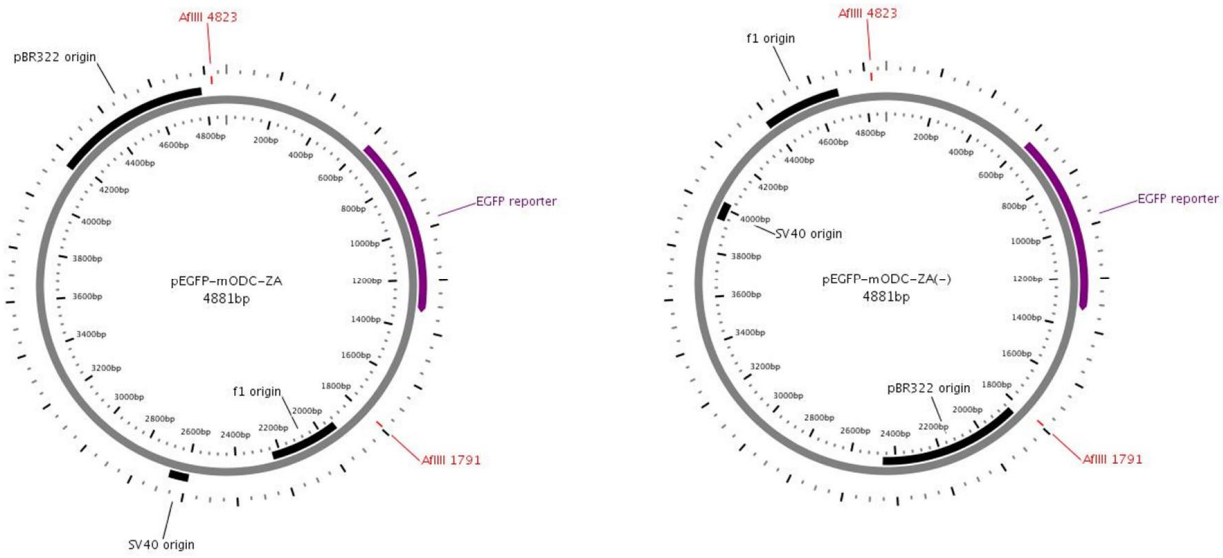

Figure 3.20 Maps of pEGFP-mODC-ZA and pEGFP-mODC-ZA(-) plasmids, which are also referred to as pZA and pZAss, respectively.

The original pEGFP-mODC-ZA plasmid contains two sites for the restriction endonuclease AflIII on both sites of the f1 ori, which can be used to invert the direction of the phage origin of replication with respect to the EGFP gene. However, as the two sequences cut by AflIII are different (5′-ACGCGT-3′ and 5′-ACATGT-3′), the overhangs produced by the enzyme can be ligated only in one orientation. Blunting the DNA ends with the T4 DNA polymerase and subsequent ligation did not produce viable clones with a desired orientation of the f1 ori after transformation of the SCS-8 strain of *E. coli*. In order to achieve a directional ligation of the two vector fragments, it was therefore necessary to invert the polarity of the AflIII sites. This was achieved by amplifying a bigger fragment of the vector (containing the f1 ori) by PCR with the Pfu Turbo proofreading DNA polymerase, using primers containing the inverted AflIII sites on their 5′ ends (section 2.2.5.1). The PCR products were purified by the MinElute Reaction Cleanup Kit and cut with the restriction endonuclease AflIII to produce sticky ends for ligation. The digested PCR products were then purified from the agarose gels, combined with the 1849 bp. AflIII digested fragment containing the EGFP gene and ligated at different temperatures (25°C and 16°C). A three-fold molar excess of the fragment containing the EGFP gene was used for ligation. Direction of ligation was set by the asymmetric AflII sites. 10 colonies were obtained after ligation at 25°C, and 7 colonies by ligation at 16°C. After PCR screening of 10 colonies obtained by ligation at 25°C (section 2.2.5.2), a single clone was identified which was assembled in the desired “minus” orientation of the f1 ori with respect to the EGFP coding strand **Figure 3.21.** All other clones demonstrated the band of 173 bp with the primers designed for the “plus” orientation and a non-specific band of

approximately 500 bp with the primers designed for the “minus” orientation, indicating that these clones contained rearranged plasmids.

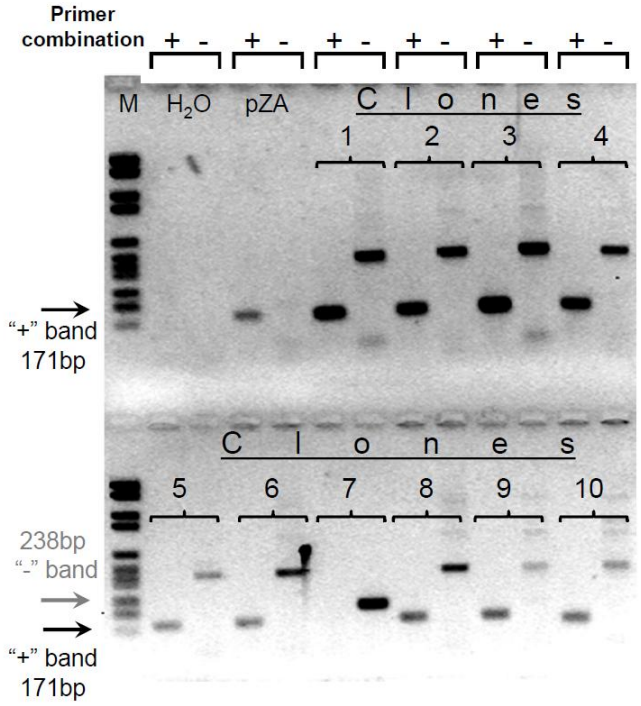

Figure 3.21 Agarose gel electrophoresis in TAE buffer of the PCR screening products obtained with primers for the “minus” and “plus” orientations. The PCR product of 238 bp, which is characteristic for the “minus” orientation, appears only with the template DNA from clone 7.

The correct clone (number 7 in **Figure 3.21**) of the obtained pZA(-) plasmid was cut with AflIII to check for possible degradation of DNA ends at the ligation sites. Appearance of specifics bands of 3032 bp and 1849 bp in agarose gel electrophoresis after digestion with AflIII (**Figure 3.22**) demonstrate the presence of AflIII recognition sites. The correct orientation of the f1 ori in the newly produced pZA(-) plasmid was further verified by sequencing. Sequencing of the f1 replication origin is shown in Appendix Finally, the pZA(-) plasmid was checked for efficient expression of the EGFP gene by transfection into HeLa cells. As shown in **Figure 3.23**, both pZA(-) clone 7 (later called pZAss) and pEGFP-mODC-ZA (later called pZA) plasmids demonstrate similarly high levels of EGFP expression (middle and right dot plots).

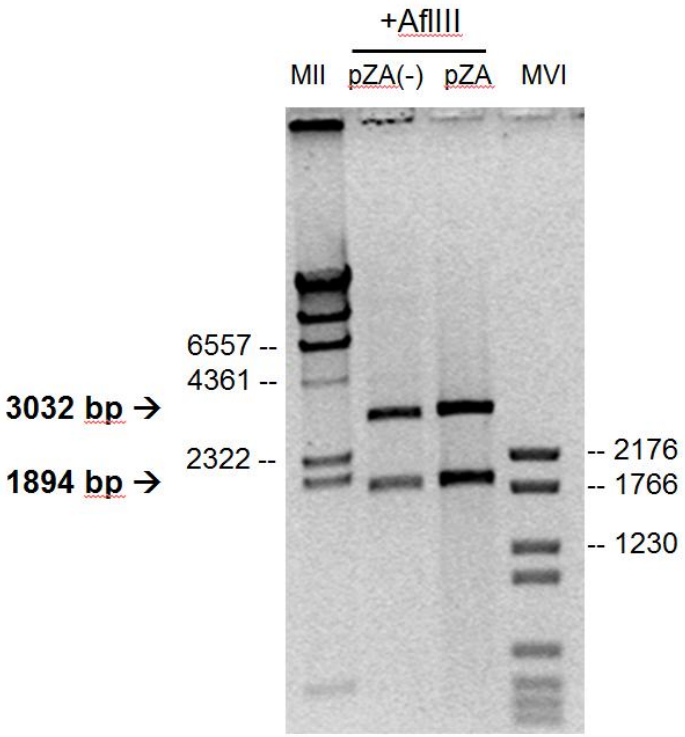

Figure 3.22 Digestion of the pZA(-) plasmid with AflIII as a control for unaltered DNA sequence at the ligation sites.

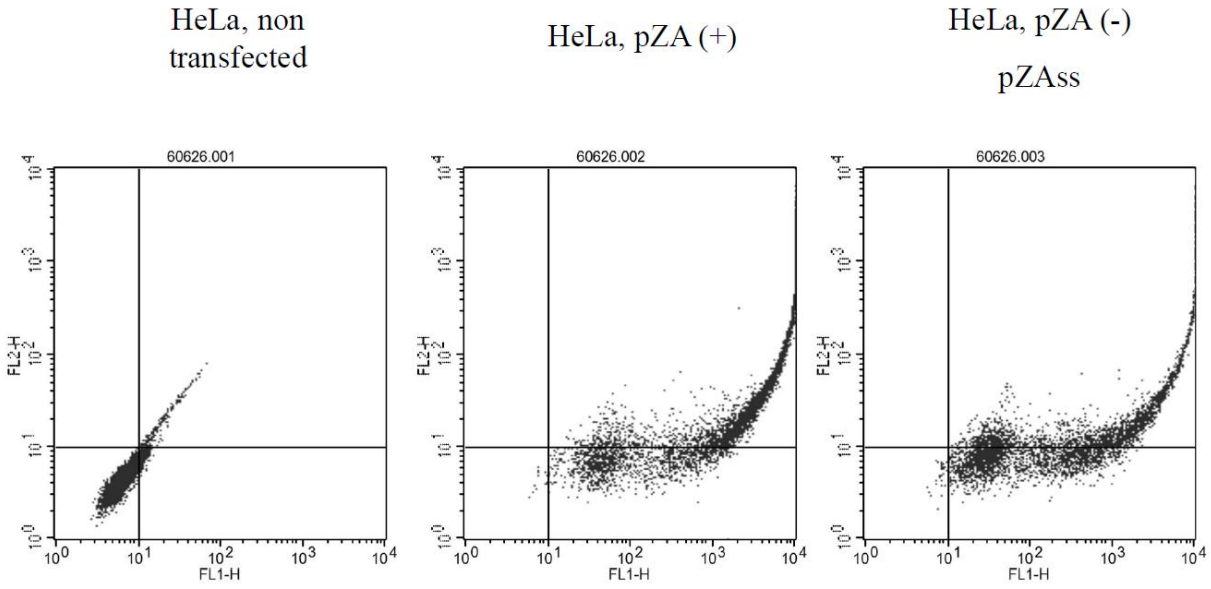

Figure 3.23 Dot density plots obtained by flow cytometry of HeLa cell transfected with pZAss or pZA plasmids. Left panel is HeLa cells without plasmid.

Following the sequence analyses and verification of the EGFP expression, we expanded pZAss in the *E. coli* SCS-8 strain, but found extensive rearrangements of the isolated plasmid, as detected by appearance of multiple bands in the agarose gel (not shown). To avoid the plasmid rearrangements, a bacterial strain with deficient recombination had to be used for propagation of the pZAss plasmid. The *E. coli* strain JM109 that carries the *rec*A1 and *end*A1 mutations was chosen. The *rec*A1-deficient genotype provides minimized recombination and aids in plasmid stability, while *end*A1 is helpful for isolation of endotoxin-free plasmid DNA. Bacteria of the strain JM109 are also suitable for the production of ss-DNA from the filamentous phage particles, because they contain an F' episome that allows infection with the M13 helper phages (Yanisch-Perron et al, 1985). This made *E. coli* JM109 the host strain of choice for this scope in the subsequent experiments.

3.3.1.2 Production of single-stranded circular DNA from the filamentous phage

Two methods were compared for isolation of the circular single stranded plasmid DNA (ssDNA), namely isolation from the M13 helper phage and degradation of one DNA strand starting from a nick introduced in the plasmid DNA isolated from bacteria.

For isolation of single stranded circular DNA from the M13 helper phage, I first verified the packaging of the correct DNA strands of the plasmids pZAss and pZA by the phage. The two plasmids contain the M13 origin of replication in opposing direction with respect to the EGFP gene. The DNA strands produced by the phage from different plasmids are partially complimentary and can be annealed together forming a heteroduplex in the regions encompassing the EGFP gene. Heating and slow annealing of a mixture of equal amounts of single-stranded circular pZAss and pZA (containing “plus” and “minus” strands of the EGFP gene, respectively) resulted in an additional band, migrating like a circular double stranded plasmid in the agarose gel (**Figure 3.24**). To verify that this band corresponded to a structure with partial annealing between the strands, we incubated this DNA with HindIII and obtained another band that migrates like a linear plasmid. Specific cutting by HindIII thus confirmed the presence of a duplex DNA region, indicating that pZAss and pZA circular ssDNA isolated from the phage contain the opposing DNA strands of the EGFP gene.

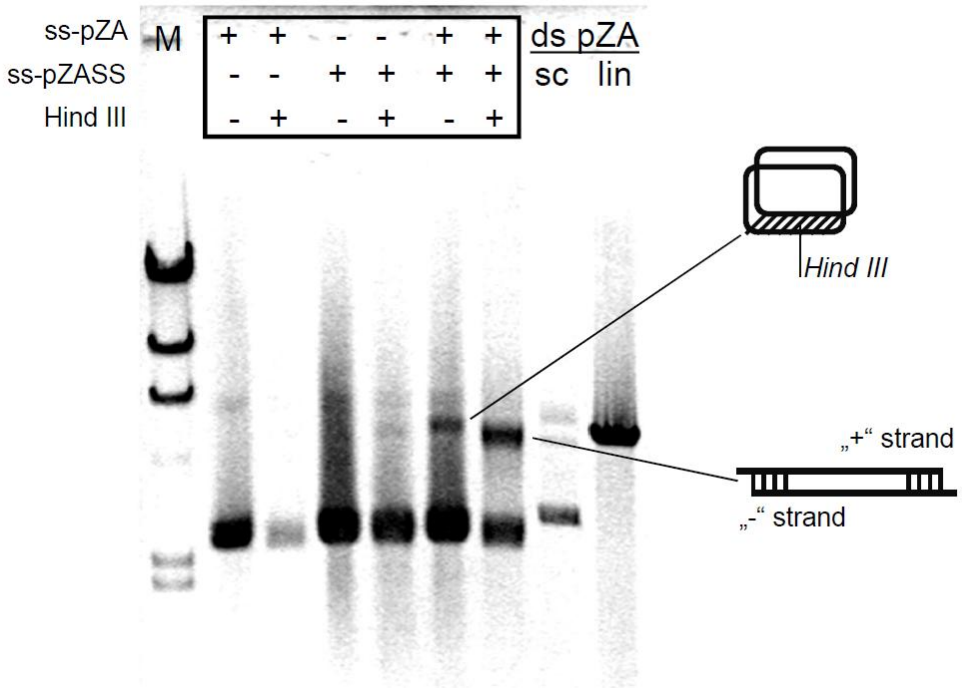

Figure 3.24 Verification of production of ssDNA containing the EGFP “+” and “-“ DNA strands by the M13 helper phage. Pictograms on the right represent the configurations of DNA found in the bands.

Next, I performed large-scale isolations of ssDNA. Several modifications have been done with respect to the previously described protocols in order to eliminate the RNA and protein contamination and thus increase the purity of DNA. Namely, RNAse A and Protease K treatments of the precipitated phage DNA were included as described in Section 2.2.6.1. Nevertheless, after performing multiple isolations of ssDNA from *E. coli* infected with the helper phage, it was noticed that the recoveries of circular ssDNA were highly variable. Besides that, most of the ssDNA preparations were not homogenous. Various degrees of contamination with the phage genome DNA were detected in all experiments. Moreover, ssDNA preparations sometimes contained significant amounts of linear and supercoiled forms of double-stranded plasmid DNA (**Figure 3.25**).

Contamination with double-stranded plasmid DNA was highly undesirable, because the unmodified supercoiled plasmid would be carried over to the final preparation of the 8-oxoG-containing covalently closed plasmid DNA. Therefore, this contamination had to be removed by treatments with the EcoRI restriction endonuclease prior to the second strand synthesis reaction. This enzyme cuts double-stranded DNA in the sequence 5′-GAATTC-3′, converting the supercoiled pZAss into the linear form. The presence of the linearised plasmid in the second strand synthesis reactions did not disturb their efficiency (data not shown). The unmodified linear DNA was left behind during the final gel purification step of the ligated plasmid DNA following the second strand synthesis (Methods section 2.2.6.6)

Altogether, the procedure of isolation of single strand circular DNA from the phage particles, although efficient, suffers from high variability and often requires additional purification steps. Moreover, there is a concern that a fraction of ssDNA molecules isolated from the phage particles could contain deletions and other types of genetic rearrangements. This stimulated us to seek for an alternative, more efficient method for production of single-stranded circular DNA templates.

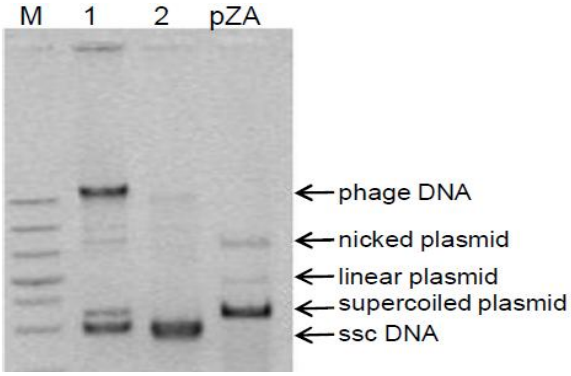

Figure 3.25 Two independent isolations of ssDNA from the M13K07 helper phage (1 and 2) containing different levels of contamination with phage genome DNA and double-stranded plasmid. Agarose gel electrophoresis (0.8% in TAE). M: 1 kb DNA Ladder (Fermentas); pZA: double-stranded plasmid DNA.

3.3.1.3 Production of single-stranded circular DNA with the help of a nicking endonuclease and exonuclease III

Exonuclease III (ExoIII) is widely used in molecular cloning for generation of targeted deletions, thanks to its 3′→5′ exodeoxyribonuclease activity specific for double-stranded DNA. ExoIII degrades dsDNA from blunt ends, 5′-overhangs or nicks, releasing 5′-mononucleotides from the 3′-ends of DNA strands and producing stretches of single-stranded DNA (Rogers & Weiss, 1980).

We reasoned that single-stranded circular DNA can be produced by degradation of one DNA strand of a plasmid with ExoIII starting from a nick (section 2.2.6.4) and (**Figure 3.26 panel A**). We therefore analysed both pZA and pZAss (pNK) plasmid sequences for the presence of recognition sites for the commercially available endonucleases, which have DNA strand specific nicking activities. We have found that both plasmids contain a BsrDI recognition site, which can be nicked by the mutant restriction endonuclease Nb.BsrDI (NEB), cutting only one DNA strand within a double-stranded DNA sequence 5′-ᵛCATTGC-3′. The DNA strand nicked by the enzyme corresponds to the transcribed strand of the EGFP gene in the pZA plasmid, and to the non-transcribed strand in pZAss. The enzymatically nicked DNA can be treated with ExoIII, which is capable of degradation of the nicked DNA strand. Thus, an extensive treatment of circular DNA, containing nick in the strand of choice, is expected to

result in homogenous single-stranded circular DNA. Indeed, it was shown that nicking by BsrDI is very specific and efficient (Khobta et al, 2010b) (**Figure 3.26**, panels B and C). This is of critical importance for the whole procedure, because the plasmid DNA without a nick cannot be degraded by ExoIII and would subsequently contaminate the final preparation of the 8-oxoG-containing ccc-DNA. After optimisation of the Exonuclease III digestion, the procedure resulted in high yields of ssDNA which was free of contamination with double-stranded plasmid DNA (**Figure. 3.26**, panels B and C). The method requires only two enzymatic steps starting from plasmid DNA that can be easily isolated in large amounts from bacteria, thus being very cost- and time-efficient in comparison to the procedure of ssDNA isolation from the recombinant M13 phage. Moreover, in the subsequent step of production of the covalently closed circular DNA incorporating the modified oligonucleotides, we repeatedly documented higher efficiencies of primer annealing and increased rates of DNA synthesis and ligation with these ssDNA templates in comparison with the templates extracted from the M13 phage. This could be attributed to a higher purity of the template DNA, particularly the absence of the phage envelope proteins.

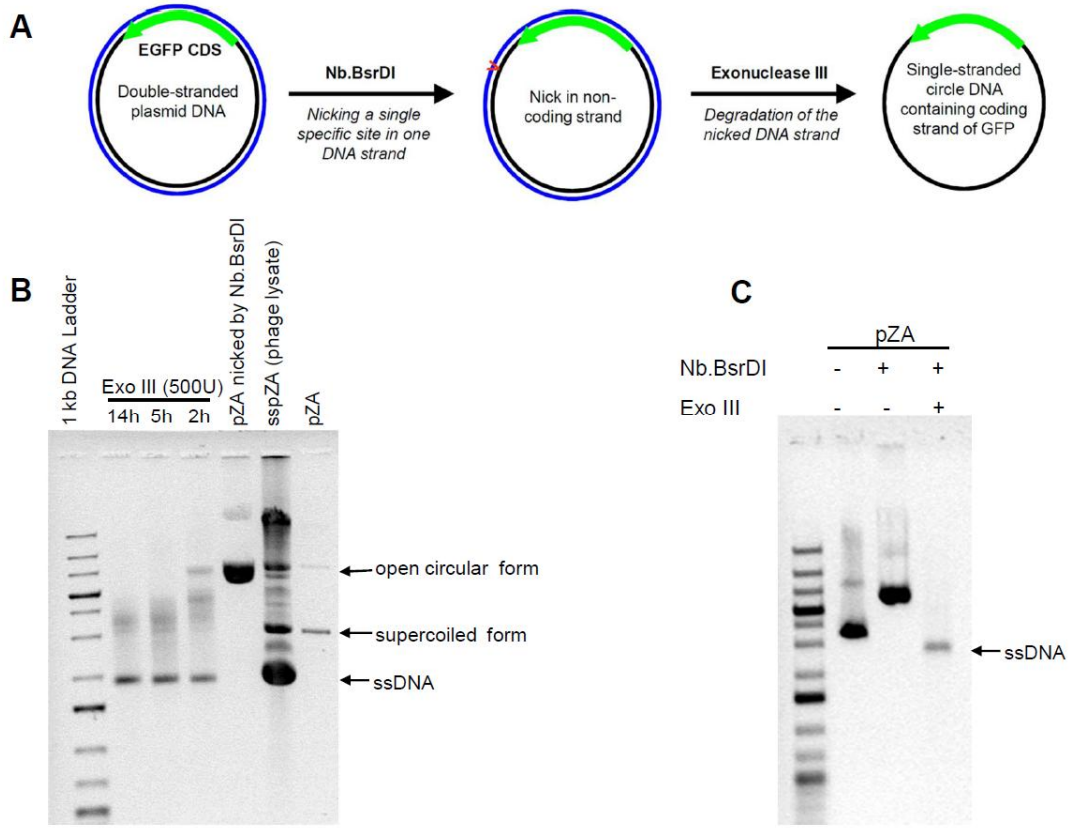

Figure 3.26 Production of ssDNA by Exo III method. (A) The scheme of the procedure. (B) Analyses of the nicked plasmid DNA before and after incubations with exonuclease III (ExoIII). Covalently closed pZA and M13 phage lysate are loaded as references for the electrophoretic mobility of different forms of the plasmid DNA. (C) Generation of circular ssDNA under the optimised conditions.

3.3.1.4 Production and purification of covalently closed plasmid vector DNA enclosing a synthetic nucleotide

To obtain covalently closed circular plasmid DNA, containing a single lesion in the defined strand and position, we performed primer extension around a circular single-stranded DNA templates followed by ligation (**Figure 3.19**).The ssDNA templates obtained either from the M13 phage (Methods section 2.2.6.1) or by the ExoIII digestions of the circular plasmid DNA starting from the nicks (Methods section 2.2.6.4) were used.

In order to obtain maximal yield of the modified plasmids, every steps of the procedure was optimised. For optimisation of the second strand synthesis (extension by T4 DNA Polymerase) and to test the buffer compatibility with the subsequent ligation step, I performed the reactions in the T4 DNA polymerase and the T4 DNA ligase buffers, trying various dNTP concentrations in the reactions (0 mM; 0.5 mM; 1 mM and 2 mM). High efficiencies of the DNA synthesis were consistently observed in the T4 DNA polymerase buffer (**Figure 3.27**). However, the subsequent ligation reaction did not take place in this buffer (not shown). In the T4 ligase buffer, the highest efficiency of the second strand synthesis was observed in the presence of 1 mM dNTPs, which corresponded to a yield of dsDNA of at least 90% (**Figure 3.27**, lane 4). These conditions were kept for all subsequent preparative reactions.

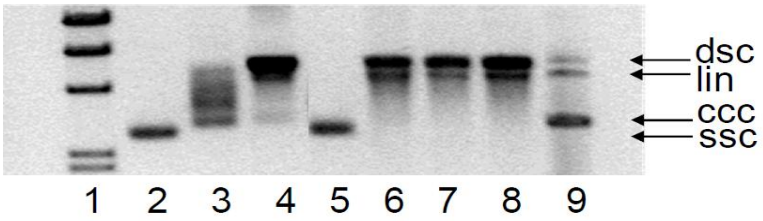

Figure 3.27 Optimization of dNTP concentration in the second strand synthesis reaction. Agarose gel electrophoresis (0.8%) in TAE buffer of products of the primer extension reactions. Lane 1: Lambda DNA/HindIII DNA marker; lanes 2−4: reactions in T4 DNA Polymerase buffer supplemented with various dNTP concentrations (0 mM; 0.5 mM; 1 mM); lanes 5−8: reactions in T4 DNA Ligase buffer supplemented with various dNTP concentrations (0 mM; 0.5 mM; 1 mM, 2 mM). Lane 9 is supercoiled pZA plasmid from bacteria. Plasmid forms by topology are indicated on the right: double stranded circular (dsc), linear (lin), covalently closed circular (ccc), single stranded circular (ssc).

Next, to optimise the concentration of primers, the extension reactions were performed in the presence of a range of increasing concentrations of the pNK1_synth915-t_G906 primer on the ssDNA template (**Figure 3.28**). The best efficiency of the second strand synthesis and

the minimal amount of the incompletely extended products were observed in the reactions performed with 0.8 µM of the primer. These reaction conditions were further kept for all primers and templates (2.2.6.5).

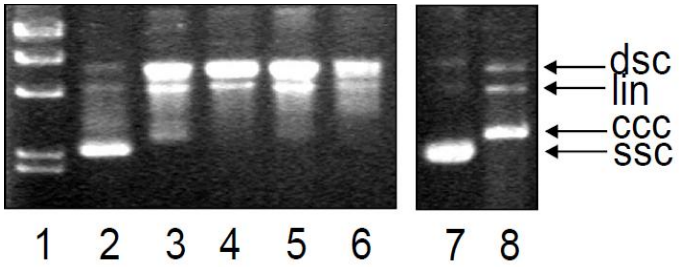

Figure 3.28 Optimization of primer concentration for the second strand synthesis reactions on the ssDNA (pZAss) template with the pNK1_synth915-t_G906 primer. Agarose gel electrophoresis (0.8%) in TAE buffer. Lane 1: DNA Ladder; lane 2: a reaction incubated without primer; lanes 3–6: products of reactions performed with primer concentrations of 0.4 µM, 0.8 µM, 1.6 µM, and 3.2 µM; lane 7: single-stranded circular plasmid DNA isolated from phage; lane 8: supercoiled pZA plasmid from bacteria. Different forms of plasmid DNA are: double stranded circular (dsc), linear (lin), covalently closed circular (ccc), single stranded circular (ssc).

In the next step, the temperature and duration of the ligation reactions were optimised, by comparison of the ratios of the amounts of the covalently closed double-stranded DNA to the amounts of the single stranded circular and linear DNA forms in the alkaline agarose gels. Only the latter two bands were present in the absence of ligase and also in the reactions performed at 16°C (**Figure 3.29**). Covalently closed double-stranded DNA (labelled “ccc” in **Figure 3.29**) was only generated in the reactions performed at 37°C, constituting 24% and 31% of total DNA in the samples incubated for 6 and 20 hours, respectively (**Figure 3.29**). Further increase of the ligation time did not improve the yield of the covalently closed DNA.

The relatively inefficient ligation could be explained either by the lack of a few nucleotides in the newly synthesized DNA strand or by a poor alignment of the DNA ends for ligation. In attempt to increase the fraction of covalently closed double stranded DNA in the final product, we tested several additives, which have been reported to improve the efficiencies of the polymerisation and ligation. Other researchers have shown that high concentrations of polyethylene glycol (PEG 6000) in the reaction buffer increased the T4 DNA ligase efficiency (Pheiffer & Zimmerman, 1983). Besides that, Brégeon and Doetsch have demonstrated that addition of PEG 8000 to a final concentration of 7.5% greatly increased the yield of ccDNA produced during the polymerization/ligation reaction (Bregeon & Doetsch, 2004). We

therefore performed the DNA second strand synthesis and ligation reactions in the presence of this compound. However, PEG 8000 failed to improve the yields of the double stranded covalently closed circular DNA in our hands (**Figure 3.30**). For this reason, further reactions were always performed in the absence of PEG and the single strand binding protein.

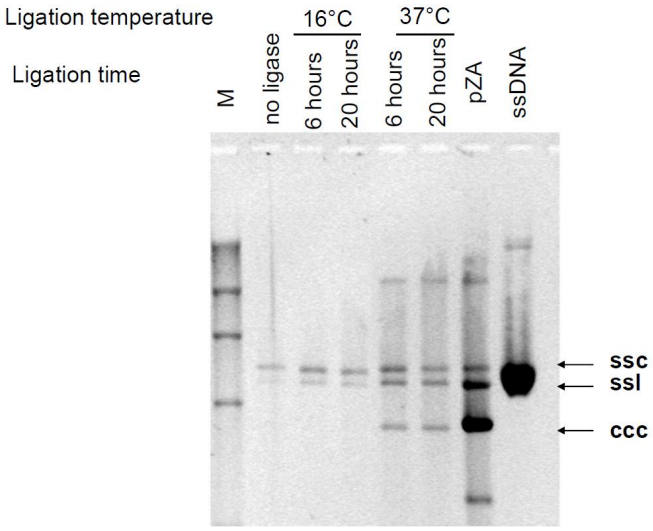

Figure 3.29 Optimisation of second strand ligation. Alkaline agarose gel (0.8%) of the products obtained at different ligation temperatures.

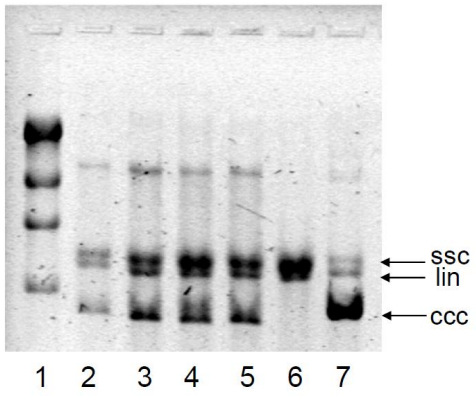

Figure 3.30 Testing the effect of addition of PEG 8000 on the yield of cccDNA produced during the polymerization/ligation reaction. MinElute Spin Columns (Qiagen) were used to remove PEG after the reactions. Alkaline agarose gel electrophoresis (0.8%). Lane 1: DNA Ladder, lanes 2 – 3: ligation reaction without PEG before and after columns; lanes 4 – 5: reactions with 5% PEG before and after columns; lane 6: single-stranded circular plasmid DNA isolated from phage; and lane 7 is pZA plasmid isolated from bacteria. Different forms of the plasmid DNA are: linear (lin); covalently closed circular (ccc); single stranded circular (ssc).

In spite of the performed optimisation steps, the yield of ccc plasmid DNA did not exceed 30% of DNA in the whole reaction products (**Figures 3.29** and **3.30**). Therefore, additional purification was necessary in order to obtain homogeneous covalently closed double stranded circular DNA suitable for transfections. Covalently closed circular plasmid DNA in ethidium bromide agarose gel migrates as supercoiled DNA and can be resolved from single-stranded closed circular and nicked double-stranded plasmids (Bregeon & Doetsch, 2004). I have compared the efficiencies of several methods for isolation of the covalently closed plasmid DNA from the excised band in the agarose gel.

In a first option, the DNA extraction kit (Fermentas) was employed. The procedure involves solubilisation of the DNA-containing agarose slice in a sodium iodide-containing buffer, absorption of DNA to glass milk and elution with the low ionic strength buffer. The method results in good yield of the extracted DNA (60-70% of the total DNA in the band). However, the OD260/OD280 ratio of the obtained DNA preparations was always lower than 1.8, possibly indicating a significant degree of contamination with agarose. Unfortunately, the procedure also generated single strand breaks in a significant fraction (up to 50%) of the isolated plasmid molecules (not shown). We deemed that some strand breaks could arise from decomposition of abasic sites, which could be generated by the exposure to a weakly acidic pH in the binding solution. However, changing pH from 5.0 to 7.0 and decreasing the incubation temperature from 55°C to 50°C did not greatly reduce the formation of strand breaks whilst had a negative effect on the overall yields of DNA. Altogether, it is hard to exclude that the quality of the plasmid DNA prepared by this method could be insufficient to allow the judgment about the specific effects of 8-oxoG in the subsequent transfection experiments, i.e. care should be taken with interpretation of results.

As alternative options for DNA isolation from the excised bands of the preparative agarose gels, we tested electroelution *(*using D-Tube Dialyzers, Novagen) and squeezing of the excised agarose slice containing the desired band of covalently closed circular DNA by ultracentrifugation. Both procedures were followed by extraction with phenol:chlorophorm and precipitation of DNA with ethanol or isopropanol (2.2.1.2). Although both methods suffered from low efficiencies (approximately 5-10% yield) and poor OD260 to OD280 ratios (similar to the DNA produced by the NaI method), the recovered DNA mostly consisted of the covalently closed plasmid (**Figure 3.31**) and could thus be suitable for transfections and the quantitative gene expression analyses. Ultracentrifugation was a method of choice, for its simplicity, low cost and time efficiency.

The amounts of the obtained covalently closed circular plasmid DNA were relatively low. For this reason we had to use a more sensitive quantification method than the commonly used spectrophotometric quantification of plasmid DNA. Another reason was the presence of impurities, which could interfere with the spectrophotometric quantification if absorb at 260 nm. An alternative way to estimate DNA concentration is to measure the fluorescence intensity of dyes that bind to nucleic acids. We quantified plasmid DNA in solution using one of the two different fluorescent dyes (Hoechst 33258 or SYBRGreen, Methods sections 2.2.6.10). Measurement of DNA concentration by SYBR green fluorescence in capillaries using a built-in fluorimeter of the LightCycler 1.5 instrument resulted to be more sensitive, allowing precise quantification of <1 ng DNA in 10 µl (**Figure 3.31**). DNA quantification was then verified by running the samples in agarose gels, containing EtBr along with the known amounts of pure plasmid DNA standards.

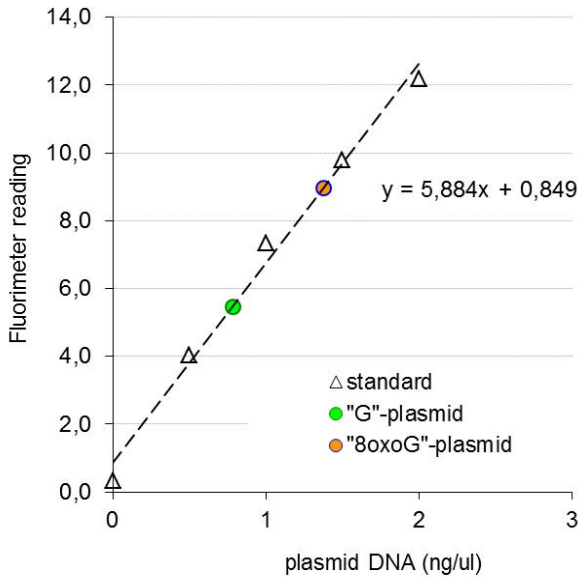

Figure 3.31 Representative results of quantification of plasmid DNA by built-in fluorimeter of the LightCycler 1.5 instrument. Calibration curve built with known concentration of pure pZA plasmid DNA (triangles) was used for determination of DNA concentration of two preparations of plasmids, constructed by the incorporation of the indicated synthetic oligonucleotides (circles).

3.3.1.5 Detection of 8-oxoG within the covalently closed plasmid DNA

Presence of 8-oxoG in the constructed plasmid substrates was verified by conversion of the covalently closed circular plasmid DNA to the open circular form by incubation with an excess of the specific DNA glycosylase Fpg, as described elsewhere (Epe et al, 1993), (Will

et al, 1999). Formamidopyrimidine [fapy]-DNA glycosylase (Fpg) is a bifunctional DNA glycosylase/lyase, the glycosylase activity of which releases damaged purines from double stranded DNA, generating an apurinic (AP site). The AP-lyase activity cleaves both 3′ and 5′ to the AP site thereby removing the AP site and leaving a 1 base gap (Tchou et al, 1994). Examples of the 8-oxoG detection by relaxation of the plasmid substrates, containing single 8-oxoG or a cluster of three 8-oxoG residues in the transcribed strand of the EGFP gene are shown in **Figure 3.32**. pZA plasmid damaged with visible light in the presence of methylene blue was used as a control for Fpg activity. The control substrates contained no or three oxidised guanines per plasmids molecule (Khobta et al, 2009). Typically, 75-95% of the plasmid substrates containing single synthetic 8-oxoG were converted into the open circular form by the Fpg treatment, while in case of the plasmid substrates containing the clusters of three 8-oxoG residues we observed almost complete cleavage (**Figure 3.32**, lanes “8oxoG=3, Fpg+” in panels A and B). Occasional incomplete cleavage of the plasmid substrates containing single 8-oxoG might be explained by contamination of plasmid with agarose that could inhibit the Fpg activity.

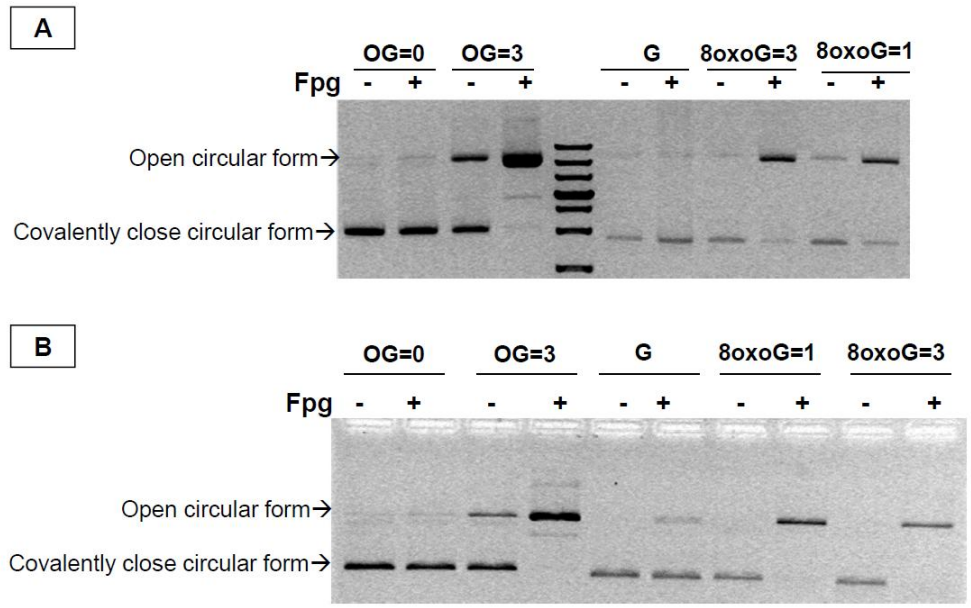

Figure 3.32 Detection of 8-oxoG within the covalently closed plasmid DNA. Ethidium bromide agarose gel electrophoresis of plasmid substrates incubated with excess of Fpg. (A) and (B) show independent plasmid preparations.

3.3.2 Incorporation of synthetic 8-oxoG by annealing and ligation of modified oligonucleotides between two nicks in the DNA strand of choice

Despite the attempts to optimize the methods of incorporation of synthetic 8-oxoG into the plasmid DNA by annealing and extension of the modified oligonucleotides on a single-stranded circular DNA template, these methods still suffer from low efficiencies (final yield of the modified plasmid of ~5-10%). Besides that, expression of the modified plasmids obtained by these methods turned out to be highly variable in the subsequent transfection experiments (data not shown). This probably was due to the presence of impurities in the obtained plasmid DNA preparations. Therefore, a more efficient method that would produce the necessary amount of topologically homogeneous modified plasmid DNA was desired.

Plasmid substrates containing a single DNA base modification can be efficiently produced by annealing and ligation of a modified synthetic oligonucleotide with a circular DNA containing the matching single-stranded gap [Donahue, 1994 #4]. It was previously demonstrated that such gapped DNA circles could be efficiently generated by treatments of a plasmid DNA with sequence-specific nicking endonucleases [Wang, 2001 #6], which are the catalytic mutants of one subunit of some heterodimeric restriction enzymes (Chan et al, 2011). If adapted for the scope of DNA strand-specific incorporation of 8-oxoG into the EGFP gene within the plasmid DNA, this approach could provide a promising alternative to the protocols based on the re-synthesis of the entire DNA strand starting from the 8-oxoG-containing primer. The search for the recognition sites for the commercially available nicking endonucleases in the in the pEGFP-mODC-ZA plasmid had revealed two Bpu10I sites within the transcribed sequence of the EGFP gene, tandemly located with an interval of 18 nucleotides. This was a lucky event, because Bpu10I is the only restriction nuclease whose catalytic mutants with the opposing DNA strand specificities are available on the market. These two mutants: Nt.Bpu10I (cutting the sequence 5′-CCvTnAGC-3′, termed as the “top strand”) and Nb.Bpu10I, which cuts the complementary sequence 5′-GCvTnAGG-3′ (Stankevicius et al, 1998), thus could be used for introducing two nicks in either the transcribed strand (Nb.Bpu10I) or the non-transcribed strand (Nt.Bpu10I) of the EGFP gene (Figure 3.33).

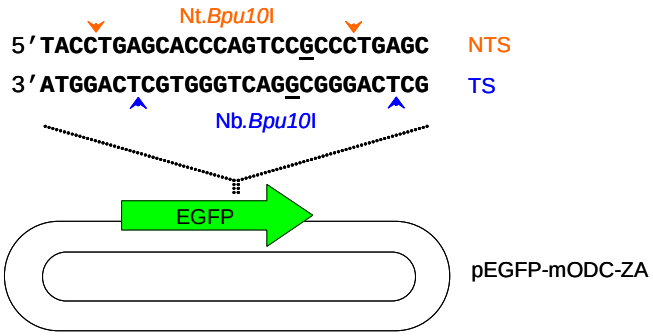

Figure 3.33 A fragment of DNA sequence of the EGFP gene with positions of the nicks induced by the Bpu10I mutant enzymes with the strand-specific nicking activities.

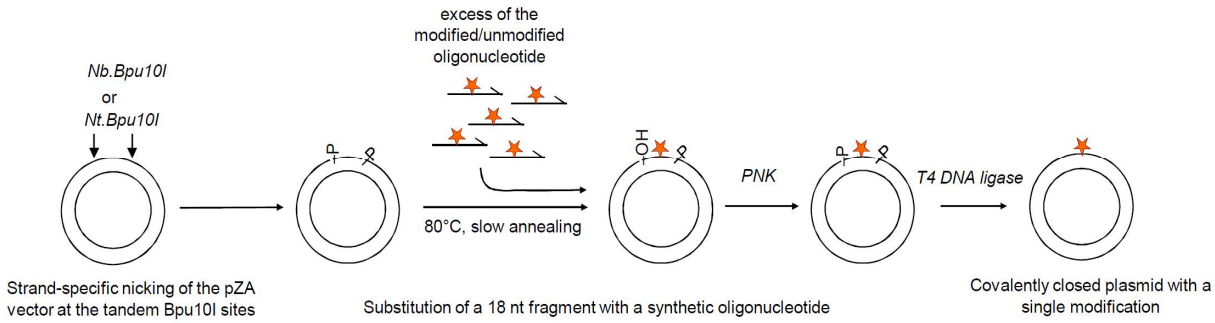

Figure 3.34 The principle of construction of plasmid substrates containing a single DNA base modification in the transcribed or the non-transcribed DNA strand of the EGFP gene. Insertion of a single 8-oxoG (star) is achieved by strand-specific excision of the 18-nt fragment and its substitution with a synthetic oligonucleotide.

The principle of construction of the plasmid substrates containing 8oxoG in different strands of EGFP gene is described in **Figure 3.34**. The first step is introduction of two strand-specific nicks in the EGFP gene with the mutants of Bpu10I restriction endonuclease with DNA strand-specific nicking activities (Nt.Bpu10I or Nb.Bpu10I). As pEGFP-mODC-ZA plasmid contains two tandemly located Bpu10I sites, this specifically introduces two nicks in either the transcribed or the non-transcribed DNA strand of the EGFP gene, depending on the enzyme used. Native single-stranded DNA 18-mers between the Bpu10I sites are melted out from the doubled stranded plasmid DNA by heating and substituted by the synthetic 18-mer

oligonucleotides containing G or 8-oxoG by means of slow annealing in the presence of excess of the synthetic competitor oligonucleotides, which can be 5′-phosphorylated by T4 polynucleotide kinase (PNK). Finally, ligation by T4 DNA ligase is performed to generate the covalently closed plasmids with a single 8-oxoG in the transcribed or the non-transcribed DNA strands of the EGFP gene or control plasmids with G in the same positions. Careful optimisation of the concentrations of the competing oligonucleotides and other reaction conditions resulted in the experimental protocol which is described in detail in Methods (2.2.7.3).

As a control for complete replacement of native single-stranded fragment by the synthetic oligonucleotides, ligations of the reaction products that were incubated in the absence of PNK were performed (**Figure 3.35**). Aliquots incubated without competitor, PNK and T4 DNA ligase showed a single band of open circular plasmid form (oc) obtained from digestion with nicking endonucleases and a minor band corresponding to the nicked dimer of the plasmid DNA. Ligation in the absence of competitor oligonucleotides resulted in the covalently closed circular DNA (ccc) as the most prominent reaction product. This was completely inhibited by the addition of the non-phosphorylated synthetic oligonucleotides, containing either G or 8-oxoG, since only the band corresponding to the open circular plasmid was present in these samples. This confirmed that the native Bpu10I-excised DNA fragments without a 5′-phosphate required for ligation were completely by outcompeted by the synthetic DNA oligonucleotides. Finally, re-appearance of the covalently closed circular plasmid form in the reactions, which contained the synthetic competitor oligonucleotides in the presence of both T4 PNK and DNA ligase, demonstrates that incorporation of synthetic oligonucleotides was very efficient (**Figure 3.35**).

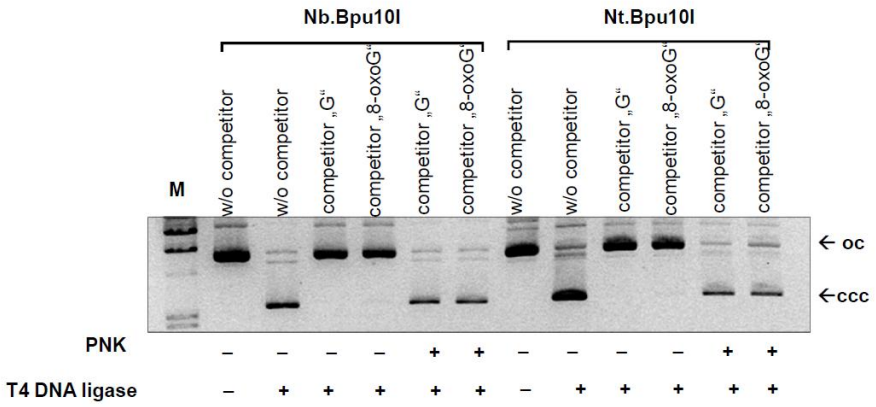

Figure 3.35 Verification of substitution of the native 18-mer oligonucleotides excised by Nt.Bpu10I or by Nb.Bpu10I with the synthetic oligonucleotides containing guanine (G) or 8-oxo-7,8-dihydroguanine (8-oxoG) by ligation in the absence of polynucleotide kinase (PNK). Agarose gel of the ligation products in the presence of ethidium bromide.

Transcription through 8-oxoG can lead to a considerable fraction of mutant RNA molecules resulting from erroneous incorporation of adenine instead of cytosine by RNA polymerase II (Saxowsky et al, 2008), (Bregeon et al, 2009).To avoid the potential miscoding by the mutant transcripts, 8-oxoG in the transcribed DNA strand was placed it in the third position of the EGFP codon 206 (Materials 2.1.5). The resulting UCC and UCA codons in mRNA both encode for serine, which would prevent the synthesis of erroneous EGFP protein in the event of misincorporation of adenine into RNA during transcription. Moreover, the frequencies of the UCC and UCA codons in human genes are similar, according to the Codon Usage Database (Ye & Maniatis, 2011), indicating that these codons are translated with similar efficiencies by the ribosome. The position of an 8-oxoG in the non-transcribed DNA strand was then intentionally selected next to the 8-oxoG site in the transcribed strand in order to minimise the possible effect of the DNA sequence context on the cellular processing of the base modification (**Figure 3.33**).

The modified plasmid preparations produced by the ligation of the G- or 8-oxoG-containing oligonucleotides typically contained at least 90% of pEGFP-mODC-ZA in the covalently closed form. This was considered to be a very good result, taking into account a high yield of the covalently closed plasmid DNA, relative simplicity of the whole procedure and the absence of the need for additional plasmid purification steps. Next, we used the Fpg DNA glycosylase of *E. coli* as the probe and the plasmid relaxation assay for verification of the presence of 8-oxoG in the constructed plasmid molecules (Methods 2.2.7.4). Incubation of the plasmid substrates containing 8-oxoG with the recombinant Fpg DNA glycosylase should result in DNA strand scission. Indeed, the agarose gel analyses in the presence of ethidium bromide of the plasmids containing 8-oxoG in the transcribed or the non-transcribed DNA strands showed that incubation with Fpg resulted in almost complete conversion of covalently closed circular form into the open circular form. Less than 3% of plasmid DNA remained uncut, thus indicating that the lesion was present in at least 97% of the plasmid molecules (**Figure 3.36**). Plasmid DNA damaged by photosensitization in the presence of methylene blue (MB) was used as control for the Fpg activity. Plasmids substrates containing guanine were used as control for unspecific cleavage by FPG and as an indicator of the overall quality of the obtained plasmid DNA.

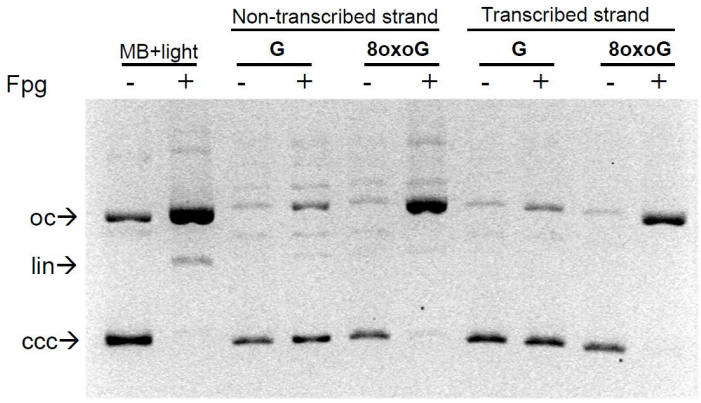

Figure 3.36 Detection of 8-oxoG in plasmid substrates by relaxation assay with recombinant Fpg DNA glycosylase.

Altogether, the data show that annealing and ligation of the modified oligonucleotides between the two nicks in one of the DNA strands is an efficient procedure for the incorporation of synthetic 8-oxoG, which allows to obtain tens of micrograms of transfection grade topologically homogeneous vector DNA. A comparison of this method with the previously used methods, which employ single stranded circular DNA, is provided in **Table 3.1**. The main advantage of the new procedure proposed here is a high efficiency of generation of topologically homogenous covalently closed plasmid DNA in a simple and straightforward procedure. High content of the covalently closed DNA in the plasmid preparations eliminates the necessity for further purification steps. Thus, both possible secondary damage to DNA and contaminations with agarose or CsCl are avoided. The high yields of the obtained modified plasmid DNA allow an accurate quantification with conventional spectrophotometry. All these factors argued for adoption of this procedure as the basic method of production of the modified plasmids for the subsequent quantitative gene expression analyses aimed on characterisation of the effects of single 8-oxoG residues on gene expression. The procedure is ideally suited for studies of the DNA strand-specific effects of 8-oxoG on transcription, because of equally efficient incorporation of 8-oxoG into the transcribed and the non-transcribed DNA strands. On another hand, this method cannot be applied for analyses of the effects of position of 8-oxoG along the whole range of the DNA sequence. Therefore, one has to use the modified plasmids produced from the single-stranded DNA while addressing this question.

Table 3.31 Comparison of two experimental procedures used for incorporation of synthetic base lesions: applicability and quality considerations.

Method	**Annealing and extension of the modified oligonucleotides on a single-stranded circular DNA template**	**Annealing and ligation of the modified oligonucleotides between the two nicks in one of the DNA strands**
Research applicability		
Position of the base modification	Any	Defined by the available Bpu10I sites
DNA strand modified	TS or NTS, depending on the available nicking sites or the strand packaged by the phage	TS or NTS
Potential for introduction of multiple base modifications (“clusters”)	Broad, but restricted to one DNA strand for a given plasmid	Limited to a short DNA sequence
Production and quality considerations		
Preparatory steps	Relatively complex (preparation of single-stranded circular DNA from the filamentous phage or by the enzymatic treatments of the plasmid DNA)	Simple (preparation of plasmid DNA from bacteria)
Costs of reagents	Relatively high (either phage or large amounts of ExoIII are needed; a proofreading DNA polymerase is required)	Low (reasonable amounts of standard molecular cloning enzymes are used)
Final purification procedures	Gel-electrophoresis or ultracentrifugation in the presence of ethidium bromide, followed by isolation and clean-up of covalently closed plasmid DNA	Not required at all or a single-step column clean-up
Final yield of the modified plasmid	Very low (5–10%)	Very high (>90%), tens of micrograms are easy to produce
Purity of the final product	Problematic for transfection and quantitative gene expression analyses	Good, suitable for transfections and quantitative gene expression analyses
Time requirements	At least two days	1 day or less

3.4 Influence of synthetic 8-oxoG on gene expression

3.4.1 Expression of pZA plasmids containing single synthetic 8-oxoG in HeLa cells

The results of host cell reactivation experiments of plasmid DNA damaged by photosensitisation in the presence of methylene blue have provided clear indications that oxidative DNA base damage leads to a strong decrease of the transcription of reporter genes (Spivak & Hanawalt, 2006), (Khobta et al, 2009), (Khobta et al, 2010a). The results obtained in the course of the present investigation show that the inhibitory effect on transcription is present in all tested cell types, including the fully repair-proficient cells (Section 3.2). The inhibition of gene expression by the oxidative DNA damage induced by photosensitisers is persistent in time and is stronger manifested in the absence of the Cockayne syndrome group B protein (CSB). Interestingly, the data also suggested that OGG1 protein, which is the most important DNA glycosylase implicated in excision of 8-oxoG in the initiatory step of base excision repair, can cause decrease of gene expression of oxidatively damaged DNA, at least in the absence of CSB (Khobta et al, 2009) Randomly distributed 8-oxoG lesions are the most abundant base modifications generated in plasmid DNA by damaging with light in the presence of methylene blue as a photosensitiser. Therefore, it was logical to suppose that the observed decrease of transcription should be due to the presence of 8-oxoG residues or the products of their processing (e.g., by DNA repair enzymes) within the reporter gene. Still, one cannot ignore the possibility that inhibition of transcription could be caused by some other DNA lesions induced by this damage mode in minor amounts, such as single strand breaks (SSB), sites of base loss, 5,6-dihydropyrimidines, or some uncharacterised base modifications

To clarify the effect of 8-oxoG on transcription, I constructed the plasmid substrates containing single synthetic 8-oxoG in the transcribed (TS) and the non-transcribed (NTS) DNA strands of EGFP gene (Chapter 3.3.2) and co-transfected them together with a tracer plasmid encoding for DsRed-Monomer into HeLa cells. Expression analyses of plasmids incorporating the synthetic oligonucleotides containing either 8-oxoG or guanine (G) in the same position were performed in HeLa cells 24 hours after transfections. The specific fluorescent signals of EGFP (FL-1H) and DsRed-Monomer (FL-2H) are independently detected and well distinguished in the two-dimensional fluorescence scatter plots obtained by flow cytometry. Transfected cells are easy to identify by their localisation in the upper two quadrants of the fluorescence scatter plots (**Figure 3.37,** on the left), which is attributed to significant levels of expressions of the DsRed-Monomer protein from the reference pDsRed plasmid. This region was chosen as a gate, delimiting the cells for analyses of the EGFP expression, as described in Methods (2.2.7.6). This was applied to produce the FL-1H fluorescence distribution plots (**Figure 3.37,** overlaid on the right), which thus specifically

show the transfected cells, excluding all the non-transfected cells. In this way it was possible to detect inhibition of transcription by the lesion in plasmid DNA as a decrease of FL1-H fluorescent signal in cells transfected with 8-oxoG-containing plasmid with respect to those transfected with the plasmid containing G. An about twofold decrease of EGFP fluorescence (compared to the control plasmid) was detected after transfections with the plasmid containing synthetic 8-oxoG in the non-transcribed DNA strand. At the same time, parallel transfections of plasmid DNA with 8-oxoG positioned in the transcribed strand of the EGFP gene, detected no detectable influence of the base modification on the expression of reporter protein compare to the control plasmid. These findings indicate that 8-oxoG inhibits transcription in a mechanism that is distinct from direct blocking of the elongating RNA polymerase, because such direct elongation block can only be caused by DNA damage in the transcribed DNA strand.

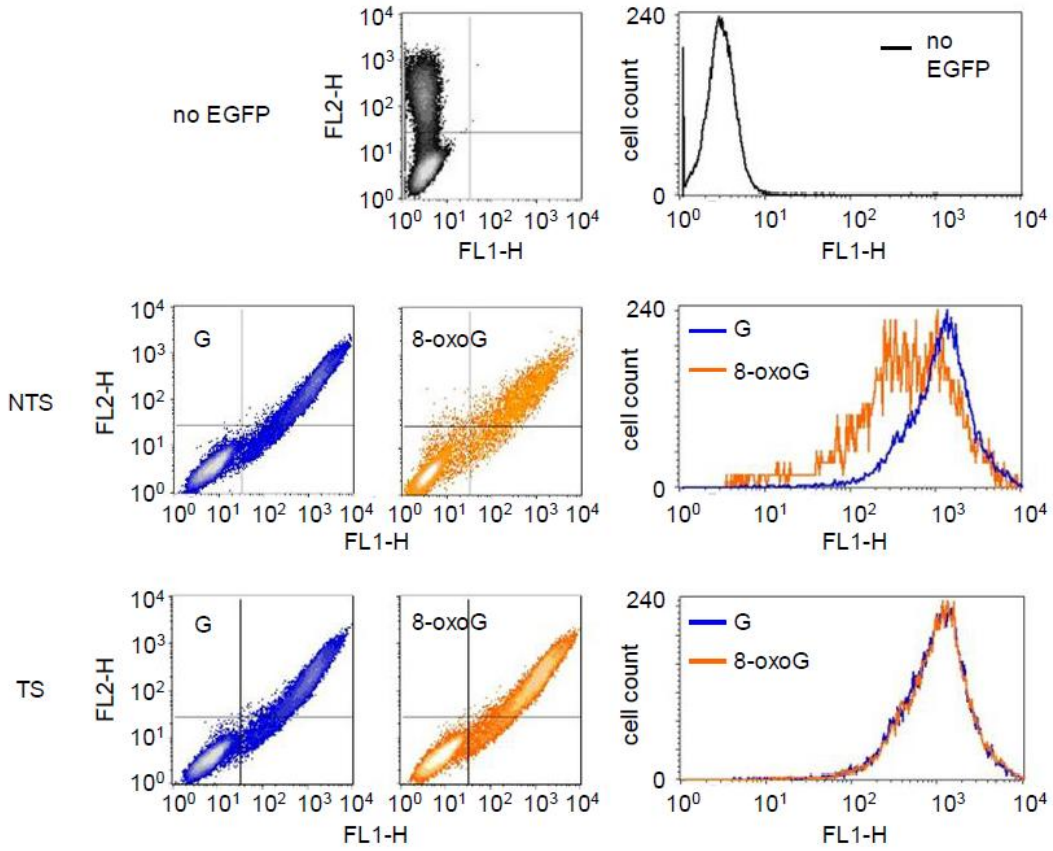

Figure 3.37 Expression of DsRed-Monomer and EGFP in HeLa cells 24 hours after co-transfection with equal amounts of the corresponding plasmids. Flow cytometry of cells transfected with the EGFP-encoding plasmid constructs containing guanine (G) and 8-oxo-7,8-dihydroguanine (8-oxoG) either in the transcribed (TS) or the non-transcribed (NTS) DNA strands. The overlaid fluorescence distribution plots (right panels) specifically show only transfected cells, marked by the expression of DsRed-Monomer, which localise in the upper two quadrants of the dot density plots

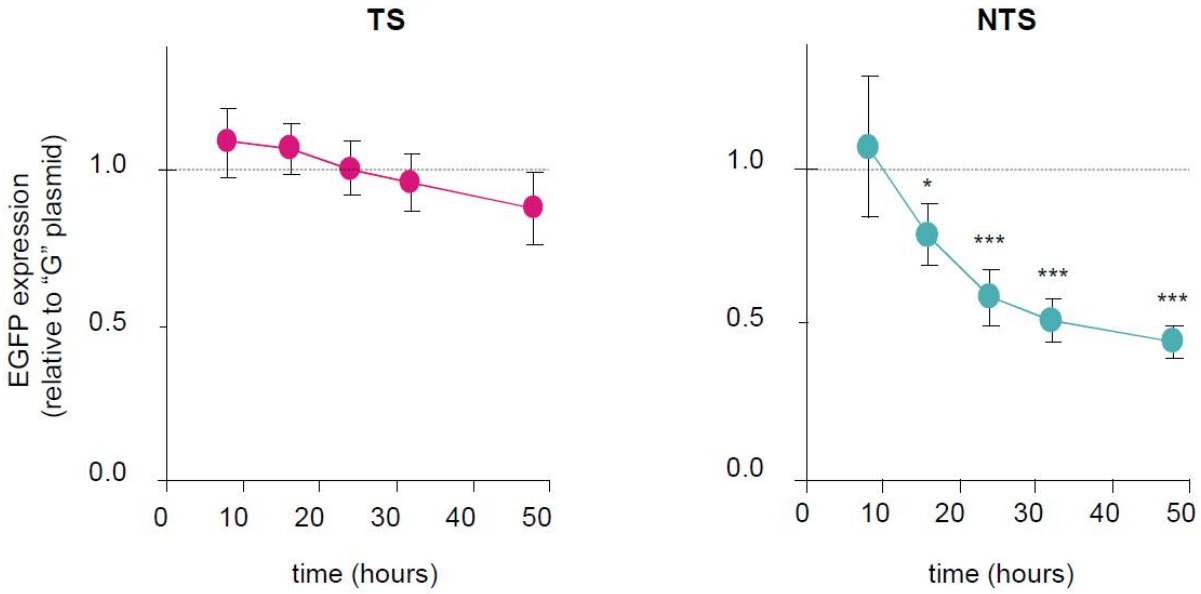

Figure 3.38 Time-dependent decrease of expression of the plasmid EGFP gene containing a single 8-oxo-7.8-dihydroguanine (8-oxoG) in the transcribed (TS) or the non-transcribed DNA strand (NTS) in transfected HeLa cells (mean for five separate plasmid preparations ±SD). Paired Student's two-tailed t-test: * $p<0.05$, *** $p<0.001$

To prove this, the expression of plasmids substrate contained single synthetic 8-oxoG in transcribed and non-transcribed strand of EGFP gene was measured at different time intervals after transfection (**Figure 3.38**). The EGFP expression from the plasmids containing 8-oxoG was determined as median FL-1 fluorescence among the transfected cells and expressed as relative to median FL-1 fluorescence of the respective plasmid substrates containing guanine in the same position at each time point in every experiment. If the decrease of the EGFP gene expression was a consequence of a direct effect of 8-oxoG on transcriptional elongation, DNA repair would lead to reactivation of the damaged gene; i.e., the inhibitory effect on transcription should decrease in time, as it has been previously reported for the UV-damaged plasmids (Khobta et al, 2009). In contrast to this expectation, the data show that there was no difference in the EGFP expression between the G- and 8-oxoG-containing plasmids 8 hours after transfections, while a pronounced decrease of EGFP expression was observed between 8 and 32 hours post-transfection in cells transfected with the plasmid containing 8-oxoG in NTS. Some decrease of gene expression was also caused by 8-oxoG in the transcribed strand (TS), but in the latter case it was delayed for even longer time and was much smaller in magnitude (**Figure 3.38**).

Altogether, the absence of a detectable reduction of the EGFP gene expression by 8-oxoG in the transcribed DNA strand at the early time points, as well as the absence of strong effect of

the damage in the transcribed DNA strand at all times tested, once again support the assumption that the lesions do not constitute an elongation block for RNA polymerase II under the tested conditions. However, if repair of the transcribed DNA strand was very fast and efficient, this could preclude the detection of transcription block in the reporter gene expression assay. To investigate the effects of unprocessed 8-oxoG on the gene expression, further transfection experiments were performed in immortalized mouse embryonic fibroblast (MEF) cell lines carrying either wild-type or disrupted copies of the *Ogg1* gene, which encodes for the OGG1 DNA glycosylase. Since the *Ogg1* null mouse cells are deficient in fast base excision repair of 8-oxoG, this experimentation would specifically address the direct effects of 8-oxoG on transfection. In addition, we also considered the use of the available *Csb* null cell lines, because CSB is required for transcription recovery after DNA damage in mice and humans (van der Horst et al, 1997) and also because human CSB protein was previously shown to improve transcription of the 8-oxoG-containing DNA template in the reconstituted transcription systems (Kuraoka et al, 2003), (Charlet-Berguerand et al, 2006) (Laine & Egly, 2006b).

3.4.2 Expression of plasmids containing single synthetic 8-oxoG in mouse embryonic fibroblasts: the effects of CSB and OGG1 deficiency

Base excision repair of 8-oxoG is absent or, at least, very inefficient in mouse embryonic fibroblast (MEF) cell lines with a homozygously disrupted *Ogg1* gene, which encodes for the OGG1 DNA glycosylase (Osterod et al, 2001), (Saxowsky et al, 2008). This makes these cells an optimal model system to study the direct effects of 8-oxoG on transcription in the absence of repair. Plasmid substrates containing G or 8-oxoG in different strands of the EGFP gene were therefore co-transfected together with the tracer pDsRed-Monomer-N1 plasmid into the *Ogg1*$^{-/-}$ MEFs. Wild type F11.1 MEFs (*Ogg1*$^{wt/wt}$) were transfected in parallel following the same scheme. Expression of the EGFP gene in the transfected cells was measured by flow cytometry at 24 hours after transfection, as described in Section 2.2.7.6. Relative EGFP expression in the presence of 8-oxoG in the transcribed or the non-transcribed DNA strands of the gene was then calculated as a ratio of the median EGFP fluorescence in cells transfected with the modified plasmid containing a single 8-oxoG to the fluorescence in cells transfected with the control plasmids (“G”), containing the unmodified synthetic oligonucleotide in the corresponding DNA strand (**Figure 3.39**).

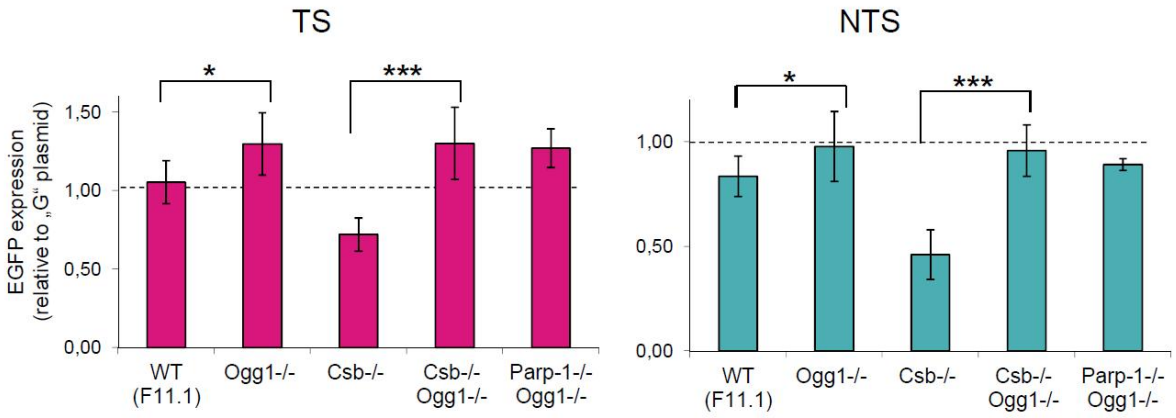

Figure 3.39 Effects of a single 8-oxo-7.8-dihydroguanine (8-oxoG) on EGFP expression in mouse embryonic fibroblasts of the indicated genotypes. TS: transcribed strand, NTS: non-transcribed strand. EGFP fluorescence was measured by flow cytometry 24 hours after transfections (mean±SD for 3 - 7 independently prepared "G" and "8-oxoG" plasmid couples). Paired Student's two-tailed t-test: *p<0.05, *** p<0.001

The results of transfections of the *Ogg1*$^{wt/wt}$ F11.1 MEFs demonstrated that 8-oxoG located in non-transcribed strand of the EGFP gene leads to a significant (p=0.002, n=7) decrease of the gene expression. At the same time, the base modification located in the transcribed strand had no effect on transcription of the reporter gene in this MEF cell line (**Figure 3.39**). The results thus show that the effects of 8-oxoG on gene expression in the wild type mouse cell line are qualitatively similar to the effects in HeLa cells, discussed in the Section 3.4.1 Remarkably, transfections of the OGG1-null MEFs (*Ogg1*$^{-/-}$) showed that also in this cell line the presence of 8-oxoG in the transcribed strand of the plasmid DNA does not cause any decrease of expression of the reporter gene in comparison with the unmodified plasmid, once again indicating the absence of the transcription blockage by this DNA base modification (**Figure 3.39**). In contrast to the results in the *Ogg1*$^{wt/wt}$ MEFs, the inhibition of transcription caused by 8-oxoG positioned in the non-transcribed DNA strand was no more detected in the parallel transfection experiments of the *Ogg1*$^{-/-}$ MEF cell line. The results thus demonstrate that functional OGG1 protein is needed for the inhibition of transcription by 8-oxoG. This finding further suggests that the decrease of transcription observed in the OGG1-proficient cells could be caused not by the base modification itself, but rather by the DNA single-strand breaks (SSBs) produced by the OGG1 DNA glycosylase in the process of excision of the 8-oxoG residues. The absence of inhibitory effect of 8-oxoG in the transcribed DNA strand on gene expression in the *Ogg1*$^{-/-}$ cell line indicates that this base modification can be efficiently bypassed by the elongating RNA polymerase II, which recapitulates the

behaviour of the mammalian RNA polymerase II in reconstituted transcription systems (Tornaletti et al, 2006).

Since transcriptional bypass of 8-oxoG was shown to be enhanced by supplementation of the transcription reactions with CSB protein (Charlet-Berguerand et al, 2006), it was interesting to test the requirement of CSB for the transcriptional bypass of 8-oxoG in cells. To test the capability of an unrepaired 8-oxoG to block gene transcription in the absence of CSB protein, we transfected the plasmid substrates containing single 8-oxoG in each of the DNA strands into the available *Csb*$^{-/-}$*Ogg1*$^{-/-}$ MEFs (Osterod et al, 2002) Quantitative analyses of EGFP expression clearly demonstrated the absence of inhibitory effects of 8-oxoG in either transcribed or the non-transcribed DNA strand on transcription of the EGFP gene in the *Csb*$^{-/-}$ *Ogg1*$^{-/-}$ cells. The expression levels of the 8-oxoG-containing plasmids were the same as in the *Ogg1*$^{-/-}$ MEFs cell lines carrying both functional alleles of the *Csb* gene (**Figure 3.39**). As the synthetic 8-oxoG in the transcribed strand causes no decrease of EGFP expression in double knockout (*Csb*$^{-/-}$*Ogg1*$^{-/-}$) MEF cell line, the results prove that unrepaired 8-oxoG is efficiently bypassed during transcription in cells, in addition demonstrating that CSB is dispensable for transcription in the presence of 8-oxoG. Efficient transcriptional bypass of 8-oxoG was once again supported by transfection results of the *Parp1*$^{-/-}$*Ogg1*$^{-/-}$ MEF cell line (n=3). Also in this independent model cell line with OGG1 deficiency, a single 8-oxoG in the transcribed DNA strand did not inhibit the reporter gene expression, and the relative expression levels of “G” and “8-oxoG” plasmids for both strands of the EGFP gene were very similar in the *Ogg1*$^{-/-}$, *Csb*$^{-/-}$*Ogg1*$^{-/-}$ and *Parp1*$^{-/-}$*Ogg1*$^{-/-}$ cells lines. Altogether, the results of transfections of three independent OGG1 deficient MEF cell lines lead to conclusion that inhibition of transcription by 8-oxoG does not take place in the absence of the OGG1 DNA glycosylase.

As the inhibition of expression of the reporter gene by 8-oxoG required the presence of the OGG1 DNA glycosylase in host cells, we hypothesized that inhibition of transcription could be caused by SSBs arising from the excision of 8-oxoG. SSBs were shown to be extremely harmful for gene expression, particularly in the cells with disrupted *Csb* gene (Khobta et al, 2010b) To elucidate the role of 8-oxoG excision in the absence of CSB on transcription, the MEF cell line lacking a functional CSB protein, but expressing the wild-type OGG1, was transfected with the plasmids containing single 8-oxoG. Under these conditions, 8-oxoG located in the non-transcribed strand of the gene caused a more than twofold decrease of EGFP expression in *Csb*-deficient mouse cells, relative to the expression level of the G-containing plasmid ($p<0.001$, n=6). Moreover, the EGFP expression was also significantly decreased by 8-oxoG in the transcribed strand ($p=0.003$, n=6) in this MEF cell line. It is important to underline that decrease of the gene expression in both cases could not be a

direct effect of 8-oxoG on transcription, because the EGFP expression was complete restored in *Csb*$^{-/-}$*Ogg1*$^{-/-}$ MEFs ($p<0.001$, $n=6$) (**Figure 3.39**).

In summary, the results of transfection experiments conducted in the different OGG1- and/or CSB-deficient MEF cell lines lead to conclusions that 8-oxoG is efficient bypassed by RNA polymerase II in absence of functional CSB protein and that excision of the base modification by OGG1 leads to inhibition of transcription. This possibly happens due to collisions of RNA polymerase II with SSB, resulting in blockage of transcription, which can be relieved by functional CSB protein.

3.5 Determination of mRNA expression levels and of plasmid survival in the transfected cells

The decrease of EGFP expression can be caused by inhibition of transcription by 8-oxoG or by synthesis of truncated or mutated mRNA in the presence of 8-oxoG or its excision products. mRNA was isolated 24 hours after co-transfection of Hela cells tracer pDsRed-Monomer plasmids together with the plasmids containing 8-oxoG in different strands of EGFP or with control “G” plasmids. The mRNA was quantitatively analysed by reverse transcription and real-time PCR with primer for two different regions within the EGFP coding sequence. One pair of primers (“EGFP1”) was selected to amplify the DNA fragment upstream from the base modification site. The second pair (“EGFP2”) contained a forward primer 3′ from the base modification site and the reverse primer 5′ from the base modification site (**Figure 3.40**). In this way comparison of cDNA quantities corresponding to these two DNA fragments should enable the detection of truncated transcripts in the case of transcription elongation block at the DNA damage site. To enable the quantitative comparison between the independently processed samples, the levels of the DsRed-Monomer expression were also determined in all samples to normalize for the efficiencies of the reverse transcription reactions.

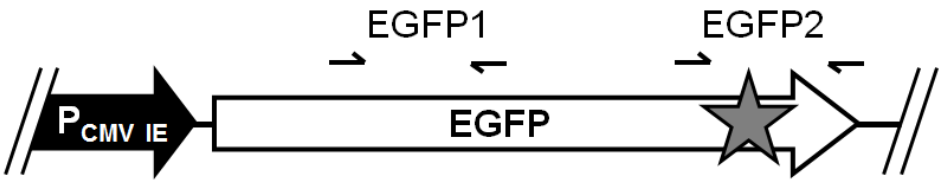

Figure 3.40 Locations of the EGFP1 and EGFP2 PCR amplicons with respect to the 8-oxoG position (star). White arrow shows the EGFP coding sequence.

Parallel isolation of DNA was performed for following two reasons. First, this allowed quantification of the mRNA expression relative to the gene amount, which is present in the same transfected cells. Second, the comparison of residual quantity of plasmid DNA in the cells in presence or absence of damage was done to check for the plasmid DNA degradation (both in terms of overall quantity of the plasmid DNA and for comparison of the quantities of the PCR fragments encompassing the damage site versus the gene region outside from the base modification). Recovery of the DsRed-Monomer gene present in the reference plasmid

was measured to normalize for the transfection efficiencies between the independently processed samples.

The analyses of DNA showed that the plasmid substrates containing G and 8-oxoG were retained with the same efficiencies in the host cells, regardless of the DNA strand modified (**Figure 3.41**). Moreover, the recovered amounts of all EGFP-encoding plasmids produced by insertion of synthetic oligonucleotides were proportional to the amounts of the unmodified pDsRed-Monomer plasmid. The mRNA analyses showed an approximately twofold decrease of the EGFP transcript in case of the cells transfected with a plasmid containing a single 8-oxoG in the non-transcribed DNA strand, which is in agreement with the protein expression data (section 3.4.1). Once again in concordance with the protein fluorescence analyses, 8-oxoG in the transcribed strand did not affect the transcript levels (**Figure 3.41**). Comparison of cDNA quantities corresponding to two DNA fragments, quantified with the primers upstream and downstream from the 8-oxoG site, provided no indication for the presence of transcripts truncated at the damage site. Therefore, the effect on transcription appears to be not caused by the interrupted elongation, but by down-regulation of the initiation stage of transcription.

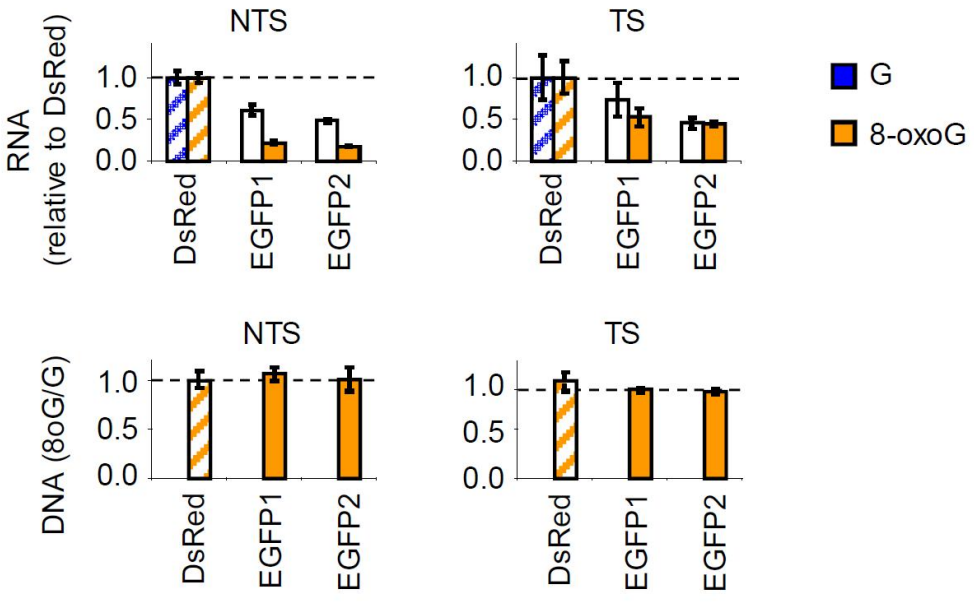

Figure 3.41 Quantification of DNA and RNA, corresponding to two distinct fragments (EGFP1 and EGFP2) of the EGFP transgene, recovered from the cells. Recoveries of the DsRed fragment from the same cell samples are shown for reference (mean±SD).

3.6 Efficiency of excision of 8-oxoG in different DNA sequence contexts

It was noticed in previous chapters that the negative effects of single 8-oxoG on expression of the EGFP gene in cells had different magnitudes, depending on the DNA strand containing the modified base. There can be several reasons for the observed differences. One possibility is that repair intermediates (e.g., SSB) are more harmful when localised in the non-transcribed DNA strand. On the other hand, repair of the two tested plasmid substrates may differ qualitatively or quantitatively. For instance, different repair efficiencies (or mechanisms) between the transcribed and non-transcribed DNA strands of the gene or modulation of excision efficiency of 8-oxoG by neighbouring DNA bases could contribute to results reported in chapters 3.4 and 3.5.

To test the possible influence of neighbouring bases on excision of 8-oxoG by OGG1, I incubated the plasmids containing 8-oxoG with a range of concentrations of purified OGG1 protein. The immediate DNA microsequence contexts of the base modification were 5′-C[8-oxoG]C in the “top” strand and 5′-C[8-oxoG]G in the “bottom” strand (see Section 2.1.5) for the full sequences). The results of plasmids relaxation assay showed that the efficiencies of 8-oxoG excision by OGG1 are different between the two tested plasmid substrates (**Figure 3.42**). Quantification of the results showed that 8-oxoG located in “top“ DNA strand (NTS) is excised with a several fold (3<X<10) higher efficiency than 8-oxoG in the “bottom” strand (TS).

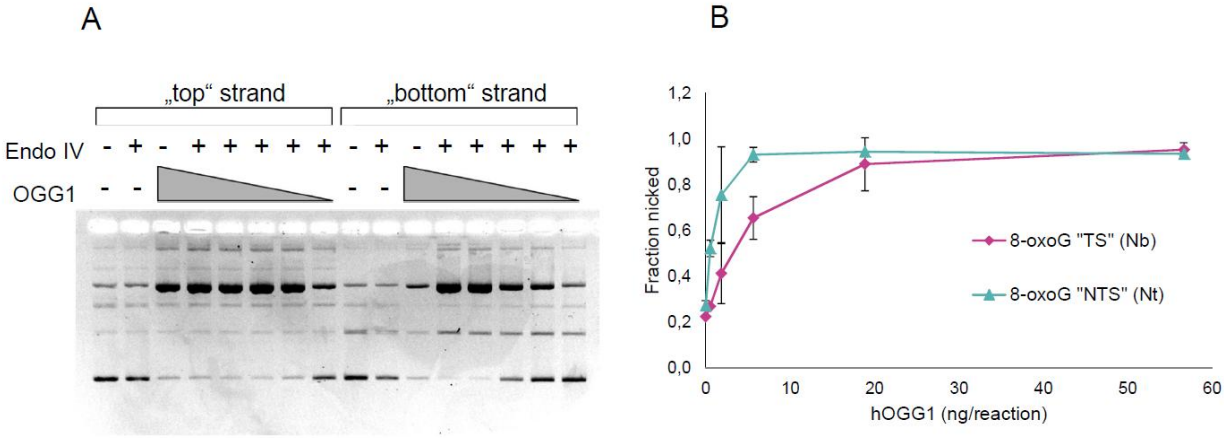

Figure 3.42 Plasmid relaxation of covalently closed DNA substrates containing single 8-oxoG in different sequence contexts (“top” and “bottom” DNA strands) after incubation with OGG1 in the presence of endonuclease IV. A: a representative agarose gel; B: quantification of fractions of nicked DNA (mean±S.D. of two experiments with the same preparation of OGG1 protein).

3.7 Analysis of replication of plasmid DNA in HeLa cells

The vectors employed in the host cell reactivation assays (chapters 3.3 – 3.6) are not supposed to replicate in the host cells used in this study, because the present SV40 origin of replication is inactive in the absence of the viral large T-antigen that is not expressed in the investigated cell lines. However, the mechanisms driving the replication of plasmid vectors in mammalian cell lines are not fully understood. If extensive replication of plasmids occurred in the host cells, this could result in dilution of DNA damage and, in consequence, underestimation of its effects on the gene transcription. Therefore, it was important to test the capability of the vectors used in the experimentation to replicate in the host cells.

The capacity of bacteria do methylate adenine within the GATC sequences can be exploited to distinguish between the DNA molecules synthesized in bacterial and mammalian cells by differential digestion with the DpnI restriction endonuclease, that specifically cuts GATC-methylated DNA (Burns et al, 2010), (Taylor & Morgan, 2003). Adenine methylation depends on the dam methylase in bacterial cells, while mammalian cells are devoid of adenine-specific DNA-methylases. Therefore, DNA synthesized in mammalian cells does not contain the adenine methylation in GATC sequences and is resistant to DpnI.

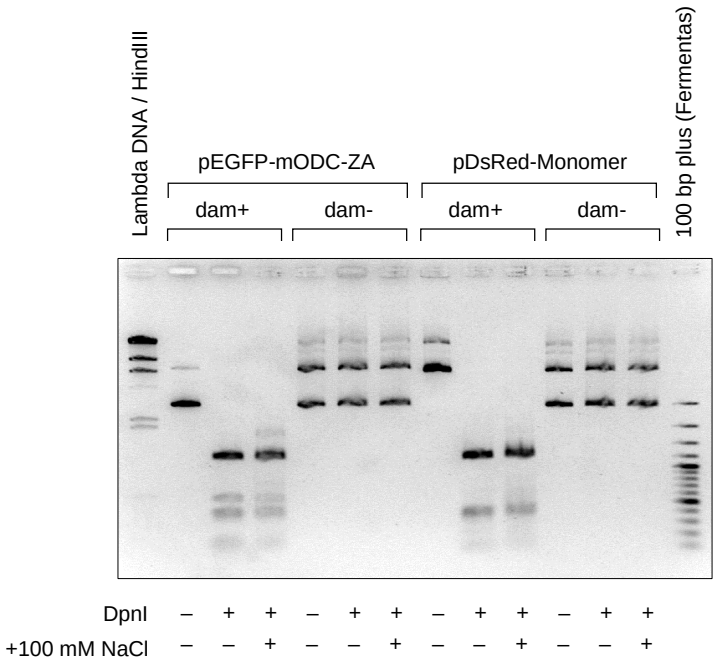

Figure 3.43 Digestion of the plasmids isolated from the dam- and dam+ bacteria with DpnI in the presence of different concentrations of NaCl.

I isolated pDsRed-MonomerN-1 and pZA vectors from the bacterial strains SCS-8 (dam+) and SCS-110 (dam-) and treated the plasmid DNA with a range of concentrations of DpnI to find the conditions when DNA from the dam+ strain is completely cut, while no digestion of the plasmid isolated from the dam- strain is observed. This corresponded to 5 units of DpnI per reaction containing 500 ng plasmid DNA. The DpnI digestions were then tested under the increased ionic strength conditions by supplementing the reactions with additional 100 µM NaCl, because such modification of the reaction conditions has been reported to inhibit cutting of the hemi-methylated DNA (Sanchez et al, 1992). The plasmids isolated from the dam+ bacteria were almost completely digested in the presence of the increased NaCl concentration (**Figure 3.43**). The increased salt concentration did not have any effect on the products of incubation of the plasmid isolated from the dam- strain, which remained intact under all tested conditions.

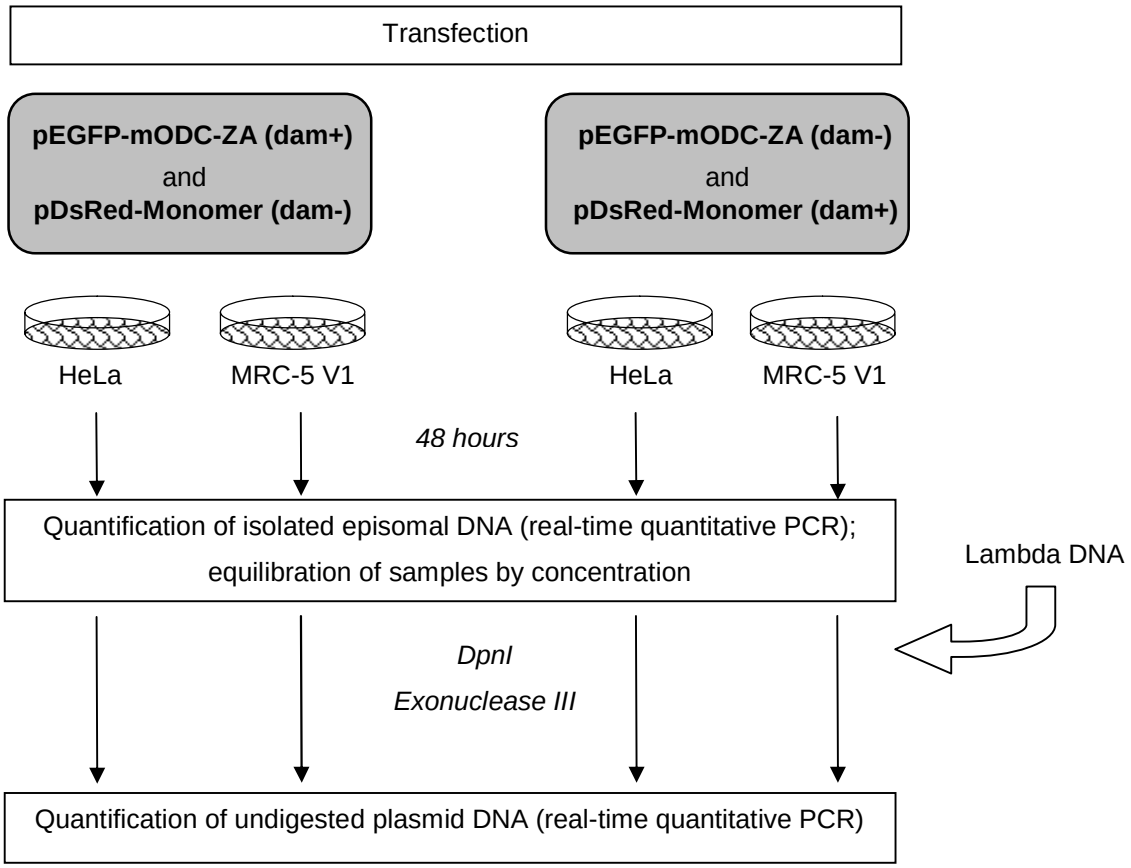

Figure 3.44 Detection of vector replication in mammalian host cells by the loss of dam-methylation detected as resistance of DNA to DpnI digestion.

Next, I transfected HeLa cells with different combinations of two plasmids, one of which has been isolated from the dam- strain, while another has been derived from the dam+ bacteria. The flowchart of the experiment is shown in **Figure 3.44.** If the plasmids do not replicate, the adenine methylation patters will be retained after prolonged incubations in the mammalian host cells. However, if the dam-methylated plasmid replicates in mammalian host, this will lead to loss of adenine methylation in the GATC sequences thus rendering the plasmid resistant to DpnI digestion. To model this situation in the experiment, I transfected MRC5-V1 cells in parallel with the HeLa cells, because this cell line was previously shown to support low levels of replication of episomal DNA containing the SV40 origin of replication (Le Page et al, 1998). The loss of GATC-methylation in the replicating plasmid can be quantified by real-time PCR with primers encompassing several DpnI sites. To avoid that short DNA fragments interfere with PCR reactions, the DpnI digestions are additionally followed by the exonuclease III treatments, which eliminates linear DNA (Burns et al, 2010) Considering that the amounts of plasmid DNA persisting in the host cells can be very low, the DpnI and exonuclease III treatments were performed in the presence of lambda DNA as a carrier to avoid the non-specific enzymatic cleavage of vector DNA (see the Methods section 2.2.8 for details). Incomplete digestion of lambda DNA was observed in all samples because phage DNA is only partially methylated (**Figure 3.45**). Similar pattern of molecular size fragments were obtained in different samples, indicating the same extent of digestion.

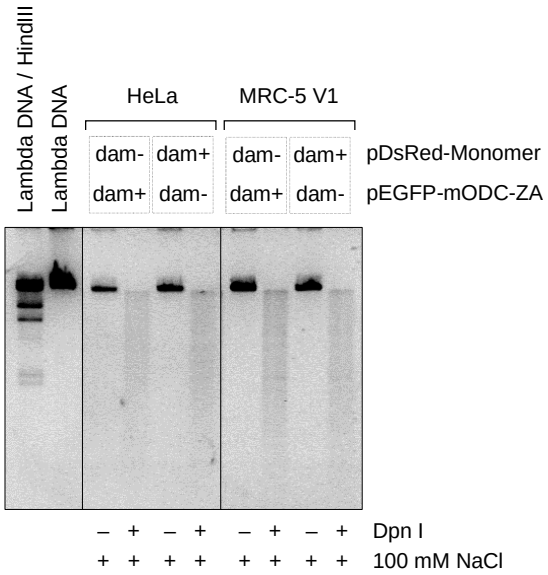

Figure 3.45 Digestion of episomal DNA isolated from HeLa and MRC-5 V1 cells 48 hours after transfections with DpnI in the presence of lambda DNA as a carrier.

Incubation of DNA recovered from HeLa cells with DpnI and exonuclease III resulted in almost complete degradation of plasmids originating from the dam+ bacteria. The remaining amounts of DNA capable to support PCR amplification, relative to the samples incubated with exonuclease III in the absence of DpnI digestion, were 4.0±0.5% for pEGFP-mODC-ZA and 8.7±4.3% for pDsRed-Monomer (the data of real-time quantitative PCR). Thus the GATC-adenine methylation was retained in the vast majority of plasmid molecules, showing that they did not undergo replication in HeLa cells during 48 hours after transfections. In contrast, a substantial loss of the dam methylation pattern occurred in the plasmids incubated in MRC-5 V1 cells, which express the large T antigen of SV40 (**Figure 3.46**). The fractions of PCR-competent DNA in this cell line were 17±1.8% for pEGFP-mODC-ZA and 39.9±3.4% for pDsRed-Monomer, indicating that some plasmid replication takes place in this cell line.

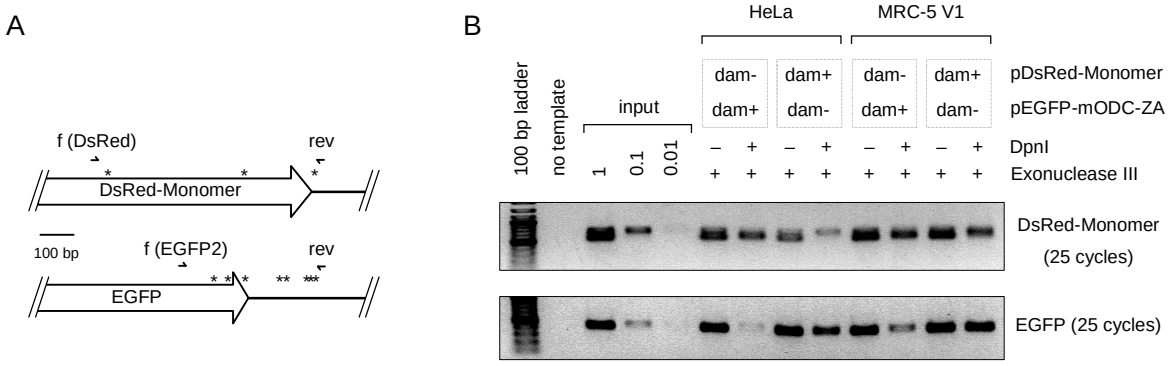

Figure 3.46 Determination of fractions of DpnI resistant DNA in the plasmid vectors re-isolated from HeLa or MRC-5 V1 cells 48 hours after transfection. (A) Positions of primers (arrows) and DpnI sites (asterisks) in the plasmid-borne DsRed-Monomer and EGFP genes. (B) Agarose gel analyses of products of PCR reactions stopped in the exponential amplification phase (25 cycles), as detected by in-reaction SYBR green fluorescence.

Altogether, the persistence of dam-specific adenine methylation in vector DNA re-isolated from HeLa cells indicate that the vectors do not replicate as episomes in this cell line. On the other hand, some episomal replication was detected in the MRC5-V1 cell line expressing the SV40 large T-antigen. Small fractions of the DpnI-resistant DNA detected in HeLa cells might reflect actual replication of plasmid DNA, but also could reflect the unavoidable experimental background, because some fraction of digested DNA could still serve as template for PCR.

This was less likely to occur with the EGFP template containing eight DpnI sites (**Figure 3.46 A**), but could be the case for the DsRed-Monomer template, which contained only three DpnI sites.

4 DISCUSSION

Many DNA damaging agents cause inhibition of transcription, because they induce DNA base modifications, which act as roadblocks for elongating RNA polymerases if occur in transcribed DNA strand of active genes. Stalling of RNA polymerases at the damage sites has double physiological importance for cells suffering genotoxic stress. First, expression of individual damaged genes can be directly affected. This can be especially relevant for those genes whose products have high clearance rates in the cells. As the subset of genes with unstable mRNA and proteins is enriched in transcription factors, chromatin modifying enzymes and the genes which products function in signaling and cell cycle-specific processes (Schwanhausser et al, 2011), it is plausible that the block of transcription can compromise the expression of these vital groups of genes at sufficiently high loads of DNA damage. Second, stalling of the RNA polymerase II at bulky DNA base adducts (cyclobutane pyrimidine dimers and some others) initiates a specialised transcription-coupled nucleotide excision repair (TCR) pathway (Mellon et al, 1987), (Laine & Egly, 2006a) and thus assists a rapid recovery of gene function and protects the cell from accumulation of mutations in functional genes.

For the reasons listed above, the mechanism underlying the establishment of a transcription block at DNA damage sites, as well as its subsequent release, are intensely studied. Reporter proteins can be highly useful in addressing these mechanisms acting at the specific genes. Enhanced green fluorescent protein (EGFP) has been chosen in the present study as a reporter because of its bright and stable fluorophore, low toxicity and ease of direct quantitative detection at single-cell level (Chalfie et al, 1994), (Cormack et al, 1996), (Kitsera et al, 2007), (Khobta et al, 2010a). One of two different strategies, applied in this work with the aim to clarify the effect of 8-oxoG on transcription of this reporter gene was to expose cells permanently transfected with the EGFP and EGFP-ODC reporter genes to damaging agents that induce well defined specific spectra of DNA base modifications (chapter 3.1.3). So the effects of irradiation with visible light in the presence of Ro19-8022 as a photosensitiser (a mode of damage which has been shown to induce predominantly 8-oxoG in DNA) could be compared with the effects of UV (which induces transcription-blocking cyclobutane pyrimidine dimers and 6-4 photoproducts).

The data showed that the UV-induced DNA damage negatively influenced the EGFP fluorescence readout in individual cells in a dose-dependent manner. Decrease of expression of the EGFP-ODC protein, whose lifetime in the cells is significantly reduced by the C-terminal fusion of the mouse ornithine decarboxylase polypeptide containing the PEST-sequence, was a much more sensitive indicator of the presence of transcription-

blocking DNA damage than the expression of unmodified EGFP (**Figure 3.11**). These results justify the application of the cell lines permanently transfected with destabilised EGFP-ODC for all tasks addressing dynamical changes in gene expression, including detection of the transcription-blocking effects of DNA damage. Moreover the EFGP-ODC reporter construct designed here is suited for detection of the gene-specific recovery of transcription following the genotoxic damage (**Figure 3.12**). Given that global recovery of transcription after UV-damage is an established marker of DNA repair, we assume that recovery of expression of the EGFP-ODC protein is an indicator of removal of transcription-blocking DNA damage in this gene (Kitsera et al, 2007).

Whilst appropriate for detection of the UV-induced transcription blocks, the cells carrying the EGFP-ODC transgene apparently cannot be adapted for detection of the effects of oxidative DNA damage (8-oxoG) on transcription. The lack of evidence for a decrease of expression of the EGFP-ODC gene following the exposures of the cells to the Ro19-8022 photosensitiser plus light (**Figure 3.13**) cannot yet be interpreted as the absence of transcription blocks, because simple calculations show that the damage densities achieved in DNA of cells at the applicable damage doses were not sufficient to affect a significant fraction of the EGFP-ODC transcription units in the whole cell population. Thus, irradiation in the presence of 50 nM Ro19-8022 induces approximately one oxidized purine modification per 2 million base pairs (Will et al, 1999). Extrapolation of this value to the maximal tolerable doses gives $\leq 1\times10^{-5}$ 8-oxoG/bp. Under these conditions, the probability of 8-oxoG to occur in the transcribed strand of the EGFP-ODC gene (≈1.9 kb) is negligibly small (<0.01). Even assuming that a single 8-oxoG in the transcribed DNA strand would lead to a complete transcription block (which is very unlikely in the light of published observations, see Chapter 1.4 for the detailed review), this would result in less than 1% decrease in the fraction of fluorescent cells, which is not possible to detect by current flow cytometry protocols within the reasonable experimental error margins.

An alternative approach to study the effects of given types of DNA damage on transcription and also repair of the transcription-blocking damage lies in exposure of a vector encoding for a reporter gene to a DNA damaging agent and subsequent delivery of the damaged gene to appropriate host cells. This experimental strategy termed “host cell reactivation” is widely applied to study the capacity of cells to repair transcription-blocking damage present in DNA (Barrett et al, 1991), proving its usefulness by successful cloning of an important nucleotide excision repair gene ERCC8, which is associated with Cockayne syndrome A (Henning et al, 1995). As an experimental approach for studying gene specific DNA repair and the direct influence of DNA damage on transcription, host cell reactivation of the transiently transfected plasmid vectors

provides several important practical advantages over the stably transfected cell lines discussed above. Since host cell reactivation does not require the establishment of stable cell lines, it is much more time-efficient and can be easily suited for use in cell lines with different genetic backgrounds or primary cells isolated from patients. Moreover, host cell reactivation permits investigation of direct effects of DNA damage on the expression of damaged reporter genes in the absence of any damage to the cell, thus excluding possible systemic effects of the genotoxic exposure on transcription (e.g., those arising from the general stress response). However, there are concerns that transcription and repair of plasmid DNA transfected to cells can substantially differ from these processes in chromosomal DNA, because the capacity of transiently transfected DNA to form a chromatin structure has not been extensively studied yet. Recent study of the Archer's group (Hebbar & Archer, 2008) and the results obtained in our lab (Khobta et al, 2010a) provided evidence that plasmid DNA delivered to nucleus rapidly associates with chromatin and adopts nucleosomal structure shortly thereafter, thus indicating that DNA repair and transcription in the plasmid DNA are probably very similar to these processes in chromosomes.

For a transcription-blocking DNA damage (such as UV-induced base modifications), there is convincing evidence in the literature that host cell reactivation is a robust indicator of DNA repair. This view is confirmed by the results obtained in our lab showing that reactivation of the UV-damaged plasmid gradually occurs in fully repair-proficient cells but not in the CSB-null MEFs, which are deficient in transcription-coupled nucleotide excision repair of the UV-induced pyrimidine photoproducts (Khobta et al, 2009). It was thus surprising to find out that transcription recovery does not take place following the transfections of the fully repair-proficient host cells with plasmids containing relatively small numbers of oxidative purine modifications (predominantly 8-oxoG), generated by photosensitisation (Section 3.2), even though the impaired host cell reactivation of the heavily damaged plasmids and viruses have been reported in the literature previously (Kassam & Rainbow, 2007), (Leach & Rainbow, 2011), (Nocentini, 1992), (Spivak & Hanawalt, 2006). Those results were subsequently interpreted by many authors as manifestation of transcription-blocking potential of oxidative DNA base modifications. However, the data presented here (**Figures 3.16**, **3.17**) questioned this interpretation, because at the employed densities of the DNA lesions (on average, 3 Fpg-sensitive DNA base modifications per plasmid molecule), the damage within the coding region of the EGFP gene would occur in only a minor fraction of the plasmid molecules (<17%) and therefore could not produce a strong effect on gene expression over the whole population of transfected cells.

A key to understanding of the different behaviour of UV-damaged reporter plasmids and those damaged with photosensitiser plus light is provided here by time-course studies of gene expression in HeLa cells (**Figure 3.16**) and by the complementary data of host cell reactivation in the MEF cell lines (Khobta et al, 2009), obtained by my contribution. These results showed that inhibition of transcription is minimal (if any) at early times after transfection of the damaged plasmids into the repair-proficient host cells. However, the inhibitory effect of the photosensitiser-induced DNA damage builds up starting from approximately 8 hours after transfection. Such a delayed inactivation of the damaged reporter gene provided an indication that, unlike direct blockage of transcribing RNA polymerase complexes by UV-induced pyrimidine dimers, the great part of the negative effect of the photosensitiser-induced oxidative damage on transcription is probably not direct, but rather mediated by the intracellular processing of the primary damage or, possibly, elimination of the damaged plasmid DNA. Further experiments (own published data not included in this thesis) showed that the cellular content of the plasmid DNA is indeed significantly (up to 50%) reduced in the presence of the photosensitiser-induced oxidative damage (Khobta et al, 2010a). It is therefore possible that incision of the oxidised DNA bases by specialised DNA repair endonucleases could promote degradation of the plasmid DNA by exonucleases in the nucleus. Alternatively, degradation of a fraction of the damaged vector DNA could be promoted by some other DNA modifications, which could be induced by the photosensitiser.

Because of the observed decreased content on the methylene blue-damaged DNA in the transfected cells, the results of FACS analyses of the protein expression were not sufficient to conclude with confidence about the negative effect of the photosensitiser-induced DNA damage on transcription. For this reason I performed quantitative mRNA analyses in MEFs (**Figure 3.18**), (Khobta et al, 2009) and in HeLa cells (Khobta et al, 2010a). The results confirmed that the remaining DNA produced several fold less transcripts than the same number of undamaged DNA molecules. Nevertheless, we still could not be absolutely sure that the negative effects on transcription were fully attributed to 8-oxoG, since photosensitisation in the presence of methylene blue was shown to induce minor amounts of other oxidative base modifications, abasic sites and strand breaks in addition to 8-oxoG. In addition, a possibility of generation of some uncharacterised DNA modifications was hard to exclude with certainty. These considerations urged us to replace the plasmid randomly damaged by photosensitisation with the plasmid DNA containing a single 8-oxoG specifically positioned in the DNA sequence of the reporter EGFP gene.

The method for construction of circular DNA molecules containing single synthetic 8-oxoG specifically positioned within the desired nucleotide sequence has been previously

described by Bregeon and Doetsch (Bregeon & Doetsch, 2004). Here I have adapted this method to insert synthetic 8-oxoG into the transcribed DNA strand of the plasmid-encoded EGFP gene by inverting the direction of the f1 replication origin available in the pEGRP-mODC-ZA vector. This allowed production of single-stranded circular plasmid DNA in the filamentous phage, annealing of the complementary oligonucleotides containing either G or 8-oxoG, synthesis of the second DNA strand with the help of the T4 DNA polymerase and ligation of the newly synthesized DNA strand (chapter 3.3.1). I overall succeeded in production of plasmid DNA containing single 8-oxoG in the desired position and of control plasmid with the incorporated G-containing synthetic oligonucleotide, as verified by the incubations with the Fpg DNA glycosylase (**Figure 3.32**). However, the yield of the covalently closed DNA produced with this method was far too low to feed the extensive transfection experiments required to study the effects of 8-oxoG on the gene expression in mammalian cells. Another serious drawback of the method is that the fraction of covalently closed DNA produced after the ligation step typically does not exceed 25−30%. Therefore, its further separation from other topological forms of the plasmid DNA (nicked circular, linear, and single-stranded circular) is always required. This is associated with unavoidable losses of the covalently closed DNA product. Moreover, the methods of DNA isolation from agarose gels or caesium chloride equilibrium centrifugation gradients containing ethidium bromide are associated with risks of induction of undesired additional DNA damage (particularly, strand breaks) and often result in contamination of the end product with agarose or caesium chloride, depending on the separation method used. In consequence, transfections of the plasmids constructed by the method of Bregeon and Doetsch (as described in chapter 3.3.1) demonstrated a very high interexperimental variation of the EGFP expression, independently of the presence or absence of 8-oxoG. This could be caused either by impurities in the final DNA preparation or by uncontrolled induction of undesired DNA modifications during the purification procedures.

The alternative technique for the DNA strand-specific incorporation of synthetic 8-oxoG into the plasmid-borne EGFP gene, which is based on selective enzymatic generation of two nicks in the DNA strand of choice and substitution of the excised single-stranded 18-nt fragment with a synthetic oligonucleotide (chapter 3.3.2), has a number of advantages over the procedures described previously. First, it avoids alterations of DNA sequence, which can occur during the phage replication, and incorporation of wrong nucleotides during the in vitro synthesis of the second DNA strand. Second, the ligation product consists of at least 90% covalently closed circular DNA with a built-in synthetic oligonucleotide, thus eliminating the need for further purifications. The excess of the oligonucleotide present in the reaction is efficiently removed during precipitation of

plasmid DNA with ethanol or can be washed away in a single step using a standard microconcentrator centrifugation device. Third, the amounts of modified DNA (tens of micrograms) produced in a single batch are sufficient for multiple transfections into various cell lines. Finally, the use of nicking endonucleases of different DNA strand polarity (Nb.Bpu10I and Nt.Bpu10I) allows incorporation of 8-oxoG in either the transcribed or the non-transcribed strand of the same vector, while only one DNA strand can be modified in the phage DNA-based procedure, depending on the direction of the f1 replication origin.

The invention of an efficient method for the DNA strand-specific incorporation of single 8-oxoG into the EGFP gene situated in a plasmid vector made it possible to address the long standing question about the potential of 8-oxoG to block gene transcription by RNA polymerase II. Resolving this problem was especially important, because conflicting data has been previously published on this topic, nourishing the speculations about potential relevance of the transcription-coupled NER as a repair pathway for 8-oxoG. Thus, Pastorizza-Gallego and Sarasin reported that 8-oxoG induces significant transcription block in CSB-deficient mouse cells, especially in the OGG1-null genetic background, and interpreted this effect as an indication for the CSB-mediated repair of 8-oxoG localised in the transcribed DNA strand (Pastoriza-Gallego et al, 2007). The conclusions of these authors were based on the assumption that unrepaired 8-oxoG significantly blocks the elongating RNA polymerase II. However blockage of transcription by 8-oxoG has not been detected neither in an earlier analogous host cell reactivation study of Klungland and co-workers (Larsen et al, 2004), nor in the in vitro transcription systems, reconstituted with cell extracts (Kathe et al, 2004), (Larsen et al, 2004) or purified RNA polymerases (Tornaletti et al, 2004), (Larsen et al, 2004), (Kuraoka et al, 2003), (Kuraoka et al, 2007) (Charlet-Berguerand et al, 2006), (Damsma & Cramer, 2009). Based on the own experience with quantitative gene expression analyses of 8-oxoG-containing plasmid vectors constructed by the method of Bregeon and Doetsch, I presume that poor quality or insufficient purity of the plasmid substrates could likely contribute to conflicting results of the host cell reactivations previously performed by different groups. Another problem of the previous experiments is that, because of limited amounts of the available 8-oxoG-containing plasmid, the authors were confined to very small amounts of DNA available for transfections. So, as little as 10 ng of the plasmid containing 8-oxoG together with 500 ng of the reference reporter plasmid were used in the study of Pastorizza-Gallego and Sarasin for transfection of MEFs (Pastoriza-Gallego et al, 2007). Other experimentators used 500 ng of total plasmid DNA without reporting the proportion of the 8-oxoG-containing plasmid in the co-transfection experiments (Larsen et al, 2004). For comparison, we use 1500 ng of the EGFP-encoding plasmid containing 8-oxoG and

equal amount of the reference plasmid encoding for DsRed-Monomer for co-transfections of MEFs using a magnet assisted transfection method, which is non-toxic and very efficient. Importantly, the use of EGFP in the experimentation presented in this thesis allows easy monitoring of the actual transfection efficiencies in every experiment. Moreover, only transfected cells marked by expression of the reference reporter gene are considered here for the quantification of the EGFP expression, while all the non-transfected cells are efficiently excluded from the analyses. This approach is truly quantitative as has been shown by an excellent linear correlation between the amount of the EGFP-encoding plasmid used for transfections and median EGFP fluorescence in the transfected cells (own published data, (Khobta et al, 2010a)). In contrast, the earlier studies measured the luciferase expression over the whole cell population without reporting the transfection efficiencies, which could be very different between the cell lines. Yet another source of discrepancies between the published results could be that the expression of the artificially produced plasmids containing 8-oxoG was compared with the expression of supercoiled plasmid isolated from bacteria in one of the studies (Pastoriza-Gallego et al, 2007), while it would be necessary to use a plasmid containing an unmodified synthetic oligonucleotide, produced and purified in the same way. All these experimental problems are overcome in the experimental procedure developed in our lab by improving the quality and yield of the artificial plasmid substrates, including the necessary controls, monitoring the transfection efficiencies, and by application of the robust quantification procedure for the EGFP expression.

The results of transfections of the plasmids containing single 8-oxoG into HeLa cells are coherent with the host cell reactivation results of the methylene blue-damaged plasmids discussed above. There are, however, some peculiarities that show which of the observed effects are caused specifically by 8-oxoG and which are not. For instance, quantification of plasmid DNA recovered from the transfected cells 24 hours after transfection showed that the plasmid constructs containing G and 8-oxoG were present in the host cells in the same amounts (**Figure 3.41**). Thus, the decreased content of the methylene blue-damaged DNA in comparison with the undamaged plasmid in earlier experiments was not caused by 8-oxoG but by some other DNA modifications. At the same time, the decrease of transcription observed in the presence of single 8-oxoG overall resembled the effect of the damage induced by methylene blue plus light (**Figures 3.16** and **3.38)**. Most importantly, time course data of the EGFP gene expression in HeLa cells clearly demonstrate the absence of a direct transcription block in the presence of a single 8-oxoG, since there was no difference between the expression on the plasmids containing G and 8-oxoG at early times after transfection (**Figure 3.38**). The subsequent decrease of the expression of the plasmids containing 8-oxoG paralleled the behaviour of

the methylene blue-damaged DNA, strengthening the hypothesis that the delayed inactivation of the gene transcription could be a result of excision of 8-oxoG in the process of DNA repair.

The availability of OGG1-null MEF cell lines (Klungland et al, 1999), (Osterod et al, 2001) made it possible to dissect the mechanism of the delayed inhibition of transcription by single 8-oxoG in the transcribed gene region. The *Ogg1*$^{-/-}$ and *Csb*$^{-/-}$*Ogg1*$^{-/-}$ MEFs employed in the gene expression studies are deficient in 8-oxoG removal from both chromosomal and plasmid DNA (Osterod et al, 2002), (Saxowsky et al, 2008). The absence of any negative effect of 8-oxoG on the reporter gene expression in these cells (**Figure 3.39**) thus implies that unprocessed 8-oxoG does not block transcription, in line with the normal gene expression in HeLa cells early after transfections (**Figure 3.38**).

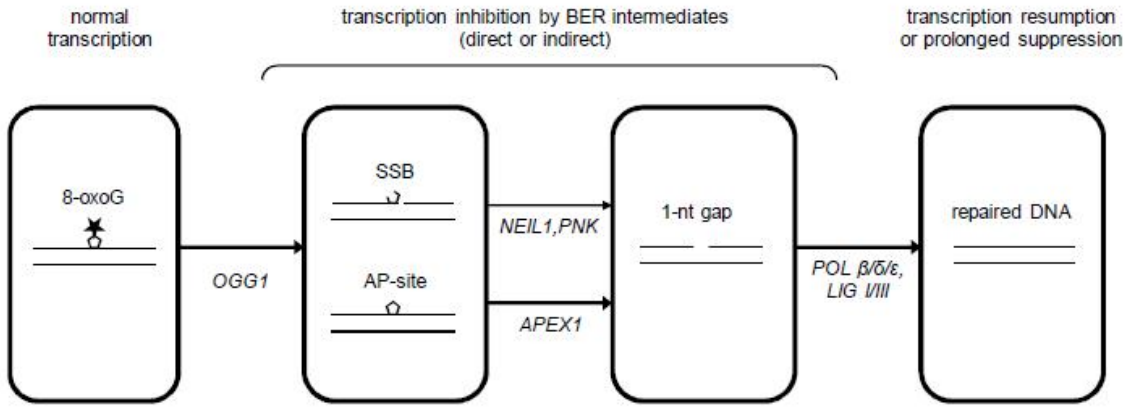

Figure 4.1 A model explaining the inhibition of transcription by 8-oxoG, in spite of the absence of a direct transcription elongation block by this DNA base modification.

The results of my experimentation further show that, in spite of the absence of a direct transcription-blocking capacity, 8-oxoG clearly can cause great negative effects on gene transcription in the cells with functional OGG1. It is therefore logical to propose that transcription of the damaged genes is most likely inhibited by BER intermediates, such as single-strand breaks (SSBs) or abasic sites, generated by the OGG1 DNA glycosylase during the excision of 8-oxoG. It has been reported that abasic sites in the transcribed DNA strand (but not in the opposing strand of the template DNA) cause an almost complete block of transcription by RNA polymerase II (Tornaletti et al, 2006). On the other hand, single-strand nicks favour formation of R-loops, i.e., persisting RNA-DNA hybrids which involving the transcribed DNA strand and efficiently block transcription. Interestingly, R-loops appear to be induced only by nicks situated in the non-transcribed

DNA strand (Roy et al, 2010), while the nicks in the transcribed DNA strand can be bypassed by elongating RNA polymerases with relatively high efficiencies (Smith & Savery, 2008). In summary, based on the host cell reactivation data presented in this work, I conclude that excision of 8-oxoG by OGG1 in cells but by no means the base modification itself interferes with transcription by RNA polymerase II. Herein I propose a model for the mechanism of inhibition of transcription by single 8-oxoG (**Figure 4.1**). As an increasing fraction of DNA modifications is recognised and processed by BER in the course of time, such model explains the gradual decrease in transcription of damaged DNA detected in the BER-proficient cell lines, but not in the OGG1-null cells.

With regard to the question about the structural nature of secondary transcription-blocking DNA lesion induced by the intracellular processing of 8-oxoG, it is interesting to note that the magnitude of the inhibitory effect of 8-oxoG on transcription was different, depending on the DNA strand containing the base modification (**Figures 3.38**, **3.39**). In view of the fact that 8-oxoG had generally a stronger negative effect on transcription when situated in the non-transcribed rather than transcribed DNA strand, it is tempting to speculate that the repair-induced SSBs could be the lesion responsible for the cessation of transcription, because the observed DNA strand bias in the inhibition of transcription by 8-oxoG is remarkably similar to the strand-specific inhibition of transcription by SSBs, recently reported by our lab members (Khobta et al, 2010b). Intriguingly, Bork Lühnsdorf in our lab found that incorporation of single uracil also caused inhibition of gene transcription, however without a notable DNA strand-specificity (Kitsera et al, 2011). This finding might reflect the structural differences between the repair intermediates formed after the damage excision by monofunctional DNA glycosylases (which initiate BER of uracil) and the bifunctional DNA glycosylase OGG1. Alternatively, the observed DNA strand bias in the effects of 8-oxoG on transcription could arise if the rates of excision and repair are different between the transcribed and non-transcribed DNA strands, or if the OGG1-dependent repair pathway is confined to the non-transcribed DNA strand. However, compelling experimental evidence for these both theses is currently lacking, as long as the contributions of different DNA repair pathways to repair of 8-oxoG in transcribed genes have not been sufficiently clarified.

Besides the functional non-equivalence of “top” and “bottom” DNA strands in transcription, the plasmid substrates employed in the present study differ in base sequence surrounding 8-oxoG. In attempt to predict to which extent DNA sequence context could influence the repair efficiency of the base modification, the efficiencies of excision of different DNA strands were tested by incubation of 8-oxoG-containing plasmid DNA with purified human OGG1 under cell free conditions (**Figure 3.42**). Intriguingly, three- to tenfold different OGG1 concentrations were required in the plasmid relaxation

assay to achieve equivalent cleavage efficiencies between the diverse DNA sequences of “top” and “bottom” DNA strands. A significantly higher cleavage rate of the “top” strand by pure OGG1 is coherent with a stronger effect of 8-oxoG in this DNA strand (NTS) on gene expression in the cell transfection experiments (chapter 3.6). Dependence of excision activity of OGG1 upon DNA sequence can thus explain the observed DNA strand-specific effect of 8-oxoG on transcription. However, kinetic analyses of sequence specific DNA repair in cells would be required to affirm this thesis.

In summary, the present study provides important insights into interactions of 8-oxoG with transcription in cells, rejecting the thesis about direct transcription-blocking potential of 8-oxoG and, instead, supplying new evidence for a critical role of BER intermediates in inhibition of transcription. The results shed light on dual role of OGG1, which initiates repair, but also transcriptional inactivation of the damaged gene. Besides of these principal findings, the research presented here revealed several intriguing facts, which lay outside of the main tasks of this work, but still merit discussion as being potentially highly relevant to the problem of interaction of DNA damage and repair with transcription in general. The finding of persistent transcriptional silencing of the methylene-blue damaged plasmids (Khobta et al, 2010a) can reflect a physiological mechanism of transcriptional regulation and epigenetic gene silencing initiated by DNA damage. 8-oxoG is a candidate DNA base modification, which can trigger such epigenetic alterations, as suggested by the abrogation of the inhibitory effect of 8-oxoG on the EGFP gene expression by incubation with a HDAC-inhibitor trichostatin A (preliminary data not shown). Given the high frequency of endogenously-generated 8-oxoG in cells under normal physiological conditions, it is important to reconstitute the full chain of molecular events that mediate transcriptional repression at the damaged DNA and identify the proteins engaged in this mechanism. Candidate factors could involve components of machineries dealing with chromatin maintenance, transcription, or DNA repair.

With respect to the underlying mechanism of transcriptional repression induced by DNA base damage, such as 8-oxoG, it would be important to further investigate the role of CSB protein in transcription of damaged DNA, since our data show that CSB improves transcription of genes containing 8-oxoG (**Figure 3.39**) and single-strand breaks (Khobta et al, 2010b). CSB has been implicated in transcriptional elongation (Charlet-Berguerand et al, 2006), (Kuraoka et al, 2007) and chromatin remodelling (Citterio et al, 2000). Moreover, CSB is associated with histone-modifying activities and thus has been linked to the epigenetic chromatin modifications (Yuan et al, 2007). In view of these activities, CSB could either promote elongation over the DNA damage site or counteract the mechanism of epigenetic gene silencing induced by DNA damage.

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APPENDIX I: sequences of plasmid vectors in FASTA format

Nucleotide sequences of pMAZ-ODC and pMZA-ODC episomal vectors are available in Genbank (accession numbers EU421131 and EU421132, respectively). The sequences of other vectors follow.

>pEGFP-mODC-ZA(+) |descr=pZA|len=4881

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AGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTA
CATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTGATGCGGTT
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>pEGFP-mODC-ZA(-) |descr=pZAss|len=4889

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APPENDIX II: publications in peer-reviewed journals which included data of this work

Kitsera N, Khobta A, Epe B (2007) Destabilized green fluorescent protein detects rapid removal of transcription blocks after genotoxic exposure. *Biotechniques* **43:** 222-227

Khobta A, **Kitsera N**, Speckmann B, Epe B (2009) 8-Oxoguanine DNA glycosylase (Ogg1) causes a transcriptional inactivation of damaged DNA in the absence of functional Cockayne syndrome B (Csb) protein. *DNA Repair (Amst)* **8:** 309-317

Khobta A, Anderhub S, **Kitsera N**, Epe B (2010a) Gene silencing induced by oxidative DNA base damage: association with local decrease of histone H4 acetylation in the promoter region. *Nucleic acids research* **38:** 4285-4295

Khobta A, Lingg T, Schulz I, Warken D, **Kitsera N**, Epe B (2010b) Mouse CSB protein is important for gene expression in the presence of a single-strand break in the non-transcribed DNA strand. *DNA Repair (Amst)* **9:** 985-993

Kitsera N, Stathis D, Luhnsdorf B, Muller H, Carell T, Epe B, Khobta A (2011) 8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1. *Nucleic acids research* **39:** 5926-5934

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DECLARATION OF AUTHORSHIP

I hereby confirm that I have authored this PhD thesis independently and without use of other than the indicated resources.

Mainz, December 20th 2011

(Nataliya KITSERA)

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