Transcription Bypass or Blockage at Single-Strand Breaks on the DNA Template Strand: Effect of Different 3′ and 5′ Flanking Groups on the T7 RNA Polymerase Elongation Complex†

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ABSTRACT: We have studied the effects of single-strand breaks present on the template strand during T7 RNA polymerase transcription elongation. A synthetic DNA template with a T7 promoter was designed to contain a one-nucleotide gap at a defined location on the template strand. This gap, surprisingly, was efficiently bypassed by T7 RNA polymerase during transcription elongation, and the full-length transcript (FLT37) generated from the bypass event was shortened by one nucleotide compared to the full-length transcript (FLT38) generated from an intact, unbroken template strand. FLT37 did not contain any nucleotide insertions opposite to the gap, so that the RNA sequence downstream from the gap, although accurately transcribed, contained a single base deletion compared to FLT38. This, to our knowledge, is the first demonstration that the continuity of the DNA template strand is not a necessary requirement for DNA-dependent RNA polymerase transcription elongation. DNA templates with different 3′ and 5′ termini at the single-strand break site were also investigated in this study. One of these templates, 1/3P-4P, which contained 3′- and 5′-phosphoryl termini at the break site, efficiently blocked T7 RNA polymerase. A single phosphoryl group present on either the 3′ or the 5′ terminus of the break site did not efficiently block RNA polymerase progression, suggesting that the blockage observed with template 1/3P-4P is due to the repulsion between the two phosphoryl termini in the vicinity of the polymerase active site.

For cells to convert the genetic information stored in DNA into functional proteins, DNA-dependent transcription processes are utilized to synthesize mRNA which is later translated. DNA-dependent transcription consists of three steps: initiation, elongation, and termination. During transcription elongation, a ribonucleotide triphosphate (rNTP),[1] complementary to a base present on the DNA template strand, is required to bind next to the 3′ end of the nascent RNA molecule (Kumar, 1981; von Hippel et al., 1984). The active site of the RNA polymerase then catalyzes internucleotide bond formation to extend the RNA by one nucleotide together with pyrophosphate release. These steps are thought to be repeated continuously during the entire transcription elongation process. In general, this process will be carried out on undamaged DNA templates. However, cellular DNA is continuously modified by a variety of agents *in vivo* (Lindahl, 1993), and it is possible for RNA polymerase to encounter a damaged DNA base on the template strand during transcription. For example, a cyclobutane pyrimidine dimer on the template strand can block *Escherichia coli* RNA polymerase during transcription elongation both *in vivo* (Sauerbier, 1976) and *in vitro* (Selby & Sancar, 1989). This type of RNA polymerase blockage is thought to be the signal for initiating preferential repair of bulky DNA lesions from the transcribed strands of actively transcribed genes by the nucleotide excision repair system (Selby & Sancar, 1993). Less bulky lesions, such as 8-oxoguanine, do not block transcription elongation by T7 RNA polymerase, and sequence analysis of the resulting transcripts reveals that a misinsertion is made opposite to this lesion (Chen & Bogenhagen, 1993). Thus, 8-oxoguanine mispairs with adenine or guanine, forming a stable configuration that allows RNA synthesis to continue. Other small lesions such as abasic sites (AP sites) and single-strand breaks (ssb) at AP sites present a different problem to the transcription elongation complex because these lesions contain no base pairing information. A number of *in vitro* studies have been carried out with various DNA polymerases and templates containing AP sites (Lawrence et al., 1990; Kunkel et al., 1982; Strauss et al., 1982; Boiteux & Laval 1982). Such studies have shown that DNA polymerases are effectively blocked at AP sites while in some cases the polymerase infrequently reads through the lesion, usually with the insertion of an incorrect nucleotide. Unlike DNA polymerases, SP6 and *E. coli* RNA polymerases can bypass AP sites efficiently *in vitro* and insert adenine during such bypass events. However, both of these RNA polymerases are completely blocked by ssb (Zhou & Doetsch, 1993). Since the major difference between an AP site and a ssb at an AP site is phosphodiester DNA backbone breakage, it might be assumed that the break itself is responsible for blocking transcription elongation. Recently, T7 RNA polymerase was shown to be capable of bypassing

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[1] Abbreviations: AP sites, abasic or apurinic/apyrimidinic sites; bp, base pair(s); ddATP, dideoxy-ATP; HPLC, high-performance liquid chromatography; nt, nucleotide(s); oligo, oligonucleotide; PAGE, polyacrylamide gel electrophoresis; rNTP, ribonucleotide triphosphate; RNase, ribonuclease; ssb, single-strand breaks.

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an AP site and a ssb at such AP sites *in vitro* (Chen & Bogenhagen, 1993). In that study, DNA strand breaks were introduced enzymatically, and in the absence of direct template analysis, the extent of strand cleavage was unknown. Hence, the T7 RNA polymerase bypass which was observed over these putative strand breaks might be attributable to bypass of intact AP sites remaining on a residual population of unbroken DNA templates.

To directly determine whether or not DNA backbone breakage could block transcription elongation, we constructed a DNA template by T7 RNA polymerase containing different types of one-nucleotide gaps present on the template strand. A defined, single-round *in vitro* transcription assay that eliminated all non-promoter-driven transcription events was also developed for T7 RNA polymerase. This assay was performed on the synthetic templates to determine the effects of one-nucleotide gaps on T7 RNA polymerase during transcription elongation. Similar studies were carried out on gapped templates containing different 3′ and 5′ termini flanking the gap in order to investigate their effects on the elongation process.

EXPERIMENTAL PROCEDURES

Reagents. Uracil−DNA glycosylase and *E. coli* endonuclease III were gifts from Dr. Richard P. Cunningham (Albany, NY). *E. coli* endonuclease IV was a gift from Dr. Davis Chen (Atlanta, GA). Synthetic DNA oligonucleotides were purified by polyacrylamide gel electrophoresis (Maniatas et al., 1982). The sequence of each oligonucleotide was verified by base-specific chemical cleavage DNA sequencing (Maxam & Gilbert, 1980). The following reagents were purchased from the sources noted: T7 RNA polymerase (Promega); T4 polynucleotide kinase (New England Biolabs); calf intestinal phosphatase (Boehringer Mannhem); terminal transferase (Promega); [α-^{32}P]CTP (Amersham); [γ-^{32}P]ATP (Amersham); [α-^{32}P]dideoxy-ATP (Amersham); HPLC-purified nucleoside triphosphates (Pharmacia); heparin (Sigma); RNA sequence kit (Nuclease Method, United States Biochemicals).

DNA Templates. To construct the one-nucleotide gap-containing DNA template (1/3-4), oligo 1 was first 5′-end-labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP (Maniatas et al., 1982). Then 10 μM 5′-end-labeled oligo 1 was added to 30 μM oligo 3 and 50 μM oligo 4 in 10 mM $MgCl_2$. The mixture was heated to 70 °C for 10 min and cooled to room temperature over 4 h (standard annealing conditions). DNA template 1/3-4 was purified from a 20% nondenaturing gel as previously described (Zhou & Doetsch, 1993). Templates 1/2T, 1/3, and 1/4 were constructed by a similar approach under standard annealing conditions (Figure 1).

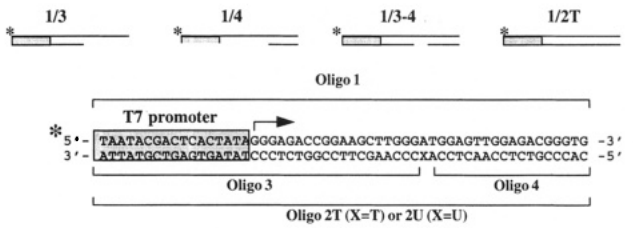
To construct ssb-containing DNA templates with different 3′- and 5′-phosphoryl termini at the break site, thymine at position X in oligo 2T was replaced with uracil to generate oligo 2U (Figure 1A). This oligo was first 5′-end-labeled as described above and then 3′-end-labeled by terminal transferase and [α-^{32}P]dideoxy-ATP (Maniatas et al., 1982). Uracil at position X in oligo 2U was removed by uracil−DNA glycosylase to generate an AP site (Lindahl, 1980), and subsequent treatment with hot piperidine converted the AP site into a ssb at this position (Doetsch & Cunningham, 1990). This treatment produced two oligonucleotide fragments; a 3′-^{32}P end-labeled species with the same sequence as oligo 3 plus a 5′-terminal phosphoryl group and a 3′-[α-^{32}P]ddAMP (oligo 3P), and a 5′-^{32}P end-labeled species with the same sequence as oligo 4 plus a 3′-terminal phosphoryl group (oligo 4P). The 5′-phosphoryl terminal group in oligo 3P was removed by calf intestinal phosphatase to generate oligo 3OH (Maniatas et al., 1982), and the 3′-terminal phosphoryl group in oligo 4P was removed by the 3′-phosphatase activity of T4 polynucleotide kinase (Richardson, 1981) to generate oligo 4OH. Templates 1/2U, 1/3P-4P, 1/3P-4OH, 1/3OH-4P, and 1/3OH-4OH were constructed by the standard annealing conditions described above (Figure 5, top panel). The generation of AP site or ssb-containing templates was carried out as described previously using template 1/2U as the starting material (Zhou & Doetsch, 1993).

Single- and Multiple-Round Transcription Experiments. Single-round transcription experiments were carried out in three stages. In stage 1, 0.05 μM T7 RNA polymerase was added to each 0.05 μM DNA template in 40 mM Hepes, pH 8.0, 10 mM NaCl, 6 mM $MgCl_2$, 10 mM dithiothreitol (transcription buffer), 500 μM ATP and CTP, and 3 μCi of [α-^{32}P]-CTP (3000 Ci/mmol) to form the transcription initiation complex. Aliquots were removed from the reaction mixture for the identification of ^{32}P end-labeled DNA template. In stage 2, 500 μM GTP and 1.25 mg/mL heparin were added to the stage 1 reaction mixture to allow transcription elongation to proceed until T7 RNA polymerase is stalled immediately before the incorporation of the first uracil (Figure 1A), and aliquots were removed to identify the stalled transcripts. In stage 3, 500 μM UTP was added to the stage 2 reaction mixture, and aliquots were removed in order to identify the runoff transcription products. To generate the RNA size marker ladder, transcription experiments were carried out without heparin at 37 °C for 30 min on template 1/2 (multiple-round transcription conditions). The resulting reaction mixture was then subjected to alkaline hydrolysis with 50 mM Na_3PO_4, pH 12, for 20 min at 70 °C.

Transcript Sequencing. Multiple-round transcription conditions were used except that the reactions were carried out in absence of [α-^{32}P]CTP. The resulting transcripts were dephosphorylated by calf intestinal phosphatase, gel-purified, and 5′-^{32}P end-labeled (Maniatas et al., 1982). RNA sequencing was carried out with base-specific ribonucleases (RNases) as described in the RNA Sequence Kit (Nuclease Method, United States Biochemicals).

Quantitation of DNA Template Strand Cleavage and RNA Polymerase Bypass Efficiency. DNA template strand cleavage analysis was performed as described previously (Zhou & Doetsch, 1993). ^{32}P-labeled DNA fragments corresponding to single-strand, full-length template and cleaved templates were identified by autoradiography and excised from the gel, and the amount of radioactivity in each band was determined by liquid scintillation counting. The percentage of DNA template cleavage was calculated as (nicked template)/(nicked template + single-stranded full-length template) × 100. To calculate T7 RNA polymerase bypass efficiency, ^{32}P-labeled RNA transcripts were excised from the gel, and the radioactivity was determined by liquid scintillation counting. FLT38 (38 nt) and FLT37 (37 nt) contain 4 cytosine residues and the truncated transcripts, TT19 (19 nt), TT20 (20 nt), and TT21 (21 nt), contain 3 cytosine residues. The percentage of T7 RNA polymerase

A. DNA templates



B. RNA transcripts

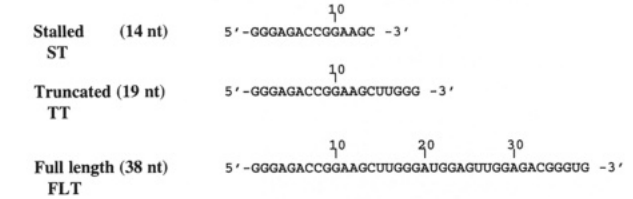


FIGURE 1: DNA templates and predicted RNA transcripts. (A) Schematic drawings of templates 1/3, 1/4, 1/3-4, and 1/2T are shown at the top of the panel. Asterisks indicate the 5′-^{32}P end-labeled terminus of the nontemplate strand oligo 1. Oligos 2T, 2U, 3, and 4 are components of template strands. The shaded portions correspond to the T7 promoter region. The arrow indicates the transcription start site and the direction of transcription. Position X is 20 nt downstream from the transcription start site and corresponds to thymine in oligo 2T, uracil in oligo 2U, and a one-nucleotide gap in template 1/3-4. (B) Stalled transcript, ST, at nt position 14 is generated under single-round transcription conditions in the absence of UTP (stage 2 reaction conditions). Truncated transcript, TT (19 nt), is generated when transcription is blocked at position X (stage 3 reaction conditions). Full-length transcript, FLT (38 nt), is generated from template 1/2T (stage 3 reaction conditions).

bypass efficiency was calculated as (full-length transcripts)/[full-length transcripts + (truncated transcripts) × 4/3] × 100.

RESULTS

Generation of DNA Templates. Figure 1A depicts the DNA templates used in this study. Template 1/3-4, containing a one-nucleotide gap, was constructed by annealing oligo 1 with oligo 3 and oligo 4. This template contains a 17 bp T7 promoter region and will support T7 RNA polymerase transcription. The resulting T7 promoter-driven transcript incorporates the first uracil at position 15, and, in the absence of UTP, transcription by T7 RNA polymerase will generate a "stalled" transcript (ST14) 14 nt in length (Figure 1B). Unlke the chemical or enzymatic cleavage methods used previously for generating DNA templates containing strand breaks (Zhou & Doetsch, 1993; Chen & Bogenhagen, 1993), the annealing procedure used here resulted in a homogeneous template containing a single nucleotide gap on the template strand at position X, 20 nt downstream from the start of transcription. Hence, blockage of RNA polymerase by the resulting gap should generate a truncated transcript (TT19) 19 nt in length (Figure 1B). Template 1/2T (Figure 1A) contains an unbroken, intact template strand and was used as a positive control. Transcription of this template with T7 RNA polymerase should generate a full-length, runoff transcript (FLT38) 38 nt in length (Figure 1B). Template 1/3 (Figure 1A) contains a shortened template strand terminating at position X and should produce a shortened transcript 19 nt in length, equivalent to transcript TT19.

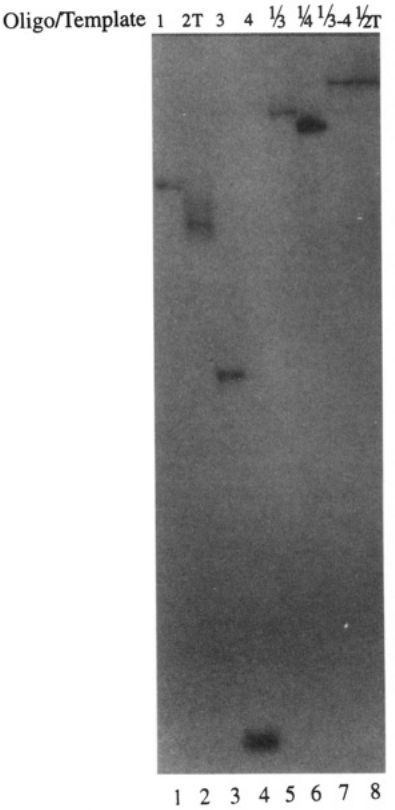


FIGURE 2: DNA template analysis. Each 5′-end-labeled oligonucleotide and DNA template construct was analyzed on a 20% nondenaturing gel as described under Experimental Procedures: oligo 1 (lane 1), oligo 2T (lane 2), oligo 3 (lane 3), oligo 4 (lane 4), template 1/3 (lane 5), template 1/4 (lane 6), template 1/3-4 (lane 7), and template 1/2T (lane 8).

Template 1/4 lacks duplex DNA in the promoter region and should not support promoter-driven transcription.

Oligo 1 was 5′-end-labeled and annealed with different oligonucleotides to generate DNA templates 1/3, 1/4, 1/3-4, and 1/2T. The DNA templates were gel-purified, and their identities were confirmed by analysis on a 20% nondenaturing gel (Figure 2, lanes 5−8). Each oligonucleotide was also 5′-end-labeled and utilized as a DNA size marker (Figure 2, lanes 1−4). The annealed product of oligos 1 and 4 (template 1/4) possessed a slower electrophoretic mobility compared to oligo 1 alone. Because oligo 3 was longer than oligo 4, template 1/3 migrated more slowly than template 1/4. Templates 1/3-4 and 1/2T contained the longest DNA duplex regions and resulted in species with the slowest electrophoretic mobilities (Figure 2). Therefore, the nature of the resulting template constructs was consistent with their observed electrophoretic mobilities. Since oligo 1 was 5′-end-labeled and used as a component for the above four DNA template constructs, the resulting templates all possess the same specific activities, a feature which allowed for precise control of the amount of DNA used in the different transcription experiments.

Single-Round Transcription Experiments. Single-round transcription experiments were conducted with the templates shown in Figure 1A. Full-length transcripts generated under multiple-round transcription conditions were hydrolyzed to generate RNA size markers (Figure 3, lane 1). Single-round transcription experiments were conducted in three stages. In the first stage, an aliquot was removed from the reaction mixture after the formation of the transcription initiation

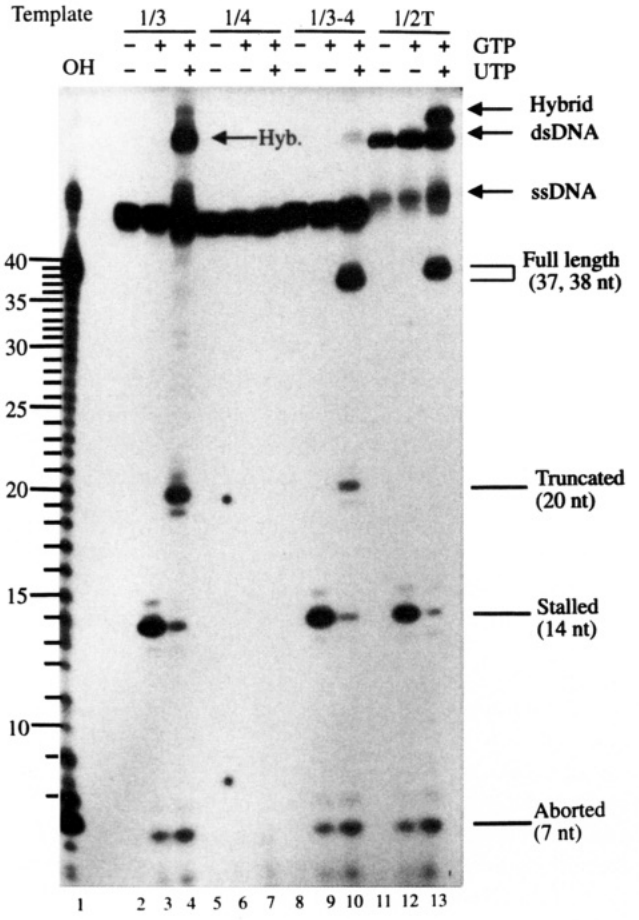


FIGURE 3: Single-round transcription experiments (stage 1, 2, and 3 reactions) with T7 RNA polymerase. Transcription experiments were carried out on templates 1/3 (lanes 2−4), 1/4 (lanes 5−7), 1/3-4 (lanes 8−10), and 1/2T (lanes 11−13) as described under Experimental Procedures. Lanes 2, 5, 8, and 11 correspond to the initiation complex (stage 1 reaction) containing only ^{32}P-labeled DNA template. Lanes 3, 6, 9, and 12 correspond to the stage 1 reaction mixture after the addition (+) of GTP and heparin (stage 2 reaction). Lanes 4, 7, 10, and 13 correspond to the stage 2 reaction mixture after the addition of UTP (+) (stage 3 reaction). Lane 1 contains RNA size markers. Arrow "hybrid" indicates a DNA−RNA hybrid species. Migration positions and sizes of full-length, truncated, stalled, and aborted transcripts are indicated.

complex and the addition of ATP, CTP, and [α-^{32}P]CTP. Since GTP was required for the synthesis of the first three nucleotides, transcription was inhibited, and the ^{32}P-labeled species observed correspond to the 5′-end-labeled DNA templates (lanes 2, 5, 8, and 11). Template 1/2T contained a 55 bp uninterrupted DNA duplex region and was not completely denatured under the gel electrophoresis conditions; thus, lane 11 contained two bands corresponding to both the duplex (arrow dsDNA) and single-strand DNA templates (arrow ssDNA). In stage 2, an aliquot was removed from the reaction mixture after the addition of GTP and heparin. Inclusion of GTP allowed promoter-driven transcription to proceed (Figure 3, lanes 3, 9, and 12), and the concentration of heparin used in our assay completely inhibited transcription reinitiation (data not shown). This type of inhibition is not observed when the transcription buffer contained 1 mM spermidine (data not shown). Template 1/4 did not contain duplex DNA in the T7 promoter region and did not result in the generation of transcription products (lane 6). Templates 1/3, 1/3-4, and 1/2T generated a transcript 14 nt in length, which corresponded to a 14 nt promoter-driven transcript (ST14) stalled at the position immediately prior to the incorporation of the first uracil (lanes 3, 9, and 12). The selective generation of the 14 nt transcript (ST14) indicated that transcription in this system was essentially promoter-dependent. In stage 3, an aliquot was removed from the reaction mixture after the addition of UTP. Although a small amount of ST14 remained following the addition of UTP, the majority of this transcript was converted into longer species (lanes 4, 10, and 13). Therefore, ST14 was not an aborted transcript but a stalled product capable of further RNA chain extension.

Previous studies suggested that strand breaks were strong barriers to the progression of RNA polymerases (Zhou & Doetsch, 1993). However, the transcription experiments carried out with a one-nucleotide gap-containing template (1/3-4) yielded unexpected results (Figure 3). Further addition of UTP to the stage 2 reaction mixture containing template 1/3 generated 19, 20, and 21 nt runoff transcripts (lane 4). Two of these products, TT20 and TT21, most likely correspond to truncated species resulting from transcription termination at nt position 19 (Figure 1B) followed by a one-nucleotide—and, to a lesser extent, two-nucleotide—non-template addition to TT19 (Jacques & Kolakofsky, 1991). We also observed the generation of a slow-migrating species (Figure 3, arrow "hybrid"). DNA and RNA sequencing of this species indicated that it contains an unexpected, covalently linked DNA−RNA hybrid containing oligo 1 in the 5′ region and a promoter-dependent transcription product in the 3′ region (unpublished results). The exact mechanism for the generation of such a hybrid species is unknown and is currently under investigation. Addition of UTP to the stage 2 reaction mixture containing template 1/2T generated the expected 38 nt full-length transcript (FLT38). A DNA−RNA hybrid was also generated with this template (arrow "hybrid"). The addition of UTP to the stage 2 reaction mixture containing template 1/3-4 generated not only TT20 but also a 37 nt full-length transcript (lane 10). This 37 nt transcript (FLT37) was one nucleotide shorter than the control, full-length transcript (FLT38) generated from template 1/2T (lane 13). Quantitation of the amounts of truncated (TT19, -20, and -21) and full-length (FLT37) transcripts produced from template 1/3-4 indicated that T7 RNA polymerase is capable of bypassing a one-nucleotide gap present on the template strand with approximately 75% efficiency (Table 1). Therefore, the physical breakage of the DNA backbone does not pose a strong block to transcription elongation by T7 RNA polymerase under stage 3 reaction conditions.

Sequence Analysis of Transcripts. We wished to determine the sequence of the transcript resulting from T7 RNA polymerase bypass of the gapped DNA template 1/3-4. Multiple-round transcription experiments were carried out with this template, and the resulting full-length transcripts were gel-purified and subjected to RNA sequence analysis (Experimental Procedures). Transcripts generated from template 1/2T produced full-length transcripts containing the predicted sequence (Figures 1B and 4A). The full-length transcript generated from template 1/3-4 possessed the same sequence as that generated from template 1/2 except that adenine at nt position 20 was deleted (arrow), resulting in a one-nucleotide deletion in this transcript (Figures 1B and 4B). We conclude from these results that when T7 RNA polymerase bypasses the one-nucleotide gap present on

Table 1: Bypass Efficiency on Different Templates by T7 RNA Polymerase[a]

DNA template[e]	5′-terminus flanking gap	3′-terminus flanking gap	template cleavage efficiency (%)[b]	polymerase bypass efficiency (%)	
				exptl 1[c]	expt 2[d]
1/3-4	5′-hydroxyl	3′-hydroxyl	100	75	75
1/2U			100	94	94
1/3P-4P	5′-phosphoryl	3′-phosphoryl	100	6	6
1/3OH-4P	5′-hydroxyl	3′-phosphoryl	100	77	71
1/3P-4OH	5′-phosphoryl	3′-hydroxyl	100	60	59
1/3OH-4OH	5′-hydroxyl	3′-hydroxyl	100	81	92
uracil				100	100
abasic site				84	73
N/E3	5′-phosphoryl	4-hydroxy-2-pentenal	90	20	23
N/E3+CIP	5′-hydroxyl	4-hydroxy-2-pentenal	90	31	27
N/E4 type I + type II	5′-deoxyribose phosphate + 5′-phosphoryl	3′-hydroxyl	72	84	85

[a] Polymerase bypass efficiency and [b]*template* cleavage efficiency were determined as described under Experimental Procedures. Bypass efficiency from gels shown in [c]Figure 5 (bottom right panel) and Figure 6 (bottom panel) and [d]not shown. [e] The indicated DNA templates were utilized in two different sets of transcription experiments (stage 3 reaction conditions).

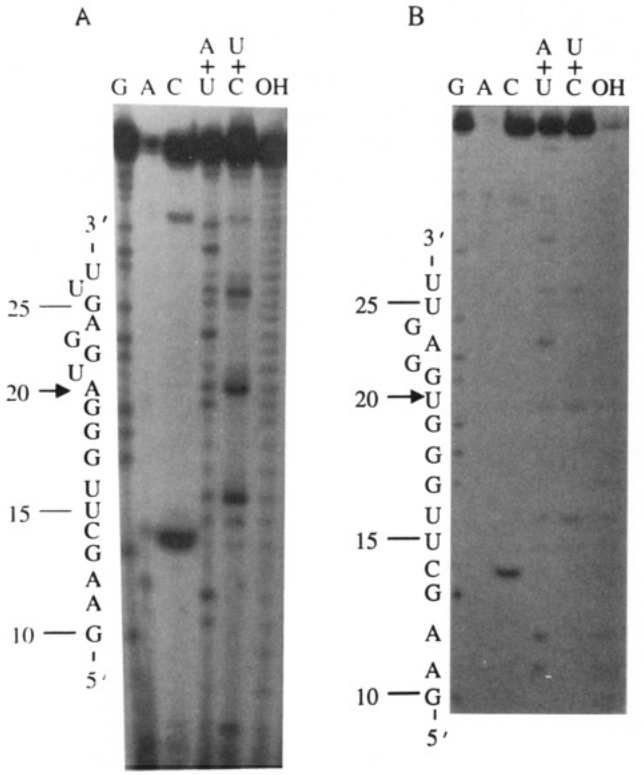


FIGURE 4: Sequence analysis of transcripts. 5′-end-labeled transcripts were subjected to RNA sequence analysis employing base-specific RNase digestions (lanes G, A, C, A+U, and U+C) as described under Experimental Procedures. Alkaline hydrolysis reactions were also carried out on these transcripts to generate RNA size markers (lane OH). (A) Sequence of full-length transcript generated from intact, unbroken template 1/2T. (B) Sequence of full-length transcript generated from one-nucleotide gap-containing template 1/3-4. The arrow indicates the presence (panel A) or absence (panel B) of an adenine in the full-length transcript at nt position 20.

template 1/3-4, transcription still proceeds in a template-dependent manner that includes faithful transcription of the template nucleotides flanking the gap.

Effect of 3′- and 5′-Termini Modifications Flanking the Template Gap Site. DNA single-strand breaks containing phosphoryl groups on the 3′ and 5′ termini have been shown to be capable of blocking SP6 and *E. coli* RNA polymerase during transcription elongation when such breaks are located on the template strand (Zhou & Doetsch, 1993). We wished to determine if this type of strand break could exert a similar effect on T7 RNA polymerase. For these studies, 3′-end-labeled olgio 3 containing a 5′-terminal phosphoryl group (oligo 3P) and 5′-end-labeled oligo 4 containing a 3′-terminal phosphoryl group (oligo 4P) were generated from oligo 2U (Figure 5, top panel, and Experimental Procedures). The 3′-phosphoryl group present on oligo 3P and the 5′-phosphoryl group present on oligo 4P were removed and replaced with hydroxyl groups to generate oligos 3OH and 4OH. Oligos 3P and 4P were utilized to generate single-strand break-containing templates with phosphoryl groups on both the 3′ and 5′ termini flanking the single nucleotide gap site (Figure 5, top, template 1/3P-4P). DNA templates containing phosphoryl and hydroxyl groups on either termini as well as DNA templates containing hydroxyl groups on both termini were also constructed (Figure 5, top). Template 1/2U containing an intact template strand was also generated as a positive control. The nature of these templates was verified by analysis on a 20% denaturing gel as shown in Figure 5, bottom left panel. The presence of a 3′- and a 5′-phosphoryl group increased the electrophoretic mobilities of oligos 3P and 4P which migrated faster than oligos 3OH and 4OH, respectively (Figure 5, bottom left). Single-round transcription experiments carried out on template 1/3P-4P resulted in primarily generation of truncated transcripts TT19, TT20, and TT21 which were 19, 20, and 21 nt in length, respectively (Figure 5, bottom right panel, lane 3). This result indicated that T7 RNA polymerase was nearly completely blocked by a single nucleotide gap flanked on both sides by a phosphoryl group. This notion was confirmed following quantitation of the gel lane (Table 1). We conclude that the presence of a 3′- and a 5′-phosphoryl group on the break termini constitutes a strong block to T7 RNA polymerase progression at the breakage site. To determine which phosphoryl group flanking the gap might be responsible for polymerase blockage, we utilized DNA templates containing either a 5′-phosphoryl group and a 3′-hydroxyl group flanking the gap (template 1/3P-4OH) or a 5′-hydroxyl group and a 3′-phosphoryl group flanking the gap (template 1/3OH-4P) (Figure 5, top). With both of these templates, we observed the generation of full-length transcript FLT37 in addition to the truncated transcripts TT19, TT20, and TT21 (Figure 5, bottom right, lanes 4 and 5). This particular distribution of transcription products was similar to that generated from the one-nucleotide gap-containing DNA template 1/3OH-4OH (Figure 5, bottom right, lane 5). Quantitation of the transcription products also indicated that the bypass efficiencies of T7 RNA polymerase over

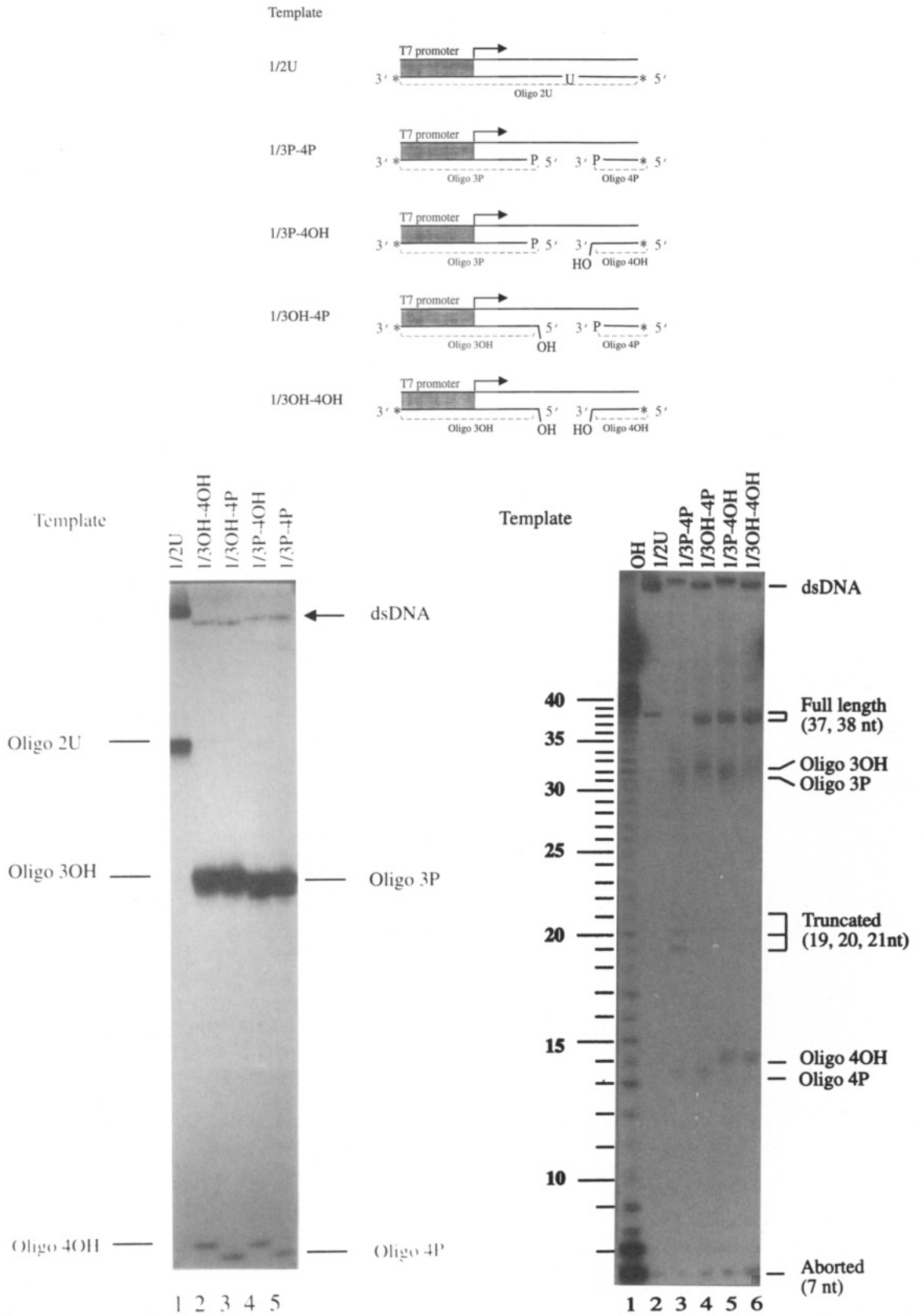


FIGURE 5: T7 RNA polymerase transcription of templates containing 3′- and 5′-terminal phosphoryl or hydroxyl groups flanking the gap site. (Top panel) DNA templates containing different termini at position X (Figure 1A) that were used in transcription experiments depicted in the bottom right panel. DNA templates: 1/2U, 1/3OH-4OH, 1/3P-4OH, 1/3OH-4P, and 1/3P-4P; P, phosphoryl group; OH, hydroxyl group. Asterisks indicate the positions of 3′- and 5′-^{32}P end-label on DNA templates, and arrows indicate the direction of transcription. The 3′ and 5′ termini of the template strand are indicated, and the size of the single-nucleotide gap site has been exaggerated. (Bottom left panel) Analysis of DNA templates. Templates 1/2U (lane 1), 1/3OH-4OH (lane 2), 1/3OH-4P (lane 3), 1/3P-4OH (lane 4), and 1/3P-4P (lane 5) were analyzed on a 20% denaturing polyacrylamide gel. Arrow dsDNA indicates residual, undenatured duplex DNA. The migration positions for oligos 2U, 3OH, 3P, 4OH, and 4P are indicated. (Bottom right panel) single-round transcription experiments (stage 3 reactions) were carried out on templates 1/2U (lane 2), 1/3P-4P (lane 3), 1/3OH-4P (lane 4), 1/3P-4OH (lane 5), and 1/3OH-4OH (lane 6), and the resulting transcripts were analyzed on a 15% denaturing gel. The RNA size marker is run in lane 1 (OH). Migration positions of full-length, truncated, stalled, and aborted transcripts as well as oligos 3P, 3OH, 4P, and 4OH and undenatured duplex DNA (dsDNA) are indicated.

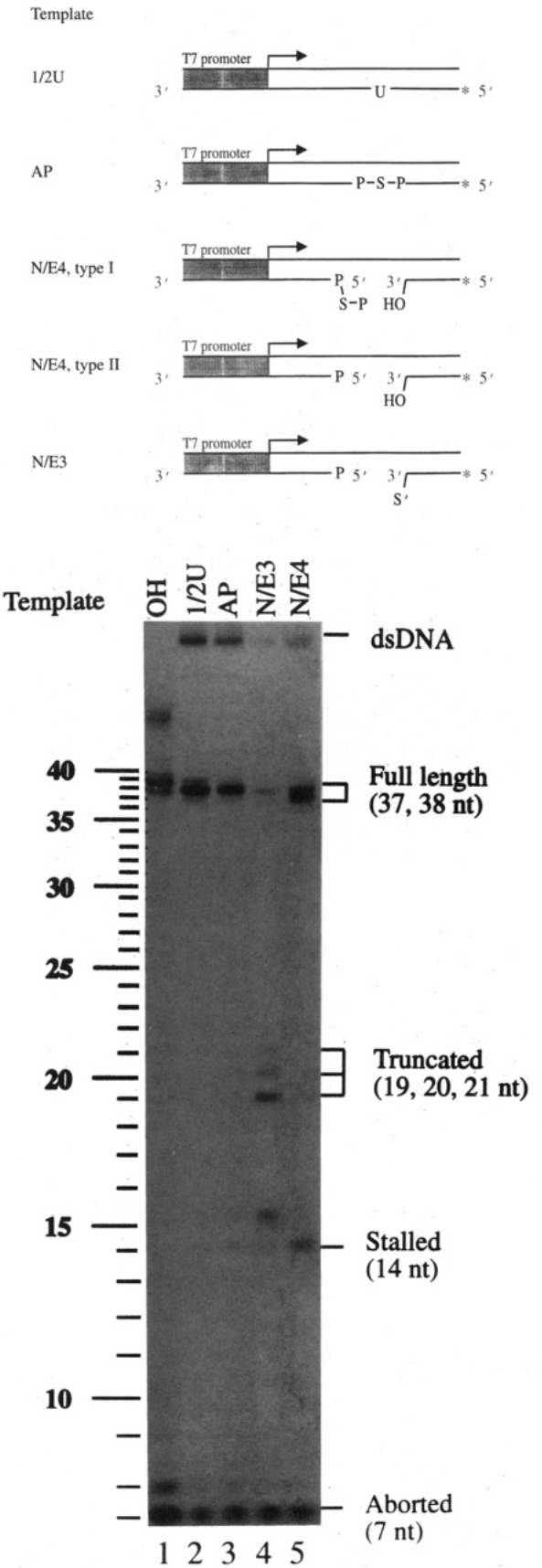


FIGURE 6: T7 RNA polymerase transcription of templates AP, N/E4, and N/E3. (Top panel) DNA templates containing different types of ssb at position X (nt position 20, Figure 1A). DNA templates: AP, N/E4 (type I and type II), and N/E3; P, phosphoryl group; S, deoxyribose; S′, 4-hydroxyl-2-pentenal group. Arrows indicate the direction of transcription, and asterisks indicate ^{32}P end-label on DNA templates. Termini of the template strand are indicated. (Bottom panel) Single-round transcription experiments (stage 3 reaction conditions) were carried out on templates 1/2U (lane 2), AP (lane 3), N/E4 (lane 4), and N/E3 (lane 5) and were analyzed on a 15% denaturing gel. RNA size markers (OH) were run in lane 1. Migration positions for full-length, truncated, stalled, and aborted transcripts are indicated.

the template strand gaps were similar among templates 1/3P-4OH, 1/3OH-4P, and 1/3OH-4OH (Table 1). We conclude that the presence of a single phosphoryl group on either terminus flanking the gap is not sufficient for blockage of the T7 RNA polymerase elongation complex. Therefore, the strong blockage of transcription elongation on template 1/3P-4P is due to the effect of the presence of phosphoryl groups on both termini flanking the gap (Figure 5, bottom right panel, and Table 1).

Effects of Different Types of Single-Strand Breaks Flanking AP Sites on T7 RNA Polymerase. Since the chemical nature of the termini could affect T7 RNA polymerase bypass efficiency over strand breaks, DNA templates with several different types of strand break termini at position X were investigated (Figure 6, top panel). These DNA templates were generated enzymatically, and the extent of DNA template strand breakage was determined by analysis on a denaturing polyacrylamide gel (Experimental Procedures and Table 1). Template AP possesses an intact DNA phosphodiester backbone with an AP site at position X (Figure 6, top). T7 RNA polymerase efficiently bypassed this AP site, producing the full-length transcript FLT38 (Figure 6, bottom, and Table 1). Template N/E4 (type I) was derived from template AP by treatment with *E. coli* endonuclease IV which introduced a strand break on the DNA backbone 5′ to the AP site, producing a 5′-deoxyribose phosphate-containing terminus and a 3′-hydroxyl terminus (Figure 6, top). The deoxyribose phosphate at the 5′ terminus is somewhat unstable, and a portion will be converted into a terminus containing a 5′-phosphoryl via a spontaneous β-elimination reaction (Bailly & Verly, 1989). The resulting template N/E4 (type II) will contain the same type of termini at the strand break site as that of template 1/3P-4OH (Figure 6, top). T7 RNA polymerase bypassed both of these types of ssb efficiently and produced two full-length transcripts, FLT37 and FLT38 (Figure 6, bottom, and Table 1). Treatment of template AP with *E. coli* endonuclease III would produce a strand break 3′ to the deoxyribose, generating template N/E3 with a 3′ terminus containing 4-hydroxy-2-pentenal and a 5′-phosphoryl terminus (Figure 6, top). This type of strand break efficiently blocked T7 RNA polymerase progression, resulting in the production of truncated transcripts TT19, TT20, and TT 21 (Figure 6, bottom, and Table 1). A low level of the full-length transcript FLT38 was produced with template N/E3 which can be ascribed to transcription of the residual, 10% unbroken DNA template that was not cleaved by *E. coli* endonuclease III (Table 1). The removal of the 5′-terminal phosphoryl group from this type of strand break had little effect on the bypass efficiency, indicating that the modified sugar present on the 3′ terminus was responsible for RNA polymerase blockage by template N/E3 (Table 1). Therefore, T7 RNA polymerase is capable of bypassing an intact deoxyribose, generating a full-length transcript FLT38, as in the case for transcription of templates AP and N/E4 type I. The 3′-terminal modified sugar in template N/E3, however, interferes with transcription elongation, and complete removal of the sugar residue allows bypass to occur, resulting in the generation of full-length transcript FLT37.

DISCUSSION

Several *in vitro* transcription systems have been utilized to determine the effects of damaged DNA templates on RNA polymerase during the transcription elongation process. The majority of these have employed DNA templates damaged with either chemical or physical agents which, in many cases, randomly introduce a variety of different base modifications distributed throughout the entire template (Flamée & Verly, 1985; Cullinane & Philips, 1992; Thrall et al., 1992; Sanchez & Mamet-Bratley, 1994). Transcription of such templates usually generates a spectrum of transcripts of different lengths, thus preventing reliable assignment of a particular transcription product to a specific type and location of DNA damage. Recently, we and several other laboratories have attempted to address this difficulty by utilizing DNA templates containing single, chemically-defined adducts at specific locations (Selby & Sancar, 1990; Sastry & Hearst, 1991; Zhou & Doetsch, 1993; Chen & Bogenhagen, 1993). In this study, we have directly compared the effects of AP sites, ssb at AP sites, and single-nucleotide gaps by employing template construction strategies that allow the placement of these lesions at the same location in the template strand. An additional advantage of our approach is that transcription elongation is carried out under single-round transcription conditions which simplifies the analysis of transcripts. We have also modified the system to eliminate nonspecific, promoter-independent transcription events. Furthermore, we have determined the lengths of the transcripts at the level of single-nucleotide resolution, which allows the determination of the exact extent of nascent RNA chain extension along the DNA template relative to the position of DNA damage. The *in vitro* transcription system utilized in the present study should be generally applicable for determining the effects of many different types of DNA damage on various RNA polymerases.

Abasic sites are thought to be one of the most frequently occurring cellular DNA lesions, and are generated spontaneously or as a result of exposure to various chemical or physical agents (Lindahl & Nygerg, 1972; Doetsch & Cuningham, 1990). Ssb are frequently occurring DNA lesions that are chemically diverse with regard to their 3′ and 5′ termini and can be produced by ionizing radiation, oxygen radicals, and certain chemicals as well as by DNA repair enzymes and various nucleic acid processing events (Henner et al., 1982; Hecht, 1986; von Sonntag, 1987; Doetsch & Cunningham, 1990). The results of our studies allow us to precisely define the consequences of RNA polymerase transcription of DNA templates containing several different types of ssb. It is evident that different 3′ and 5′ termini flanking the breakage site can significantly alter the ability of ssb to block RNA polymerase during the elongation step. Thus, the exact chemical nature of a particular ssb located on the template strand may have significant effects on gene expression *in vivo* as well as on how such damages are processed by the DNA repair machinery.

Previously, we have determined that SP6 and *E. coli* RNA polymerases can bypass AP sites but not ssb during transcription elongation, suggesting that template strand breaks are the cause of such polymerase blockage (Zhou & Doetsch, 1993). Chen and Bogenhagen, however, have proposed that AP-endonuclease-generated ssb do not block T7 RNA polymerase (Chen & Bogenhagen, 1993). Our results in this study demonstrate that physical breakage of the template strand does not constitute a strong block for T7 RNA polymerase progression. When bypass occurs over a one-nucleotide gap, this results in a transcript that is one nucleotide shorter than the transcript produced from an intact, unbroken template. The sequence of this shortened transcript indicates that correct complementary base pairing of, and chain extension over, the two template nucleotides flanking the gap does occur. If similar RNA polymerase bypass over a one-nucleotide gap occurs *in vivo*, the resulting transcripts will contain a one-nucleotide deletion (frameshift mutation).

Transcription of DNA templates containing AP sites results in the generation of 38 nt full-length transcripts (FLT38), whereas transcription of templates containing a one-nucleotide gap results in transcripts which are shortened by one nucleotide. A possible mechanism for generation of the shortened, FLT37 transcript is for the RNA polymerase to actively bring the 3′ and 5′ termini flanking the gap on the template strand into close proximity. This "gap closing" event would allow the incoming rNTP to bind immediately next to the 3′ end of the nascent RNA chain for internucleotide bond formation and subsequent chain extension. For the transcription of template AP, an intact, unbroken backbone prevented a similar gap-closing event from occuring and resulted in RNA polymerase insertion of a ribonucleotide opposite to the AP site, producing the full-length transcript FLT38. To obtain further insight into the possibility of gap-closing on broken templates, we utilized template 1/3P-4P which contains phosphoryl groups on both the 3′ and 5′ termini flanking the gap. Such termini efficiently blocked T7 RNA polymerase bypass of one-nucleotide gaps, suggesting that the termini flanking the gap site cannot be actively brought into close proximity due to electrostatic repulsion between the two phosphoryl groups. The behavior of T7 RNA polymerase on the collection of different DNA templates used in these experiments suggests that during elongation, proper spacing must exist between adjacent bases present on the template strand in the vicinity of the catalytic site of the polymerase. This distance requirement is met with templates containing either an intact phosphodiester backbone, or single combinations of phosphoryl and hydroxyl groups, or pairs of hydroxyl groups present on the termini flanking the one-nucleotide gap. Transcription elongation is blocked with templates that cannot fulfill this distance requirement, such as those that contain phosphoryl group pairs flanking the one-nucleotide gap or a 3′-modified deoxyribose group, such as that produced by *E. coli* endonuclease III.

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