DNA base damage products either formed spontaneously or as a result of exposure to various genotoxic agents were examined for their effects on *Escherichia coli* RNA polymerase-mediated transcription *in vitro*. Uracil, O6-methylguanine (O6-meG), and 8-oxoguanine (8-oxoG) were placed at specific sites downstream from the transcriptional start site on the transcribed strand of a duplex template under the control of the strong tac promoter. *In vitro*, single-round transcription experiments carried out with purified *E. coli* RNA polymerase revealed efficient bypass at the three lesions examined and subsequent generation of full-length runoff transcripts. Transcript sequence analysis revealed that *E. coli* RNA polymerase inserted primarily adenine into the transcript opposite to uracil, uracil opposite to O6-meG, and either adenine or cytosine opposite to 8-oxoG. Thus, a uracil in the DNA template resulted in a G-to-A transition mutation in the lesion bypass product whereas O6-meG produced a C-to-U transition mutation and 8-oxoG generated either the correct transcriptional product or a C-to-A transition mutation. When 8-oxoG was placed within close proximity to the transcriptional start site (within the region required for effective promoter clearance), a reduced of full-length, runoff transcript was observed, indicative of lower promoter clearance. Taken together, these results demonstrate that the DNA base damages studied here may exert significant *in vivo* effects on gene expression and DNA repair with respect to the production of mutant proteins (transcriptional mutagenesis), or decreased levels of expressed proteins.

There are three major steps for *Escherichia coli* RNA polymerase (RNAP) to properly effect DNA template-dependent transcription: promoter binding and initiation, RNA chain elongation, and chain termination (1). Any of these steps could potentially be perturbed by DNA base damage occurring on the template strands of transcribed gene sequences, leading to various deleterious consequences for a cell. For example, certain bulky lesions such as UV light-induced cyclobutane pyrimidine dimers and psoralen adducts if located on the transcribed strand of DNA have the ability to permanently arrest RNA polymerase at the site of damage during elongation (2–4). If left un repaired, these lesions block gene expression. In *E. coli*, it is has been demonstrated that RNAP arrested at a DNA lesion is recognized by factors that may displace the arrested polymerase and recruit the appropriate repair machinery components to the damaged site in order to facilitate nucleotide excision repair (2, 5, 6). It has also been shown that damages such as UV-light-induced cyclobutane pyrimidine dimers, when located on the template strands of actively transcribed genes, are repaired preferentially compared with the non-template strand as well as nonexpressed regions of the genome (7–9). Thus, transcription-coupled repair is dependent on the ability of a specific DNA lesion to arrest an elongating RNAP. However, there exist several examples of DNA lesions such as abasic sites, uracil, dihydrouracil, and certain types of strand breaks that are efficiently bypassed by the *E. coli* transcription elongation complex, and thus presumably not subject to transcription-coupled repair (10–13). Furthermore, these damages have been shown to be miscoding lesions for RNAP *in vitro*, causing base substitutions in the resulting transcripts.

In addition to effects on RNAP elongation, it can be envisioned that certain base damages could also affect transcriptional initiation by altering the efficiency of the transition of the RNAP from the initiation state to an elongation complex (promoter clearance). Several reports have shown that ability of RNAP to clear the promoter may be dependent on the nucleotide sequence of the initially transcribed region of DNA (14–16). Damaged bases within this initial region could affect promoter clearance and subsequently production of a full-length transcript. The effects of DNA lesions on this process are unknown.

The goal of this work was to extend our previous studies concerning the effects of nonbulky DNA base lesions on elongation of various phage RNA polymerases by both expanding the range of lesions studied, and also by examining these effects on the more complex, multi-subunit *E. coli* RNAP. Uracil, 8-oxoG, and O6-meG are important DNA base damage products formed cytosine deamination, oxygen radical attack of guanine, and alkylating agent-induced methylation of guanine, respectively (17). Each of the lesions studied are repaired by distinct base excision repair enzymes (uracil and 8-oxoG) or direct reversal proteins (O6-meG) with specificity for that base damage (17). We examined whether these lesions allowed bypass of RNAP and, if so, whether these lesions were mutagenic at the level of transcription. In addition to further generalizing our previous elongation studies, we wished to study the effects of these damages on promoter clearance. From these experiments, we find that (i) uracil, 8-oxoG, and O6-meG are lesions that do not block *E. coli* RNAP during elongation, (ii) these lesions cause mutagenic base insertion RNAP during transcription, and (iii) when placed near the transcriptional start site, 8-oxoG decreases promoter clearance. These findings have broad implications for the effects of DNA damage on gene expression.

Anand Viswanathan‡ and Paul W. Doetsch§

*From the ‡Graduate Program in Genetics and Molecular Biology and §Departments of Biochemistry and Radiation Oncology, Emory University School of Medicine, Atlanta, Georgia 30322. The abbreviations used are: RNAP, RNA polymerase; O6-meG, O6-methylguanine; 8-oxoG, 8-oxoguanine; Fpg, formamidopyrimidine-DNA glycosylase; PFR, polymerase chain reaction; bp, base pair(s); nt, nucleotide(s); ITIS, initial transcribed sequence.

This paper is available on line at http://www.jbc.org
expression, transcription-coupled repair, and transcriptional mutagenesis.

**EXPERIMENTAL PROCEDURES**

**Materials**—The pGEM-2 in *vitro* transcription vector was purchased from Promega. Undamaged oligonucleotides for use as PCR primers for transcription duplex DNA template synthesis were synthesized by the Emery Microchemical Facility. Oligonucleotides containing uracil, 8-oxoG, and O6-methylG were obtained from Life Technologies, Inc. and National Biosciences, Inc. The 5-propynyl cytosine-containing oligonucleotide was obtained from Genosys, Inc. Heparin and RNase inhibitor were purchased from Sigma and Promega, respectively. High performance liquid chromatography-purified nucleotide triphosphates, [α-32P]UTP (3000 Ci/mmol), and [γ-32P]ATP (3000 Ci/mmol) were from Amersham Pharmacia Biotech. QIAEX DNA extraction kit was from Qiagen.

**Enzymes**—Purified *E. coli* RNAP (21) was a generous gift from Charles Turnbough (Birmingham, AL). Moloney murine leukemia virus reverse transcriptase was purchased from Stratagene. T4 polymerase kinase was purchased from New England Biolabs. *E. coli* endonuclease III was a gift from Richard P. Cunningham (Albany, NY). Uracil DNA N-glycosylase was purchased from Epicenter Technologies. *E. coli* endonuclease IV and Fpg protein were gifts from Yoke W. Kow (Atlanta, GA). Tqg DNA polymerase for PCR amplifications was purchased from Promega.

**Construction of *E. coli* RNA Polymerase Transcription Templates**—A series of oligonucleotides were used as PCR primers to generate linear duplex transcription templates for elongation and promoter clearance experiments. For elongation studies, four different 139-bp duplex transcription templates were generated via PCR amplification using a modification of a method originally described by Zhou and Doetsch (10) employing the plasmid pGEM-2, an upstream primer oligonucleotide (68-mer, containing the tac promoter) and a downstream primer containing either no damage (ND-38-mer), 5-propynyl cytosine (PROC-38-mer), uracil (UR-38-mer), 8-oxoG (8OG-31-mer), or O6-methylG (O6MEG-31-mer) 4 nt downstream from the transcription start site. The products (Fig. 1A) contained either no damage (template ND-elong), 5-propynyl cytosine (template PROC-elong), or uracil (template UR-elong) 4 nt downstream from the transcription start site or 8-oxoG (template 8OG-elong) or O6-methylG (template O6MEG-elong) 63 nt downstream from the transcription start site. For promoter clearance studies, two different 94-bp duplex transcription templates were generated via a similar strategy except that a segment of duplex template ND-elong was PCR-amplified with upstream primer oligonucleotide 21-mer and downstream undamaged oligonucleotide C-45-mer or 8-oxoG-containing oligonucleotide 8OG-45-mer. The products (Fig. 1B) contained either no damage (template ND-clear) or 8-oxoG (template 8OG-clear) 4 nt downstream from the transcription start site.

**Trancription Experiments**—Single-round transcription reactions with *E. coli* RNAP were carried out as described previously (18) with several modifications. Briefly, the transcription buffer contained 40 mM Tris-HCl, pH 7.95, 50 mM KCl, 10 mM MgCl2, 2 mM ATP, 5 mM dithiothreitol, and RNasin at 0.8 unit/μl. Reaction mixtures containing 0.5 pmol of DNA template, RNAP, and 1.5 μl [α-32P]UTP were incubated at 37 °C for 10 min to form initiation complexes. After preincubation, heparin (250 μg/ml) and GTP, CTP, and cold UTP (0.4 μM each) were added (20 μl final reaction volume), and 4-μl aliquots were removed at various time intervals. Transcripts were analyzed on denaturing 10% polyacrylamide gels and detected by autoradiography and quantified by PhosphorImager analysis (Molecular Dynamics model 445 SI).

**RNA Sequencing**—Full-length RNA transcripts were gel-purified and cDNAs were generated with MMLV reverse transcriptase and a primer covering the 5′ end of the RNA transcript under conditions recommended by the supplier (Stratagene). The resulting cDNAs were PCR-amplified with both 5′ and 3′ primers covering the 5′ and 3′ ends of the transcript using Tqg DNA polymerase. These amplified products were subsequently sequenced by dideoxy sequencing using the [32P]-labeled 5′ primer under conditions recommended by the supplier (U. S. Biochemical Corp.) and as described previously (10).

**RESULTS**

**Construction of Transcription Templates Containing Various Base Damages at Specific Sites**—Duplex DNA templates were constructed by PCR amplification of a segment of the pGEM-2 vector (see “Experimental Procedures”). Transcription templates for elongation studies (139 bp) contained the *E. coli* tac promoter (49 bp) and a transcribable segment (90 bp) with various base modifications on the transcribed strand (Fig. 1A). Transcription templates for promoter clearance experiments were similar to the elongation templates except that the transcribable segment was shorter (45 bp) and contained 8-oxoG 4 nt downstream from the transcription start site (Fig. 1B). To confirm the identity of each of the templates, PCR reactions were conducted with 5′ end labeled downstream primers in order to generate labeled DNA templates containing no damage, uracil, 8-oxoG, or O6-methylG. Template URA-elong was treated with uracil glycosylase and subsequently with *E. coli* endonuclease IV, which generated the appropriate DNA cleavage product when analyzed on a DNA sequencing gel (data not shown). Likewise, templates 8OG-elong and 8OG-clear were treated with *E. coli* Fpg, which resulted in DNA cleavage at the sites of 8-oxoG and generation of the predicted, appropriate length DNA strand scission products. Template O6MEG-elong was confirmed by primer extension sequencing by DNA polymerase which yielded the appropriate misinserted base (T) opposite to O6-meG into the extended fragment (19). Furthermore, both the damaged primer and template exhibited altered gel mobilities due to the presence of O6-meG lesion and the melting temperature of the primer was significantly reduced as would be expected in the presence of this base damage product (20).

**E. coli RNAP Bypass of Nonbulky Base Damage during Elongation**—Damaged DNA templates were used in *in vitro* transcription experiments with purified *E. coli* RNAP (21). In order to determine the interaction of *E. coli* RNAP with each of the damaged bases during elongation, comparative single-round transcription experiments were carried out with templates CON-, PROC-, URA-, 8OG-, and O6MEG-elong (Fig. 1A). In these single-round transcription experiments, each template molecule is utilized only once by a single molecule of RNAP and the transcription products represent a single, promoter-dependent elongation event (10–12). Measurement of transcript formation at various times during a single round of elongation will directly reveal template sites that cause temporary pausing or permanent arrest of RNAP at a lesion site (10). The amount of transcript generated was monitored from 0 to 2 min following the start of the transcription elongation phase. The undamaged control template CON-elong, which contains cytosine 56 nt downstream and guanine 63 nt downstream from the transcriptional start site, produced a 90-nt full-length runoff transcript as expected (Fig. 2A, lanes 1–5). Base damage-containing templates URA-, 8OG-, and O6MEG-elong were also efficiently transcribed and generated full-length runoff transcription products (Fig. 2, lanes 6–20), indicating efficient bypass of uracil, 8-oxoG, and O6-methylG by *E. coli* RNAP. No temporary pausing or permanent arrest of RNAP at the lesion sites was observed as determined by the absence of shortened transcripts in the 56–63 nt size range. However, a template (PROC-elong) containing 5-propynyl cytosine at nt position 56 efficiently blocks transcription at this site indicating that this type of base modification is an effective block to transcriptional elongation under single-round transcription conditions (Fig. 2B). With the exception of PROC-elong, each template generated the 90-nt full length, runoff transcript with similar kinetics (Fig. 3). Thus, unlike our previous studies, which examined abasic sites and dihydrouracil effects on phage and *E. coli* RNAP (10), there was no evidence of temporary pausing of the polymerase at sites of uracil, 8-oxoG, and O6-methylG. We conclude that, when encountered by an elongating *E. coli* RNAP, uracil, 8-oxoG, and O6-methylG are efficiently bypassed with no detectable pausing or arrest.

**Sequence Analysis of Transcripts from Elongation Stud-
ies—In living cells, uracil is formed through the spontaneous deamination of cytosine (17). Therefore, if any base other than guanine is inserted opposite to uracil by RNAP, the resulting transcript will contain a base substitution mutation (10). Sequence analysis of transcripts generated from template URA-elong indicated the expected adenine insertion opposite to uracil (Fig. 4B). In the case of 8-oxoG, transcript sequence analysis revealed that E. coli RNAP inserted either adenine or cytosine opposite to this lesion with approximately equal frequencies (Fig. 4C). This result is similar to that previously observed for T7 RNA polymerase and 8-oxoG (22), indicating that this base damage may affect both types of RNA polymerases in a similar fashion. Sequence analysis of transcripts generated from template O6MEG-elong indicated preferential insertion of uracil opposed to uracil (Fig. 4B). In the case of 8-oxoG, transcript sequence analysis revealed that E. coli RNAP inserted either adenine or cytosine opposite to this lesion with approximately equal frequencies (Fig. 4C). This result is similar to that previously observed for T7 RNA polymerase and 8-oxoG (22), indicating that this base damage may affect both types of RNA polymerases in a similar fashion. Sequence analysis of transcripts generated from template O6MEG-elong indicated preferential insertion of uracil...
opposite to O6-meG (Fig. 4D). This insertion preference is similar to the effect of O6-meG on DNA polymerases (19). Thus, these three DNA base damages are highly mutagenic at the level of transcription in vitro, with uracil causing G → A transition mutations, 8-oxoG causing C → A transversion mutations or normal base insertions, and O6-meG causing C → U transition mutations.

Promoter Clearance Studies—An important step in transcriptional initiation is promoter clearance, the transition step where RNAP switches from an initial transcribing stage to an...
elaboration stage. During this step, the holoenzyme is involved in generating short RNA oligomers from the promoter-proximal sequence (initial transcribed sequence (ITS)) in abortive initiation. In order to determine whether nonbulky DNA base damages in the ITS could affect promoter escape and consequently the extent of gene expression, a transcription template containing 8-oxoG 4 nt downstream from the start of transcription was constructed (Fig. 1B). This damage was placed in close proximity to the promoter because it had been shown previously that alterations in the ITS contribute to variations in promoter activity (1, 16). In vitro transcription experiments were carried out to assess the effect of 8-oxoG on the extent of transcription associated with efficient promoter clearance. In order to accurately compare transcription in damaged versus undamaged templates, the amount of DNA used in each reaction was carefully controlled (see “Experimental Procedures”). The extent of promoter clearance was assessed as a function of the amount of full-length runoff transcript that was generated from either the undamaged (CON-clear) or damaged (8OG-clear) template. Transcription products were quantified by polyacrylamide gel electrophoresis followed by PhosphorImager analysis (see “Experimental Procedures”). It was found that runoff transcript, and hence promoter clearance, was reduced by approximately 50% when 8-oxoG was placed near the promoter (Fig. 5). From these data, we conclude that oxidative base damage, if located near the promoter, can significantly reduce gene expression at the level of transcription.

**DISCUSSION**

The results of this study demonstrate that various types of DNA damage can have different effects on transcription by *E. coli* RNAP when such damages are located on the template strand of the transcribed DNA segment. First, we have shown that several nonbulky lesions affect the quality of the transcription products (i.e. mutation-containg) by allowing for efficient bypass and mutagenic base insertion during elongation. We have also demonstrated that when certain base damages such as 8-oxoG are located close to the transcriptional start site, there is a significant negative effect on promoter clearance by RNAP and a corresponding decrease in the level of transcript produced. It is conceivable that these lesions, which are efficiently bypassed by RNAP, may negatively influence their own repair if situated on the template strand of a transcribed segment of DNA. Therefore, if lesions that do not block RNAP accumulate and/or remain unrepaird, they could alter gene expression in several ways. As each of the lesions studied in this work are recognized by different repair enzymes, it is possible that a defect in any one of these enzymes could lead to the generation of significant levels of mutant protein or reduced gene expression.

Uracil, 8-oxoG, and O6-mG are all efficiently bypassed by *E. coli* RNAP during elongation in vitro. Transcriptional bypass is accompanied by mutagenic base insertion events opposite to these lesions. Specifically, uracil inserts an adenine (assuming cytosine to be in the undamaged template), whereas 8-oxoguanine also frequently miscodes for adenine, and O6-methylguanine allows for the insertion of uracil. All of these events are equivalent to a base substitution mutation at the level of RNA synthesis (transcriptional mutagenesis). These findings have several important implications for DNA repair and mutagenesis in general. More bulky lesions such as cyclobutane pyrimidine dimers cause the permanent arrest of RNAP at the dam-

![Fig. 4. Transcript sequence analysis.](image)

**Fig. 4. Transcript sequence analysis.** *E. coli* RNA polymerase-generated transcripts were produced under single-round transcription conditions, and cDNA was generated through reverse transcriptase PCR and subsequent sequencing as described under “Experimental Procedures.” Arrows indicate base insertions at nucleotide positions 56 and 63 from the 5' end of the transcripts and correspond to base insertion events opposite cytosine (template CON-elong, nt 56) and guanine (template CON-elong, nt 63) (A), uracil (template URA-elong, nt 56) (B), 8-oxoG (template SOG-elong, nt 63) (C), and O6-meG (template O6MEG-elong, nt 63) (D) on the transcribed strand.

![Fig. 5. Effect of 8-oxoG on promoter clearance.](image)

**Fig. 5. Effect of 8-oxoG on promoter clearance.** A, single-round transcription experiments with undamaged control (CON-clear) and 8-oxoG-containing (SOG-clear) templates. Arrow RO indicates full-length runoff transcript (90 nt). Levels of transcription products were determined at various times (0–2 min) following the start of elongation. B, kinetics of full-length transcript formation between templates containing control (●) and 8-oxoguanine (○). Three separate single-round transcription experiments were conducted for each template, and the error bars represent standard deviations.
age site, thus signaling the cellular proteins which direct various components of the DNA repair machinery to the damage site in order to carry out repair (2). It is the arrest of an elongating RNAP that is thought to be an important signal for initiating transcription-coupled repair of DNA damages located on the template strands of actively transcribed genes (7–9). However, the nonbulky base damages studied in this work as well as other lesions previously studied in our laboratory (10, 11) do not arrest an elongating RNAP, but rather allow for efficient bypass. This leads to the prediction that these lesions may not be subject to the transcription-coupled repair system involved in removing other DNA lesions that cause permanent RNAP arrest.

With regard to transcriptional mutagenesis, it is evident that if these lesions remain unrepaired, a population of mutant RNA transcripts will be generated, subsequently leading to translation and production of mutant protein that could have toxic or other types of effects on cellular physiology. For example, transcriptional mutagenesis could lead to the production of a mutant protein that could cause the aberrant initiation of replication and cell division in a previously quiescent, non-dividing cell. As these lesions also cause mutagenic insertions for DNA polymerases, a mutagenic lesion for RNAP that leads to mutant protein through transcription could force a cell to divide, which subsequently, through the miscoding of DNA polymerase, could fix the mutation permanently into the cellular genome (10, 23).

We have also shown that 8-oxoG, if located near the promoter of an actively transcribed DNA segment, causes decreased promoter clearance and hence lower amounts of expressed full-length transcript. Promoter clearance is a target of gene regulation. There are several implications for decreased promoter clearance due to DNA damage. DNA damage could lower gene expression, which could potentially lead to toxic effects or a myriad of other effects due to inadequate amounts of an essential cellular protein. These results highlight the fact that ways in which specific DNA base damages are able to affect gene expression will depend on their exact location on the template strand of a transcribed gene. The extent to which transcriptional mutagenesis may affect gene expression in living cells is currently unknown and will be an important avenue of pursuit for future studies.

REFERENCES
1. Chamberlin, M. J. (1995) Harvey Lect. 88, 1–21
2. Selby, C. P., and Sancar, A. (1993) Science 260, 53–58
3. Shi, Y., Gamper, H., and Hearst, J. E. (1988) J. Biol. Chem. 263, 527–534
4. Donahue, B. A., Yin, S., Taylor, J., Reines, D., and Hanawalt, P. C. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 8502–8507
5. Mellon, I., Rajal, D. K., Kol, M., Boland, C. R., and Champe, G. N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1292–1297
6. Mellon, I., and Champe, G. N. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 1292–1297
7. Mellon, I., Bohr, V. A., Smith, C. A., and Hanawalt, P. C. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 8878–8882
8. Mellon, I., Spivak, G., and Hanawalt, P. C. (1987) Cell 51, 241–249
9. Bohr, V. A., and Hanawalt, P. C. (1987) Carcinogenesis 8, 1333–1336
10. Zhou, W., and Doetsch, P. W. (1993) Proc. Natl. Acad. Sci. U. S. A. 90, 6601–6606
11. Liu, J. Z., and Doetsch, P. W. (1995) Mol. Cell. Biol. 15, 6729–6735
12. Liu, J., and Doetsch, P. W. (1996) Biochemistry 35, 14999–15008
13. Liu, J., and Doetsch, P. W. (1998) Nucleic Acids Res. 26, 1707–1712
14. Jacques, J.-P., and Susskind, M. M. (1990) Genes Dev. 4, 1801–1810
15. Jin, D. J. (1996) J. Biol. Chem. 271, 11659–11667
16. Hernandez, V. J., Hsu, L. M., and Cashel, M. (1996) J. Biol. Chem. 271, 18775–18779
17. Friedberg, E. C., Walker, G. C., and Siede, W. (1995) DNA Repair and Mutagenesis, ASM Press, Washington, DC
18. Selby, C. P., and Sancar, A. (1990) J. Biol. Chem. 265, 21330–21336
19. Voigt, J. M., and Topal, M. D. (1995) Carcinogenesis 16, 1775–1782
20. Snow, E. T., Poole, R. S., and Mitra, S. (1984) J. Biol. Chem. 259, 8095–8100
21. Turnbough, C. L., Hicks, K. L., and Donahue, J. P. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 368–372
22. Chen, Y. H., and Bogdenogen, D. F. (1993) J. Biol. Chem. 268, 5849–5855
23. Bridges, B. A. (1995) Nature 375, 741
24. Altman, A. W., Cisowski, D. E., and Chamberlin, M. J. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 3784–3788
25. Qi, F., and Turnbough, C. L. (1995) J. Mol. Biol. 254, 552–565
26. Qi, F., Liu, C., Heath, L. S., and Turnbough, C. L. (1996) Methods Enzyomol. 273, 71–85
27. Ellinger, T., Behnke, D., Bujard, H., and Gralla, J. D. (1994) J. Mol. Biol. 239, 455–65
28. Liu, C., Heath, L. S., and Turnbough, C. L. (1994) Genes Dev. 8, 2904–2912