

8-Oxo-7,8-dihydroguanine in DNA does not constitute a barrier to transcription, but is converted into transcription-blocking damage by OGG1

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ABSTRACT

The common DNA base modification 8-oxo-7,8-dihydroguanine (8-oxo-G) affects the efficiency and fidelity of transcription. We constructed plasmid substrates carrying single 8-oxo-G residues, specifically positioned in the transcribed or the non-transcribed DNA strands, to investigate their effects on the expression of an EGFP reporter gene and to explore the role of base excision repair in the mechanism of transcription inhibition. We report that 8-oxo-G does not directly block transcription in cells, since a single 8-oxo-G in the transcribed DNA strand did not reduce the EGFP expression levels in repair-deficient (OGG1-null) mouse embryonic fibroblast cell lines. Rather, inhibition of transcription by 8-oxo-G fully depends on 8-oxoguanine DNA glycosylase (OGG1) and, at the same time, does not require the localization of the lesion in the transcribed DNA strand. We propose that the interruption of transcription is induced by base excision repair intermediates and, therefore, could be a common consequence of various DNA base modifications. Concordantly, the non-blocking DNA modification uracil was also found to inhibit transcription, but in an OGG1-independent manner.

INTRODUCTION

The 8-oxo-7,8-dihydroguanine (8-oxo-G) is a major product of intracellular DNA oxidation and one of the most common types of endogenously generated mutagenic base damage (1). Removal of 8-oxo-G from mammalian

chromosomal DNA occurs with half-times of several hours predominantly via a very efficient base excision repair (BER) pathway initiated by the 8-oxoguanine DNA glycosylase (OGG1) (2–4). The excision of 8-oxo-G catalysed by human OGG1 is accompanied by cleavage of DNA strand via β -elimination of the deoxyribose residue (5). The efficiency of strand cleavage by OGG1 can be modulated by other BER proteins, such as AP endonuclease and NEIL1 DNA glycosylase (6,7). Catalytic activities of these proteins result in BER intermediates with different chemical structures of DNA ends and largely define the subsequent steps of repair (8).

In the absence of timely repair, bypass of 8-oxo-G by replicative DNA polymerases leads to high rates of misincorporation of adenine, which forms a stable Hoogsteen base pair with 8-oxo-G (9–11). A Hoogsteen base pairing also occurs during transcription by RNA polymerase II between adenine in RNA and 8-oxo-G in the DNA template (12), although the fraction of the mutant transcripts with C to A substitutions does not exceed 10% (13,14). In addition, elongation by RNA polymerases is inhibited in the presence of 8-oxo-G in the template strand of DNA (15). However, the lesion does not cause a permanent block or termination of elongation but rather a temporary stalling of the elongating human RNA polymerase II complexes (16,17). The blocking effects of 8-oxo-G observed in the reconstituted *in vitro* transcription reactions can be overcome by addition of the purified TFIIS, Elongin or CSB proteins (13,18).

Despite the absence of an irreversible elongation block *in vitro*, in the host cell reactivation assays 8-oxo-G seems to account for strong decrease of expression of plasmid-encoded genes caused by oxidative DNA damage induced

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by photosensitizers (19,20), because 8-oxo-G was the prevailing DNA lesion under that conditions. Since transcription was most strongly affected in cell lines with mutations in the *CSA* or *CSB* genes, which lack transcription-coupled repair and are thought to be defective in the damage bypass by RNA polymerase II, it was proposed that 8-oxo-G could directly block transcriptional elongation (19,21). However, this was subsequently questioned by different kinetics of host cell reactivation of the oxidatively damaged plasmids and of plasmids that contained transcription blocking UV-induced lesions (20). To understand the mechanism of transcription inhibition, we now placed synthetic 8-oxo-G in defined positions in either the transcribed or the non-transcribed DNA strand of a plasmid-borne *EGFP* gene. We found that 8-oxo-G does not exhibit a direct elongation block and obtained evidence that inhibition of transcription results from the generation of transcription-blocking structures by BER.

MATERIALS AND METHODS

Oligonucleotides

Unmodified and uracil-containing oligonucleotides were from Eurofins MWG Operon. 8-oxo-G-containing oligonucleotides were synthesized on an Amersham Äkta oligopilot 10 synthesizer (GE Healthcare Europe, München, Germany). Benzylthiotetrazole (0.25 M) was used as coupling reagent with the coupling times of 10 min for 8-oxo-G and 4 min for other amidites. Final detritylation and deprotection were achieved by 18-h incubation with 50% ammonium hydroxide and 20% methylamine in water at room temperature. All oligonucleotides employed for construction of the plasmid substrates (Supplementary Table S1) were purified by reversed phase HPLC, desalted on SepPaks cartridges (Waters, Eschborn, Germany) and verified by analytical HPLC and MALDI-TOF spectrometry.

Plasmid substrates

The pDsRed-Monomer-N1 plasmid was from Clontech (Saint-Germain-en-Laye, France). pEGFP-mODC-ZA that encodes for enhanced green fluorescent protein has been described previously (20). The vectors are of similar size and share the same backbone containing an SV40 origin for replication in mammalian cells and a pUC origin of replication for propagation in *Escherichia coli*. The vectors differ only in the portion between the immediate early promoter of CMV and the SV40 early mRNA polyadenylation signal, which contain either the DsRed-Monomer or the EGFP coding sequences. The vectors cannot autonomously replicate in mammalian cells used in the present study because of the absence of the SV40 large T antigen.

Two nicks were produced in either of the EGFP strands by the Nt.Bpu10I or Nb.Bpu10I nicking endonucleases (Fermentas, St Leon-Rot, Germany). The excised 18 nt oligonucleotides were substituted by synthetic oligonucleotides containing 8-oxo-G or uracil or no base modifications. To this end, 30 µg of double-nicked plasmids

were supplemented with the T4 DNA ligase Buffer (Fermentas) and 1.7 pmol of a synthetic competitor oligonucleotides (~180-fold molar excess) and incubated with T4 polynucleotide kinase (Fermentas) at 37°C 30 min in a reaction volume of 1125 µl. The native 18-mers were melted out at 80°C for 10 min, followed by cooling to 50°C at a rate of 0.02°C/s. Reactions were transferred to ice. Ligation was done with 150 U T4 DNA ligase 1 h at 22°C followed by heat-inactivation of the enzyme for 15 min at 65°C. To verify the complete replacement of the excised 18-mers, one aliquot was processed without the phosphorylation step (Figure 1A). Modified plasmids were concentrated by Amicon Ultra Ultracel® 30 centrifugal filters (Millipore, Schwalbach am Taunus, Germany), followed by phenol-chloroform extraction and ethanol precipitation. The concentration step can be omitted if smaller plasmid amounts are to be produced.

Recombinant Fpg protein (22)—a kind gift from Serge Boiteux—was used to verify the presence of 8-oxo-G by conversion of plasmid DNA to the open circular form as described previously (20). Similarly, the combination of Ung and Endonuclease IV (NEB GmbH, Frankfurt am Main, Germany) was used to excise uracils and cleave the resulting apyrimidinic sites (AP sites). Agarose gel electrophoresis was performed 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).

Mouse embryonic fibroblast cell lines

Spontaneously immortalized *Ogg1*^{-/-} and *Csb*^{-/-}*Ogg1*^{-/-} mouse embryonic fibroblasts (MEFs) were described previously (4,23); *Ung*^{-/-} and wild-type (F11.1) MEFs were kindly provided by Deborah Barnes (24). Other independently obtained wild-type MEF cell lines were tested in several experiments with very similar results (data not shown). 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.

Transfections and protein expression analyses by flow cytometry

MATra-A system (PromoKine, Heidelberg, Germany) was employed for transfection of MEFs as described previously (20) and Effectene (Qiagen, Hilden, Germany) was used for HeLa cells. Equal amounts of the EGFP-encoding plasmid, produced by insertion of the synthetic oligonucleotides containing the specified bases or base modifications and of the plasmid encoding for DsRed-Monomer were transfected. 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 µg (for the transfections with MATra-A) of total plasmid DNA. Cells were harvested and fixed with 1% formaldehyde at indicated times after transfections and analysed by FACSCalibur™ flow cytometer and CellQuest™ Pro software (Beckton Dickinson, GmbH, Heidelberg, Germany) as described previously

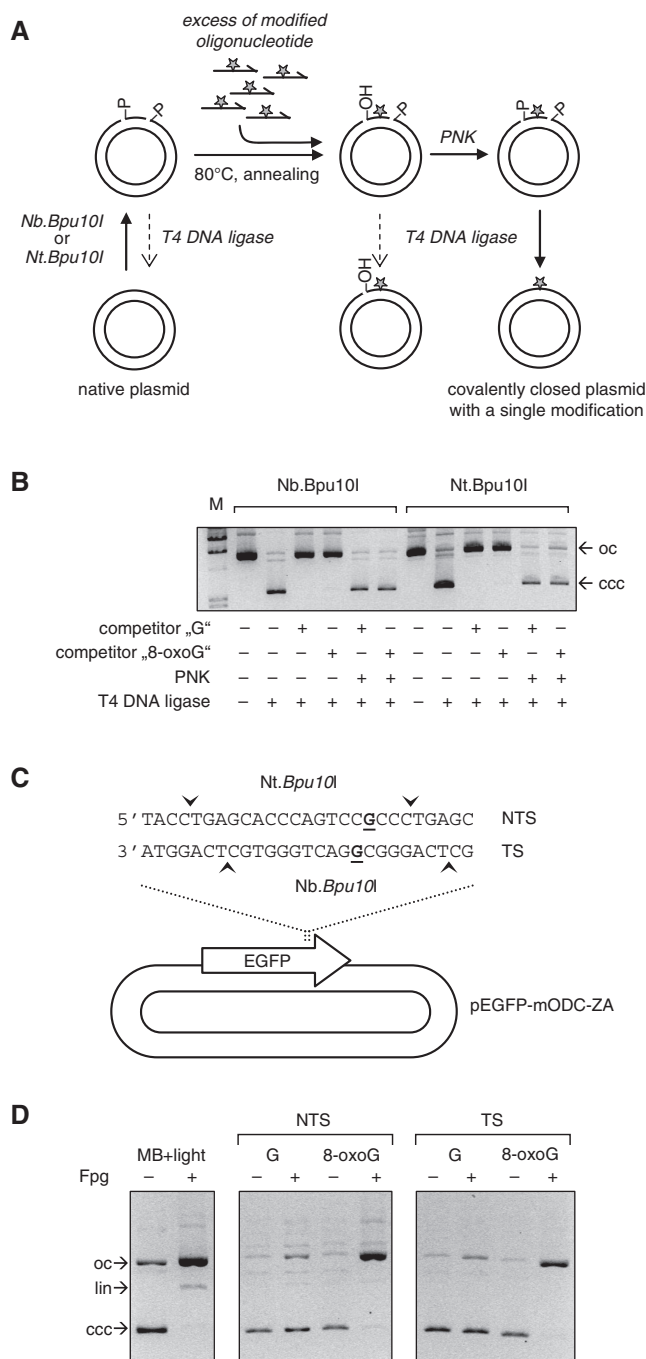


Figure 1. Construction of the plasmid substrates containing a specifically positioned 8-oxo-G in the transcribed or the non-transcribed DNA strands (TS and NTS, respectively) of the *EGFP* gene. (A) The strategy for production of the covalently closed plasmid DNA containing a single base modification (star) by strand-specific excision of the 18 nt fragment and its substitution with a synthetic oligonucleotide. (B) 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-G by ligation in the absence of polynucleotide kinase (PNK) and agarose gel electrophoresis. (C) Positions of the nicking sites (arrowheads) of the *Bpu10I* mutant variants in both DNA strands and of 8-oxo-G (underlined "G" in the DNA sequence) in the pEGFP-mODC-ZA plasmid. Arrow shows the EGFP complementary DNA sequence. (D) Agarose gel electrophoresis of the plasmid substrates after incubation with recombinant Fpg DNA glycosylase. Left panel shows plasmid DNA damaged by photosensitization in the presence of methylene blue (MB).

(25). EGFP expression was determined as median FL-1 fluorescence among the transfected cells, defined by a significant content of DsRed-Monomer (FL-2 >30).

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

Cells were split in three equal parts (for DNA, RNA and protein expression analyses) 8 h after transfections and incubated in 6-well plates for additional 16 h. For isolation of total DNA, cells were incubated 3 h at 50°C in 0.5% sodium dodecyl sulphate supplemented with 100 mg/l Proteinase K, proceeding with standard phenol-chloroform extraction procedures and ethanol precipitation. The plasmid copy numbers in DNA samples were determined by real-time quantitative PCR. Total 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) for 30 min at room temperature. RNA integrity was verified by denaturing agarose gel electrophoresis. Reverse transcription (RT) reactions were performed with a Revert Aid™ First Strand cDNA Synthesis Kit (Fermentas) using random hexamer primers. 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. Absence of contaminating plasmid DNA was confirmed by PCR of aliquots removed prior to RT.

Real-time quantitative PCR

Light Cycler 1.5 and FastStart DNA Master^{PLUS} SYBR Green I kit (Roche Diagnostics, Mannheim, Germany) were used according to the manufacturer's instructions. Primers were controlled by melting curve analyses and agarose gel electrophoresis to produce a single specific PCR product. The measurements were done in quadruplicates. Standard curves obtained by amplification of at least four DNA standards had linear regression coefficients ≥ 0.99 . The oligonucleotides employed as PCR primers are listed in Supplementary Table S2.

RESULTS

Design and construction of the plasmid substrates containing a single 8-oxo-G

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 (26). The gapped DNA circles can be conveniently generated by treatments of a plasmid DNA with sequence-specific nicking endonucleases (27). We used the mutants of *Bpu10I* restriction endonuclease with DNA strand-specific nicking activities (28) and the pEGFP-mODC-ZA vector, which contains two tandemly located *Bpu10I* sites, to specifically introduce two nicks in the transcribed or the non-transcribed DNA strands of the *EGFP* gene. Native single-stranded DNA 18-mers between the *Bpu10I* sites were melted out and replaced with the synthetic

oligonucleotides containing G or 8-oxo-G (Figure 1). Complete inhibition of ligation in control aliquots processed in the absence of the T4 polynucleotide kinase (Figure 1B) confirmed the efficient incorporation of synthetic oligonucleotides, which, in contrast to the native Bpu10I-excised DNA fragments, lack a 5'-phosphate required for ligation.

Transcription through 8-oxo-G can lead to a considerable fraction of mutant RNA molecules resulting from erroneous incorporation of adenine instead of cytosine by RNA polymerase II (14,29). To avoid miscoding transcripts, 8-oxo-G in the transcribed DNA strand was placed in the third position of the EGFP codon 206 (Table 1). The resulting UCC and UCA codons in mRNA both encode for serine and occur with similar frequencies in human genes, according to the Codon Usage Database (30). The position of an 8-oxo-G in the non-transcribed DNA strand was chosen next to the 8-oxo-G site in the transcribed strand (Figure 1C). Ligation of the G- or 8-oxo-G-containing oligonucleotides typically produced at least 90% of pEGFP-mODC-ZA in the covalently closed form. Incubation of the 8-oxo-G containing plasmids with Fpg DNA glycosylase resulted in complete conversion into the open circular form, indicating that the lesion was present in >97% of the plasmid molecules (Figure 1D).

Effect of a single 8-oxo-G on transcription does not require its localization in the transcribed DNA strand

To quantitatively analyse the effect of single positioned 8-oxo-G bases on expression of the *EGFP* gene, we co-transfected HeLa cells with the pEGFP-mODC-ZA plasmid, containing either G or 8-oxo-G, together with a tracer plasmid encoding for DsRed-Monomer. It was previously shown that DsRed-Monomer does not interfere with detection of EGFP by flow cytometry and allows a sensitive quantification of EGFP fluorescence in transfected cells with a very good signal linearity (25). Replication of the pEGFP-mODC-ZA and pDsRed-Monomer does not occur in HeLa cells. This notion was confirmed by transfections of *dam*-methylated plasmids and digestion of reisolated episomal DNA with the DpnI restriction endonuclease and Exonuclease III (Supplementary Figure S1). The DpnI resistant fractions of the plasmid DNA recovered 48 h after transfections

Table 1. Choice of position for the incorporation of 8-oxo-G into the TS of the *EGFP* gene

Codon	202	203	204	205	206	207	208	209	210
DNA sequence (TS)	3'-GAC	<u>TCG</u>	<u>TGG</u>	<u>GTC</u>	<u>AGG</u>	<u>CGG</u>	<u>GAC</u>	<u>TCG</u>	TTT
mRNA	5'-CUG	AGC	ACC	CAG	UCC	GCC	CUG	AGC	AAA
Mutant transcript	5'-CUG	AGC	ACa	CAG	UCa	GCa	CUG	AGC	AAA
Protein	L	S	T	Q	S	A	L	S	K

The 18 nt DNA fragment excised by Nb.Bpu10I is underlined. Three of the guanines (boldface) within this fragment can be substituted for 8-oxo-G without causing an amino acid change in the EGFP protein in case of misincorporation of adenine during transcription.

were minimal, indicating that >90% of the plasmid molecules retained the *dam* methylation pattern and therefore did not replicate in HeLa cells.

Expression analyses of the modified plasmids 24 h after transfections showed that 8-oxo-G in the transcribed DNA strand did not have any detectable influence on the expression of EGFP protein. At the same time, 8-oxo-G in the non-transcribed strand of the *EGFP* gene caused a ~2-fold decrease of the gene expression, compared to the plasmid substrate containing guanine in the analysed position (Figure 2A and B). Quantitative mRNA analyses by reverse transcription and real-time PCR confirmed a strong decrease of EGFP transcript in the presence of a single 8-oxo-G in the non-transcribed DNA strand, while 8-oxo-G in the transcribed strand did not affect the transcript levels (Figure 2C). Separate quantification of the cDNA fragments with the primers upstream and downstream from the 8-oxo-G site provided no indication for the presence of transcripts truncated at the damage site. Parallel analyses of DNA showed that the plasmid substrates containing G and 8-oxo-G were retained with the same efficiencies in the host cells, regardless of the DNA strand modified (Figure 2D). 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. In summary, RNA and protein expression analyses demonstrate that 8-oxo-G inhibits transcription in a mechanism that is distinct from direct blocking of the elongating RNA polymerase II.

Inhibition of gene expression by 8-oxo-G occurs only in the presence of functional *Ogg1* gene

If the *EGFP* gene expression in repair-proficient HeLa cells is modulated due to the direct effect of 8-oxo-G on transcriptional elongation, this effect should decrease in time, reflecting the removal of damage from the transcribed gene sequence, as reported for the UV-damaged plasmids (20). However, this did not happen with the plasmids containing 8-oxo-G (Figure 3). Curiously, there were no differences in the EGFP expression between the G- and 8-oxo-G-containing plasmids 8 h after transfections, while the subsequent time course showed that expression of the '8-oxo-G' plasmid was steadily decreasing in comparison with the 'G' plasmid, especially when the base modification was present in the non-transcribed strand (NTS) of the gene (Figure 3). The effect caused by the presence of 8-oxo-G in the TS was much weaker and appeared only after a significant delay. We interpret the absence of a detectable reduction of gene expression in the presence of 8-oxo-G in the transcribed DNA strand at the early time points as a clear indication of an efficient bypass of the lesion by RNA polymerase II.

It was recently shown that single-strand breaks induced by the nicking endonucleases strongly affect transcription, particularly if located in the non-template strand of the transcribed DNA (31,32). We hypothesized that a similar mechanism of transcription inhibition could also operate at the strand breaks induced by a DNA glycosylase/AP lyase in the process of base excision repair. To address the

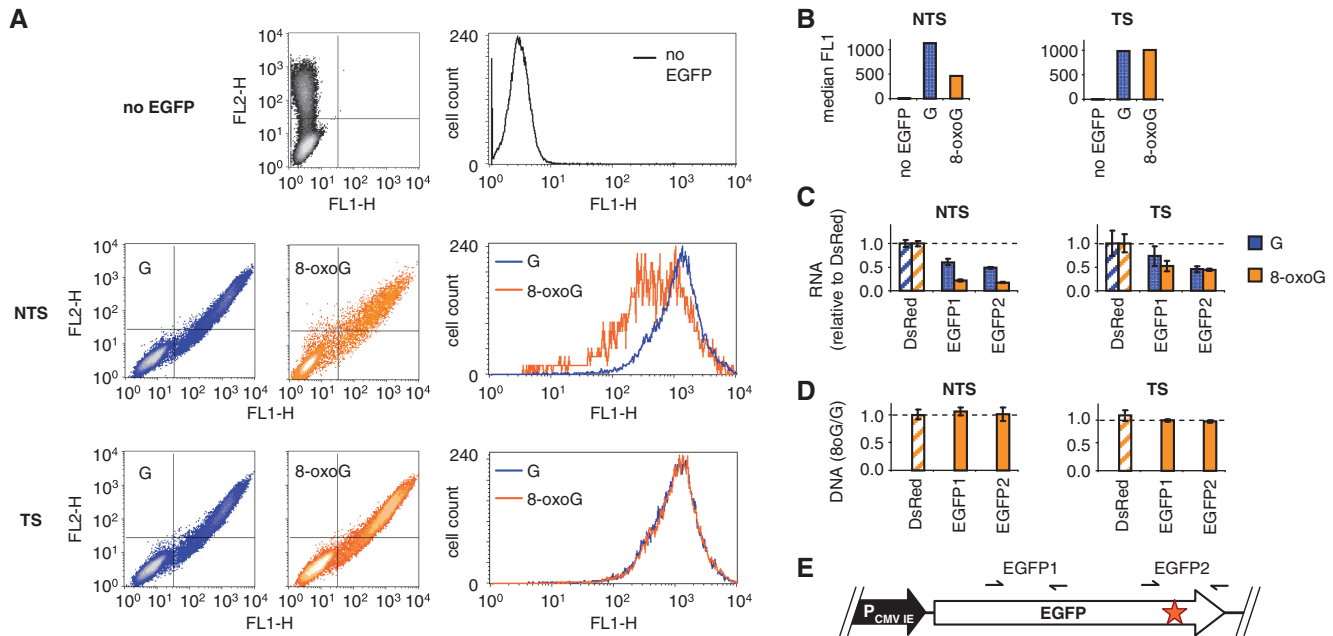


Figure 2. Expression of DsRed-Monomer and EGFP in HeLa cells 24 h after cotransfection with equal amounts of the corresponding plasmid DNAs. EGFP-encoding plasmid substrates contained guanine (G, blue colours) or 8-oxo-G (amber colours) (A) Flow cytometry of cells transfected in parallel with 'G' and '8-oxo-G' plasmids. Detected are the fluorescent signals of EGFP (FL1-H) and DsRed-Monomer (FL2-H). Overlaid fluorescence distribution plots (on the right) specifically show transfected cells, which localize in the upper two quadrants of the dot density plots. (B) Quantitative analyses of the data presented in A. (C and D) 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 \pm SD). (E) Locations of the EGFP1 and EGFP2 regions with respect to the 8-oxo-G position (a star).

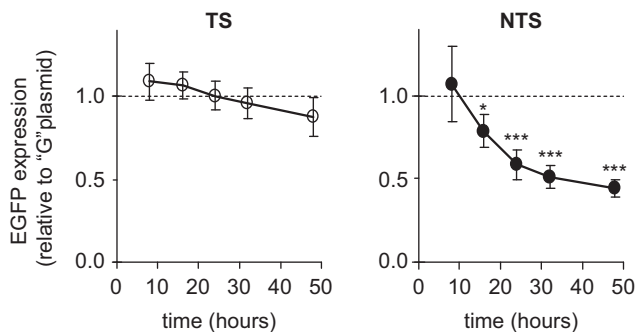


Figure 3. Time-dependent decrease of expression of the plasmid EGFP gene containing a single 8-oxo-G in the TS or NTS in transfected HeLa cells (mean for five separate plasmid preparations \pm SD). Paired Student's two-tailed *t*-test: * $P < 0.05$, *** $P < 0.001$.

role of the OGG1 DNA glycosylase, we performed further transfection experiments in immortalized MEF cell lines carrying either wild-type or disrupted copies of the *Ogg1* gene. Comparison between the 'G' and '8-oxo-G' plasmid substrates in the *Ogg1*^{+/+} MEFs confirmed the inhibition of transcription by a single 8-oxo-G in the non-transcribed DNA strand ($P = 0.002$ for seven independent preparations of each 'G' and '8-oxo-G' plasmid substrates), although the effect was somewhat weaker than in HeLa cells. The inhibition of EGFP gene expression by 8-oxo-G was completely abolished in *Ogg1*^{-/-} MEFs (Figure 4), thus indicating that functional OGG1 is required for the inhibition of transcription.

CSB protein was previously implicated in the bypass of 8-oxo-G by RNA polymerase II *in vitro* (13). On the other hand, CSB was shown to be important for transcription in cells in the presence of single-strand breaks (31). We therefore analysed the expression of the 8-oxo-G-containing plasmids in *Csb*^{-/-} MEFs, which were either *Ogg1*^{+/+} or *Ogg1*^{-/-}. In *Csb*^{-/-} *Ogg1*^{+/+} cells, EGFP expression was significantly decreased by 8-oxo-G in either transcribed ($P = 0.003$, $n = 6$) or non-transcribed ($P < 0.001$, $n = 6$) DNA strand of the gene. The negative effect of 8-oxo-G on transcription was quantitatively much stronger in *Csb*^{-/-} than in *Csb*^{+/+} cells (Figure 4). Most strikingly, this was again completely abrogated by the *Ogg1* knockout (Figure 4), confirming that inhibition of transcription fully depends on OGG1 and showing that CSB is dispensable for transcriptional bypass of the unprocessed 8-oxo-G in the cells.

Effects of uracils and AP sites in different DNA strands on transcription

If inhibition of transcription by 8-oxo-G is mediated by a BER intermediate, excision of other base modifications is expected to have analogous effects. To test this presumption, we constructed the plasmids containing uracil (Figure 5) as a DNA base modification that does not have an appreciable transcription-blocking or miscoding potential (33). In the wild-type cells, there was no detectable effect of uracil on EGFP expression (Figure 5C). The same result was obtained in cells lacking the major uracil DNA glycosylase UNG2, in agreement with the notion

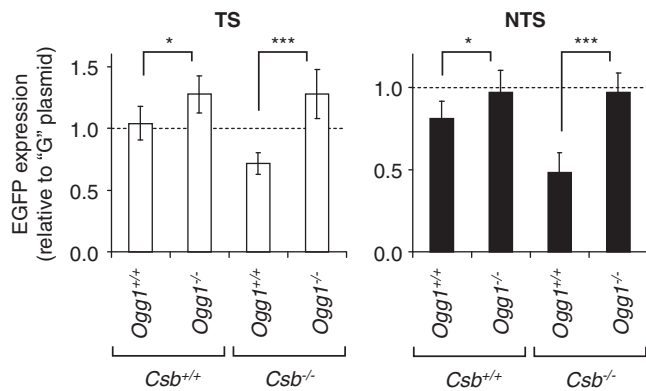


Figure 4. Effects of a single 8-oxo-G on EGFP expression in mouse embryonic fibroblasts of the indicated genotypes. EGFP fluorescence was measured by flow cytometry 24 h after transfections (mean \pm SD for six independently prepared 'G' and '8-oxo-G' plasmid couples). Paired Student's two-tailed *t*-test: **P* < 0.05, ****P* < 0.001.

that uracil does not block RNA polymerase II. Nonetheless, a \sim 2-fold decrease of EGFP expression was caused by uracil in the *Csb*^{-/-} cells, regardless of the DNA strand concerned. Importantly, unlike 8-oxo-G, uracil caused a pronounced decrease of EGFP expression also in *Csb*^{-/-} *Ogg1*^{-/-} cells, which are proficient in incision of uracil but not of 8-oxo-G. There was no major difference between the effects of uracil in the *Csb*^{-/-} and *Csb*^{-/-} *Ogg1*^{-/-} cells. The results thus demonstrate that inhibition of gene expression by uracil occurs independently of OGG1 and further support the notion that inhibition of transcription depends on incision of the modified bases.

Excision of uracil in the process of BER results in AP site as a primary intermediate product. Therefore, we further investigated the effects of AP site on the *EGFP* gene expression in cells. We treated the uracil-containing plasmids with Ung protein *in vitro* to obtain the plasmids containing single AP sites. The AP sites in both DNA strands showed inhibitory effects on EGFP expression, which were observable in wild-type MEFs and were even more pronounced in CSB-deficient MEF cell lines (Figure 6). As in case of uracil, the effects of the lesions in the T and the NT DNA strands were similar. We think that the observed decrease of gene expression could be caused in part by direct interference of the AP site in the TS with elongating RNA polymerase II and in part by generation of DNA strand breaks by downstream BER enzymes.

DISCUSSION

Transcription-blocking potential of 8-oxo-G is debated for more than a decade (34). Recent experimental data largely support the existence of a very weak block of elongation in cell free transcription systems, what is hard to reconcile with strong inhibition of transcription observed in cells. The studies reporting a clear inhibition of transcription by photochemically induced oxidative damage to plasmid DNA (19) and the ability of such damage to trigger persistent gene silencing (25) did not

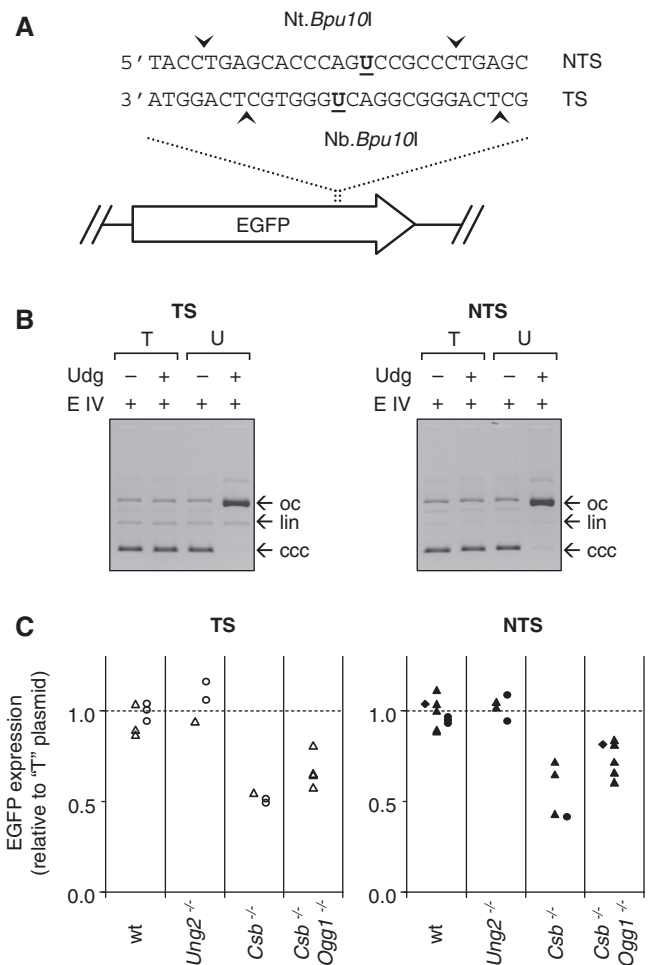


Figure 5. Effects of uracil in the TS or the NTS on EGFP expression. (A) Positions of the analysed uracils (underlined) in the *EGFP* gene. (B) Agarose gel analyses of the thymine and uracil-containing plasmids ('T' and 'U', respectively) after treatments with Udg and Endonuclease IV. (C) Expression of 'U' plasmid substrates in mouse embryonic fibroblasts of the indicated genotypes measured by flow cytometry 24 h after transfections. Different symbols indicate three independent preparations of the plasmid substrates.

firmly identify 8-oxo-G in the transcribed sequences of the genes as the lesion responsible, since the damage in the gene promoter regions could contribute to the observed effects. Also, previous investigations of the effects of synthetic 8-oxo-G positioned within the transcribed gene regions did not unambiguously answer the question about the capability of this base modification to block transcription in cells, because the independent studies in MEF cell lines reported the results as different as no effect at all (35) and the very strong effects (up to 90% reduction in gene expression, depending on the genotype) (21). In the latter case, it was proposed that the magnitude of the transcription inhibition could depend on the microsequence context at the DNA damage site and that the effect of 8-oxo-G could require a strong promoter. However, we think that the disagreement could also arise from the limitations of the method of preparation of the expression vector with a single DNA

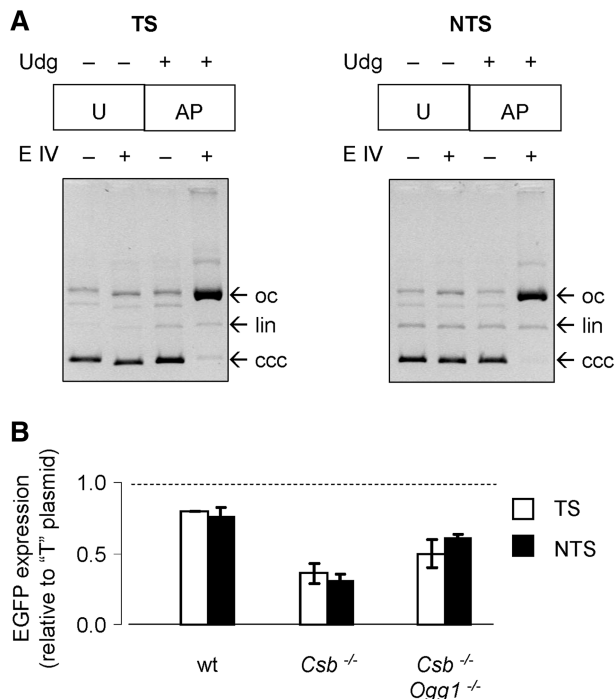


Figure 6. Effects of apyrimidinic sites (AP sites) in the TS or the NTS on EGFP expression. (A) Agarose gel analyses of the plasmids containing uracil ('U') and AP site ('AP') treated with Endonuclease IV. (B) EGFP expression in mouse embryonic fibroblasts of the indicated genotypes measured by flow cytometry 24 h after transfections. The same plasmid preparations were transfected in parallel into different cell lines (two independent transfections).

modification. For instance, possible contamination with caesium chloride (CsCl) and low yield of DNA available for transfections could compromise the sensitivity of detection and result in high experimental error values (estimated as about 20% in the previous studies). Here, we overcome these difficulties by applying a new method for construction of circular DNA templates carrying a single base modification in a DNA strand of choice (Figure 1). This allows a straightforward and highly efficient production of tens of micrograms of transfection grade topologically homogeneous vector DNA. Since there is no need for further separation of the covalently closed circular plasmid DNA in the ethidium bromide containing media (agarose gels or CsCl equilibrium density gradients), the secondary damage to DNA and possible contamination with agarose or CsCl are avoided.

Ogg1^{-/-} and *Csb*^{-/-} *Ogg1*^{-/-} MEFs employed in the gene expression studies are deficient in 8-oxo-G removal from both chromosomal and plasmid DNA (14,23). The unaffected gene expression in these cells (Figure 4) thus implies that unprocessed 8-oxo-G does not block transcription, in line with the normal gene expression in HeLa cells early after transfections (Figure 3) and with the absence of a clear effect of synthetic 8-oxo-G in transcribed DNA strand on the luciferase gene expression in one of the earlier studies (35). Curiously, in the absence of OGG1, EGFP expression even appeared slightly stimulated by 8-oxo-G in the transcribed DNA strand (Figure 4).

We did not experimentally address the molecular mechanism for this phenomenon. However, in view of the reported role of endogenously-generated 8-oxo-G in transcriptional activation of the chromosomal *BCL2* gene (36), a minor stimulatory effect of a single 8-oxo-G observed in the *EGFP* gene in our study might be attributed to a similar mechanism.

Our data show that, in spite of the absence of a direct transcription-blocking capacity, 8-oxo-G clearly has a great transcription blocking potential in the cells with functional BER. Putative mechanism of inhibition of transcription by BER intermediates in the cell can resemble the reported modes of transcription inhibition by similar DNA structures under cell free conditions. Thus single-strand nicks in the non-transcribed DNA strand favour formation of R-loops, which involve the transcribed DNA strand and efficiently block transcription (32), while the nicks in the transcribed DNA strand are weakly blocking (37). In contrast, 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 (38). Altogether, it is conceivable that DNA strand scissions and abasic sites induced in the process of BER can lead to inhibition of transcription in cells. In accordance with such a view, a strong inhibition of transcription by 8-oxo-G in the non-transcribed DNA strand and a much weaker effect of this base modification in the transcribed strand resemble the reported effects of single-strand breaks, which also inhibit gene expression to a much stronger extent if located in the non-transcribed strand (31). The observed strand bias (Figures 2–4) can thus be attributed to the BER-induced single-strand breaks rather than to 8-oxo-G. However, it should be also kept in mind that the rates of excision and repair could differ between the DNA strands, potentially resulting in different effects on transcription.

The role of BER intermediates in the inhibition of transcription is further supported by the importance of CSB, which is known to improve transcription in the presence of single-strand breaks (31) and, by analogy with its yeast homologue Rad26, might accelerate the repair of abasic sites situated in the transcribed DNA strand (39). Interestingly, we found that the effects of uracil are in several aspects different from those of 8-oxo-G. Thus, no pronounced strand bias was detected in the case of uracil and no effect was detected in the *Csb*^{+/+} MEFs (Figure 5). Generation of structurally distinct DNA repair intermediates in result of base incision by the monofunctional (UNG2) and bifunctional (OGG1) DNA glycosylases could plausibly account for the observed differences (left versus right pathways in Figure 7).

In summary, we present a model in which the excision of 8-oxo-G by OGG1 leads to the generation of a single-strand break, which on turn blocks transcription by RNA polymerase II (Figure 7). Moreover, we propose that a BER-dependent inhibition of transcription is a common feature of various types of DNA base damage and that the transcription-blocking potential of the individual DNA lesions can depend on the BER sub-pathway. As an increasing fraction of DNA

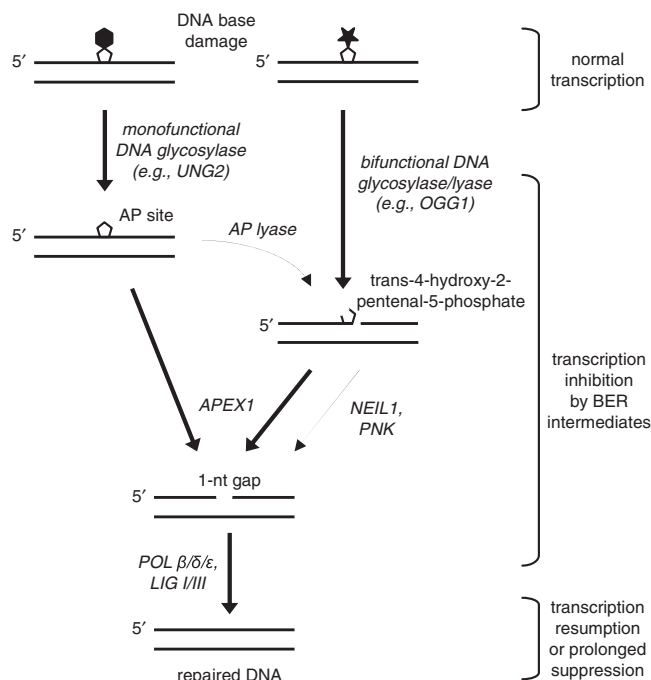


Figure 7. A model explaining inhibition of transcription by DNA base modifications, which do not directly block transcriptional elongation. Base excision repair generates a series of intermediate structures, each of which has a transcription blocking potential. The completion of DNA repair should allow full resumption of transcription. However, in a fraction of the template DNA transcription does not recover easily, remaining suppressed for long periods of time.

modifications is recognized and processed by BER in the course of time, such model explains the observed gradual decrease in transcription of damaged DNA (Figure 3). It is harder to rationalize the observation that transcription remains suppressed for many hours, because ongoing repair process should rapidly restore the DNA integrity. Indeed, quantitative analyses of DNA recovered after 24 h (Figure 2D) did not provide any indication of persistent single-strand breaks. It appears, therefore, necessary that BER intermediates lead to a prolonged suppression of transcription via a signalling mechanism, which could be initiated either by some components of the stalled (or otherwise failed) transcription or by other damage sensor proteins.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online.

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