The effects of covalent additions of a psoralen on transcription by *E. coli* RNA polymerase

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ABSTRACT

Synthetic DNA substrates containing a site-specifically engineered psoralen monoadduct or diadduct were used to characterize the response of the *E. coli* RNA polymerase elongation complex to these lesions. The psoralen derivative HMT (4'-hydroxymethyl-4, 5', 8-trimethylpsoralen) was site specifically placed into two synthetic double-stranded DNA fragments each of which contained an *E. coli* RNA polymerase promoter at one end. The HMT molecule was attached to the middle of the DNA fragments as either a furan-side monoadduct or an interstrand diadduct. Transcription off the HMT crosslinked DNA templates showed that *E. coli* RNA polymerase terminated at the HMT diadduct site, i. e., one nucleotide before the modified thymidine residue on the template strand. The furan-side monoadduct when on the template strand also blocked transcription by the polymerase. However, no effect on transcription was observed when the monoadduct was located on the non-template strand.

INTRODUCTION

Psoralens (furocoumarins) are a class of compounds which can intercalate between base pairs of double-stranded nucleic acids. Upon near UV irradiation, the intercalated psoralens can photoreact with pyrimidine bases to yield monoadducts as well as interstrand diadducts (for review see 1-3). In the first step of the photoreaction either a furan-side or pyrone-side monoadduct can be formed depending upon whether the 4', 5'-double bond or the 3, 4-double bond of the psoralen reacts with the 5, 6-double bond of a pyrimidine base. By absorbing a second photon, the furan-side monoadduct can be converted into a diadduct if there is another adjacent pyrimidine base available for photoreaction on the other strand (4-5). Psoralens react mostly with thymidine residues in DNA and uridine residues in RNA, although a minor reaction with cytosine residues also occurs (6-7). The structures of the adducts formed between the psoralen derivative HMT (4'-hydroxymethyl-4, 5', 8-trimethylpsoralen) and thymidine (T) are shown in figure 1. These psoralen-nucleic acid adducts are photoreversible upon exposure to light in their respective absorption regions (8-9).

The photoreactivity of psoralens with nucleic acids has found application in the medical and biological fields. Psoralens are used in the treatment of human skin diseases (10-11) and for probing nucleic structure (1-3, 12-14) and nucleic acid-protein interactions (15-16). The nucleic acid lesions formed by psoralen plus near UV (320-400 nm) have both mutagenic and

Figure 1. Structure of HMT (4'-hydroxymethyl-4, 5', 8-trimethylpsoralen) and HMT-thymidine photoproducts. M_{Fu}: HMT-thymidine furan-side monoadduct; M_{Py}: HMT-thymidine pyrone-side monoadduct; T-HMT-T: thymidine-HMT-thymidine diadduct.

lethal effects (17-19) and can be recognized by repair systems (20-25). It has been shown that psoralen interstrand diadducts block replication by *E. coli* DNA polymerase I, whereas psoralen monoadducts act as kinetic attenuators when located on the template strand and have no effect when located on the nick translated strand (26-27). However, psoralen monoadducts on a single-stranded DNA template terminate DNA synthesis by *E. coli* DNA polymerase I large fragment, T4 DNA polymerase, and AMV reverse transcriptase (28).

In this communication, we report the effects of HMT adducts on transcription by *E. coli* RNA polymerase. The psoralen derivative was site specifically placed in two double-stranded synthetic DNA fragments downstream from an *E. coli* RNA polymerase promoter as either a diadduct or a furan-side monoadduct. Transcription on the psoralen modified templates indicate that the diadduct blocks transcription as would be expected by preventing strand separation of

the template by the polymerase. The furan-side monoadduct also terminates transcription when located on the template strand but has no effect when attached to the non-template strand.

MATERIALS AND METHODS

Materials

HMT was a gift from HRI Associates Inc. (Berkeley, CA). *E. coli* RNA polymerase holoenzyme (40-50% active) was a gift from M. Chamberlin. All oligonucleotides were synthesized on an automated DNA synthesizer. After synthesis, the oligonucleotides were deprotected and purified by electrophoresis on polyacrylamide-urea gels followed by extraction and EtOH precipitation. T4 DNA ligase and ^{32}P labelled ribonucleotides were purchased from Amersham. T4 polynucleotide kinase was obtained from New England Biolabs. 3'-OCH₃ nucleoside triphosphate derivatives were acquired from Pharmacia Molecular Biologicals. The concentration of double-stranded DNA was measured by absorption using an extinction coefficient of $1.2 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}/\text{bp}$ (29).

Preparation of Double-stranded DNA Templates

The double-stranded 138mer shown in figure 2 contains an *E. coli* RNA polymerase *tac* promoter (30) at the left end with an HMT molecule attached to the site indicated as either an interstrand diadduct (XL-138mer) or a furan-side monoadduct on the non-template strand (M_{Fu} -138mer). The unmodified (UM-138mer) and the psoralen modified 138mer templates were prepared as described (15).

The double-stranded 140mer shown in figure 3 is similar to the double-stranded 138mer. The psoralen modified 140mer contains an HMT molecule attached to the site indicated as either an interstrand diadduct (XL-140mer) or a furan-side monoadduct on the template strand (M_{Fu} -140mer). The 140mer templates were prepared as described below.

UM-140mer. 5.7 μg of the 58mer (the oligonucleotide at the 5'-end of the bottom strand, see figure 3) was end-labelled with ^{32}P in 50 μl 1X kinase buffer (50 mM TrisHCl, 10 mM MgCl₂, 0.1 mM spermidine, 0.1 mM EDTA, and 5 mM DTT, pH 7.5) containing 0.6 mCi [γ - ^{32}P]ATP (3000 Ci/mmol, lyophilized to dryness and dissolved back in H₂O) and 20 units of T4 polynucleotide kinase at 37°C for 18 hours. The 75mer (2.9 μg) of the top strand and the 70mer (2.5 μg) and the 12mer (3 μg) of the bottom strand were 5'-phosphorylated together in 25 μl 1X kinase buffer containing 4.8 mM ATP and 20 units of T4 polynucleotide kinase at 37°C for 18 hours. Both kinased samples were extracted with phenol (two equal volume phenol extractions, pH 7.8, followed by one back extraction of the phenol phase with water to recover any DNA in the phenol phase, and then two ether extractions of the combined aqueous phase). Half of the labelled 58mer (the other half was used in the preparation of the M_{Fu} -140mer, see below) was combined with the other kinased oligonucleotides and precipitated with EtOH. These oligonucleotides were then dissolved in ligation buffer together with 2.1 μg of the top strand 65mer, heated at 65°C for 10 min., and incubated at room

temperature for at least 20 min. to allow the oligonucleotides to hybridize. BSA (to 200 $\mu\text{g/ml}$), ATP (to 1.6 mM), and 6.3 units of T4 DNA ligase were added to the mixture (final volume of 50 μl in 50 mM Tris HCl, 10 mM MgCl_2 , 8 mM EDTA, and 10 mM DTT, pH 7.5). The DNA was ligated at room temperature (22-24°C) for 2 hours and then at 4°C for 20 hours. The product was extracted with phenol, vacuum dried in a Speedvac concentrator (Savant Instrument Inc.) and purified by electrophoresis on an 8% polyacrylamide-8 M urea gel (40 cm x 20 cm x 0.5 cm, 20 W for 2-3 hours, see figure 4A). The mobilities of the two strands were identical and consequently both strands (only one was labelled) were isolated from the gel as a single band. The DNA was then electro-eluted out of the gel and purified by EtOH precipitation. To ensure that all fragments were double-stranded, the DNA was further purified on an 8% non-denaturing polyacrylamide gel (40 cm x 20 cm x 0.8 cm, 5 W for 2-3 hours) to remove any single-stranded DNA. The overall yield was 65%.

HMT-Modified 140mers. The furan-side monoadducted double-stranded 140mer ($M_{\text{Fu}}\text{-140mer}$) was prepared first. An HMT furan-side monoadduct was site specifically placed on the thymidine residue of the bottom strand 12mer as previously described (31). This monoadducted 12mer was substituted for the 12mer of the bottom strand in the ligation reaction described above to produce the double-stranded 140mer with a site specific HMT monoadduct ($M_{\text{Fu}}\text{-140mer}$). Although psoralen monoadducted short oligonucleotides run slower than the corresponding unmodified oligonucleotides (31-32), incorporation of a psoralen monoadduct into oligonucleotides longer than 60 bases does not alter their electrophoretic mobility (15). Therefore, the component strands of the $M_{\text{Fu}}\text{-140mer}$ could be isolated as a single band in a denaturing gel. The $M_{\text{Fu}}\text{-140mer}$ was purified as above with a 60% overall yield.

To generate the HMT crosslinked 140mer (XL-140mer), the purified $M_{\text{Fu}}\text{-140mer}$ (in 10 mM TrisHCl, 1 mM EDTA, and 10 mM NaCl, pH 7.4) was heated at 65°C for 5 min., cooled at room temperature for 20 min., and then irradiated at room temperature for 4 min. with 0.3 W/cm^2 of 320-380 nm light from a 2.5 kW Hg/Xe lamp, the output of which was filtered through a pyrex glass and an aqueous cobaltous nitrate solution [1.7% $\text{Co}(\text{NO}_3)_2$, 2% NaCl, 9 cm pathlength] (8). This irradiation resulted in 80% conversion of the $M_{\text{Fu}}\text{-140mer}$ to XL-140mer. The XL-140mer was separated from the $M_{\text{Fu}}\text{-140mer}$ by electrophoresis on an 8% polyacrylamide-8 M urea gel (see figure 4B) and purified as above.

Transcription and RNA Sequencing

The DNA template (1 nM) and *E. coli* RNA polymerase holoenzyme (30 nM) were preincubated together at 37°C for 5 min in transcription buffer (40 mM TrisHCl, 10 mM MgCl_2 , 100 mM KCl, 0.1 mM EDTA, and 0.1 mM DTT, pH 8.0). Transcription was initiated by the addition of 0.2 mM ATP, 0.2 mM GTP, 0.2 mM CTP, and 10 μM [$\alpha\text{-}^{32}\text{P}$]UTP (100 Ci/mmol) (final concentrations in 25 μl of transcription buffer). After incubation at 37°C for additional 10 min., the reaction was stopped by adding 100 μl stop buffer (3.75 mM EDTA, 125 mM LiCl, 3 μg carrier tRNA, and 0.05% SDS) followed by EtOH precipitation (3 fold

Top Strand: 5'-51mer-18mer-12mer-14mer-43mer-3'

12mer: 5'-GAAGCTACGAGC-3'

14mer: 5'-TGAGGCCATCGATA-3'

18mer: 5'-CAGACTCAGTACGAATTC-3'

43mer: 5'-AAGGTCTAGATCTCCCTATAGTGAGTCGTATTAATTTTCGACGT-3'

51mer: 5'-CCCATCCCCCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTG-3'

Bottom Strand: 5'-40mer-52mer-46mer-3'

40mer: 5'-TACGTCGAAATTAATACGACTCACTATAGGGAGATCTAGA-3'

46mer: 5'-TCCACACATTATACGAGCCGATGATTAATTGTCAACAGGGGGATGG-3'

52mer: 5'-CCTTTATCGATGGCCTCAGCTCGTAGCTTCGAATTCGTAAGTCTGCAAT-3'

DS 138mer:

Figure 2. Sequence of the double-stranded 138mer and the oligonucleotides from which the 138mer was synthesized. The numbers above the sequence are relative to the *E. coli* RNA polymerase transcription start site. The arrow denotes the thymidine residue on the top strand (non-template strand) to which an HMT furan-side monoadduct is attached in the M_{Fu} -138mer. The solid triangle denotes the thymidine residue on the bottom strand (template strand) which is additionally modified in the XL-138mer.

EtOH at -20°C for overnight). The precipitated RNA transcripts were dissolved in 95% deionized formamide containing 10 mM TrisHCl, 1 mM EDTA, and 10 mM NaCl, pH 7.4 and analyzed on a 20% polyacrylamide-7 M urea gel.

RNA sequencing was modeled after the protocol described by Reisbig and Hearst (33). The reactions were performed as above except for the concentration of the nucleotide being analyzed, which was 13.4 μM for ATP, 6.7-13.4 μM for GTP, 10 μM for UTP, and 13.4 μM for CTP, respectively. The corresponding 3'-OCH₃ nucleoside triphosphate analog concentrations were 0.48 mM A, 0.4 mM G, 0.48 mM U, and 0.2 mM C, respectively. The sequencing samples were treated and analyzed as described above.

RESULTS

A synthetic double-stranded 138mer with or without an HMT adduct at a specific site was constructed from eight oligonucleotides as shown in figure 2 (15). The HMT molecule was attached to T-29 of the non-template strand as a furan-side monoadduct in the M_{Fu} -138mer and

Bottom Strand: 5'-58mer-12mer-70mer-3'

12mer: 5'-GAAGCTACGAGC-3'

58mer: 5'-TACGTGCGAAATTAATACGACTCACTATAGGGAGATCTAGACCTTTATCGATGGCCTCA-3'

70mer: 5'-GAATGTTCTGTAAGTCTCAATTCACACATTATACGAGCCGATGATTAATTGTCAAC-
-AGGGGGATGG-3'

Top Strand: 5'-65mer-75mer-3'

65mer: 5'-CCCATCCCCCTGTTGACAATTAATCATCGGCTCGTATAATGTGTGGAATTGGAGAGTGAG-
-TACGA-3'

75mer: 5'-ACATTTCGTCGTAGCTTCTGAGGCCATCGATAAAGGTCTAGATCTCCCTATAGTGAGTCG-
-TATTAATTTGACGT-3'

DS 140MER:

Figure 3. Sequence of the double-stranded 140mer and the oligonucleotides from which the 140mer was synthesized. The numbers above the sequence are relative to the *E. coli* RNA polymerase transcription start site. The arrow denotes the thymidine residue on the bottom strand (template strand) to which an HMT furan-side monoadduct is attached in the M_{Pu} -140mer. The solid triangle denotes the thymidine residue on the top strand (non-template strand) which is additionally modified in the XL-140mer.

to both T-29 of the non-template strand and T-30 of the template strand as an interstrand diadduct in the XL-138mer. The effects of these HMT additions on transcription by *E. coli* RNA polymerase were investigated by analyzing the RNA transcripts produced from the unmodified and HMT-adducted templates. 3'-OCH₃ RNA sequencing on the unmodified template was used to determine the exact termination sites. It is known that during transcription the RNA polymerase transiently unwinds the DNA template and that a newly incorporated RNA chain forms a short RNA-DNA hybrid with the template strand (34). Consequently, the covalent interstrand HMT diadduct in the XL-138mer blocks forward movement of RNA polymerase by preventing DNA unwinding and strand separation. As expected, figure 5 shows that the HMT diadduct efficiently blocked elongation thus yielding a transcript terminated with U-29, i.e., one base before the adducted thymidine (T-30) on the template strand (lanes 3 and 4). In contrast, the furan-side monoadduct on the non-template strand had no effect on transcription (compare lanes 1 and 2). Both the UM-138mer and the M_{Pu} -138mer yielded runoff transcript (the slowest moving band). The band running slightly faster than the runoff

Figure 4. Preparation of double-stranded 140mer templates. A) 8% polyacrylamide-8M urea gel purification of the ligation products with ^{32}P -label on the 5'-end of the bottom strand 58mer. B) Preparation of XL-140mer from end labelled M_{Fu} -140mer. The M_{Fu} -140mer was irradiated with 320-380 nm light and the reaction mixture was electrophoresed on an 8% polyacrylamide-8 M urea gel to purify the XL-140mer.

transcript corresponds to transcription termination in the AT rich region at the right end of the template. This product was found to decrease when higher UTP concentration was used (data not shown).

These results show that the HMT diadduct blocks transcription whereas the monoadduct has no effect when it is located on the non-template strand. In a series of experiments to study the interaction of T7 RNA polymerase with DNA in elongation complexes arrested at psoralen adduct sites by DNase I footprinting techniques, we have shown that an HMT diadduct also blocks transcription by T7 RNA polymerase(16). However, in these experiments an HMT furan-side monoadduct on the template strand acted equally well as a barrier to transcription, suggesting that such a monoadduct might also block transcription by *E. coli* RNA polymerase. To test this hypothesis, a double-stranded 140mer was constructed.

As shown in figure 3, the double-stranded 140mer consists of five oligonucleotides, three on the bottom strand and two on the top strand. These oligonucleotides were hybridized together and ligated to produce the unmodified 140mer (UM-140mer) (figure 4A). To make the HMT modified templates, a bottom strand 12mer which contained an HMT furan-side monoadduct on the thymidine residue was used in the hybridization and ligation reaction to produce M_{Fu} -140mer with an HMT furan-side monoadduct attached to T-32 of the template strand (figures 3 and 4A). The M_{Fu} -140mer was then irradiated with 320-380 nm light to generate the XL-140mer with an HMT attached to both T-32 of the template strand and T-31 of the non-template strand (figures 3, 4B).

Figure 6 shows the results of transcription on the double-stranded 140mer templates. The

Figure 5. Analysis of the transcripts from UM-138mer, M_{Fu} -138mer, and XL-138mer. Lanes A, G, U, and C are the corresponding 3'-OCH₃ RNA sequencing reactions. Lane 1: UM-138mer; Lane 2: M_{Fu} -138mer; Lanes 3 and 4: XL-138mer.

Figure 6. Analysis of the transcripts from UM-140mer, M_{Fu} -140mer, and XL-140mer. Lanes A, G, U, and C are the corresponding 3'-OCH₃ RNA sequencing reactions. Lane 1: UM-140mer; Lane 2: XL-140mer; Lane 3: M_{Fu} -140mer.

UM-140mer yielded runoff transcript and some shorter transcripts (lane 1), which were reduced upon increasing the UTP concentration during transcription (data not shown). The HMT diadduct in the XL-140, as expected, stopped transcription by the polymerase (lane 2). Similar to the result obtained with T7 RNA polymerase (16), the HMT furan-side monoadduct blocked elongation by *E. coli* RNA polymerase (lane 3). Both the XL-140mer and M_{Fu}-140mer templates yielded a transcript 31 bases long, i.e., terminated one base before the HMT adducted thymidine residue (T-32) on the template strand.

DISCUSSION

DNA damages, such as thymidine glycols (35-36), abasic sites (37), UV photoproducts (38-40), and chemical lesions (40-41), often block DNA synthesis. Psoralen interstrand diadducts in double-stranded DNA (27) and psoralen monoadducts in single-stranded DNA (28) have been shown to arrest replication. In contrast, psoralen monoadducts in double-stranded DNA have a negligible effect on DNA synthesis by *E. coli* DNA polymerase I (27). Little is known about the effects of psoralen adducts on transcription. We have determined, using a double-stranded DNA substrate, that either orientational isomer of an HMT diadduct effectively blocks transcription by *E. coli* RNA polymerase whereas a furan-side HMT monoadduct arrests transcription only when on the template strand. The mechanism of transcription termination may be fundamentally different for the two lesions. While the monoadducted thymidine is no longer able to function as a template for RNA synthesis, the diadduct blocks elongation either due to the non-coding character of the adducted thymidine or by preventing DNA unwinding and strand separation. A similar pattern of inhibition has been observed by us for RNA synthesis by T7 RNA polymerase (16) and for DNA synthesis by T4 DNA polymerase (42). These results indicate that a psoralen monoadducted thymidine is usually non-coding and that the ability of *E. coli* DNA polymerase I to synthesize past such a modified base may be an example of error prone synthesis. Although we have not examined the effects of a psoralen pyrone-side monoadduct on transcription, its structural similarity to the furan-side monoadduct would imply a similar mode of action.

Though the results reported here are the *in vitro* effects of psoralen adducts on transcription, analyses of cellular RNA in cells treated with psoralen and near UV have also shown that these adducts block RNA synthesis (43, 44, 45). Hanawalt (46) has suggested that the blockage of transcription, i.e., the arrested elongation complex, could act as a signal of DNA repair. If this is true, our data would suggest that monoadducts on a template strand would be repaired efficiently, whereas those on a non-template strand would not. Recent data by Vos and Hanawalt (47) supports such a prediction.

Using 3'-OCH₃ RNA sequencing the exact transcription termination sites induced by the HMT diadduct and the HMT furan-side monoadduct on the template strand have been determined. In both cases, RNA synthesis proceeds to the 5' neighboring base of the HMT

adducted thymidine residue on the template strand. It is known that during the elongation process, RNA polymerase opens approximately 12-17 base pairs of DNA template (34, 48). Based on an unwinding angle analysis of *E. coli* RNA polymerase complexes at different stages of transcription, Gamper and Hearst (48) have proposed a topological model for transcription elongation, in which they suggest the existence of unwindase and rewindase activities on the polymerase. During elongation, the unwindase opens the DNA helix while the rewindase, lagging by 17 base pairs, displaces the RNA transcript and reseals the DNA helix. If the model is correct, our results suggest that the unwindase and the catalytic sites of the enzyme are located very close to each other since RNA synthesis proceeds up to the diadduct site in both the XL-138mer and XL-140mer templates, where the unwinding site is assumed to be arrested by the interstrand crosslink. In fact, the two enzymatic sites could be one and the same. In this case the unwindase activity would be directly linked to nascent RNA chain synthesis, which would extend an RNA-DNA helix and consequently unwind and strand separate the DNA helix. Using an in vitro transcription assay, Phillips and Crothers (49) have found that RNA synthesis proceeds up to bases adjacent to preferred intercalation sites of the drug actinomycin D. Since drug intercalation requires a double-stranded helix, this result also suggests that strand separation does not extend more than one or two bases ahead of the catalytic site of the enzyme.

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ABBREVIATIONS

HMT	4'-hydroxymethyl-4, 5', 8-trimethylpsoralen
M _{Fu}	HMT furan-side monoadduct
XL	HMT crosslink
EDTA	Ethylenediaminetetraacetic acid
EtOH	Ethanol
Tris	Tris(hydroxymethyl)aminomethane
SDS	Sodium dodecyl sulfate

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