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Review

Transcription arrest at DNA damage sites

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Abstract

Transcription arrest by RNA polymerase II at a DNA damage site on the transcribed strand is considered an essential step in initiation of transcription-coupled repair (TCR), a specialized repair pathway, which specifically removes lesions from transcribed strands of expressed genes. To understand how initiation of TCR occurs, it is necessary to characterize the properties of the transcription complex when it encounters a lesion in its path. The analysis of different types of arrested complexes should help us understand how an arrested RNA polymerase may signal the repair proteins to initiate a repair event. This article will review the recent literature describing how the presence of DNA damage along the DNA affects transcription elongation by RNA polymerase II and its implications for the initial steps of TCR.

Keywords: RNA polymerase; Transcription elongation; Transcription arrest; Transcription-coupled repair; DNA damage

Contents

Abbreviations: T7 RNAP, T7 RNA polymerase; RNAPII, RNA polymerase II; GTFs, general transcription initiation factors; CPD, cyclobutane pyrimidine dimer; (6-4)PP, (6-4) photoproduct; cisplatin, diamminedichloroplatinum(II); *cis*-1,2-d(GG), cis-[Pt-$(NH_3)_2$\{d(GpG)-N7(1),N7(2)\}]; *cis*-1,3-d(GTG), *cis*-[Pt-$(NH_3)_2$\{d(GpTpG)-N7(1),N7(3)\}]; Tg, thymine glycol; UV, ultraviolet light; AF, N-2 aminofluorene; AAF, N-2-acetylaminofluorene; M_1dG, pyrimido purin-10(3*H*)-one; N2-OpdG, N2-(3-oxo-1-propeniyl)-dG; PdG, N2-propanodeoxiguanine; TCR, transcription-coupled repair; CS, Cockayne syndrome; CHO, Chinese hamster ovary cells; Dhfr, dihydrofolate reductase

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Prologue

I have had the privilege during the past 10 years of knowing Phil Hanawalt, not only through his remarkable scientific contributions but also in the daily life at the lab at Stanford University. Phil's success not only reflects his intellectual ability but also his graciousness in personal relationships. His example has always been an inspiration and encouragement to everybody who has had the opportunity to work with him. His many gifts to science include not only his personal achievements but also the enthusiasm that he has so brilliantly been able to infuse in his many students, and for which I will always be grateful.

1. Introduction

The elongation phase of transcription is a dynamic process that is affected by the sequences transcribed. To add to the complexity of this process, messenger RNA processing occurs cotranscriptionally and involves the addition of a 5′-cap, the excision of intronic sequences by splicing factors and the addition of a 3′-poly(A) tail [1,2]. The elongation complex must also proceed through chromatin and specific DNA sequences that impede elongation. Impediments to transcription elongation include transcriptional pause, arrest and termination [3,4]. Some lesions in the transcribed strands may pause the elongation process while others may represent absolute blocks to translocation of the RNA polymerase. In the latter case, there may be a subset of lesions that actually cause the polymerase and nascent RNA product to be released from the DNA, while for other lesions, the ternary complex may be very stable and persist in the arrested state for a long time. A mammalian RNA polymerase II (RNAPII) arrested at an ultraviolet (UV)-induced cyclobutane pyrimidine dimer (CPD) in the transcribed DNA strand has been reported to have a half-life of about 20 h [5] and to prevent the repair of that lesion by photolyase [6]. Not surprisingly, mechanisms have evolved to displace the polymerase in order to facilitate repair of the lesion.

2. Transcriptional pausing and arrest

When RNAPII stops adding nucleotides to the nascent RNA for some time before resuming elongation, transcriptional pausing occurs (Fig. 1). Factors that stimulate transcription elongation include TFIIF, the ELL family, Elongins, FCP1, CSB and DSIF [7]. Transcriptional pause can evolve into arrest depending on the time the polymerase spends at the pause site [8]. Transcriptional arrest is defined as a permanent halt to RNA synthesis, where RNAPII cannot resume elongation without accessory factors. Arrest is believed to result from polymerase backtracking relative to the DNA template, causing misalignment of the catalytic active site and 3′-OH of the nascent transcript. Transcription can resume by an evolutionarily conserved mechanism that requires cleavage of the RNA transcript in a 3′- to 5′-direction, allowing proper realignment of the polymerase active site and the RNA 3′-OH, promoting readthrough of the arrest site. The elongation factor TFIIS is the eukaryotic factor that promotes RNAPII readthrough at transcription arrest sites, such as unusual DNA sequences, DNA-bound proteins and small DNA-bound drugs [9–12]. However, TFIIS alone

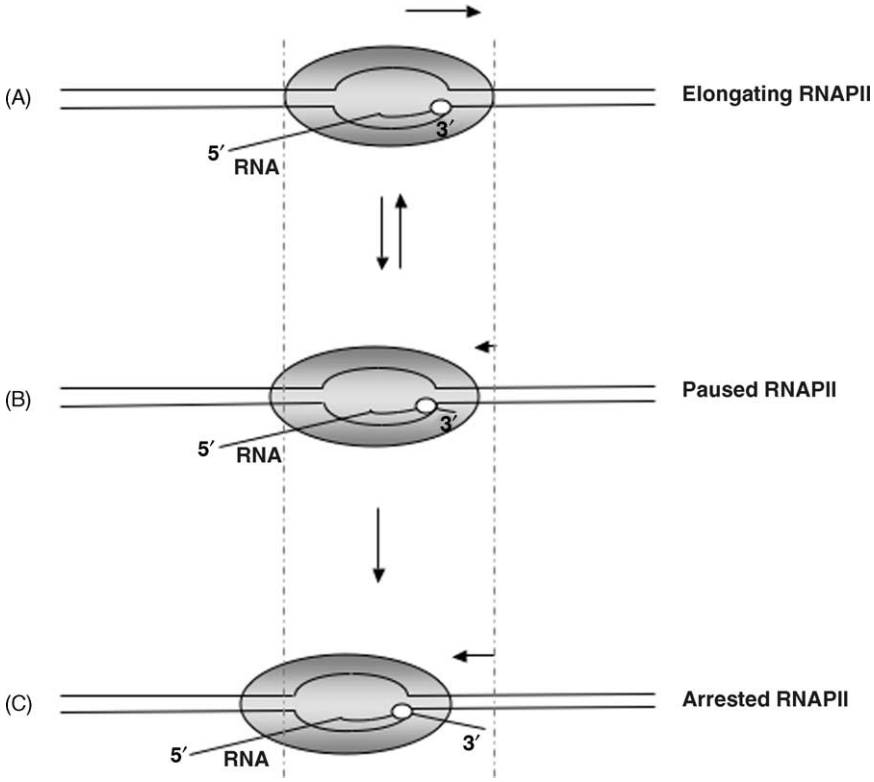

Fig. 1. Multiple conformational states of RNAPII during transcription elongation. (A) Elongating RNAPII, where the 3′-end of the nascent transcript is aligned with the catalytic site of the polymerase; (B) paused RNAPII, characterized by reversible backtracking for a few nucleotides, RNAPII can decay into an arrested state in a time-dependent fashion; (C) arrested RNAPII, characterized by longer backtracking, that results in misalignment of the 3′-OH of the nascent RNA from the polymerase catalytic site. Transcription can resume only after transcript-cleavage mediated by elongation factor TFIIS.

is unable to facilitate readthrough in vitro of complete blocks to elongation such as CPDs [5,6].

3. Transcription-coupled repair

Nucleotide excision repair is a repair pathway that recognizes and removes several structurally different lesions from DNA [13–15]. There are two general pathways for nucleotide excision repair, a global genomic repair pathway that recognizes and repairs lesions throughout the genome and a pathway termed transcription-coupled repair (TCR), that operates on lesions in the transcribed strands of expressed genes [16]. It has been proposed that TCR has three functions: (1) removal of the stalled RNA polymerase to enable repair to occur [17]; (2) recruitment of repair proteins to sites of lesions that block transcription, usually allowing them to be repaired more rapidly; (3) removal of a strong signal for apoptosis [18]. Several lines of evidence indicate that initiation of TCR requires that RNAPII is in an elongation mode. For example, TCR of the *lac operon* of *Escherichia coli* can only be observed when the operon is induced [19]. In eukaryotes, TCR operates on RNAPII transcribed genes and the polymerase must be in the actively elongating mode to initiate TCR. Treatment of mammalian cells with α-amanitin, a specific inhibitor of RNAPII, abolishes the preferential repair of CPDs in expressed genes [20–22]. In yeast with temperature sensitive mutations in the gene encoding a subunit of RNAPII, a loss of TCR is observed at the non-permissive temperature [23,24]. Mammalian ribosomal genes, transcribed by RNA polymerase I, are not preferentially repaired, al-

though more recent studies suggest that in yeast, there is TCR of ribosomal genes [25]. TCR does not vary in efficiency through the normal cell cycle in mammalian cells for a gene that is expressed continuously throughout the cycle [26]. TCR does not appear to be inducible by UV. However, in human cells, efficient global genomic repair of CPDs requires activation of the p53 tumor suppressor [27]. Furthermore, growing evidence indicates that arrested transcription (e.g. at CPDs) provides a sensitive signal for the activation and stabilization of p53 [18,28,29].

The classic and still current TCR model proposes that RNAPII arrest at a lesion is a prerequisite for initiation of TCR. This model assumes that the polymerase must be removed from the damaged site to provide access for the repair complex to the lesion [17]. In *E. coli*, the *mfd* gene product participates in this process [30]. The Mfd protein can promote the release of the RNA polymerase and the incomplete transcript from the DNA template and then can target components of NER to the site of transcription blockage [30,31]. In human cells, the *CSB* gene product is implicated in this process. However, it remains unclear whether the polymerase is translocated away from the site of damage without dissociating from the template DNA or released from the DNA [32,33], as suggested by evidence that in human fibroblasts, RNAPII becomes ubiquitinated after UV irradiation or cisplatin treatment [34,35], followed by proteasomal degradation of the large subunit [34,36].

4. Methods utilized to study in vitro transcription at lesions

4.1. Tailed templates

Duplex DNA molecules with a single strand deoxycitidine protruding from their 3′-end are efficient templates for RNAPII (Fig. 2A). Such tailed duplexes can be synthesized enzymatically using terminal transferase and dCTP [37], followed by restriction digestion to remove one end containing one tailed 3′-terminus. Tailed templates can also be formed by ligating a (dC) oligonucleotide into a linear DNA duplex containing a 5′-overhanging end. Such a tailed template may be viewed by RNAPII as half of an unwound transcription bubble. In fact, RNAPII starts transcribing a short RNA complementary to a few bases within the single-stranded region, about three bases from the junction with duplex DNA and proceeds into the double stranded region [38]. An advantage of using tailed templates to form elongation complexes is that they circumvent the requirement for additional general transcription initiation factors (GTFs) necessary to initiate transcription at a promoter. In addition, they are used very efficiently by RNAPII, in contrast with the low template occupancy (a few percent of the template) in most promoter-dependent reactions reconstituted with GTFs or nuclear extracts. However, an unusual feature of tailed templates is the propensity for the non-template strand to be displaced during RNA synthesis such that the transcript remains base paired with the template strand and a long RNA:DNA hybrid is produced [39]. In addition, the sizes of the full length RNA are very similar to the template DNA because transcription initiates at the end of the template. This can lead to confusion in identifying the reaction products since RNAPII has the ability to label the non-template strand by adding a radioactive nucleotide to the 3′-end [40]. Therefore, depending on the question asked, this method may not be appropriate.

4.2. Promoter-dependent transcription by RNA polymerase II

With the resolution and purification of the general initiation factors basal transcription by RNAPII can be reconstituted with purified proteins. RNAPII specifically binds to several promoters and initiates transcription in the presence of GTFs. Either purified systems or HeLa extracts have been successfully used to study the effect of lesions on RNAPII transcription (Table 1). One advantage of using HeLa extracts over purified systems is that it can be assumed that any activity required for transcription should be present in the extract. A drawback, however, is that some repair proteins present in the extract are active under transcription conditions and can remove the lesion from the transcription substrate, making the analysis of the transcription results more complicated [41]. To study the behavior of RNAPII at DNA lesion sites, we have developed an in vitro transcription system consisting of substrates containing a single lesion located more than 150 bp downstream of the Adenovirus major late promoter, to make sure that the RNAPII elongation

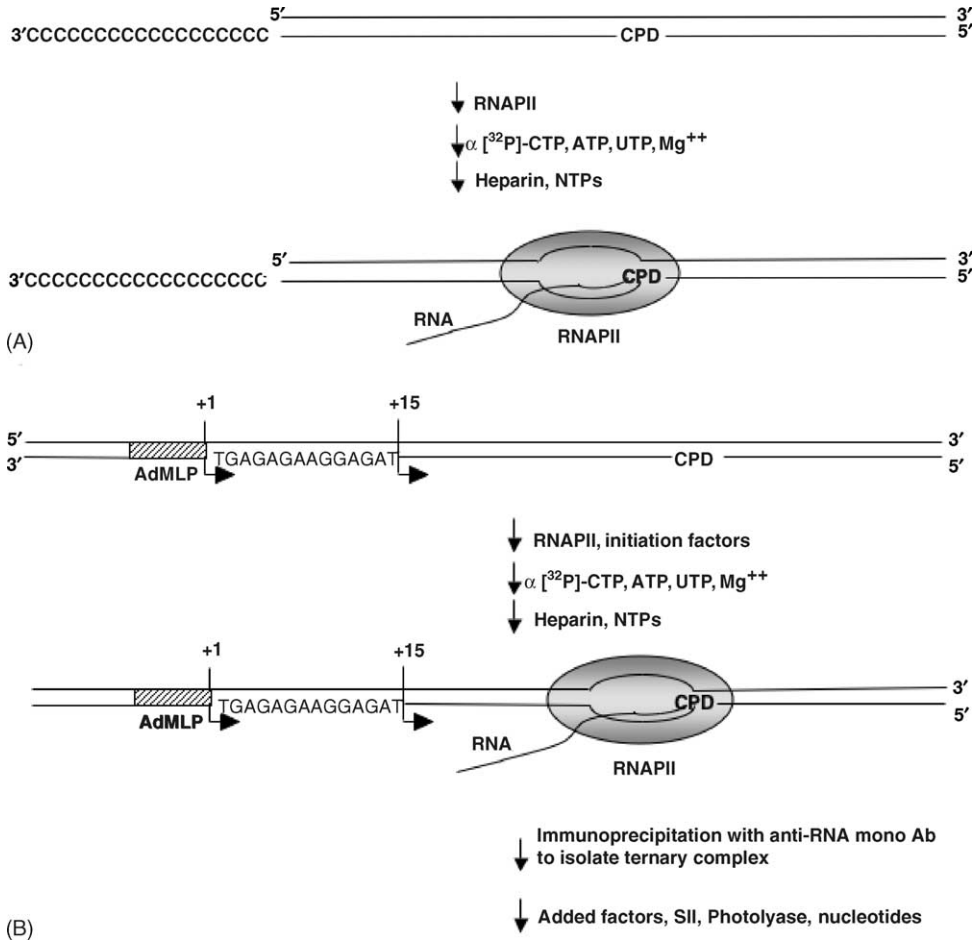

Fig. 2. Methods used to study in vitro transcription of templates containing specifically located DNA lesions. (A) dC-tailed template: RNAPII initiates transcribing about three bases from the junction between the single-stranded dC tail and the double-stranded DNA, without requirement of GTFs. (B) Promoter-dependent transcription: RNAPII transcribes from a promoter, after assembly of the initiation complex in the presence of GTFs.

complexes have completed any transitions out of the initiating state before encountering the lesion (Fig. 2B) [42,43]. RNAPII and initiation factors are purified from rat liver or recombinant sources [44,45]. Following assembly of the initiation complex, RNAPII is stalled by omission of a required nucleotide at position 15, after synthesis of a short RNA labeled with [α-^{32}P]CTP and addition of heparin to prevent further binding of the polymerase to the promoter. This allows each template to be transcribed only once by a single molecule of RNAP so that each transcription product represents a single promoter-dependent elongation event [46]. All four NTPs are then added to allow elongation to continue and further incorporation of ^{32}P is avoided by adding excess cold ribonucleotides. As a result, transcripts become labeled to the same specific activity regardless of their length or base composition. In addition, because no repair proteins are present in this reconstituted system, synthesis of full length RNA products represents bypass of the damaged base rather then removal of the adduct. In order to study the protein interactions and modifications of RNAPII complexes arrested at a lesion, we have generated homogeneous populations of arrested transcription complexes by immunoprecipitation with D44 anti-RNA antibody and formalin-fixed *S. aureus* cells [47]. This step is critical

Table 1
DNA lesions, transcription arrest in vitro and TCR in vivo

DNA lesion	Arrest of transcription	RNA polymerase	TCR	References
N-Ethylpurines	ND		+	[102]
7 Me-guanine	–	T7	–	[103]
3 Me-adenine	+	T7	–	[103]
8-OxoG	±	T7, *E. coli*, rat liver, human	±	[41,46,74,75,77,83,86,89,96]
CPD	+	T7, *E. coli*, rat liver, human	+	[5,6,30,49,104]
(6-4)PP	+	Human	+	[51,55]
Tg	+	T7		[83]
	–	Rat liver, human	±	[41,105,106]
cis-1,2-d(GG)	+	T3, T7, *E. coli*, wheat germ	+	[86]
	+	Rat liver		[60,61,63]
	–	Human		[58,62]
cis-1,3-d(GTG)	+	T3, T7, *E. coli*, rat liver	+	[60,61,63,86]
	+	Human		[58,62,89]
Malondialdehyde	+	T7, rat liver	ND	[101]
Aflatoxin B1	ND		+	[22]
CC-1065	ND		+	[107]
Psoralen monoadduct	+	T7, human	–	[70,108,109]
Psoralen interstrand crosslink	+	T7, *E. coli*, human	+	[70,108–110]
BPDE (human cells)	+[a]	T7, human	+	[72,73,111,112]
BPDE (rodent cells)			–	[113]
AAF	+	T7, rat liver	±	[71,86]
AF	+	T7, rat liver	–	[71,86,114]
Single-strand breaks	–	T7	–	[86,115]
	+	SP6, *E. coli*		[116]
	+	Human		[41,78]
Abasic sites	+	T7	ND	[86]
	–	SP6, *E. coli*		[116]
Gaps	–	T7, SP6, *E. coli*		[115,117,118]

ND, not determined.
[a] The extent of transcription arrest is dependent on the BPDE diastereomer (reviewed in [73]).

to eliminate a large background (95–99%) represented by DNA and protein molecules that do not participate in the transcription reaction and could interfere with subsequent binding of proteins and interpretation of the results. Using this system, we have analyzed the behavior of RNAPII when it encounters any of several kinds of DNA lesions along its path.

5. RNAPII and bulky adducts

5.1. Cyclobutane pirimidine dimers

Cyclobutane pirimidine dimmers (CPDs) represent the most frequent lesion produced in DNA by ultraviolet light exposure. TCR was first documented in Phil Hanawalt's laboratory in UV irradiated Chinese hamster ovary (CHO) cells, in which it was found that CPDs were more efficiently repaired in an expressed gene than in a silent sequence downstream [48]. Subsequently, it was shown that this efficient repair was due to the preferential repair of CPDs in the transcribed DNA strand [49]. The original TCR model suggests that RNAPII arrest at the site of the lesion is the first step necessary for initiation of TCR. Indeed, when in vitro transcription with mammalian RNAPII and initiation factors was carried out on templates containing a single CPD in the transcribed strand, it was found that a CPD is a complete block to mammalian RNAPII progression [6]. Furthermore, another requirement of this model, stability of the arrested complex, was indicated by the ability to be subject to the transcript cleavage reaction mediated by elongation factor SII [6,47,50]. This result was confirmed by Dnase I footprinting analysis of elongation complexes arrested at a CPD, which revealed that the complex has a half-life of about 20 h in

vitro [5]. In addition, atomic force microscopy imaging has shown that the ternary complex is arrested at a CPD [51]. The similarity in the behavior of RNAPII arrested at a CPD or at natural impediments encountered during normal transcription suggested that a change in RNA polymerase conformation similar to that resulting from naturally occurring arrest sites might also occur at the site of a lesion, and that this might be at least part of the signal required to recruit repair proteins to the damage site. To determine the likely conformation of RNAPII arrested at transcription blocking lesions, we have characterized the structural properties of transcription complexes arrested at a CPD, and we have compared them with those of complexes arrested at a well-characterized arrest site located in the first intron of the human H3.3 gene. Transcription complexes arrested at a CPD were immunoprecipitated with D44 anti-RNA antibodies, to remove most of the DNA template that did not participate in the transcription reaction and to obtain a homogeneous population of ternary complexes arrested at lesions. To map the boundaries of arrested complexes, we used *E. coli* exonuclease III (ExoIII) and T4 DNA polymerase $3' > 5'$ exonuclease (T4 exo) as footprinting agents. We found that the footprint of RNAP II arrested at a CPD covers ∼35 bp and is asymmetrical around the dimer. A similar footprint was observed when RNAPII was arrested at the well-characterized human H3.3 natural arrest site. The footprint did not change after transcript cleavage mediated by the elongation factor SII [47], probably because the distance the polymerase had backtracked was not detectable by the footprinting agent utilized.

The similarity between the footprint at the H3.3 site and at a CPD suggests that arrest at a CPD may occur with a similar mechanism. Recent models of transcription arrest postulate that arrest occurs at template locations at which RNAPII fails to continue translocation, resulting in backward translocation of the polymerase along the DNA template and as a consequence, misalignment of the transcript 3′-end from the catalytic site [3]. The arrested complex remains stably associated with the DNA, maintaining a 7–8 bp RNA–DNA hybrid upstream of the transcript 3′-end [52,53]. The strength of the RNA:DNA hybrid, and in particular the last few base pairs at the 3′-end of the transcript, is a critical feature of the elongation complex in preventing arrest. This is consistent with arrest generally occurring at T-rich sequences in the non-transcribed strand, where the dA:dT hybrid at the leading edge of the transcription bubble is energetically favored over the dA:rU hybrid [54]. Furthermore, arrest sites are often characterized by helix distortions [3]. A stable interaction in the last base pair is necessary to properly orient the transcript 3′-end with the incoming nucleotide to form a phosphodiester linkage with the nascent transcript. If the hybrid is weak, bond formation is delayed, allowing the polymerase time to translocate upstream on the template. Similarly, formation of a CPD causes a small deformation of the double helix consisting of unwinding by ∼15° and bending of at least 7° relative to the B form. The neighboring pyrimidines must rotate from their usual B form DNA alignment with overlapping of the 5, 6 bonds [13]. It is likely that the presence of a CPD in the transcribed strand affects formation of the RNA:DNA hybrid, and this in turn may shift the equilibrium from nucleotide addition toward arrest.

5.2. (6-4) Photoproducts

UV-induced (6-4) photoproducts ((6-4)PP) are also repaired by TCR [55]. The structural changes induced by this lesion in the DNA have suggested that this lesion is a likely block to RNAPII elongation. The recent availability of oligonucleotides containing a uniquely positioned (6-4)PP has revealed that indeed this lesion causes transcription arrest.

To analyze the effect of a single ((6-4)PP) on transcription elongation by RNAPII, an in vitro transcription elongation system using RNAPII and oligo(dC)-tailed templates containing a (6-4)PP at a specific site was employed [51]. It was found that the (6-4)PP was a complete block to RNAPII progression when located in the transcribed strand of template DNA. Sequencing analysis of the stalled transcripts revealed that RNAPII arrest occurred after incorporation of either the correct or the incorrect nucleotide opposite the lesion. Atomic force microscopy imaging of the ternary complex revealed that RNAPII formed a stable ternary complex consisting of RNAPII, the damaged DNA template and the nascent transcript.

5.3. Cisplatin intrastrand crosslinks

Diamminedichloroplatinum(II) (cisplatin) is an antitumor drug that preferentially reacts with purine bases in the DNA in vitro and in vivo, to form the *cis*-[Pt-

$(NH_3)_2$\{d(GpG)-N7(1),N7(2)\}] [*cis*-1,2-d(GG)], with a frequency of 65%, the *cis*-[Pt-$(NH_3)_2$\{d(ApG)-N7(1),N7(2)\}], with a frequency of 25%, the *cis*-[Pt-$(NH_3)_2$\{d(GpTpG)-N7(1),N7(3)\}] [*cis*-1,3–d(GTG)] with a frequency of 5–10%, and a small percentage of interstrand crosslinks and monofunctional adducts [56]. Cisplatin-induced intrastrand crosslinks are repaired by global genomic nucleotide excision repair and by TCR [57–59]. These adducts may impose a more serious problem for an elongating RNA polymerase compared to a CPD, since they cause substantial unwinding and bending of the DNA helix (reviewed in [56]). These lesions have been shown to block transcription by T3, *E. coli* and wheat germ RNAP [60–62].

Using our reconstituted transcription system, we found that a single *cis*-1,2-d(GG) or a single *cis*-1,3-d(GTG) located in the transcribed strand is a strong block to both T7 RNAP and RNAPII [63]. Furthermore, the efficiency of the block at a *cis*-1,2-d(GG) is not affected by the sequence context around the lesion. The arrested RNAPII complex was stable, as indicated by the ability of elongation factor TFIIS to induce transcript cleavage, producing a population of transcripts up to 30 nucleotides shorter than those arrested at the lesion, which could then be re-elongated up to the lesion when the nucleotide triphosphate precursors were added. Interestingly, we also observed partial blockage when a single *cis*-1,3-d(GTG) was located in the non-transcribed strand. A *cis*-1,3-d(GTG) in the transcribed strand also blocks RNAPII transcription in extracts of human cells [62]. In addition, the presence of cisplatin-induced lesions in plasmids transfected into human or hamster cells almost completely inhibits RNAPII transcription of a reporter gene [64].

The bulky nature of cisplatin-induced intrastrand crosslinks and the DNA structural changes induced by their presence in the double helix may be why these adducts cause RNAP arrest. Both lesions unwind the DNA, the *cis*-1,2-d(GG) by 13–25°, the *cis*-1,3-d(GTG) by 19–23° [65]. They also cause the DNA to bend towards the major groove, the *cis*-1,2-d(GG) by 32–78° [66], the *cis*-1,3-d(GTG) adduct by 25–35° [66,67]. In addition, NMR analysis of oligonucleotides containing a single *cis*-1,3-d(GTG) adduct has shown that the overall structure of the DNA is more distorted than that of DNA containing a single *cis*-1,2-d(GG) [68]. This might explain why the *cis*-1,3-d(GTG) adduct also has an effect on transcription when located in the non-transcribed strand, while the *cis*-1,2-d(GG) does not.

Several natural transcription arrest sites are characterized by DNA helix distortions [3]. It is likely that the structural changes induced by the *cis*-1,2-d(GG) and the *cis*-1,3-d(GTG) would affect the formation and/or the stability of the RNA:DNA hybrid, an essential component of the elongation complex [4]. A weak RNA:DNA hybrid has been proposed as a primary determinant of the arrest modality, as it promotes backward translocation of RNAP along the DNA template. This in turn can result in the displacement of the 3′-end of the RNA from the catalytic site, leading to polymerase arrest [69]. Similarly, transcription arrest caused by several bulky lesions including CPDs [6], psoralen intra and interstrand crosslinks [70], adducts formed by N-2 acetylaminofluorene (AAF) [71] and benzo[a]pyrene diol epoxide (BPDE) [72,73], has been attributed to weakening of the RNA:DNA.

The sequence context around a *cis*-1,2-d(GG) (5′-CT*GG*CC-3′ and 5′-TA*GG*CC-3′) did not have a significant effect on the extent of T7RNAP and mammalian RNAPII blockage at the site of the lesion [63]. This result suggests that the distortion induced by the *cis*-1,2-d(GG) is the major factor in causing transcription arrest at this lesion. Similar to our results, Corda et al. [60] reported that a *cis*-1,2-d(GG) caused complete blockage of wheat germ RNA polymerase when located in the sequence context 5′-CT*GG*CC-3′. However, Cullinane et al. [62] found that a *cis*-1,2 d(GG) in the sequence context 5′-TA*GG*CC-3′ was not a block to RNAPII transcription in HeLa cell extracts, suggesting that differences in transcription systems and/or in the source of RNA polymerase might play a role in determining the extent of arrest at this lesion.

6. RNAPII and oxidative damage

Although TCR was originally demonstrated for DNA damage induced by UV light [49], it has also been reported that oxidative damage is repaired in a transcription dependent manner in *E. coli* [74] and human cells [75], suggesting a link between base excision repair and transcription. However, TCR does not seem to be a general pathway to repair oxidative lesions. In CHO cells, preferential repair of oxidative damage produced by photosensitization and oxidizing agents was

not observed in the dihydrofolate reductase (Dhfr) and c-fos genes [76,77]. Radiation-induced strand breaks, lesions also repaired by BER, do not seem to be repaired by TCR in the Dhfr gene from CHO cells [78] or human colon cancer cells [79]. These results raise the question of how the RNAPII may react to an oxidative lesion compared to a CPD in the transcribed strand.

6.1. Thymine glycol

Thymine glycols (Tg) are formed by oxidation of the 5, 6 double bond of thymine upon exposure to ionizing radiation or oxidative stress. Tg is a strong blocking lesion for prokaryotic DNA polymerases in vitro, and can be lethal in vivo [80]. Tg is normally repaired by the base excision repair system [81]. Using our reconstituted in vitro transcription system with DNA substrates containing a single Tg in the transcribed or in the non-transcribed strand located downstream of the T7 promoter or the AdMLP, we studied the effect of Tg (5′-CGTgAC-3′) on transcription elongation by T7 RNAP and mammalian RNAPII. We found that a Tg in the transcribed strand of template DNA was a significant block to the progression of T7 RNAP, in agreement with the results of others [82,83], resulting in polymerase arrest 50% of the time. Surprisingly, a Tg in the transcribed strand was efficiently bypassed by RNAPII. A Tg in the non-transcribed strand was bypassed by both polymerases. A recent study by Kathe et al. [41], using HeLa nuclear extracts and DNA substrates containing a single Tg (5′-GGTgGG-3′) downstream of the HIV promoter, has similarly reported that a Tg located in the transcribed strand caused RNAPII to pause only transiently at the lesion. The pause was followed by subsequent readthrough.

6.2. 8-Oxoguanine

8-Oxoguanine (8-oxoG) is a major oxidative lesion produced in DNA by normal cellular metabolism or after exposure to exogenous sources such as ionizing radiation. Persistence of this lesion in DNA causes G to T transversions, with deleterious consequences for the cell [84]. As a result, several repair processes have evolved to remove this lesion from the genome. In *E. coli*, at least three proteins participate to prevent accumulation of this base damage in the cell. The mutM gene product, formamidopyrimidine DNA glycosylase excises 8-oxoG paired with cytosine, the MutY glycosylase removes adenine inserted opposite 8-oxoG by DNA polymerases, and MutT, an 8-oxodGTPase, hydrolyzes 8-oxodGTP and prevents its incorporation into DNA [80]. Mammalian homologs of these enzymes have been identified [80,85]. TCR of 8-oxoG has been reported in *E. coli* [74] and human cells, when a single 8-oxoG was located in the transcribed strand of a shuttle vector [72]. However, in CHO cells repair of 8-oxoG in the Dhfr gene, did not reveal a strand bias [77]. 8-OxoG has been shown to be bypassed by T7 RNAP with various efficiencies depending on the sequence context studied [83,86]. Similarly, elongation by *E. coli* RNA polymerase in vitro was not affected by 8-oxoG [46,74,87]. However, when a single 8-oxoG was located in the transcribed strand of a shuttle vector transfected into human cells transcription arrest at the lesion was observed [72]. The effect of 8-oxoG on RNAPII transcription in vitro has been studied in several laboratories. Using RNAPII purified from HeLa cells and oligo(dC) templates containing a single 8-oxoG in the sequence context 5′-GT-8-oxoG-CA-3′, some arrest at the lesion was observed [88]. Interestingly, a decrease in CTP concentration decreased arrest at the lesion, suggesting that incorporation of C opposite 8-oxoG was causing RNAPII to stop transcribing. Indeed, sequence analysis of the bypassed transcription products indicated that incorporation of A was increased at high ATP concentrations [88].

Using our in vitro transcription system with purified RNAPII and GTFs, and templates containing a single 8-oxoG in the sequence context 5′-CG-8-oxoG-CG-3′ downstream of the AdMLP, we found that this lesion only transiently paused RNAPII elongation. Furthermore, a decrease of the ATP concentration from 800 to 40 μM increased arrest at 8-oxoG, in agreement with Kuraoka et al. [88]. However, the extent of arrest at the lesion observed by Kuraoka et al. [88] was significantly higher than we observed when 8-oxoG was in a different sequence context. The stability of the RNA:DNA hybrid formed around the lesion, which represent a primary component of the elongation complex [4,69] might have played a role in determining the extent of bypass of 8-oxoG in our sequence context 5′-CG-8-oxoG-CG-3′ compared to that studied by Kuraoka et al. (5′-GT-8-oxoG-CA-3′). Differences in the source of RNAPII and/or in transcription systems

might also have contributed to the extent of bypass at 8-oxoG. Indeed, when the lesion was located in the same sequence context studied by Kuraoka et al., but downstream of the AdMLP, a weak 5% block was observed at the site of the lesion using either a reconstituted in vitro system with purified RNAPII and initiation factors or HeLa extracts [89]. Using the same transcription system but substrates containing a single cisplatin-induced intrastrand crosslink instead of a 8-oxoG, a strong block was detected, indicating that the experimental conditions utilized were adequate to detect transcription arrest. Similarly, Kathe et al. [41] have shown, using HeLa extracts as source of RNAPII and GTFs, that a single 8-oxoG in the sequence context 5′-GG-8-oxoG-GG-3′ located in the transcribed strand downstream of the HIV-1 promoter was not a block to RNAPII transcription. When a CPD was located in the transcription substrate instead of an 8-oxoG, a complete block of RNAPII was observed. It is interesting to note that although these studies have employed different transcription systems, promoters and sequence contexts, they seem to come to the conclusion that the presence of 8-oxoG in template DNA does not substantially affect transcription elongation by RNAPII in vitro. These results are in agreement with the minor effect on DNA structure induced by 8-oxoG. NMR and X-ray crystallographic analysis of short double-stranded oligomers containing a single 8-oxoG have shown that when 8-oxoG is paired with either cytosine or adenine, the overall structure of the DNA duplex is very similar to that of undamaged B-form DNA, where the lesion assumes a anti-conformation when correctly paired with cytidine or a syn conformation when incorrectly paired with adenine [90–92]. When a single 8-oxoG was positioned in a 25 base oligomer, however, significant changes were observed in the phospho-deoxyribose backbone immediately adjacent to the lesion, as compared to an undamaged oligomer. This may suggest that the presence of the 8-oxoG lesion in single-stranded regions, such as those found in the transcription bubble, can alter the fidelity of transcription by affecting the conformational properties of the localized DNA backbone [93]. This might explain the weak blockage observed at 8-oxoG sites.

How can the in vitro results of limited blockage of RNAPII at 8-oxoG be reconciled with the in vivo evidence of TCR of 8-oxoG? Several scenarios can be envisioned: it has been proposed [74] that RNAPII pausing instead of a complete arrest at the 8-oxoG site might be sufficient to trigger TCR. Furthermore, it was speculated that 8-oxoG could cause RNAPII arrest through binding of a protein to the lesion [75]. However, when we added the mismatch recognition protein complex MSH2–MSH6, which has been shown to be associated with 8-oxoG in DNA [94,95], we did not observe any change in the extent of RNAPII blockage at 8-oxoG, suggesting that the MSH2–MSH6 complex is probably not involved in the initial steps of TCR of 8-oxoG [96]. It is still possible that in the presence of other mismatch repair proteins different results might be obtained. Interestingly, it was found that oxidative stress-induced lesions block transcription in an MLH1-dependent manner [97]. This may suggest that MLH1 might be needed to stabilize MSH2–MSH6 complexes at sites of lesions resulting in blockage of transcription. Another alternative candidate required for RNAPII arrest at 8-oxoG and initiation of TCR in vivo could be the recently discovered mammalian DNA glycosylase NEIL2, which has been suggested to be involved in repair of lesions in DNA bubbles generated during transcription and replication [98]. It was postulated that the single strand break generated by NEIL2 at the 8-oxoG site could cause transcription arrest. Indeed, Kathe et al. [41] have recently shown that strand breaks in the transcribed strand are strong blocks to RNAPII elongation. However, it is unlikely that single strand breaks will have a sustained inhibitory effect on transcription in cells, due to the rapid repair of these lesions in vivo.

6.3. *Malondialdehyde*

Malondialdehyde (MDA) is a genotoxic byproduct of lipid peroxidation and prostaglandin biosynthesis in cells [99]. MDA reacts with guanine in DNA to form the pyrimidopurinone adduct, pyrimido purin-10(3*H*)-one (M_1dG). M_1dG formation results in two chemically distinct adducts, the N2-(3-oxo-1-propeniyl)-dG (N2-OpdG) when paired with cytosine in double-stranded DNA, resulting from a cytosine catalyzed conversion of M_1dG to an acyclic structure and the exocyclic M_1dG when mispaired with thymine or in single-stranded DNA. Interestingly, in healthy human tissues, M_1dG adducts occur at a frequency of 1 in 10^6 nucleotides, and in the pancreas they reach levels similar to those measured for 8-oxoG [100].

These adducts are highly mutagenic in bacteria and mammalian cells, inducing frameshifts and base substitutions. Exocyclic guanine adducts are removed by base excision repair, however, *E. coli* AlkA mutants, defective in the primary glycosylase for ethenopurine and ethenocitosine adducts, do not show increased mutation frequency induced by a synthetic, saturated analog of M_1dG, N2-propanodeoxiguanine (PdG). UvrA-mutants exhibit a four-fold increase in base substitutions induced by PdG, suggesting a significant role of NER in the repair of M_1dG. Mismatch repair may also contribute to the repair of MDA adducts. We have recently studied the effect of these adducts on T7 RNAP and mammalian RNAPII transcription [101]. It was found that an M_1dG or PdG adduct in the transcribed strand impeded elongation by both RNAPs, with RNAPII more significantly blocked than T7 RNAP. The extent of arrest induced by M_1dG was dependent on base pairing, where M_1dG mispaired with thymine posed a stronger block then the acyclic N2-OpdG. Furthermore, elongation complexes arrested at PdG adducts remained stable, as indicated by by RNAPII backup from the adduct and transcript cleavage in response to elongation factor SII [101]. These results suggest that MDA adducts produced in mammalian cells may block RNAPII elongation and thus, may be subject to TCR.

7. Perspectives

RNAPII clearly represents a key player in the control of excision repair in expressed genes. Several investigators have recently proposed models for TCR, its link with BER and NER and its relationship with human disease. To critically test the various hypotheses, we need a better understanding of the behavior of the RNAPII when it encounters lesions in the template DNA and the ordered assembly of the multiprotein complexes to effect repair. How does a polymerase arrested at a natural arrest site differ from a polymerase arrested at a DNA lesion? What are the features that determine whether a lesion will block transcription? What are the factors involved in the initial steps of TCR? Does the polymerase fall off the template or can it continue transcribing after repair has occurred? These are some of the challenging questions for future research.

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