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Transcription Blockage by Bulky End Termini at Single-Strand Breaks in the DNA Template: Differential Effects of 5′ and 3′ Adducts

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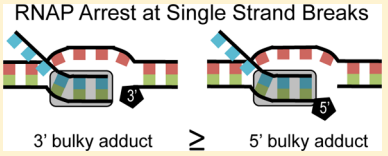
Supporting Information

ABSTRACT: RNA polymerases from phage-infected bacteria and mammalian cells have been shown to bypass single-strand breaks (SSBs) with a single-nucleotide gap in the template DNA strand during transcription elongation; however, the SSB bypass efficiency varies significantly depending upon the backbone end chemistries at the break. Using a reconstituted T7 phage transcription system (T7 RNAP) and RNA polymerase II (RNAPII) in HeLa cell nuclear extracts, we observe a slight reduction in the level of transcription arrest at SSBs with no gap as compared to those with a single-nucleotide gap. We have shown that biotin and carbon-chain moieties linked to the 3′ side, and in select cases the 5′ side, of an SSB in the template strand strongly increase the level of transcription arrest when compared to unmodified SSBs. We also find that a small carbon-chain moiety linked to the upstream side of an SSB aids transcriptional bypass of SSBs for both T7 RNAP and RNAP II. Analysis of transcription across SSBs flanked by bulky 3′ adducts reveals the ability of 3′ end chemistries to arrest T7 RNAP in a size-dependent manner. T7 RNAP is also completely arrested when 3′ adducts or 3′-phosphate groups are placed opposite 5′-phosphate groups at an SSB. We have also observed that a biotinylated thymine in the template strand (without a break) does not pose a strong block to transcription. Taken together, these results emphasize the importance of the size of 3′, but usually not 5′, end chemistries in arresting transcription at SSBs, substantiating the notion that bulky 3′ lesions (e.g., topoisomerase cleavable complexes, 3′-phosphoglycolates, and 3′-unsaturated aldehydes) pose very strong blocks to transcribing RNA polymerases. These findings have implications for the processing of DNA damage through SSB intermediates and the mechanism of SSB bypass by T7 RNAP and mammalian RNAPII.



Single-strand breaks (SSBs) are disruptions in the DNA backbone that often include the loss of one nucleotide and damage to the 3′ and 5′ termini that surround the break. They are frequently caused by reactive oxygen species (ROS), which can disintegrate backbone sugars by direct attack or initiate SSB-inducing base excision repair (BER) by damaging DNA bases (most commonly guanine). Other types of DNA base incongruities that are processed into SSB intermediates during BER include uracil, which results from cytosine deamination, and alkylated base lesions such as 3-methyladenine. Aborted topoisomerase I (TOPI) activity also results in an SSB, leaving the covalently bound TOPI on the 3′-terminus at the break. SSBs along with abasic sites, which are processed through an SSB intermediate, represent the prevalent form of endogenous DNA damage in living cells.[1−5]

SSB repair begins with the processing of DNA end groups at the break to a 5′-phosphate (5′-PO4) and a 3′-hydroxyl (3′-OH) group. There are several types of damaged DNA ends that require further processing. Direct oxidative damage of the DNA backbone by ROS results in 3′-phosphate (3′-PO4) and 3′-phosphoglycolate groups at an SSB. The removal of a damaged base during BER and the removal of covalently bound 3′-TOPI by the phosphodiesterase TDP1 also leaves a 3′-PO4 at an SSB. Other damaged SSB termini include 5′-hydroxyl (5′-OH) groups, which are products of TDP1 activity and direct oxidative backbone damage, and 3′-unsaturated aldehydes (4-hydroxy-2-pentenal), which can arise during BER.[1−3,6]

SSBs in the template strand (TS) of a transcribed sequence are bypassed during transcription in vitro by phage, prokaryotic, and eukaryotic RNA polymerases, at efficiencies that depend significantly on the 5′ and 3′ end chemistries at the break. SSBs with 5′-OH|3′-OH, 5′-PO4|3′-OH, 5′-dRP|3′-OH, and 5′-OH|3′-PO4 end chemistries have been shown to cause <50% arrest of T7 RNA polymerase (T7 RNAP) in vitro.[7] In contrast, 5′-PO4|3′-PO4 SSBs pose a very strong block to transcribing polymerases, causing nearly complete arrest of T7 RNAP and *Escherichia coli* RNA polymerase (*E. coli* RNAP) in vitro and 85% arrest of RNA polymerase II (RNAP II) in HeLa cell nuclear extracts.[7−9] SSBs with 3′-aldehyde groups are also very strong blocks to T7 RNAP, RNAP II, and *E. coli* RNAP.[7−9]

The extent of TS SSB bypass by RNA polymerases is also influenced by the size of the gap at the SSB. Most in vitro experiments that explore the effects of DNA end chemistries on polymerase bypass have been conducted on substrates with SSBs at the site of a missing nucleotide.[7−9] However, T7 RNAP has been shown to bypass SSBs in the TS with gaps of up to 24 nucleotides.[10,11] Transcription across gaps by T7 RNAP and *E. coli* RNAP in vitro results in shortened transcripts, lacking the code for the nucleotides that were missing at the SSB.[7,8,10]

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In vivo, SSBs resulting from BER of 8-oxoguanine as well as abasic sites, which arrest T7 RNAP and RNAP II transcription in vitro, enhance transcriptional arrest.[12−14] SSBs with covalently bound 3′-TOPI lesions are blocks to transcription in vitro and in mammalian cells.[15−18] When present in either the TS or the nontemplate strand (NTS), SSBs can also lead to an overall reduction in the level of gene expression.[19,20]

Because SSBs do not appear to be preferentially repaired in transcribed DNA,[21,22] their impact on transcriptional miscoding and mutation is likely significant. In contrast to the in vitro data, the results for transcription in vivo across SSBs with one-nucleotide gaps, abasic sites, uracil, or 8-oxoguanine in the TS show transcriptional miscoding opposite the lesion.[20,23,24] This process, known as transcriptional mutagenesis (TM), has been documented during the transcription of damaged plasmids in *E. coli* and mammalian cells.[20,23,24] In particular, there is evidence to suggest that SSBs in the TS with unprocessed 3′-PO4 termini, despite being strong blocks to transcription in vitro, are more likely to be bypassed and result in TM than other types of SSBs.[20]

We set out to learn the characteristics of SSBs that influence transcriptional arrest and bypass at SSBs in the TS. To accomplish this, we have tested the effects of various bulky chemical DNA end adducts at SSBs on transcription by T7 RNAP and RNAP II. Furthermore, because this had not been studied systematically in vitro, we sought to compare the effect of a single-nucleotide gap to that of a simple strand break with no gap in contributing to transcription arrest at SSBs. Our results shed light on some of the factors that contribute to the arrest of T7 RNAP and RNAP II at SSBs. In particular, we have shown that bulky adducts at 3′-damaged termini are influential in causing T7 RNAP and RNAP II transcription arrest at SSBs. These results raise the question of whether transcription-coupled DNA repair might occasionally occur at these sites.

MATERIALS AND METHODS

DNA Substrates. DNA transcription substrates were prepared using the protocol described previously (Figure 1).[25] Substrates consisted of two parts: a promoter fragment isolated from plasmid DNA and an "insert" containing an SSB in the TS. Two different promoter fragments, containing either the T7 RNAP promoter or the RNAP II cytomegalovirus (CMV) promoter, were prepared via *Eco*RI and *Bam*HI restriction of the pWT or pWT-C plasmid or *Eco*RI and *Bse*RI restriction of the pCMVβ plasmid (Clontech), respectively.[25,26] Promoter-containing DNA fragments were then purified via agarose gel purification without exposure to UV or ethidium bromide, as described previously.[25]

Annealed oligonucleotide inserts (Figure S1A of the Supporting Information) consisted of three polyacrylamide gel electrophoresis-purified oligonucleotides from Integrated DNA Technologies (IDT): a long NTS oligo, a short complementary TS oligo upstream of the SSB, and a short complementary TS oligo downstream of the SSB. Some NTS oligos contained a thymine opposite the SSB site, such that SSBs in these substrates encompassed a single-nucleotide gap [black dot (Figure S1A of the Supporting Information)]. Other NTS oligos did not include this thymine, permitting the construction of substrates containing an SSB without a gap. The TS custom oligos were made with different 5′ (for the upstream oligo) and 3′ (for the downstream oligo) end chemistries so that SSBs with various termini could be generated. Oligos were annealed by incubating the NTS oligo

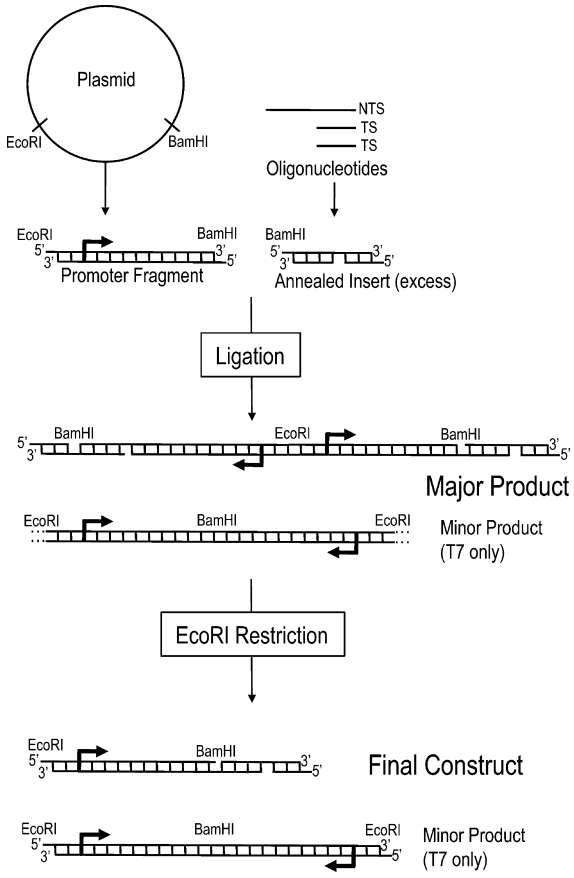

Figure 1. Protocol for making transcription substrates, which consisted of two parts: a promoter-containing fragment, which was obtained via restriction from plasmid DNA, and an insert, which consisted of three oligonucleotides that were annealed such that a site-specific SSB with various flanking termini was present in the transcribed strand. Inserts were ligated to promoter fragments via *Bam*HI sticky ends for T7 RNAP substrates and *Bse*RI sticky ends for RNAPII substrates. Ligation was followed by *Eco*RI digestion, yielding the final substrates containing one promoter fragment (designated by the arrow) and one insert. For T7 RNAP substrates, NTS oligonucleotides were not phosphorylated, to prevent insert self-ligation, but this left a nick in the NTS of final constructs. Furthermore, a minor fraction of T7 RNAP substrates, consisting of promoter fragments self-ligated through *Bam*HI sites, still appeared but did not interfere with transcription experiments. NTS oligonucleotides were phosphorylated for RNAP II substrates because *Bse*RI sticky ends were asymmetrical; thus, they did not contain a nick in the NTS. This also prevented the formation of any minor products during RNAP II substrate fabrication. Sticky ends on the downstream side of substrates were not used in this study.

(2 μM) with an excess of both TS oligos (4 μM) in a solution containing 10 mM Tris-HCl and 10 mM $MgCl_2$ at 65 °C for 20 min, followed by 1 h at room temperature.

Annealed inserts were ligated to T7 promoter fragments in reaction mixtures containing the T7 promoter fragment (14 nM), the annealed insert (565 nM), 1× T4 DNA ligase buffer (NEB), and T4 phage DNA ligase (100 units/μL) in a total volume of 20 μL overnight at 16 °C. The excess of insert was necessary during the T7 ligation reaction to reduce the probability of promoter fragments ligating to themselves via *Bam*HI sticky ends (Figure 1, minor products). Annealed inserts were ligated to CMV promoter fragments in reaction mixtures containing the CMV promoter fragment (30 nM), the

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annealed insert (100 nM), 1× T4 DNA ligase buffer (NEB), and T4 phage DNA ligase (100 units/μL) in a total volume of 20 μL overnight at 16 °C. Inserts were not present in significant excess for CMV reactions because *Bse*RI sticky ends were asymmetrical. Thus, there was no concern that CMV promoters or inserts would ligate to themselves. Following overnight incubation, both CMV and T7 reaction mixtures were heated at 65 °C for 30 min to inactivate ligase and then digested with *Eco*RI, yielding constructs containing one promoter fragment and one insert (Figure 1, final construct). In the case of T7 substrates, NTS oligos were not 5′-phosphorylated (which prevents self-ligation of the insert via symmetrical sticky ends) and final constructs contained a nick at the site of ligation in the NTS. This NTS nick was shown to have no significant effect on transcription.[25] For substrates containing 5′-PO4|3′-OH nicks, the TS oligo downstream of the SSB was added to the construct mixture after T4 DNA ligase had been heat-inactivated. This ensured that the SSB of interest would not be ligated during construction. Final constructs were analyzed by gel electrophoresis on 1.5% agarose gels and visualized by EtBr staining and exposure to UV light (Figure S2 of the Supporting Information).

The double strandedness of inserts downstream of SSB sites was confirmed by *Sal*I restriction. This quality check ensured that the majority of SSB-induced blockage during in vitro transcription was not due to incomplete annealing. Restriction assays were conducted with radiolabeled, annealed oligonucleotide inserts (not ligated to promoter fragments) except in the case of 5′-PO4|3′-OH substrates, where final constructs were used. Radiolabeling reactions were performed in a total volume of 12.5 μL containing 208 nM annealed inserts, 1× T4 kinase forward reaction buffer (Invitrogen), 12 μCi of [^{32}P]ATP, and 5 units of T4 polynucleotide kinase (Invitrogen). The reaction mixture was incubated for 10 min at 37 °C. Radiolabeled products were purified using the QIAquick nucleotide removal kit. The restriction assay was conducted in a total volume of 50 μL containing 0.83 pmol of radiolabeled annealed insert, 1× NEBuffer 3 (NEB), and 1× BSA (NEB) with or without 20 units of *Sal*I. Reaction mixtures were incubated for 2 h at 37 °C. EDTA was added to a concentration of 20 mM, and the restriction enzyme was inactivated by heat at 65 °C for 20 min. Solutions were diluted 1:20 in water and run on an 8% polyacrylamide gel containing 8 M urea for 2 h at 2000 V. Results were visualized using a Bio-Rad personal molecular imager system. The *Sal*I restriction percentage was calculated as the percent of restricted NTS oligo (Figure S1B,C of the Supporting Information).

T7 RNAP Transcription. Reactions were performed in a total volume of 12 μL, containing 2 μL of 5× transcription buffer from Promega [40 mM Tris (pH 7.9), 6 mM $MgCl_2$, 2 mM spermidine, and 10 mM NaCl], 4 mM DTT, 16 units of RNAsin (Promega), ATP, GTP, and UTP (0.17 mM each), 0.017 mM CTP, 10 μCi of [α-^{32}P]CTP, 20 units of T7 RNAP (Promega), and the corresponding DNA substrate (≈1 nM). The reactions were conducted at 37 °C for 30 min. The reaction was stopped, and transcription products were precipitated as described previously.[26] Samples were run in a 5% polyacrylamide gel containing 8 M urea for 1.5 h at 2000 V. Results were visualized using a Bio-Rad personal molecular imager system.

RNAPII Transcription in HeLa Cell Nuclear Extracts. Reactions were performed in two stages, initiation and elongation. Initiation reactions were performed in a total volume of 18 μL containing 4 nM CMV transcription substrate, 3 μL of 1× HeLa nuclear extract transcription buffer [20 mM HEPES (pH 7.9 at 25 °C), 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT, and 20% glycerol], 4.17 mM $MgCl_2$, and 8 units of HeLaScribe nuclear extract (Promega). Initiation reaction mixtures were incubated at 28 °C for 30 min; 7 μL of the elongation mixture was then added to the initiation reaction mixture for a final elongation reaction volume of 25 μL containing, in addition to the reagents present in the initiation reaction mixture, 400 nM ATP, GTP, and UTP, 40 μCi of [α-^{32}P]CTP, 40 units of RNAsin (Promega), and 4% DMSO. Elongation reactions were conducted at 28 °C for 30 min. To stop the reactions, EDTA was added to a final concentration of 6 mM and RNA transcripts were purified using the Qiagen RNeasy MinElute cleanup kit. Samples were run in a 5% polyacrylamide gel containing 8 M urea for 1.5 h at 2000 V. Results were visualized using a Bio-Rad personal molecular imager system.

To test whether the enzymatic activity of proteins in the HeLa cell nuclear extract altered the composition of SSB end chemistries, incubation was carried out under the same conditions that were used for the transcription experiments using radiolabeled annealed oligonucleotide inserts (without promoters) instead of CMV transcription substrates. Following the reaction, inserts were isolated using the Qiagen Nucleotide Removal kit and run in a 20% polyacylamide gel containing 8 M urea for 4.5 h at 2000 V. Alterations to SSB end groups were visualized as a change in gel shift in 20% gel, or a loss of radioactive 5′-PO_4 label (Figure S3 of the Supporting Information). To further test the effects of the HeLa cell nuclear extract on SSB end chemistries, RNAPII transcription initiation reaction mixtures were incubated for 1 h at 28 °C and compared side by side with reaction mixtures that underwent the standard 30 min initiation incubation. This allowed us to observe whether alterations to SSB end chemistries caused by increased incubation time with enzymes in the nuclear extract altered SSB-induced transcription arrest. All changes in transcription arrest observed from the longer initiation time were <5% (data not shown).

RESULTS

T7 Transcription Arrest at SSBs Flanked by Hydroxyl and Phosphate Groups. To test the effects of different SSB end chemistries on elongating T7 RNAP, annealed DNA oligonucleotides containing a site-specific SSB were ligated to a DNA fragment containing a T7 promoter sequence (Figure 1). In the case of double-stranded DNA substrates that did not contain an SSB, the one well-defined band corresponds to full-length transcription runoff (324 nucleotides) (Figure 2, lane 9). Truncated transcription products, which were interpreted as a consequence of transcription arrest, were observed when 5′-OH|3′-OH, 5′-PO4|3′-OH, 5′-OH|3′-PO4, and 5′-PO4|3′-PO4 SSBs were present in the TS with or without a single-nucleotide gap (Figure 2, lanes 1−8). The lengths of these truncated transcripts correspond to the site of the SSB (284 nucleotides) in the TS. While all of these SSBs were blocks to transcription, the extent to which they arrested transcription varied [measured as the percentage of truncated transcript in relation to the overall transcription products, normalized by the number of radiolabeled cytosine residues expected in each transcript (see the numbers under each lane)]. All SSBs were tested at least twice, and the average percentage arrest is indicated in Figure 2. 5′-PO4|3′-PO4 SSBs were a complete block to T7

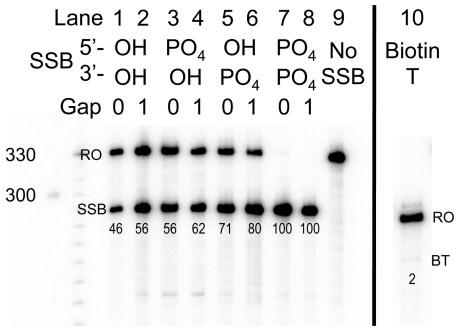

Figure 2. T7 RNAP transcription arrest at SSBs with naturally occurring end chemistries. Runoff products (324 nucleotides) are indicated by RO. Truncated transcripts (SSB) correspond to transcription arrest at the site of the SSB in the TS of transcription substrates (284 nucleotides). The percentage of transcription arrest (indicated underneath each lane) was calculated as the radioactive volume of truncated transcripts divided by that volume plus the radioactive volume of runoff products. Each radioactive volume was normalized for the number of radioactively labeled cytosine residues expected in the corresponding transcript. Lane 10 contained transcription products using a transcription substrate that contained biotinylated thymine. This substrate had a runoff (RO) that was 40 nucleotides shorter than SSB-containing substrates. Biotinylated thymine (BT) caused negligible arrest.

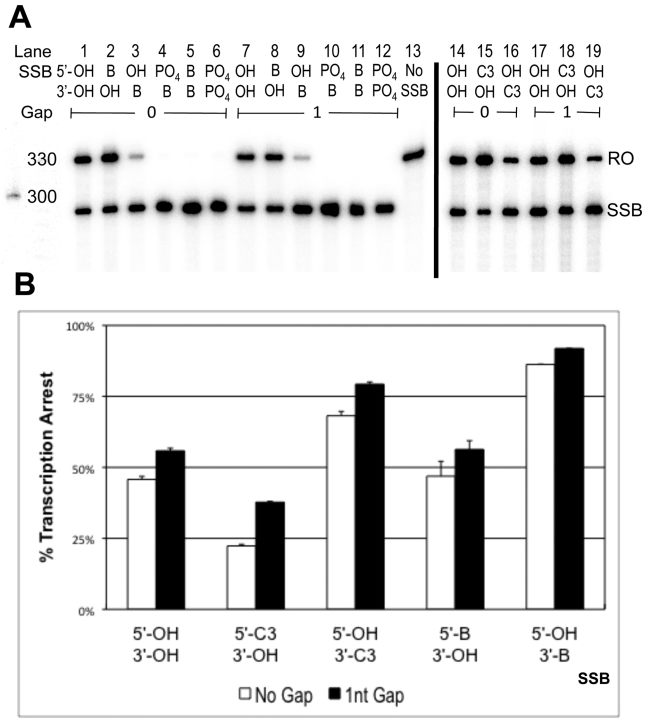

Figure 3. 3′, but not 5′, moieties increase the level of T7 transcription arrest at SSBs. (A) Radiolabeled T7 RNAP transcription products. The letters RO indicate runoff products (324 nucleotides). The letters SSB indicate truncated transcripts with lengths corresponding to arrest at the SSB in the TS of transcription substrates (284 nucleotides). (B) Percent blockage calculated as described above. Each SSB was tested at least twice, and the bar graph depicts the average arrest for the corresponding SSB end chemistries. Error bars indicate the standard error. SSBs containing 3′ bulky adducts show noticeably greater levels of arrest than their mirror images.

transcription (Figure 2, lanes 7 and 8), and 5′-OH|3′-OH SSBs arrested transcription 46% of the time when there was no gap (Figure 2, lane 1). The 5′-PO4|3′-OH SSBs arrested transcription ∼15% less than the 5′-OH|3′-PO4 SSBs (Figure 2, lanes 3−6). There was also a noticeable difference between SSBs containing a one-nucleotide gap and no gap, with no gap causing 6−10% less blockage depending on end chemistry (Figure 2).

Bulky Groups at the 3′ Side of an SSB Cause Stronger Transcription Arrest Than Those at the 5′ Side. In light of the finding that a phosphate group on the 3′ side of an SSB (5′-OH|3′- PO4) is a stronger block to T7 transcription than a phosphate group on the 5′ side (5′-PO4|3′-OH), experiments were performed to determine whether the same trend is observed for other DNA end adducts for both T7 RNAP and RNAPII. Transcription substrates were designed using two different synthetic end groups (available from IDT), such that mirror image SSBs existed in the TS. One synthetic end group consisted of a biotin group that was attached to 5′-phosphate (5′-biotin, molecular weight of 393.4) or 3′-phosphate (3′-biotin, molecular weight of 437.4) DNA ends. The other was a three-carbon-containing 1,3-propanediol end adduct that was also connected to either 5′-phosphate (5′-C3, molecular weight of 138.1) or 3′-phosphate (3′-C3, molecular weight of 138.1) DNA ends [HO-$(CH_2)_3$-O-P(=O)(O^-)-3′/5′]. For T7 RNAP, 5′-C3|3′-OH SSBs with no gap and a one-nucleotide gap caused 22 and 38% transcription arrest, respectively, while 5′-OH|3′-C3 SSBs with no gap and a one-nucleotide gap caused 68 and 79% arrest, respectively (Figure 3, lanes 15, 16, 18, and 19). Similarly, 5′-biotin|3′-OH SSBs with no gap and a one-nucleotide gap caused 47 and 56% arrest, respectively, as compared to 5′-OH|3′-biotin SSBs with no gap and a one-nucleotide gap, which caused 86 and 92% arrest, respectively (Figure 3, lanes 2, 3, 8, and 9). For RNAPII, the C3 synthetic end groups showed asymmetrical blockage patterns. 5′-C3|3′-OH SSBs with no gap and a one-nucleotide gap caused 60 and 58% arrest for RNAPII, respectively (Figure 4, lanes 2, 4, 7, and 9). In contrast, 5′-OH|3′-C3 SSBs caused 94 and 92% arrest, respectively (Figure 4, lanes 3, 5, 8, and 10). 5′-C3|3′-OH SSBs caused ∼20% less transcription arrest than 5′-OH|3′-OH SSBs for T7 RNAP and ∼5% less for RNAPII. There was also a noticeable increase in the level of arrest caused by substrates with a one-nucleotide gap compared to those with no gap (Figures 3B and 4B)

Bulky 3′ Adducts Cause T7 RNAP Transcription Arrest in a Size-Dependent Manner. To test the influence of the size of end termini on T7 RNAP transcription arrest at SSBs, we conducted transcription assays with substrates containing various 3′ synthetic groups opposite 5′-hydroxyl termini at an SSB. The modified 3′ termini consisted of the biotin and C3 groups described above, as well as a six-carbon 1,6-hexanediol adduct [HO-$(CH_2)_6$-O-P(=O)(O^-)-3′] (3′-C6) (molecular weight of 180.1) and a 1′-2′-deoxyribose phosphate (3′-dRP) (molecular weight of 180.1). The level of SSB-induced transcription arrest observed when these 3′ groups were opposite a 5′-OH at an SSB increases with an increasing length of the chain 3′ groups (C3 < C6 < biotin) (Figure 5B). Furthermore, these adducts cause 100% transcription arrest for T7 RNAP when they are located on the 3′ side of an SSB opposite a 5′-PO4 group in our system (Figure 5A, lanes 9−12). In the case of RNAPII, all synthetic 3′ groups caused very strong arrest, and their quantitative ranking is difficult to evaluate. A decrease in the level of arrest caused by one-

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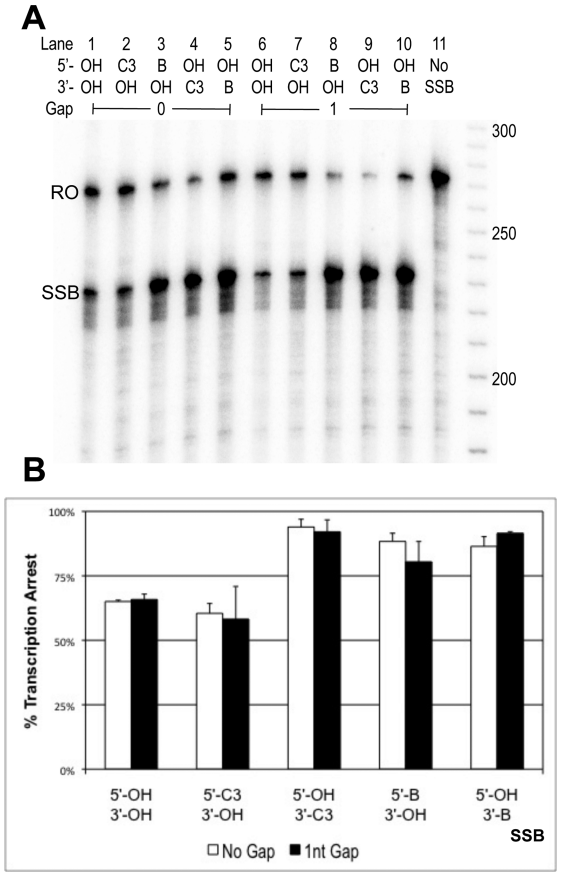

Figure 4. 3′-C3, but not 5′-C3, moieties increase the level of RNAP II transcription arrest at SSBs. (A) Radiolabeled RNAP II transcription products. The letters RO indicate runoff products (270 nucleotides). The letters SSB indicate truncated transcripts with lengths corresponding to arrest at the SSB in the TS of transcription substrates (230 nucleotides). (B) Percent blockage calculated as described above. Each SSB was tested at least twice, and the bar graph depicts the average arrest for the corresponding SSB end chemistries. Error bars indicate the standard error. SSBs containing 3′-C3 show noticeably greater levels of arrest than their mirror images, but 5′-OH|3′-biotin and 5′-biotin|3′-OH SSBs exhibit similar levels of transcription arrest.

nucleotide gap SSBs versus that caused by SSBs with no gap was observed for these substrates as well (Figure 5B).

An Internal Biotin Modification, without an SSB, Causes Very Minor Blockage for T7 RNAP. Because biotin-containing end groups caused such strong blockage at SSBs, we wanted to determine whether an internal biotin moiety (not at an SSB) in the TS would cause transcription arrest as well. To test this, we prepared T7 transcription substrates containing a biotinylated thymine (BT) in the TS. BT consisted of a thymine with the six-carbon linker biotin group described above attached to the C5 atom of thymine. These substrates were 40 nucleotides shorter than other T7 transcription substrates and did not contain an SSB in the TS. Like the linear control substrates, the one well-defined transcription product from the BT substrates corresponds to the transcription runoff (Figure 2, lane 10). There was ~2% arrest at the site of BT.

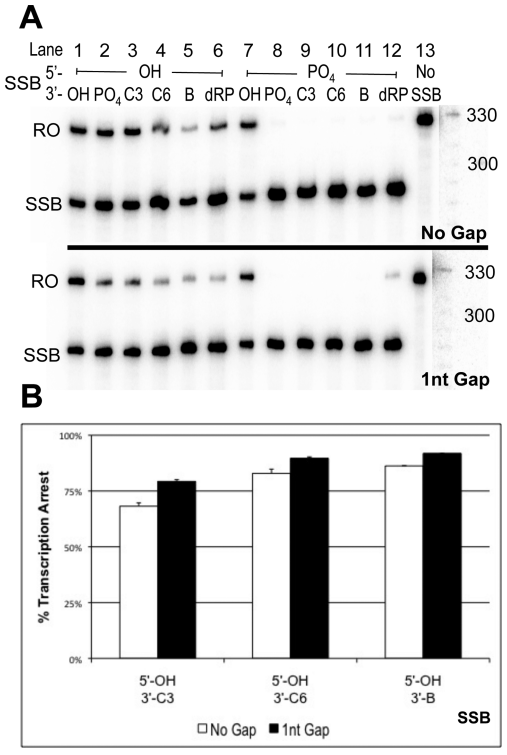

Figure 5. Level of transcription arrest increases with an increasing size of bulky 3′ groups at SSBs with 5′-OH groups. (A) In this figure, two gels containing T7 RNAP transcription products are stacked: substrates with no gap (top) and substrates with a one-nucleotide gap (bottom). Runoff products (324 nucleotides) appear as expected (RO). Truncated transcripts (SSB) have lengths corresponding to transcription arrest at the site of the SSB in the TS of transcription substrates (284 nucleotides). (B) Percent blockage calculated as described above. Each SSB was tested at least twice, and the bar graph depicts the average arrest for the corresponding SSB end chemistries. Error bars indicate the standard error. SSBs are ordered by increasing size of 3′ adducts from left to right.

DISCUSSION

We found that large adducts on the 5′ side of an SSB in the TS (5′-biotin|3′-OH and 5′-C3|3′-OH) cause considerably less transcription arrest than large 3′ adducts (5′-OH|3′-biotin and 5′-OH|3′-C3) for T7 RNAP and, to some extent, RNAP II. This result is in accord with the previous studies of Zhou and Doetsch, who reported that SSBs with bulky 3′ termini (5′-OH|3′-aldehyde and 5′-PO4|3′-aldehyde) are strong blocks to T7 RNAP, while SSBs with bulky 5′ groups (5′-dRP|3′-OH) were less significant sources of transcription arrest.[7] Thus, it is clear that bulky 3′, but not 5′, termini at an SSB have a significant influence on SSB-induced transcription arrest.

This asymmetry in the effect of bulky adducts flanking an SSB on transcription arrest might occur because the RNAP must capture the 3′ (downstream) terminus at the break and reinsert it into the catalytic site to continue transcribing. In contrast, a bulky adduct on the 5′ (upstream) side of a break may cause little interference with transcription because the RNAP is already bound and translocating along the DNA backbone on the upstream side of an SSB. It is also possible that bulky 5′ adducts do not interfere with SSB bypass because they are extruded from the transcription complex during 3′ end capture, especially if this capture occurs from the post-translocated state of the RNAP, which would leave the 5′

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terminus of the SSB farther from the catalytic center of the polymerase.[27−29] This suggests that a bulky backbone adduct, in contrast to a bulky base,[30] produces relatively little interference with transcription because it is extruded from the transcription complex. In accordance with that explanation, we found that thymine with a biotinylated linker at the C5 position (which is on the side of the base opposite Watson−Crick base pairing) within an intact template strand produces almost no transcription blockage for T7 RNAP (Figure 2, lane 10). It has also been shown that bulky aminofluorene adducts in the TS do not cause significant arrest of T7 RNAP.[31] Both thymine biotinylated at C5 and aminofluorene contain bulky and flexible adducts that face away from the region of Watson−Crick base pairing. It is possible that this orientation of a bulky base adduct allows for T7 RNAP bypass.

In the case of a 5′-OH|3′-biotin or 5′-OH|3′-C3 SSB, 3′ end capture could be inhibited because the biotin or carbon group is too large or unrecognizable to be efficiently captured and reinserted into the polymerase. In contrast, at a 5′-biotin|3′-OH or 5′-C3|3′-OH SSB, the smaller 3′-OH group is probably more easily recognized and captured by T7 RNAP, allowing for a greater extent of SSB bypass. The notion that size plays a role in 3′ terminus recognition and capture is supported by our finding that, at 5′-OH SSBs, the extent of T7 RNAP blockage increases with an increased size of 3′ end groups (C3 < C6 < biotin) (Figure 5). Our proposed model for transcription arrest at SSBs supports the hypothesis of Doetsch and co-workers, from template strand "thread-in" experiments, which suggested that T7 RNAP has difficulty restarting transcription on the downstream side of a large gap when there is a 3′-PO4 terminus because of charge and/or size.[10]

In some instances, we found that bulky 5′ adducts enhance transcription arrest at SSBs. We observed that the substitution of a 5′-PO4 for a 5′-OH group at SSBs containing bulky 3′ groups exacerbates transcription arrest for T7 RNAP (in most cases increasing the extent of blockage to 100%) (Figure 5, lanes 8−12). Thus, it seems that, in the case of inhibited 3′ end capture, the presence of a bulky 5′ adduct at a TS SSB exacerbates transcription arrest. Interestingly, 5′-C3|3′-OH SSBs caused less transcription arrest than 5′-OH|3′-OH SSBs for both T7 RNAP and RNAP II, suggesting that the 1,3-propanediol adduct aids SSB bypass when the adduct is linked to the 5′ side of a break. It is possible that the 5′-C3 adduct, which is less bulky than 5′-biotin, somehow stabilized the transcription complex, permitting more efficient bypass or tracking across the SSB. This sort of interaction may not be possible on the downstream side of the break if the polymerase must actively engage the downstream terminus to continue translocation. We also found that, in contrast to the T7 RNAP results, RNAPII was arrested to nearly the same extent at 5′-biotin|3′-OH and 5′-OH|3′-biotin SSBs. These results, however, might be affected by enzymatic reactions with biotin in HeLa extracts (Figure S3 of the Supporting Information).

In addition to our findings regarding effects of end chemistry on transcription arrest at SSBs, we detected a difference between the level of transcription arrest caused by SSBs with no gap and that caused by SSBs with a one-nucleotide gap. SSBs with no gap occur most frequently as DNA repair intermediates, either preceding the final ligation step of excision repair pathways or following TDP1 cleavage after abortive TOPI activity. Such SSBs contain 5′-PO4|3′-OH and 5′-OH|3′-PO4 end groups, respectively. In our study, 5′-PO4|3′-OH and 5′-OH|3′-PO4 SSBs with no gap blocked T7 transcription 56 and 71% of the time, respectively, suggesting such repair intermediates may pose a block to transcription in vivo. All of the gapless SSBs tested (both naturally occurring and unnatural) caused a slight decrease in the level of transcription arrest when compared to one-nucleotide gap SSBs with the same end chemistries. This consistent finding suggests that the one-nucleotide gap present at most naturally occurring SSBs increases the likelihood of transcription arrest at those sites.

At the physiological level, our findings with T7 RNAP have implications for the mechanism of arrest and bypass at SSBs by RNAPs, especially human mitochondrial RNA polymerase, which is similar in structure and mechanism to T7 RNAP.[32] This relevance is highlighted by the fact that mitochondrial DNA is prone to SSBs because of the elevated levels of ROS observed in mitochondria.[33] Furthermore, the observed patterns of enhanced arrest at SSBs with bulky 3′ adducts also arise with RNAP II. We conclude that SSB bypass by T7 RNAP and RNAP II is significantly hindered at SSBs with large 3′ end chemistries. This blockage may play a part in alterations in gene expression and transcriptional mutagenesis due to SSBs. The RNAP arrest might also trigger a gratuitous form of transcription-coupled DNA repair that could be deleterious.

■ ASSOCIATED CONTENT

Supporting Information

Figures related to the construction and confirmation of the structure of transcription substrates. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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■ ABBREVIATIONS

SSB, single-strand break; 5′-PO4, 5′-phosphate DNA end chemistry; 3′-PO4, 3′-phosphate DNA end chemistry; 5′-OH, 5′-hydroxyl DNA end chemistry; 3′-OH, 3′-hydroxyl DNA end chemistry; 5′-dRP, 5′-deoxyribose sugar DNA end chemistry; 3′-dRP, 3′-deoxyribose sugar DNA end chemistry; 3′-C3, 3′ 1,3-propanediol DNA end chemistry; 3′-C6, 3′ 1,6-hexanediol DNA end chemistry; TS, transcribed strand; NTS, non-transcribed strand; BER, base excision repair; TOPI, topoisomerase I; RNAP, RNA polymerase.

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