

Transcriptional Mutagenesis and Its Potential Roles in the Etiology of Cancer and Bacterial Antibiotic Resistance

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Most cells do not undergo continuous cell division and DNA replication, yet they can still acquire novel RNA mutations that can result in the production of mutant proteins and induce a phenotypic change. All cells are frequently subjected to genotoxic insults that give rise to damaged nucleotides which, similarly to DNA replication, can undergo base mispairing during transcription. This mutagenic lesion bypass by RNA polymerase, transcriptional mutagenesis (TM), has been studied in a variety of systems and organisms, and may be involved in diverse pathogenic processes, such as tumorigenesis and the acquisition of bacterial antibiotic resistance. Tumor cells and bacteria within the human body are subject to especially high levels of oxidative stress, which can damage DNA and consequently drive TM. Mutagenesis at the level of transcription may allow cells to escape growth arrest and undergo replication that could permanently establish mutations in DNA in a process called retromutagenesis (RM). Here, we review the broad range of DNA damages which may result in TM including a variety of non-bulky lesions and some bulky lesions, which recent studies indicate may not completely block transcription, and emerging evidence supporting the RM concept in the context of tumorigenesis and antibiotic resistance.

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DNA damage from endogenous sources, such as reactive oxygen species (ROS) produced by mitochondria during respiration, and environmental sources, including ultraviolet (UV) light, is ubiquitous, and poses a constant threat to genomic integrity. All cells, regardless of their proliferation state, are constantly exposed to DNA damage. DNA base damages can often have miscoding properties and be mutagenically bypassed by DNA polymerase. The well-established mutagenic potential of DNA damage during DNA replication has been linked to genomic instability and a variety of diseases, including cancer. However, largely quiescent cells may not be protected from the potentially deleterious consequences of DNA damage, as many of these lesions can also be bypassed by RNA polymerase (RNAP), giving rise to mutant RNA molecules. This process, termed transcriptional mutagenesis (TM), can alter the phenotype of non-proliferating cells (Viswanathan et al., 1999b; Bregeon et al., 2003; Saxowsky et al., 2008). Since analogous bases are often incorporated opposite the lesion by both DNA and RNA polymerases, if a phenotype conferred by the transcriptional mutation is cell cycle entry, one of the resulting daughter cells could acquire a permanent DNA mutation, and thus permanent establishment of the phenotype. This mechanism, which can potentially allow quiescent cells to enter the cell cycle and acquire novel mutations, has been termed retromutagenesis (RM) (Doetsch, 2002).

Two highly conserved pathways, base excision repair (BER) and nucleotide excision repair (NER), repair base damage in humans, bacteria, and yeast, as well as other organisms. BER primarily repairs non-bulky, non-helix-distorting lesions, which are generally bypassed by RNAP and may result varying levels of TM. Bulky lesions present in non-transcribed DNA are repaired by global genome repair (GGR) sub-pathway of NER, while RNAP stalled at lesions occurring in DNA undergoing

transcription serves as the first point of recognition for the transcription-coupled repair (TCR) sub-pathway. BER and NER pathway deficiencies have been associated with an increased susceptibility to cancer, neurodegenerative disease, as well as developmental defects (Cleaver et al., 2009; Fu et al., 2012), and the consequences of the interactions of unrepaired DNA damage with DNA and RNA polymerases may influence these outcomes. TM and RM may have a deleterious impact on human health by contributing to the etiology of such diseases, as well as giving rise to antibiotic-resistant pathogenic bacteria.

Transcriptional Mutagenesis Induced by Non-Bulky Base Damage

BER is initiated by lesion-specific glycosylases, which recognize and excise damaged bases, leaving an apurinic/apyrimidinic (AP)

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site, which is then further processed and replaced by an undamaged base. Such lesions arise from four primary sources of damage: alkylating agents, ROS, reactive nitrogen species, and deaminating agents (Friedberg et al., 2006). RNAP bypass of a plethora of such non-bulky lesions, as well as the BER pathway intermediates AP sites and single-strand breaks, which can also arise spontaneously, give rise to TM (Bregeon and Doetsch, 2011). Non-bulky lesions allow bypass by both RNA and DNA polymerases, and thus mediate potential transcriptional and replicative mutagenesis, respectively. TM may be important in a variety of contexts and organisms. For example, adenine is misincorporated opposite an 8-oxoguanine (8-oxoG) lesion by mammalian RNAPII, as well as yeast, bacterial, and mitochondrial RNAP (Nakanishi et al., 2013).

In the presence of oxidative DNA damage, BER deficiency can dramatically increase the levels of TM, as was found in mouse embryonic fibroblasts (MEFs) for 8-oxoG in an 8-oxoguanine glycosylase-null (*Ogg1*^{-/-}) background (Saxowsky et al., 2008). Within BER-deficient backgrounds, a TM-causative lesion may persist long enough to be mutagenically bypassed by DNA polymerase, and thus permanently establish mutations in a cell's genome through RM (Viswanathan et al., 1999a). However, although 8-oxoG can be bypassed by RNAP before repair, bypass is obstructed following interaction with *Ogg1* (Kitsera et al., 2011).

Encounters of RNAP With Bulky DNA Lesions

Unlike BER substrates, bulky lesions distorting the double helix frequently stall RNAP, which serves as an impediment to replication and transcription, and may induce apoptosis, unless repaired by TCR. However, emerging evidence indicates that the mechanisms by which RNAP stalls differ depending on the lesion and, for some damages, transcription may not be entirely prevented (Walmacq et al., 2012; You et al., 2012a,b). When the block of transcription is not complete, and a fraction of RNAPs are able to bypass the damage, TM may occur (Fig. 1).

Using a novel assay allowing the quantitative estimation of TM and lesion bypass efficiency, relative to undamaged sequence, it was recently demonstrated that this is the case for some bulky oxidative damages and thiopurine drug-induced lesions (You et al., 2012a,b). This Competitive Transcription and Adduct Bypass assay employs non-replicating vectors containing site-specific base modifications premixed with lesion-free competitor plasmids, from which run-off transcripts are reverse-transcribed, restriction-digested, and analyzed by PAGE and LC-MS/MS (You et al., 2012a). By employing the assay and HeLa nuclear extracts or transfection into human cells, it was determined that 8,5'-cyclo-2'-deoxyadenosine (cdA) and 8,5'-cyclo-2'-deoxyguanosine (cdG) prevent transcription by human RNAPII *in vitro* and strongly inhibit transcription in human cells. However, the limited bypass of the lesions resulted in TM (You et al., 2012a). Moreover, a major lesion induced by thiopurine drugs, widely used for the treatment of cancers such as leukemia and other conditions, S6-methylthioguanine, also strongly inhibits transcription, but is also transcriptionally mutagenic (You et al., 2012b). It has been therefore proposed that thiopurine drug-induced DNA damage may not only contribute to the cytotoxic effects of these agents, likely due to its transcription-blocking effects, but may also play a role in the pathogenesis of thiopurine therapy-associated cancers (You et al., 2012b).

While the structure–outcome relationship by which the above lesions inhibit transcription and result in TM are not yet known, mechanistic insight has been gained from crystallographic studies of *Saccharomyces cerevisiae* RNAP in complex with two other NER substrate lesions, cyclobutane pyrimidine dimers (CPDs) (Brueckner et al., 2007; Walmacq et al., 2012) and cisplatin lesions (Damsma et al., 2007), as well

as the BER substrate 8-oxoG (Damsma and Cramer, 2009). CPDs, which are covalently linked adjacent pyrimidines and are the most common DNA damages induced by UV light, can enter the RNAP active site but translocation is disfavored following lesion-templated misincorporation of uridine (Brueckner et al., 2007). Nevertheless, with the CPD outside the active site, a non-templated adenine can be inserted at the first thymine of a CPD lesion, allowing the entry of the lesion into the active site and non-mutagenic elongation past the lesion (Walmacq et al., 2012). In contrast, bypass has not been observed for the 1,2-d(GpG) intrastrand crosslinks induced by the chemotherapeutic drug cisplatin, which do not enter the RNAP active site and block translocation (Damsma et al., 2007). Also, similar to the encounter of RNAP or DNA polymerase with an abasic site or strand break (Clauson et al., 2010), a non-templated adenine is misincorporated by RNAP stalled at a cisplatin lesion, suggesting that the “A-rule” observed for DNA polymerases when they incorporate adenine as they replicate through a non-informative site—may also apply to transcribing RNAP (Damsma et al., 2007).

Bacterial Transcriptional Mutagenesis and Antibiotic Resistance

The first models of TM were established *in vitro*. Experiments with plasmid vectors containing site-specific abasic sites showed efficient bypass by *E. coli* RNA polymerases, giving rise to transcripts containing an adenine at the site corresponding to the template DNA abasic site (Zhou and Doetsch, 1993). Bacteriophage polymerases were also used to illustrate TM *in vitro* when bypassing dihydrouracil lesions (Liu et al., 1995), as well as uracil, O6-methylguanine, and 8-oxoguanine, each of which promoted a distinct range of RNA misinsertions (Viswanathan and Doetsch, 1998). Finally, the mutagenic specificity of lesions was compared between RNA and DNA polymerase bypass, and found to be the same for dihydrouracil (Liu and Doetsch, 1998). Analogous RNAP and DNA polymerase misinsertion at the site of a lesion is essential to the model of RM, as it would allow permanent establishment of the original transcriptional mutation.

In vivo models were later established for the study of TM. Uracil was cloned into a stop codon engineered into the firefly luciferase gene, allowing TM at this locus to permit the synthesis of a full-length luciferase transcript. When this construct was transformed into non-dividing cells, luciferase activity was used to measure TM arising from the transgene and found to be dependent on DNA base damage and uracil repair background (Viswanathan et al., 1999b). This experimental system was used to measure TM arising from uracil, as well as from 8-oxoguanine, which was found to be increased in a TCR-deficient background (Bregeon et al., 2003). The same system was used to study TM arising from abasic sites and strand breaks, which, *in vivo*, gave rise to TM (Clauson et al., 2010). One advancement to this system is the introduction of site-specific lesions into the *E. coli* chromosome, which could improve the physiological relevance of the bypass of such lesions (Pages et al., 2012). Interestingly, a recent study has illustrated an increased frequency of *lac* operon expression switching in cells with decreased RNAP fidelity, causing *lac* to undergo derepression without undergoing reversion mutations, yet also causing heritable changes in *lac* expression (Gordon et al., 2009). This reinforces the notion that mutant transcripts can confer heritable phenotypes.

TM is a mechanism by which bacteria may escape growth restrictions imposed by their environment. TM is particularly important in conditions where bacteria divide at lower rates *in vivo*; for example, *E. coli* divides four to six times slower in the gut than it does in culture (De Paepe et al., 2011). In addition, antibiotic treatment, to which bacteria in a physiological setting

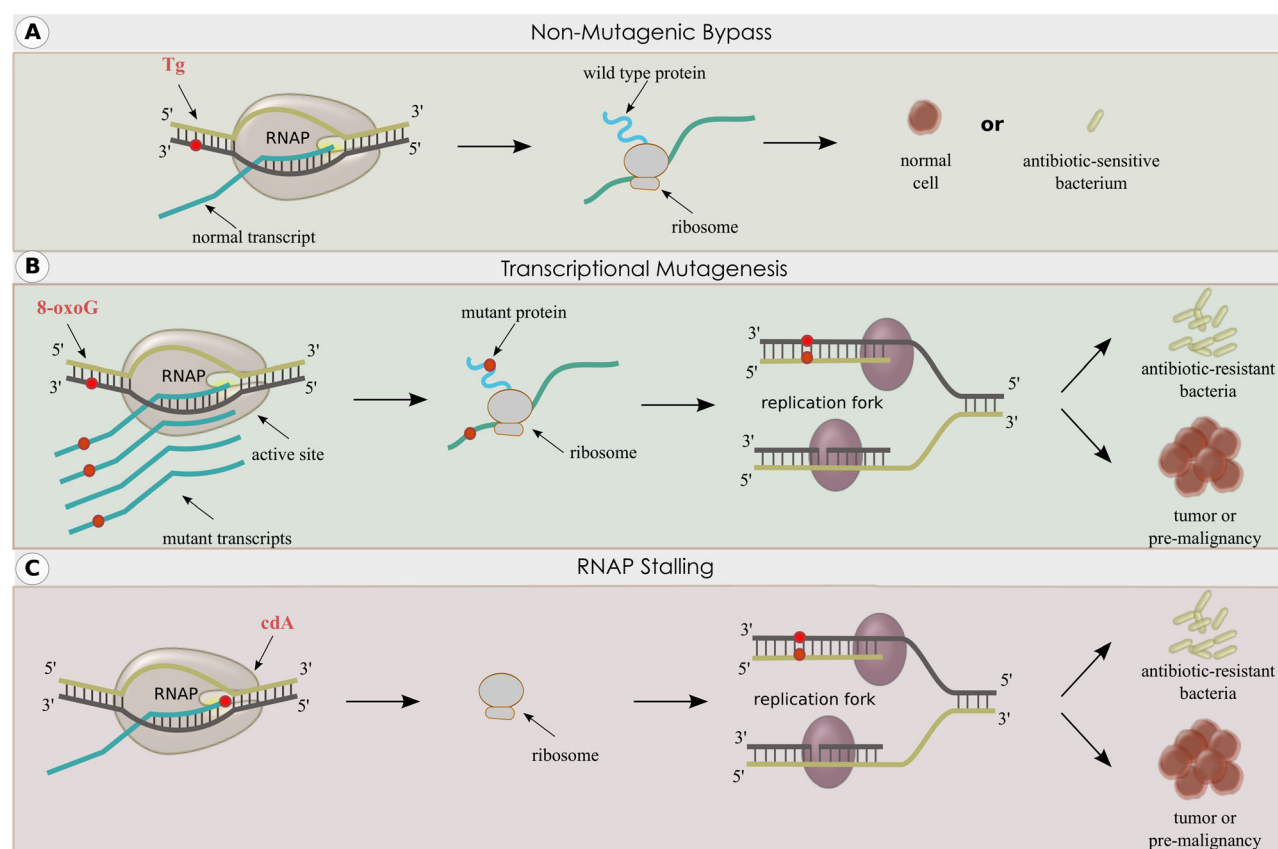


Fig. 1. Potential phenotypic consequences of DNA damage during transcription. A: Some BER substrates, such as thymine glycol (Tg) (Charlet-Berguerand et al., 2006), can be bypassed with only low levels of TM, unlikely to induce a phenotypic change. **B:** Some non-bulky BER substrates, such as 8-oxoG, can be bypassed and result in significant levels of TM. If the TM event drives a transcriptional gain-of-function mutation and aberrant activation of a proliferation-promoting oncogene, it may initiate tumorigenesis. In the case of bacteria, if the transcriptional mutation is such that it confers antibiotic resistance, it may allow the cells to evade cell death or cytostasis and give rise to a population of antibiotic-resistant bacteria. **C:** Bulky lesions that partially stall RNAP but are mutagenic may result in reduced expression of a tumor suppressor and low levels of mutant transcripts, and may allow cells to evade growth-suppressive conditions or apoptosis. Bypass of such lesions in bacteria may allow cells to acquire mutations of antibiotic target proteins, which allow them to survive antibiotic selection.

are often subjected, is typically cytostatic, and can even impair the fidelity of translation (Brakier-Gingras and Phoenix, 1984). Under such non-dividing conditions, point mutations arise that allow phenotypic reversion over the course of several days, indicating that such mutations were not present before cells were plated on selective media, and the mechanisms by which these mutations arise could include RM (Quinones-Soto and Roth, 2011). The oxidative DNA lesions from which TM arises are common among bacteria, which are often subjected to oxidative stress that may alter their phenotype. Bacteria in infection microenvironments undergo oxidative stress (Farr and Kogoma, 1991), which can give rise to lesions known to contribute to stationary-phase mutagenesis (Vidales et al., 2009). Strains with greater endogenous oxidative stress undergo more frequent adaptive mutations, possibly through TM (Aubron et al., 2012). Oxidative damage is known to facilitate *lacZ* reversion because mutating *MutT*, which sanitizes oxidative damage from dNTP pools, allows increased *lacZ* missense reversion under aerobic conditions (Taddei et al., 1997). Antibiotics induce oxidative stress, which has been correlated with but may not be necessary for their bactericidal effects (Keren et al., 2013), yet the ROS induced by sub-lethal concentrations of antibiotics can induce mutations causing antibiotic resistance (Kohanski et al., 2010). TM may allow cells to acquire antibiotic resistance through the expression of a

mutated target protein that allows the cell to survive antibiotic selection, and the daughter cells may acquire the same mutation through RM.

Phenotypic Consequences in Mammalian Cells and Tumor Development

Cancer is a disease of cellular proliferation, and perhaps some of the most notorious hallmarks of cancer include aberrant proliferative signaling and evasion of signals, that suppress growth (Hanahan and Weinberg, 2011). Yet multiple tumor types can originate from largely quiescent cells, such as adult stem cells, following the introduction of a limited number of defined genetic alterations, including single-base substitutions. For example, the G12D mutation of K-Ras leads to bronchioalveolar stem cell expansion and lung tumorigenesis (Kim et al., 2005), and the combined inactivation of p53 and Rb in stem cells of the ovarian surface epithelium results in ovarian adenocarcinoma in mice (Flesken-Nikitin et al., 2013). Since it may allow quiescent cells to acquire new oncogenic mutations, TM may serve as one possible route to tumor initiation for cancers with stem cell origins, following RM, but it may also play a role in cancers that originate from highly proliferative cells, such as progenitors. If a TM event initiates aberrant differentiation and proliferation of stem cells and the

acquisition of one out of a set of mutations required for tumorigenesis, it might initiate the acquisition of one trait required for a multistep tumorigenesis process, or a pre-malignancy. Such aberrantly proliferating cells may be rendered more susceptible to acquire subsequent mutations and more likely to become cancerous. In addition, loss-of-function mutations in tumor suppressors that can negatively regulate proliferation, such as p53 in the event of extensive DNA damage, may allow proliferative cells to evade harsh conditions that could otherwise lead to growth arrest, apoptosis, or necrosis.

Most human cancers possess somatic mutations with pro-mitogenic effects, such as loss-of-function mutations in negative regulators of proliferation, including p53, or gain-of-function mutations in oncogenes, such as the Ras family of GTPases, B-raf, or PI3-kinases, that can lead to the constitutive activation of pro-mitogenic signaling. The mutational spectra can vary between tumor types, likely due to exposure to different types of DNA damage or defects in repair (Greenman et al., 2007). Many of these mutations are consistent with those occurring due to lesions known to induce TM, supporting the hypothesis that TM is one possible route to tumor development. Importantly, more direct evidence supporting this idea came from a study using a MEF system and a non-replicating construct containing a site-specifically inserted 8-oxoG lesion in the transcribed strand of H-Ras, designed such that mutagenic bypass of the lesion would result in the production of the constitutively active Q61K mutant form of H-Ras (Saxowsky et al., 2008). Bypass of the lesion resulted in approximately 14% of the transcripts being mutant, which was sufficient to produce a measurable activation of the H-Ras downstream effector ERK, thus demonstrating the ability of TM to initiate oncogenic signaling.

Conclusions

While TM can drive Ras downstream oncogenic signaling, it remains unclear whether these molecular changes are sufficient to serve as the first step of the tumor-initiating process. It is likely that non-bulky BER substrate lesions which are efficiently bypassed by RNAP and are highly transcriptionally mutagenic play a more significant role in inducing such phenotypic changes. They may play important roles during tumor initiation or progression when they trigger the aberrant activation of a pro-proliferative oncogene or when they result in the loss-of-function mutation of a tumor suppressor. In contrast, NER substrate lesions that inhibit transcription but are mutagenic when RNA and DNA polymerase bypass occurs may be more significant in the etiology of cancers when they result in the loss-of-function mutation of a tumor suppressor, since the total transcript levels following bypass by RNAP may or may not be sufficient to drive a phenotypic change that depends on a gain-of-function mutation, and some could contribute to the cytotoxic effects of DNA damaging chemotherapeutics, such as thiopurine drugs (You et al., 2012b). However, gain-of-function mutations may be more relevant to the RM concept, as heterozygous tumor suppressor loss may not be sufficient to drive phenotypic change. While loss of heterozygosity (LOH) by point mutation of the wild type allele cannot be excluded, that is commonly not the case. For example, tumor development in p53^{+/-} mouse models of the cancer predisposition Li-Fraumeni syndrome is associated with LOH by large scale genetic aberrations, such as chromosomal nondisjunction (Jacks et al., 1994), which is not expected to arise by a TM mechanism.

Bacteria also remain an important crucible for the phenotypic consequences of TM. Although oxidative stress contributes to antibiotic resistance and adaptive mutagenesis, it remains to be seen whether oxidative base damage can drive

antibiotic resistance through RM. A major technical challenge to the study of the RM concept in physiologically relevant contexts is the development of technologies for the study of TM initiated in situ, due to DNA damage occurring in genomic DNA. A single bacterium acquiring a transcriptional mutation conferring antibiotic resistance may give rise to a population of resistant bacteria. However, studying TM before RM has occurred and given rise to a population of antibiotic resistant DNA mutants requires identifying such rare transcriptional mutant cells among a large population of bacteria that are not transcriptional mutants. The development of reliable technologies for the identification of DNA damage lesions and single-cell transcriptomics may facilitate the study of such rare transcriptional mutants within large, highly heterogeneous populations of cells. Future high-throughput studies may also help address which lesions are most transcriptionally mutagenic and thus most likely to contribute to important phenotypes such as antibiotic resistance and tumorigenesis through TM and RM.

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