Proc. Natl. Acad. Sci. USA
Vol. 90, pp. 6601–6605, July 1993
Biochemistry

Effects of abasic sites and DNA single-strand breaks on prokaryotic RNA polymerases

(apurinic/apyrimidinic sites/transcription/DNA damage)

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Communicated by Philip C. Hanawalt, April 19, 1993

ABSTRACT **Abasic sites are thought to be the most frequently occurring cellular DNA damage and are generated spontaneously or as the result of chemical or radiation damage to DNA. In contrast to the wealth of information that exists on the effects of abasic sites on DNA polymerases, very little is known about how these lesions interact with RNA polymerases. An *in vitro* transcription system was used to determine the effects of abasic sites and single-strand breaks on transcriptional elongation. DNA templates were constructed containing single abasic sites or nicks placed at unique locations downstream from two different promoters and were transcribed by SP6 and *Escherichia coli* RNA polymerases. SP6 RNA polymerase is initially stalled at abasic sites with subsequent, efficient bypass of these lesions. *E. coli* RNA polymerase also bypassed abasic sites. In contrast, single-strand breaks introduced at abasic sites completely blocked the progression of both RNA polymerases. Sequence analysis of full-length transcripts revealed that SP6 and *E. coli* RNA polymerases insert primarily, if not exclusively, adenine residues opposite to abasic sites. This finding suggests that abasic sites may be highly mutagenic *in vivo* at the level of transcription.**

The biologic end point of unrepaired DNA damage is determined primarily by its effects on the DNA and RNA synthesis machinery. Lesions that block RNA polymerases, such as UV light-induced cyclobutane-pyrimidine dimers, are repaired preferentially on the template strands of actively transcribed genes compared to other regions of the genome (1–4). Most studies investigating the effects of DNA damage on RNA polymerases have focused on relatively bulky DNA lesions and crosslinks that have the ability to block transcription elongation complexes (5–9). Currently there is very little information on how frequently occurring, spontaneous DNA damages, such as depurinations and base deaminations affect the transcriptional machinery. Abasic [apurinic/apyrimidinic (AP)] sites are produced by various chemical or radiation-induced modifications of DNA and by the action of DNA repair N-glycosylases (10). Spontaneous release of purines constitutes one of the most frequently occurring types of DNA damage under normal physiological conditions (11, 12). AP sites are both toxic and mutagenic with regard to DNA replication processes (12–14). A number of *in vitro* studies have been carried out with various DNA polymerases and templates containing AP sites (13, 15–18).

Currently, there is very little information available on the events that occur when RNA polymerase encounters an AP site during transcription. Previous studies carried out with chemically depurinated T7 phage DNA and *Escherichia coli* RNA polymerase are difficult to interpret because of the lack of knowledge regarding the exact location and stability of the AP sites within the 7-kb phage genome and the combined effects of such damage on both transcription initiation and elongation processes (19, 20). We wished to determine the effects of AP sites on the transcription elongation process by using a defined system that would provide direct information regarding the ability of RNA polymerases to bypass or be blocked by AP sites and, if bypass was observed, to determine the nature of the base insertion opposite such lesions. For these studies, we constructed a DNA template containing a single AP site placed at a unique location downstream from either the SP6 or *tac* promoter and carried out *in vitro* transcription experiments with SP6 and *E. coli* RNA polymerases. Similar experiments were also carried out with templates containing chemically or enzymatically induced nicks at the AP sites. Full-length transcripts resulting from RNA polymerase bypass of AP sites were sequenced. Our results indicate that RNA polymerase misinsertions may generate mutant proteins from unrepaired DNA damage.

MATERIALS AND METHODS

Materials. The pGEM-2 *in vitro* transcription vector was purchased from Promega. Synthetic oligonucleotide U44 corresponds to a segment of the SP6 RNA polymerase template strand contained in pGEM-2 with uracil (replacing thymine) 6 nt from the 3′ end (Fig. 1*A*). The oligonucleotides SP6-1 and TAC-1 were synthesized for construction of SP6 and *E. coli* RNA polymerase transcription templates (Fig. 1).

Enzymes. SP6 RNA polymerase (>99% purity), *E. coli* RNA polymerase (>95% purity), and restriction enzymes were purchased from Promega. DNA polymerase (Hot Tub) was purchased from Amersham. Moloney murine leukemia virus reverse transcriptase was purchased from Stratagene. Polynucleotide kinase was purchased from New England Biolabs. *E. coli* uracil-DNA glycosylase and endonuclease III were gifts from R. Cunningham (Albany, NY). *E. coli* endonuclease IV was a gift from B. Demple (Boston).

Construction of SP6 and *E. coli* RNA Polymerase Transcription Templates. DNA templates were generated by PCR amplification of a segment of the pGEM-2 vector (nt positions 2852–86) containing the SP6 promoter. Oligonucleotides SP6-1 (800 pmol) and 5′-end-labeled U44 (200 pmol) were employed with linearized pGEM-2 DNA (5.5 fmol) and Hot Tub DNA polymerase to generate a linear duplex transcription template containing a single uracil 44 nt downstream from the SP6 transcription start site. The 5′-end-labeled PCR product (104 bp) was gel-purified and was designated SP6-U (Fig. 1*B*). Transcription templates for experiments with *E. coli* RNA polymerase were produced by a modification of the procedure described above and used oligonucleotide TAC-1 in place of SP6-1. The 5′-end-labeled PCR product (139 bp) was designated ECO-U (Fig. 1*C*).

Generation of Transcription Templates Containing AP Sites and Single-Strand Breaks. SP6-U and ECO-U (20 pmol) were

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Abbreviation: AP, apurinic/apyrimidinic.

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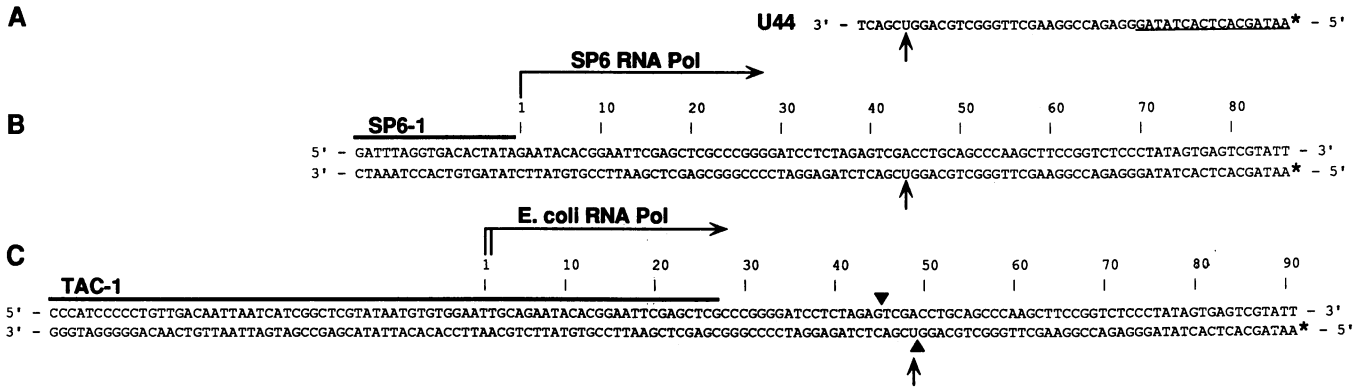

FIG. 1. Oligonucleotides and DNA templates. (*A*) Oligonucleotide U44 is 5'-^{32}P-end-labeled (star) and contains a single uracil (arrow) and a 5' segment (underlined) corresponding to the T7 promoter region. (*B*) DNA template SP6-U was generated from PCR primers U44 and SP6-1 (overlined) with pGEM-2 as described in the text. SP6-U contains the SP6 RNA polymerase promoter and a unique uracil (arrow) located on the template (bottom) strand 44 nt downstream from the transcription start site (nt position 1, vertical bar). (*C*) DNA template ECO-U was generated from PCR primers U44 and TAC-1 (overlined) with pGEM-2 as described in the text. ECO-U contains the *tac* promoter for *E. coli* RNA polymerase, a unique uracil (arrow) on the template (bottom) strand 48 or 49 nt downstream from the two transcription start sites (nt positions 1 and 2, vertical bars), and a single *Sal* I restriction site (solid triangles).

incubated with 5 units of *E. coli* uracil-DNA glycosylase as described (21) to generate SP6-AP and ECO-AP. Templates SP6-AP and ECO-AP were treated with *E. coli* endonuclease III to generate templates SP6-N/E3 and ECO-N/E3 [with breaks containing a 3'-modified deoxyribose and 5'-phosphoryl group at the AP site (10)], *E. coli* endonuclease IV to generate SP6-N/E4 [with breaks containing a 3'-hydroxyl and 5'-phosphoryl group at the AP site (10)], and hot piperidine (22) followed by reannealing (23) to generate SP6-N/HA and ECO-N/HA [with breaks containing a 3'-phosphoryl and 5'-phosphoryl group at the AP site (10)]. ECO-U was also treated with *Sal* I to produce a shortened transcription template. The generation of these transcription templates is summarized in Fig. 2. Chemical cleavage DNA sequencing reactions were used to indicate the nucleotide site of cleavage on the nicked DNA template (22).

Transcription Experiments. *In vitro* transcription reactions with SP6 RNA polymerase (10 units) were carried out as described (24) with 1 pmol of transcription template in 40 mM Tris·HCl (pH 7.9), 10 mM NaCl, 6 mM $MgCl_2$ (SP6 buffer) and either 0.5 mM ATP, 0.5 mM UTP, 50 μM GTP, and 10 μM CTP (standard NTP concentrations) or 0.5 mM NTPs (high NTP concentrations) and 10 μCi (1 Ci = 37 GBq) of [α-^{32}P]CTP (3000 Ci/mmol) at 37°C for 60 min (20-μl reaction volume). The ^{32}P-labeled transcripts were analyzed on denaturing 12% polyacrylamide gels without sample heating prior to loading and detected by autoradiography.

Single-round transcription reactions were carried out by preincubation of 1 pmol of transcription template with SP6 buffer, standard NTP concentrations minus CTP, and 10 units of SP6 RNA polymerase at 20°C for 8 min (25). After preincubation, heparin (250 μg/ml), CTP (10 μM), and 10 μCi of [α-^{32}P]CTP were added (20-μl final reaction volume), and 2-μl aliquots were removed at various time intervals. Transcripts were analyzed as described above and quantified by densitometry. *In vitro* transcription reactions with *E. coli* RNA polymerase were carried out as described previously by Selby and Sancar (9) except that 1 pmol of DNA template and 40 mM Hepes/KOH (pH 8.0) buffer were used in the reactions.

RNA Sequencing. Full-length RNA transcripts were gel-purified, and cDNAs were generated with Moloney murine leukemia virus reverse transcriptase and T7 primer under conditions recommended by the supplier (Stratagene). The resulting cDNAs were PCR-amplified with T7 and SP6-2 (5'-GAATACACGGAATTCGAGC-3') primers using Hot Tub DNA polymerase and sequenced using the *fmol* DNA sequencing system (Promega).

RESULTS

Construction of Transcription Templates Containing a Unique AP Site or Single-Strand Break. Uracil-DNA glycosylase was used for removal of uracil from SP6-U and ECO-U to generate transcription templates SP6-AP and ECO-AP containing single AP sites (Fig. 2). Three different nicked transcription templates (N/E3, N/E4, and N/HA) containing single-strand breaks with different 5' and 3' termini were enzymatically or chemically produced. The resulting collection of 5'-end-labeled RNA polymerase substrates allowed for the precise control of the amount of DNA template used in each transcription experiment as well as allowing for the direct analysis of the lesion-containing template strand. Each type of DNA template used in the SP6 RNA polymerase transcription experiments was analyzed on a DNA sequencing gel to verify the nature and location of the damage. Treatment of SP6-AP with either hot alkali or endonuclease III to generate SP6-N/E3 and SP6-N/HA (Fig. 3*A*), respectively, or treatment with endonuclease IV to generate SP6-

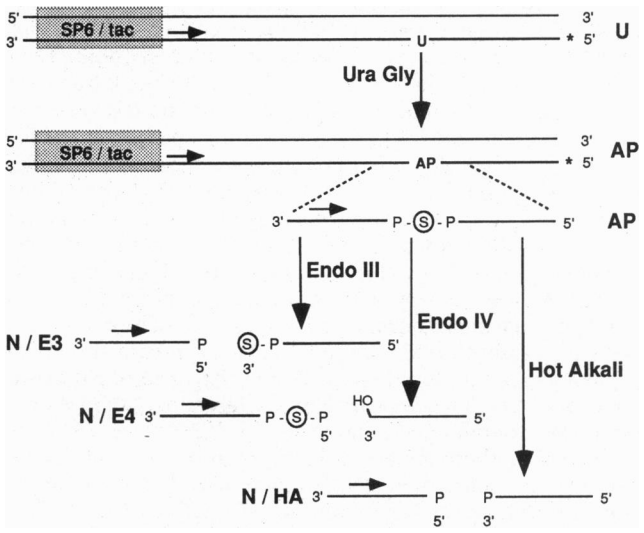

FIG. 2. Generation of DNA templates containing AP sites and single-strand breaks. Templates SP6-U and ECO-U (U) were treated with uracil-DNA glycosylase (Ura Gly) to generate a unique AP site at the position of uracil on the template strands of U (location and sequence depicted in Fig. 1) to produce SP6-AP and ECO-AP (AP). AP templates were subsequently treated with AP endonucleases (Endo) or hot piperidine to generate nicked DNA templates N/E3, N/E4, and N/HA. S, sugar (deoxyribose).

N/E4 (data not shown) resulted in quantitative cleavage at the position of the AP site. A similar analysis of ECO-U, ECO-AP, ECO-N/E3, and ECO-N/HA verified the nature of these DNA templates (data not shown).

SP6 RNA Polymerase Transcription of DNA Templates Containing AP Sites and Single-Strand Breaks. Initial transcription experiments were carried out under conditions allowing multiple rounds of transcription per DNA template. DNA template SP6-U containing a single uracil 44 nt downstream from the transcription start site was transcribed by SP6 RNA polymerase, producing the expected 86 nt, full-length transcript (Fig. 3*B*). The full-length nature and expected sequence of this transcript were verified by direct RNA sequence analysis (data not shown). Replacement of the DNA template uracil with an AP site (SP6-AP) also resulted in the production of full-length transcripts, indicating bypass by RNA polymerase. In addition, faint bands were observed corresponding to truncated transcripts (Fig. 3*B*, lane AP), suggesting that RNA polymerase might be stalled or terminated in the vicinity of the AP site. In contrast, SP6 RNA polymerase transcription of the DNA templates SP6-N/E3, SP6-N/E4, and SP6-N/HA containing single-strand breaks resulted in the exclusive production of truncated transcripts 43–47 nt in length and indicated that such strand breaks completely blocked progression of RNA polymerase.

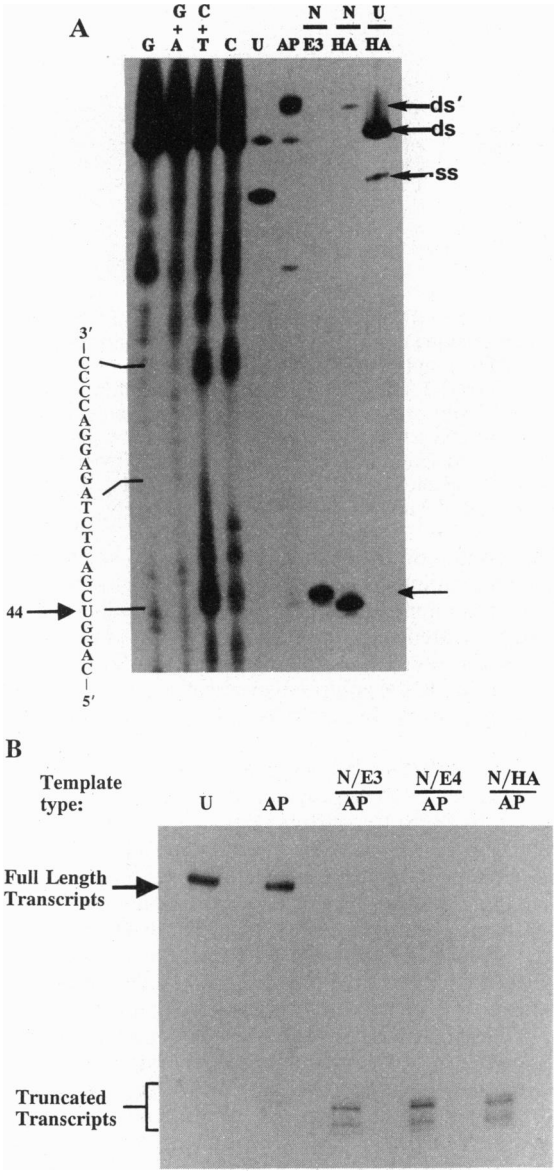

FIG. 3. SP6 RNA polymerase transcription of damaged DNA templates. (*A*) 5'-End-labeled DNA templates SP6-U (lane U), SP6-AP (lane AP), SP6-N/HA (lane N/HA), SP6-N/E3 (lane N/E3), or SP6-U treated with hot alkali (lane U/HA) were analyzed on a denaturing 20% polyacrylamide gel alongside the base-specific chemical cleavage DNA sequencing reactions of SP6-U. The sequence displayed corresponds to the template strand. Arrows at U44 indicate the strand cleavage location of nicked templates at the AP site. Full-length, single-stranded (ss), undenatured double-stranded (ds), and partially denatured double-stranded (ds') fragments are located at the top of the gel. (*B*) Transcripts from DNA templates SP6-U (lane U), SP6-AP (lane AP), SP6-N/E3 (lane N/E3), SP6-N/E4 (lane N/E4), and SP6-N/HA (lane N/HA) were analyzed on a denaturing gel. The arrow indicates the position of full-length transcripts (86 nt); bars indicate positions of truncated transcripts (43–47 nt).

The possibility that SP6 RNA polymerase might be stalling in the vicinity of AP sites was further examined by carrying out single-round transcription experiments under conditions that prevented reinitiation from occurring (25). Transcription of SP6-U was essentially complete 60 sec after the start of elongation (Fig. 4 *A* and *C*). SP6-AP was transcribed more slowly than SP6-U, and during the early stages of elongation, shortened transcript bands appeared, suggesting that SP6 RNA polymerase was stalling at the AP site. These stalled transcript bands disappeared 60 sec after the start of elongation (Fig. 4*C*). These results suggest that SP6 RNA polymerase pauses near the AP site but subsequently bypasses this lesion. Similar experiments were carried out with the single-strand-break-containing DNA templates SP6-N/E3 and SP6-N/HA, which completely blocked RNA polymerase progression at the break site and resulted in the production of at least three different truncated transcripts (Fig. 4*B*). The truncated transcripts, T1, T2, and T3, were sized by comparison with base-specific RNase cleavage products of a 5'-end-labeled full-length transcript on a denaturing polyacrylamide gel and corresponded to transcription products of 43, 44, and 45 nt in length, respectively (data not shown). The distribution of T1, T2, and T3 changed during the course of elongation and suggested that SP6 RNA polymerase stops at the template nucleotide immediately preceding the nick (nt position 43) to generate T1 (Fig. 4*D*). The formation of transcripts T2 and T3 can be attributed to nontemplated addition of nucleotides to the 3' end of T1 and is a general property of many RNA polymerases (26).

***E. coli* RNA Polymerase Transcription of Damaged DNA Templates.** The transcribed sequence used in the SP6 RNA polymerase experiments was placed downstream from the strong *E. coli* RNA polymerase *tac* promoter (Fig. 1*C*), and DNA templates containing a single uracil (ECO-U), AP site (ECO-AP), or single-strand break (ECO-N/E3 and ECO-N/HA) 49 nt downstream from the transcription start site were utilized (Fig. 2). ECO-U was efficiently transcribed by *E. coli* RNA polymerase and resulted in the generation of full-length transcripts of 90 and 91 nt (Fig. 5). *E. coli* RNA polymerase transcription of ECO-AP produced both full-length transcripts and a series of truncated transcripts. Although this result indicates that *E. coli* RNA polymerase also bypasses AP sites, a fraction of the transcripts generated corresponded to products resulting from termination of elongation in the vicinity of the AP site. It was observed that ECO-AP was nicked during the course of these transcription experiments, and we attribute at least some of the shortened transcripts produced to arrest of *E. coli* RNA polymerase at these single-strand break sites. In a separate experiment, incubation with ECO-AP and *E. coli* RNA polymerase in the absence of NTPs resulted in a time-dependent cleavage of this DNA template at the AP site (data not shown). It is not known whether the nicking of the ECO-AP DNA template was due to *E. coli* RNA polymerase itself or an unidentified component in the enzyme preparation.

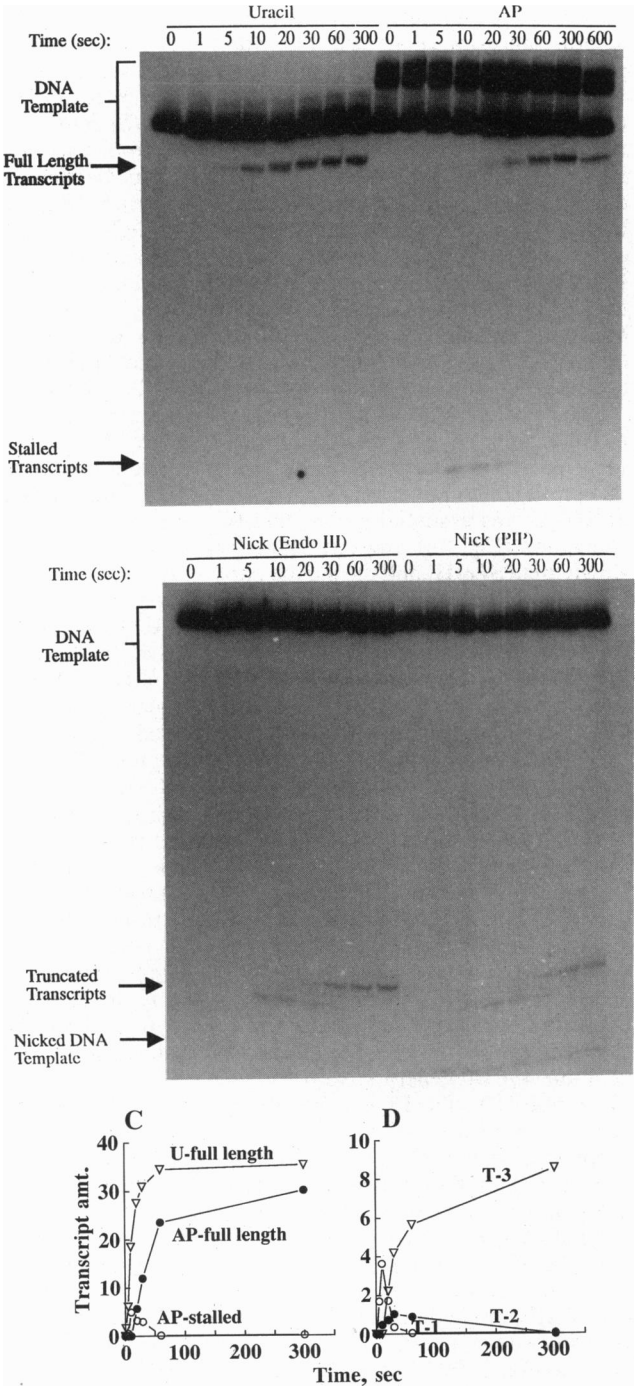

FIG. 4. SP6 RNA polymerase temporarily stalls at AP sites but is blocked at single-strand breaks. (*A*) SP6 RNA polymerase transcription of DNA templates SP6-U (Uracil lanes) and SP6-AP (AP lanes). (*B*) SP6 RNA polymerase transcription of DNA templates SP6-N/E3 [Nick (Endo III) lanes] and SP6-N/HA [Nick (PIP) lanes]. Arrows indicate positions of full-length, stalled, or truncated transcripts and nicked, single-stranded 5′-end-labeled DNA templates. Bands at top of gel are the 5′-end-labeled, full-length DNA templates: upper bands, partially denatured species; lower bands, fully denatured and native species. (*C*) Time course of full-length transcript synthesis from DNA templates SP6-U (▽) and SP6-AP (●) and stalled transcript synthesis from SP6-AP (○) shown in *A*. (*D*) Time course of truncated transcripts T1 (○), T2 (●), and T3 (▽) synthesis from DNA template SP6-N/E3 shown in *B*. Quantitation of transcripts (densitometer density units) was carried out as described in the text.

E. coli RNA polymerase transcription of the nicked DNA templates ECO-N/E3 and ECO-N/HA resulted in the exclusive production of truncated transcripts, suggesting that this enzyme cannot bypass this type of single-strand break located on the template strand (Fig. 5). The size distribution of these truncated transcripts was identical to that obtained with the shortened DNA template ECO-SalI (Fig. 1*C*), suggesting that RNA polymerase arrest occurs in the vicinity of the single-strand break followed by nontemplated additions.

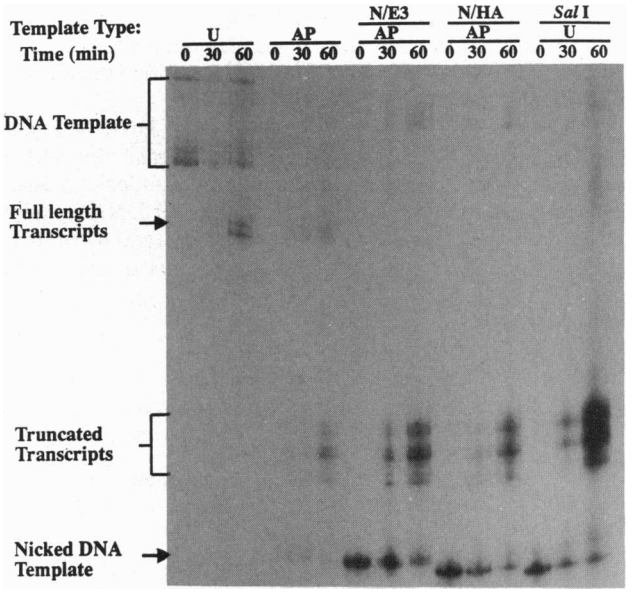

FIG. 5. *E. coli* RNA polymerase transcription of DNA templates containing a unique uracil or AP site or single-strand break. 5′-End-labeled DNA templates ECO-U (U lanes), ECO-AP (AP lanes), ECO-N/E3 (N/E3 lanes), ECO-N/HA (N/HA lanes) or ECO-SalI (*Sal* I lanes) were transcribed with *E. coli* RNA polymerase. Full-length transcripts correspond to species of 90 and 91 nt. Truncated transcripts correspond to species ranging in size from 49 to 56 nt. Bands at the top and bottom of the gel correspond to 5′-end-labeled full-length and nicked DNA templates, respectively.

SP6 and *E. coli* RNA Polymerase Insertions at AP Sites. Both SP6 and *E. coli* RNA polymerases bypassed AP sites to produce full-length transcripts. Because AP sites are noninstructive lesions, it was of interest to determine the nature of the inserted nucleotide(s). As expected, adenine was inserted at nucleotide position 44 opposite to uracil by SP6 RNA polymerase transcription of SP6-U (Fig. 6*A*). For template SP6-AP, SP6 RNA polymerase inserted adenine opposite to the AP site with no other predominant nucleotide insertions or frameshifts detected at this position with either standard or high NTP concentrations. The presence of adenine at nucleotide position 44 on the transcript generated from SP6-AP was further confirmed by direct RNA sequence analysis using base-specific RNases (data not shown). Sequence analysis of transcript cDNAs produced from *E. coli* RNA polymerase transcription of templates ECO-U and ECO-AP revealed that adenine insertions also occurred opposite to the uracil and to the AP site on these templates (Fig. 6*B*). We cannot rule out the possibility that SP6 and *E. coli* RNA polymerases insert bases other than adenine opposite to AP sites because the sensitivity of the methods employed here would not detect low-frequency insertion events.

DISCUSSION

The results of these studies indicate that AP sites, when located on the template strand of a transcribed sequence, can be efficiently bypassed by two different prokaryotic RNA polymerases, which insert adenine opposite to this lesion. These findings have several implications for DNA repair and mutagenesis. The current model for the preferential removal

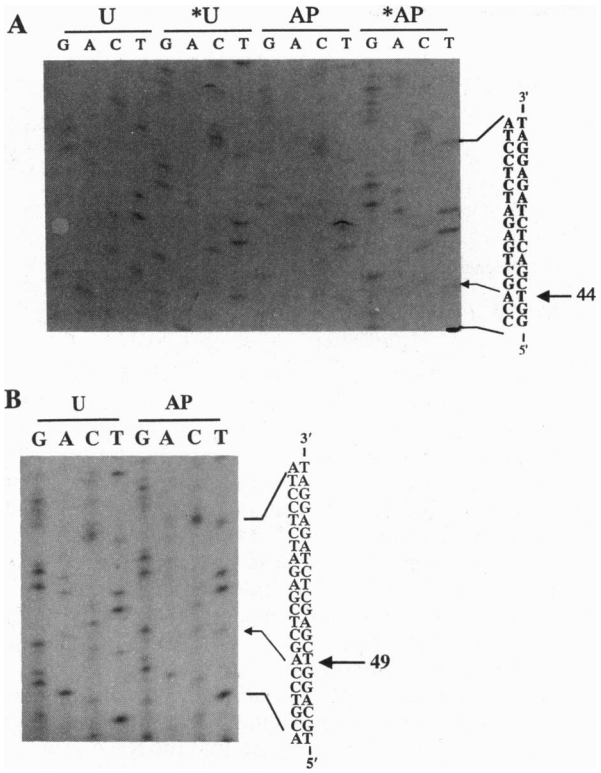

FIG. 6. Sequence analysis of transcripts. The DNA sequence corresponding to the transcript sequence is displayed as the inside strand. The arrows indicate the positions on the transcript resulting from nucleotide insertion opposite to the uracil or AP site by RNA polymerase. (*A*) Sequencing of cDNAs of full-length transcripts produced by SP6 RNA polymerase transcription of DNA template SP6-U (U and *U lanes) or SP6-AP (AP and *AP lanes) under standard (U and AP lanes) or high (*U and *AP lanes) NTP concentrations. (*B*) Sequencing of cDNAs of full-length transcripts produced by *E. coli* RNA polymerase transcription of DNA template ECO-U (U lanes) or ECO-AP (AP lanes).

of DNA damages from the template strands of actively transcribed genes involves arrest of RNA polymerase progression (1, 4). *In vitro* transcription studies with DNA templates containing unique cyclobutane-pyrimidine dimers (9) on the transcribed strands show that RNA polymerase is blocked at the damage site. Cyclobutane-pyrimidine dimers are preferentially removed from the transcribed strands of genes (2, 3), and a stalled elongation complex is thought to be part of the signaling system that directs the repair machinery to the damage (1, 4). Our results clearly indicate that single-strand breaks block progression of RNA polymerases, but it is not known at present whether or not the enzyme remains stalled in the vicinity of the break before dissociating from the DNA template. RNA polymerases bypass AP sites, suggesting that these lesions may not be preferentially repaired.

Our findings also indicate that prokaryotic DNA and RNA polymerases exhibit a similar property with respect to the preferential insertion of adenine opposite to AP sites (13, 15–18). A major difference, however, is that AP sites effectively block DNA polymerases and are bypassed only infrequently. Transcription of the uracil-containing templates resulted in the expected insertion of adenine opposite to uracil and, although not a surprising finding, indicates that cytosine deamination to uracil will result in a mutated transcript if such damage is left unrepaired.

The ability of SP6 and *E. coli* RNA polymerases to efficiently bypass AP sites suggests that if this event occurs *in vivo*, unrepaired AP sites may be considerably mutagenic at the level of transcription. AP sites are most frequently generated in cells as a result of spontaneous depurination at sites of guanine (12). RNA polymerase bypass of depurinated guanine residues resulting in insertion of adenine residues opposite to these AP sites would be equivalent to a C → A transversion mutation on the resulting transcript. Based on *E. coli* codon usage (27), if such an event occurred with an equal probability at any guanine site on the template strand of a gene, it would lead to a transcript missense mutation in about 74% of the cases. If the precursors to AP sites are either adenine or cytosine, the majority of codon changes would also lead to missense mutations. Thymine would be the least frequently occurring AP site precursor (12) but would not result in the generation of a mutant transcript. Hence, the most likely result of RNA polymerase bypass of an AP site will be a transcript containing a missense mutation. If such a situation occurs *in vivo*, it may be a major source of mutant proteins, particularly in nondividing cells.

We wish to thank R. P. Cunningham and B. Demple for their gifts of DNA repair enzymes. We would also like to thank D. Brash, N. Drinkwater, and D. Reines for helpful discussions and K. Hamilton, L. Augeri, and M. Cooney for critical reading of the manuscript. This work was supported by grants from the National Cancer Institute (CA42607 and CA55896). P.W.D. is a recipient of a Research Career Development Award (CA01441) from the National Cancer Institute.

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