WHEN DNA POLYMERASE COLLIDES WITH RNA POLYMERASE

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Degree Conferred: . . . . . .

I dedicate this thesis to my parents

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When DNA polymerase collides with RNA polymerase

Bin Liu

Abstract

Replication proteins encoded by bacteriophage T4 generate DNA replication forks that can pass a molecule of *Escherichia coli* RNA polymerase moving in either direction relative to the fork in an *in vitro* assay. The bypassed RNA polymerase remains bound to the replicated, double-stranded DNA and can resume faithful RNA synthesis. During a head-on collision, the bypassed RNA polymerase switches its template DNA strand.

Further studies on the behavior of RNA polymerase encountering model branched and nicked DNA templates reveal that a template switch by RNA polymerase may be a rather common event during elongation of RNA chains. A related reaction pathway may help the RNA polymerase survive its collisions with the DNA replication apparatus.

Bruce M. Alberts

Chairperson of the committee

WHEN DNA POLYMERASE COLLIDES WITH RNA POLYMERASE

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

Chapter 4

Appendix 3

Chapter 1

Introduction

The question:

DNA replication and DNA transcription are two fundamental biological processes that take place on a common DNA molecule at the same time. In *E. coli*, the rate of replication is ten to twenty times faster than the rate of transcription (Bremer and Dennis, 1987; Hirose et al., 1983), so that collisions between the two types of polymerase are inevitable, even when they move in the same direction. How does a cell resolve such a collision? The answer to this question is relevant to understanding the general mechanism of reactions taking place on DNA.

The strategy for an *in vitro* study:

In principle, a polymerase collision could be resolved in one of three ways: a replication fork could knock an RNA polymerase molecule and its nascent transcript out of its way; a replication fork could slow down and passively follow behind a co-directionally moving transcription complex; or a replication fork could pass a transcribing RNA polymerase molecule without displacing it from the template.

Preservation of the nascent transcript when a replication fork passes would be advantageous since RNA chains are generated by an energy-consuming multi-step process (von Hippel et al., 1984; Wang et al., 1992). Is this possible chemically? To answer this question, we have examined the consequences of a collision (in either direction) between a replication fork

and a transcribing RNA polymerase, using the highly purified *in vitro* T4 bacteriophage DNA replication system to replicate through a precisely placed *E. coli* RNA polymerase transcription complex. In this completely defined system, the fate of the nascent transcript after replication can be determined without ambiguity.

Components of the *in vitro* experimental system:

1. The templates and the formation of a ternary transcription complex:

A uniquely nicked circular DNA molecule containing a strong T4 late promoter superimposed with an *E. coli* σ^{-70} promoter has been used as a DNA template that supports concurrent DNA replication and DNA transcription (Fig. 1). The direction of a polymerase collision is controlled by placing the nick on appropriate DNA strand, i.e., the template strand for a head-on collision and the non-template strand for a co-directional collision. By withholding CTP, we stalled the RNA polymerase at a specific downstream site, creating a stable *ternary transcription complex* composed of RNA polymerase, an 18-nt nascent RNA transcript, and the DNA template.

2. The proteins:

The core RNA polymerase used in this study is from *E. coli;* either the *E. coli* σ^{-70} protein or the T4 gene 55 σ-family protein is used for appropriate

promoter recognition (Liu et al., 1993).

We chose the bacteriophage T4 DNA replication machinery for this study because it has been completely reconstituted *in vitro* (the only replication system at present that is understood at the level of each individual subunit). In addition, it is functionally analogous to the DNA polymerase of *E. coli* and eukaryotic replication systems (reviewed in Stillman, 1994; also see Table 1).

Seven highly purified bacteriophage T4-encoded proteins reconstitute an *in vitro* replication system that catalyzes efficient leading-strand DNA synthesis. The proteins involved are the T4 DNA polymerase holoenzyme (consisting of the products of T4 genes 43, 44, 62, and 45), a helix-destabilizing single-stranded DNA-binding protein (gene 32 protein), the highly processive DNA helicase (gene 41 protein), and the gene 59 protein that greatly facilitates the loading of the gene 41 protein onto DNA at a replication fork (Barry & Alberts, 1994). Gene 61 protein (primase) along with gene 41 protein constitutes the primosome, making primers for lagging-strand DNA (Okazaki fragment) synthesis. Altogether, those eight proteins constitute the complete T4 replication apparatus, catalyzing coupled leading- and lagging-strand DNA synthesis at a rate comparable to that observed *in vivo* (reviewed in Alberts, 1987; see Figure 2).

3. Purification of the ternary complex:

To reduce the binding of RNA polymerase to the nick and to weak variant T4 late promoters on the plasmid, we purified the ternary complex on

Sepharose CL-2B after exposure to high salt (0.5 M NaCl) (Liu et al., 1993). The Sepharose CL-2B column excludes the ternary complex (which elutes in the void volume), but includes free core RNA polymerase, gene 55 protein and nucleotides (which are thereby removed). Promoter-bound RNA polymerase and other, less stable ternary complexes dissociate from DNA in 0.5 M NaCl (Williams et al., 1987). Thus, the desired ternary complex is highly enriched after passage through the CL-2B column. Moreover, since the gene 55 protein and ribonucleoside triphosphates (NTPs) have been removed, there can be no reinitiation by RNA polymerase during the subsequent DNA replication reaction.

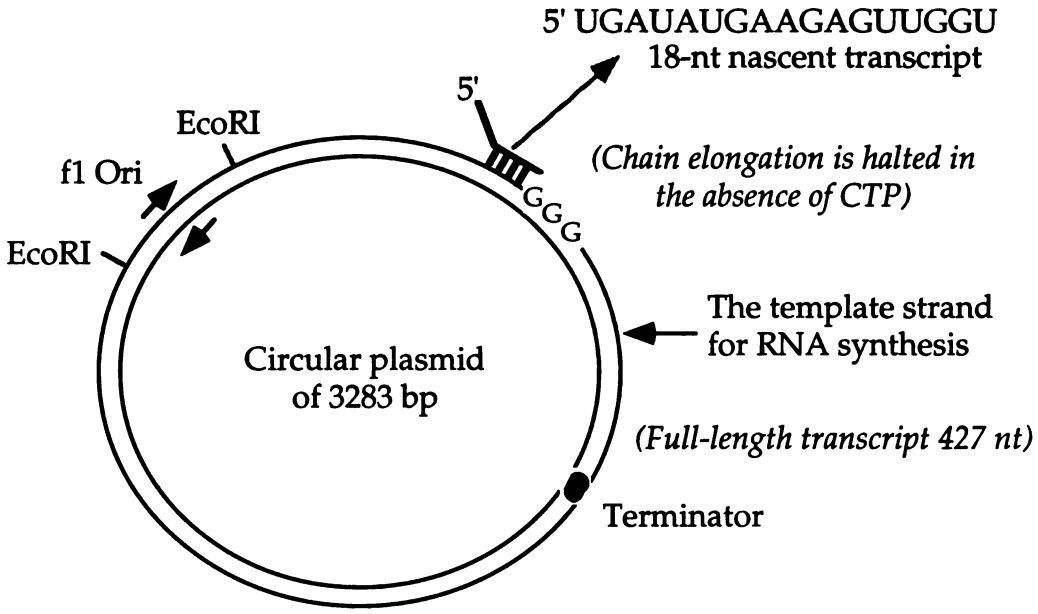

Figure 1: Templates for studying a polymerase collision. A superimposed E. coli σ^{70} and T4 late promoter initiates RNA synthesis with either E. coli RNA polymerase holoenzyme or core E. coli RNA polymerase supplemented with T4 gene 55 protein, gene 33 protein, gene 45 protein and gene 44/62 proteins. Three consecutive G nucleotides are present on the template strand 17, 18, and 19 bps downstream of the initiation site, so that RNA synthesis is halted after 18 nt when CTP is omitted. The f1 origin is nicked with fgII endonuclease to initiate DNA synthesis. By inverting the EcoRI-EcoRI fragment, the nick can be placed at either of the two DNA strands. When it is placed on the non-template strand for RNA synthesis, DNA polymerase collides with RNA polymerase co-directionally; when it is placed on the template strand for RNA synthesis, the two polymerases collide head to head.

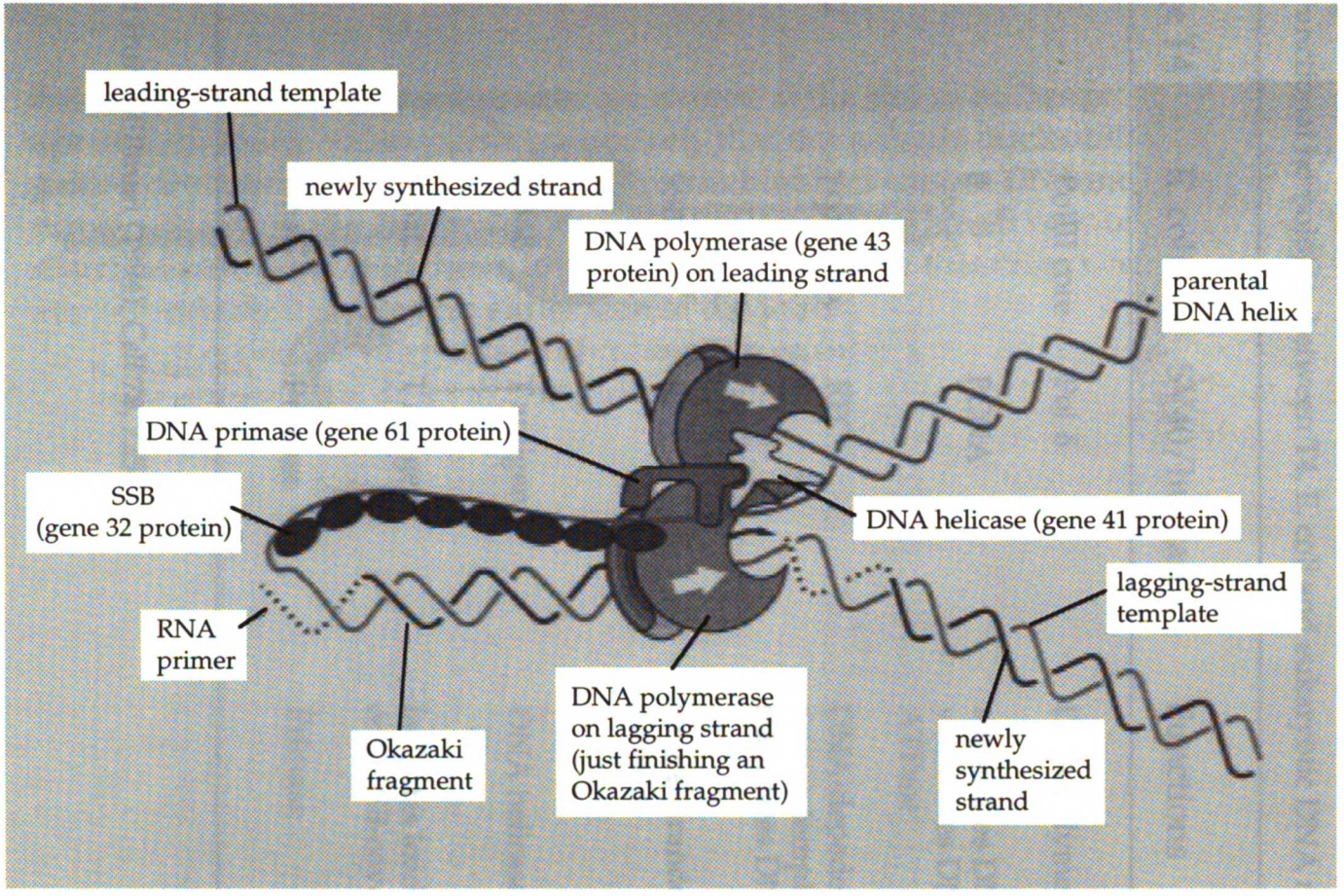

Figure 2: Coupled leading- and lagging- strand DNA synthesis with T4 replication proteins.

Table 1. Functional homologies between T4, E. coli and eukaryotic DNA replication proteins

Phage T4	E. coli	SV40/human	Functions
43	PolIII core	Pol δ	DNA polymerase
45	β	PCNA	stimulates DNA polymerase; stimulates DNA-depedent ATPase
44/62	γ complex	RFC	DNA-depedent ATPase; primer-template binding; stimulates DNA polymerase
32	SSB	RPA	single-stranded DNA binding protein
41	DnaB	T antigen	DNA helicase
59	DnaC	T antigen	faciliates loading of helicase onto SSB-covered DNA
61	DnaG	Primase	Primase

Adopted from Stillman (1994), *Cell* **78**, 725

Chapter 2

Consequences of a co-directional polymerase collision

This chapter is a reprint of the material as it appears in Nature 366, 33-39 (1993). I performed all the experiments shown except Fig. 5, which is a collaborative effort between Mei Lie Wong (EM lab, HHMI and Dept. Biochemistry, UCSF) and myself. Rachel Tinker and Prof. E. Peter Geiduschek (Dept. Biology, UCSD) provided plasmid pRT510-C+18, T4 gene 33 protein, T4 gene 55 protein and E. coli RNA polymerase. They also provided valuable advice on purification and KMnO4 footprint of the ternary complex. I drafted the manuscript that was edited and polished carefully by Prof. Alberts and Prof. Geiduschek.

The DNA replication fork can pass RNA polymerase without displacing the nascent transcript

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Replication proteins encoded by bacteriophage T4 generate DNA replication forks that can pass a molecule of *Escherichia coli* RNA polymerase moving in the same direction as the fork *in vitro*. The RNA polymerase ternary transcription complex remains bound to the DNA and retains a transcription bubble after the fork passes. The by-passed ternary complex can resume faithful RNA synthesis, suggesting that the multisubunit RNA polymerase of *E. coli* has evolved to retain its transcript after DNA replication, allowing partially completed transcripts to be elongated into full-length RNA molecules.

No known mechanism prevents DNA replication and transcription from taking place on a DNA molecule concurrently; when they move in the same direction, the respective polymerases must use the same DNA single strand as template. In *E. coli*, the rate of replication is 10–15 times faster than the rate of transcription[1,2], so that collisions between the two types of polymerase are inevitable, even when they move in the same direction. Collisions could be resolved in one of three ways: a replication fork could knock an RNA polymerase molecule and its nascent transcript out of its way; a replication fork could slow down and passively follow behind a transcription complex; or a replication fork could pass a transcribing RNA polymerase molecule without displacing it from the template.

Preservation of the nascent transcript when a replication fork passes would be advantageous because RNA chains are generated by an energy-consuming multistep process[3–5]. Is this possible chemically? To answer this question, we examined the consequences of a collision between a replication fork and co-directionally transcribing RNA polymerase. We used the highly purified *in vitro* T4 bacteriophage DNA replication system to replicate through a precisely placed *E. coli* RNA polymerase transcription complex. In this completely defined system, the fate of the nascent transcript after replication can be determined unambiguously. Surprisingly, a replication fork can pass through a transcription complex without displacing it, leaving intact its ability to resume RNA chain elongation.

A template for investigating the collision

A uniquely nicked circular DNA molecule containing an appropriately oriented strong T4 late promoter[6–8] was used as a DNA template that supports co-directional replication and transcription (Fig. 1*a*, left side; note that the template strand for transcription is also the template for leading-strand DNA synthesis). By withholding rCTP, we stalled the RNA polymerase at a specific downstream site, creating a stable ternary transcription complex composed of RNA polymerase, an 18-nucleotide (nt) nascent RNA transcript, and the DNA template. The core RNA polymerase was from *E. coli*; the T4 gene 55 σ-family protein enables it to recognize the T4 late promoter[9].

To reduce the binding of RNA polymerase to the nick[10] and to weak variant T4 late promoters on the plasmid, we either used a low molar ratio of RNA polymerase to template DNA (for example, 4 : 1), or purified the ternary complex on Sepharose CL-2B after exposure to high salt (0.5 M NaCl), as specified in each experiment. The Sepharose CL-2B column excludes the ternary complex, but includes free core RNA polymerase, gene 55 protein and nucleotides. Promoter-bound RNA polymerase and other, less stable ternary complexes dissociate from DNA in 0.5 M NaCl (ref. 11). Thus, the desired ternary complex is highly enriched after passage through the CL-2B column. Moreover, because the gene 55 protein and ribonucleoside triphosphates (rNTPs) have been removed, there is no reinitiation by RNA polymerase during the subsequent DNA replication reaction.

The replication fork passes the ternary complex

Seven highly purified bacteriophage T4-encoded proteins reconstitute an *in vitro* replication system that catalyses efficient leading strand DNA synthesis[12–15]. The proteins involved are the T4 DNA polymerase holoenzyme (consisting of the products of T4 genes 43, 44, 62 and 45), a helix-destabilizing single-stranded DNA-binding protein (gene 32 protein), the highly processive DNA helicase (gene 41 protein), and the gene 59 protein that greatly facilitates the loading of the gene 41 protein onto DNA at a replication fork (J. Barry and B.M.A., manuscript in preparation).

We analysed the effect of stalled RNA polymerase ternary complexes on the movement of replication forks by alkaline agarose gel electrophoresis. As the DNA template, we used either mock-treated DNA, or CL-2B-purified ternary complexes. Even though about 70–80% of the DNA molecules bear a bound ternary complex (determined by a gel shift assay), there is no strong blockage of DNA synthesis, with or without DNA helicase (gene 41 protein) (Fig. 1*b*). Thus, the ability to pass the RNA polymerase ternary complex is intrinsic to the DNA polymerase holoenzyme (DNA polymerase plus accessory proteins). When helicase is included in the reaction, the replication fork speeds up, and it advances at a slightly reduced rate on templates bearing the ternary complex (compare lanes 7, 8 with lanes 11, 12), suggesting that the fork pauses transiently before passing stalled RNA polymerase. Without a helicase, the fork pauses at many sites, making it difficult to detect any additional pausing

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caused by the RNA polymerase ternary complex (compare lanes 1–3 with lanes 4–6).

The complex stays bound to DNA

The experiment in Fig. 1*b* shows the DNA replication fork readily passing a DNA template-bound RNA polymerase molecule that carries a transcript. To distinguish between the possible fates of this RNA polymerase (Fig. 1*a*), we designed the experiment illustrated in Fig. 2*a*. We used RNA-labelled ternary complexes as templates for replication with dUTP as one of the four dNTP substrates. DNA containing dUMP on one strand is resistant to double-strand cleavage by the restriction enzyme *Dra*I, which recognizes the sequence TTTAAA. The sensitivity of the RNA-labelled replication products to *Dra*I, as analysed by non-denaturing polyacrylamide gel electrophoresis, can therefore be used to analyse whether the replication fork has passed the ternary complex without displacing it (see Fig. 2*a*).

The analysis of such an experiment is shown in Fig. 2*b* (replication with DNA helicase) and Fig. 2*c* (replication without DNA helicase). Because the same amount of RNA-labelled ternary complex is seen in lane 1 (no replication) and lane 2 (after replication), it is evident that the ternary complex is not displaced from the template by the replication fork (quantification of the radioactivity typically shows <5% difference). The slowly migrating, branched structures that would be expected for replication forks stalled behind the ternary complex are not seen. Lane 4 shows the *Dra*I-resistant products, proving that the replication fork has passed through the ternary transcription complex. About 30–40% of the DNA in these RNA-labelled complexes is cut by *Dra*I, in agreement with other results indicating that 60–70% of the DNA template molecules replicate in this experiment. Lanes 3 in Fig. 2*b* and *c* show that *Dra*I digestion goes to completion when DNA is replicated with dTTP. Note that the same results are obtained with or without DNA helicase present.

Retention of a transcription bubble

The experiment outlined in Fig. 3*a* examines whether the ternary complex retains its original position after passage of a replication fork by separately marking DNA for the presence of a ternary complex and for downstream penetration of the replication fork. Enhanced reactivity of the DNA with $KMnO_4$ (a footprint)

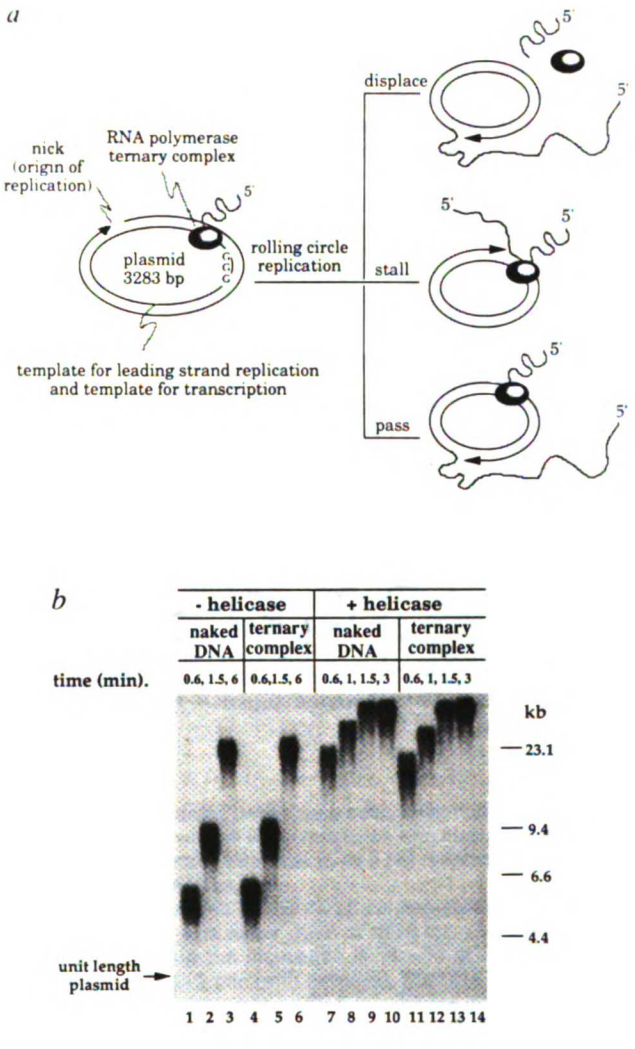


FIG. 1 *a*, The experimental system. The template for *in vitro* replication by the bacteriophage T4 replication proteins is a 3.3-kilobase-pair (kb) circular plasmid containing the replication origin of bacteriophage M13, located ~170 nt behind the stalled RNA polymerase. Nicking this origin with the filamentous bacteriophage gene 2 endonuclease provides a unique DNA 3′ end that serves as a starting site for initiation of rolling circle DNA synthesis *in vitro*[30]. Three consecutive G nucleotides were placed on the template strand 17, 18 and 19 base pairs (bp) downstream of the transcription initiation site. Using the dinucleotide UpG to initiate transcription at bp −1 (that is, one bp upstream of the normally initiating G) in the presence of rATP, rGTP and rUTP and withholding rCTP, we stalled the RNA polymerase at the triple G site with an 18-nt nascent transcript. Because rCTP is withheld, lagging strand DNA synthesis is very inefficient, and we have generally omitted the DNA primase (gene 61 protein)[36,37] from replication reactions, leaving the template for lagging strand DNA synthesis as a displaced single strand. *b*, Effect of the ternary complex on movement of the replication fork. The products of *in vitro* DNA synthesis, using either naked DNA (as control) or column-purified ternary complexes as the DNA template, were analysed by alkaline agarose gel electrophoresis, followed by autoradiography. METHODS. *a*, The plasmid pRT510-C+18, which is derived from pDH310 (ref. 38) through two rounds of mutagenesis[39], contains a −35 σ^{70} consensus sequence placed upstream of the −10 T4 late promoter consensus sequence of gene 23. The resulting promoter, P′23, is efficiently used *in vitro* by both σ^{70}-containing and gene 55 protein-containing RNA polymerases. The P′23 sequence was further changed to −1GATATGAAGAGTTGGATCCC, where +1 designates the start site of transcription (non-template strand; the entire sequence of plasmid pRT510-C+18 is available on request). To initiate DNA synthesis on circular pRT510-C+18, the DNA was specifically nicked at the M13 bacteriophage gene 2 protein recognition site, as described[40]. To prepare the ternary complex, 0.2 pmol nicked DNA was incubated with the following reagents in 40 µl for 30 min at 37 °C: 6 pmol *E. coli* RNA polymerase core, 30 pmol gene 55 protein, 9 pmol gene 33 protein, 27 pmol gene 44/62 protein, 98 pmol gene 45 protein, 1 mM dATP; 100 µM UpG, 4 µM rATP, 4 µM rGTP, 4 µM [α-^{32}P]rUTP (specific activity ~50,000–100,000 c.p.m. pmol^{-1}), 33 mM Tris–acetate (pH 7.8), 250 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol (DTT), 100 µg ml^{-1} nuclease-free BSA as protein carrier. The reaction was stopped by chilling the sample on ice, followed by the addition of NaCl to 0.5 M and gel filtration through a 1 ml CL-2B column with a 200-µl 0.5 M NaCl loading zone, and elution with replication buffer (33 mM Tris–acetate (pH 7.8), 66 mM potassium acetate, 10 mM magnesium acetate, 100 µg ml^{-1} BSA and 0.5 mM DTT) in the presence of 3–5% glycerol. Radioactive fractions were pooled for the subsequent replication reactions. Typically 70–80% of the DNA templates were occupied by a ternary complex (determined by gel shift assay). *b*, Replication in 40 µl of the replication buffer with 0.02 pmol of the column-purified ternary complex or control naked DNA, 3 µg ml^{-1} gene 43 protein, 80 µg ml^{-1} gene 32 protein, 40 µg ml^{-1} gene 44/62 protein, 20 µg ml^{-1} gene 45 protein, 20 µg ml^{-1} gene 41 protein and 1.2 µg ml^{-1} gene 59 protein (whenever the gene 41 protein was omitted, so was the gene 59 protein), 25–50 µg ml^{-1} rifampicin, 0.5 mM dATP, 0.5 mM dGTP, 0.2 mM dCTP, and 0.08 mM [α-^{32}P]dTTP (~25,000 c.p.m. pmol^{-1}). After 30 s at 37 °C, non-radioactive dTTP was added to 1 mM to stop the labelling. Aliquots were taken at the indicated times, mixed with Na_3-EDTA (20 mM final concentration), loaded on a 0.6% agarose alkaline denaturing gel, and run in 30 mM NaOH, 1 mM Na_3-EDTA for 18 h at 2 V cm^{-1}.

marks the transcription bubble of the ternary complex[5,16]; incorporating 5'-methyl dCMP (dmCMP) in place of dCMP into the newly synthesized DNA generates resistance to cutting by the restriction enzyme *Alu*I. Any *Alu*I-resistant DNA that retains the $KMnO_4$ footprint of the ternary complex can only be generated by replication forks that have replicated past the ternary complex without permanently displacing this complex.

An analysis of the ternary complex footprint by primer extension is shown in Fig. 3*b*. A comparison of lanes 2 and 5 reveals no significant reduction of the footprint signal after replication (typically <5% difference). Proof that the ternary complex is not displaced from the template after replication comes from the demonstration (lane 4) that 40–50% of the molecules that carry a ternary complex also resist *Alu*I cutting (and therefore must have replicated). When dCMP instead of dmCMP is incorporated into DNA, *Alu*I is fully active and the footprint disappears as expected (lane 3). Because the position of the footprint is unchanged after replication (lanes 2, 4 and 5), the by-passed ternary complex retains its place on DNA and its transcription bubble.

A by-passed complex remains fully functional

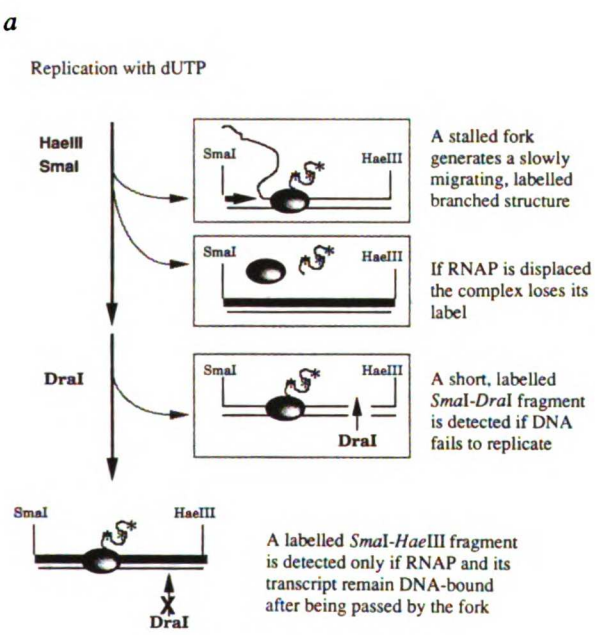
We next assessed the functional competence of ternary complexes after replication forks have passed through them. Ternary complexes bearing nascent transcripts labelled with [α-^{32}P]rUTP were purified through CL-2B. Replication proteins were added and replication was allowed to proceed until the fork had travelled several times around the circular DNA template. Non-radioactive rNTPs were then added to permit the elongation of any nascent transcripts. If the ternary complexes are inactivated by the passage of the replication fork, the pre-labelled, 18-nt nascent transcripts should not be elongated into full-length RNA. No new ternary complexes should form under our experimental conditions (no rNTPs or gene 55 protein present during replication; no gene 55 protein present during the chase); moreover, any newly initiated transcripts would not be radioactively labelled.

The results of the above experiments are shown in Fig. 4*a*. Lane 1 shows the expected 18-nt nascent transcript before a chase. Lane 2 shows that, as expected, the nascent transcripts on column-purified ternary complexes chase into 427-nt full-length RNA in the absence of DNA replication. The important result is that the 18-nt transcripts are also nearly completely converted to full-length transcripts following replication without or with DNA helicase (lanes 4 and 6, respectively). When no rNTPs are added after replication, a 'mock' 6–8 min incubation leaves the nascent 18-nt transcript unchanged (lanes 3 and 5).

To assess the fidelity of RNA synthesis after replication, we repeated the chase experiment on a DNA template cut with *Alu*I to generate only a ~33-nt run-off transcript. Identical run-off transcripts were observed before and after replication (Fig. 4*b*), demonstrating the precise retention of position by the functional ternary complex.

To test whether a nascent transcript that has been released into solution can reassociate with DNA to be further elongated, we added purified 18-nt ^{32}P-labelled RNA to a reaction mixture containing the components of the chase experiment shown in Fig. 4*a*. When incubated with RNA polymerase core (with or without gene 55 protein) and cold rNTPs (either alone or with DNA replication proteins and dNTPs), no 18-nt RNA was elongated, and most of this RNA remained detectable as a radioactive band in the 18-nt position (data not shown).

The above chase experiments are significant if a major fraction of the DNA molecules bearing ternary complexes have been



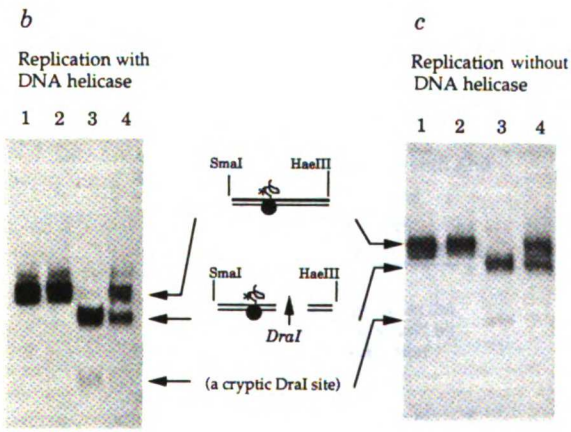


FIG. 2 A test for retention of the RNA polymerase (RNAP) ternary complex, identified by its radioactive nascent transcript, after replication. *a*, Outline of the experiment. After DNA is replicated with dUTP in place of dTTP, a *Sma*I–*Hae*III fragment bearing the ternary complex is tested for its susceptibility to *Dra*I. *b*, Gel autoradiograph after replication through RNAP ternary complexes with DNA helicase (gene 41 protein) present. Lane 1, control ternary complex on the *Sma*I–*Hae*III fragment (no replication); lane 2, control ternary complex on the *Sma*I–*Hae*III fragment after replication with dUTP; lane 3, replication with dTTP and cutting with *Dra*I (the same bands were seen when non-replicated molecules were digested with all three restriction enzymes); lane 4, replication with dUTP and cutting with *Dra*I. c, As *b* but replication was without the DNA helicase. Lanes as in *b*.

METHODS. After replication on the CL-2B column-purified ternary complexes (~0.01 pmol) with either dUTP or dTTP for ~30 s, 10 units of *Hae*III were added and the incubation continued at 37 °C for 4 min (without helicase) or 1–2 min (with helicase). The DNA was cut with 10 units of *Sma*I at room temperature for 5 min. Where indicated, 10 units of *Dra*I were then added for another 5 min at 37 °C. The reaction was stopped by chilling on ice; heparin and Ficoll were added to 100 μg ml^{-1} and 3%, respectively. Samples were loaded on a 3% (Fig. 2*b*) or 4% (Fig. 2c) non-denaturing, neutral polyacrylamide gel (37.5: 1 acrylamide: bisacrylamide in 1 × TBE (89 mM Tris base, 89 mM boric acid, 2.5 mM EDTA)) for electrophoresis at room temperature for ~5 h at 11 V cm^{-1}. The gel was dried and autoradiographed. *In vitro* replication was done as described in Fig. 1*b* except that 0.2 mM non-radioactive dTTP or dUTP was used instead of [α-^{32}P]dTTP.

replicated. To determine this fraction, nascent RNA was labelled with [α-^{32}P]rUTP and ternary complexes were purified by gel filtration. Non-replicated circular DNA templates run as a defined band during electrophoresis on neutral agarose gel. Replication converts these molecules to circular molecules with long single-stranded tails, which migrate more slowly. Because only the RNA is labelled, the changing distribution of radioactive signals in the gel as a function of time reflects the efficiency of replication on templates bearing ternary complexes (Fig. 4*c*). Quantification of radioactivity at the position corresponding to the non-replicated template reveals that ~70% of the templates bearing a ternary complex have been replicated. Moreover, there appears to be no blockage of replication fork movement by the ternary complex, because no discrete bands corresponding to stalled structures appear on the gel, even in the absence of DNA helicase. We conclude that most of our DNA templates have undergone extensive DNA synthesis, and that the ternary transcription complexes bound to them remain functional for RNA chain elongation after the passage of replication forks.

Electron microscopic examination

As an independent test of our conclusions, we have used electron microscopy to examine the fate of the ternary complex after replication. The analysis should also reveal unanticipated products of replication, if any are formed. For each DNA molecule that undergoes rolling-circle replication, the extent of such replication is easily assessed by the length of its single-stranded DNA tail. In principle, the replication fork must have passed the ternary complex without displacing it from the template if a DNA molecule bearing such a complex has a single-stranded tail that is longer than the distance from the nick to the ternary complex (~170 nt).

The template for these replication reactions was the ternary complex purified on the CL-2B column. Before visualization, replication products were re-treated with 0.5 M NaCl and passed again through CL-2B to remove replication proteins. As a control, Fig. 5*a* shows a globular particle associated with the non-replicated circular template. Several lines of evidence suggest that this particle is the ternary complex: (1) it survives high-salt (0.5 M NaCl) treatment and CL-2B gel filtration; (2) it occupies the expected place on DNA cut at unique restriction enzyme sites (such as *Ssp*I in Fig. 5*b* and *Hin*dIII in Fig. 5*c*); (3) it disappears when rNTPs are added for several minutes (not shown); (4) nascent transcripts can be seen on brief incubation (30 s) with a low concentration of rNTPs (1 μM each) (Fig. 5*d*).

Replicated DNA molecules (Fig. 5*e–g*) bearing the ternary complex have tails of varying lengths that can exceed the size of the circular template. We randomly sampled 180 molecules to obtain the data in Fig. 5*i*, *j*. A similar fraction of the DNA molecules bear the ternary complex before and after replication (Fig. 5*i*), consistent with the finding that the by-passed ternary complex remains DNA-bound. Moreover, a significant fraction of templates bearing the ternary complex have tails longer than the size of the circular template (Fig. 5*j*), proving that at least

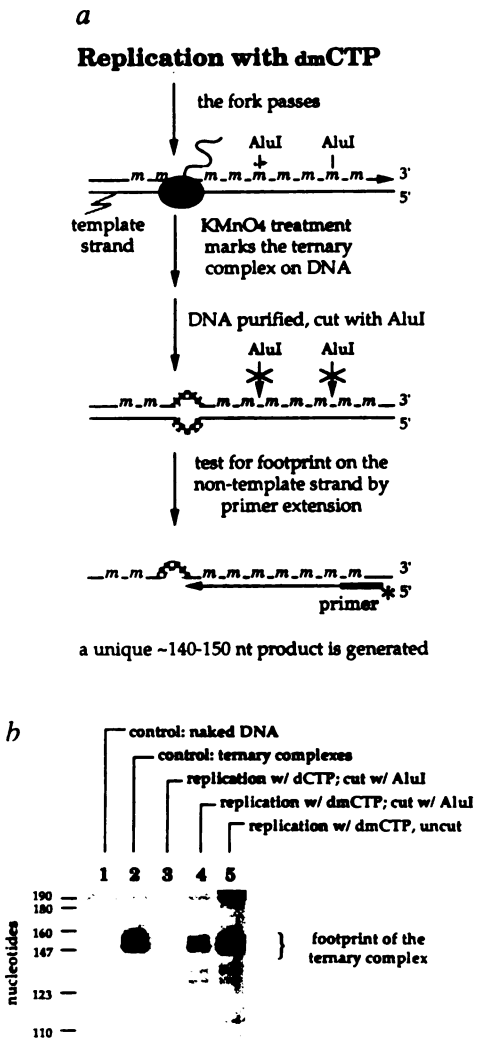


FIG. 3 Determination of the location of the ternary complex after replication. *a*, Outline of the experiment. $KMnO_4$ oxidizes regions of single-stranded DNA[41] in the ternary complex, and this footprint of the transcription bubble is observable by primer extension analysis only if DNA is resistant to cutting by *AluI* at the sites shown. Resistance is conferred by incorporation of dmCMP ('m'). The asterisk represents the ^{32}P-label at the 5′ end of the primer. *b*, Primer extension analysis. Lane 1, naked DNA control; lane 2, ternary complex control, showing the position and signal intensity of the ternary complex in unreplicated DNA not cut with *AluI*; lane 3, replication with dCTP and cut with *AluI*; lane 4 replication with dmCTP and cut with *AluI*; Lane 5 replicated with dmCTP, but not cut with *AluI*.

METHODS. Nicked DNA (0.1 pmol) was incubated with 0.4 pmol RNA polymerase core supplemented with 2 pmol gene 55 protein and 0.8 pmol gene 33 protein; 40 μg ml^{-1} gene 44/62 protein, 20 μg ml^{-1} gene 45 protein, 1 mM dATP, 120 μM UpG, 5 μM rATP, rGTP, and rUTP, 220 μM 3′-*O*-methyl rCTP as chain terminator, 5% polyethylene glycol (3.3K), 33 mM Tris–acetate (pH 7.8), 250 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT and 100 μg ml^{-1} nuclease-free BSA. After incubation at 37 °C for 15 min, potassium acetate was diluted to 120 mM and replication was allowed to proceed at 37 °C for 4 min by adding 3 μg ml^{-1} gene 43 protein, 60 μg ml^{-1} gene 32 protein, 20 μg ml^{-1} gene 41 protein, 0.2 μg ml^{-1} gene 59 protein, 0.5 mM dGTP, 0.2 mM dCTP or dmCTP, 0.2 mM dTTP, with 50 μg ml^{-1} rifampicin present to prevent re-formation of ternary complexes by way of newly initiated transcription, and 10 units of *Hae*III added to limit the extent of DNA synthesis by linearizing the DNA template. $KMnO_4$ was then added to a final concentration of 5.1 mM. After 1 min at 37 °C, the $KMnO_4$ reaction was quenched with 5 μl 14 M β-mercaptoethanol. The sample was treated with 80 μg ml^{-1} proteinase K in the presence of 0.5% SDS for 30 min at 37 °C, followed by phenol–chloroform extraction and ethanol precipitation with 15 μg ml^{-1} glycogen as carrier. The pellet was dissolved in a buffer (10 mM Bis-Tris–propane–HCl, 10 mM $MgCl_2$, 1 mM DTT, pH 7.0) that allowed optimal digestion by *AluI* (10 units) during 8 min at 37 °C. Primer extension was done with a 5′ end-labelled 19-nt single-stranded DNA complementary to the non-template strand; subsequent sample preparation and electrophoresis on a 10% polyacrylamide gel (37.5: 1 acrylamide: bisacrylamide) with 8 M urea in 1 × TBE were performed as described[42–44].

one round of replication has occurred. Thus, the replication fork is indeed able to pass the ternary complex without displacing it.

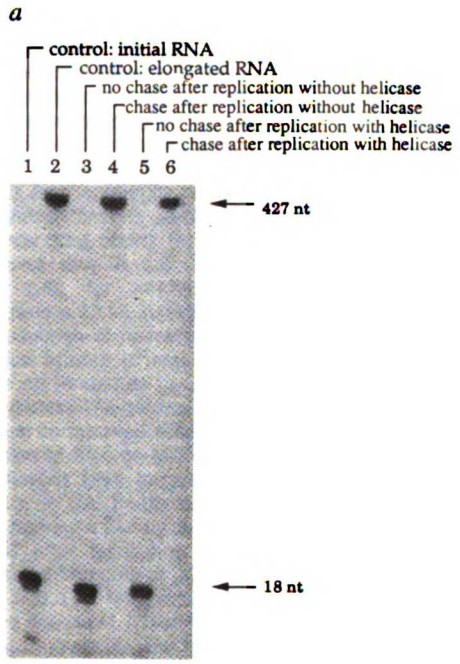
When we briefly added rNTPs to mixtures that had finished replication, nascent RNA was detected on many extensively replicated DNA templates (Fig. 5*h*), indicating that by-passed ternary complexes are functional. Finally, DNA structures other than those expected from rolling-circle replication were not observed, arguing against the possibility that any of the findings in this article are explained by some unanticipated replication mechanism.

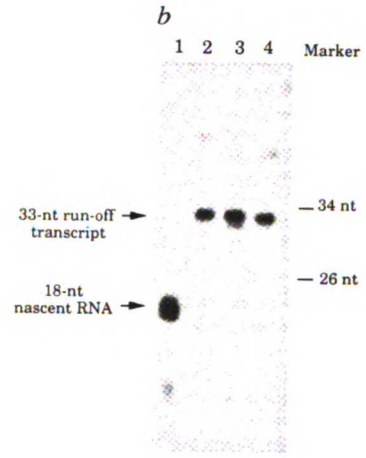
Discussion

Our examination of the consequences of a collision between a replication fork and a codirectionally orientated, stalled RNA polymerase ternary transcription complex yields a surprising result: the replication fork passes the ternary complex after only a brief pause (estimated to last <1 s; Fig. 1*b*); the by-passed ternary complex not only remains bound to the DNA (Fig. 2) with a transcription bubble at its original DNA site (Fig. 3), but it is fully competent to resume RNA synthesis (Fig. 4). Electron microscope examination of the reaction products supports this conclusion at the single macromolecule level (Fig. 5).

Our results do not merely reflect a special property of T4 late gene transcription. We have repeated the experiment shown in Fig. 4 with a ternary transcription complex derived from initiation at a σ^{70} promoter by *E. coli* RNA polymerase (in the absence of any T4 protein) and have obtained the same result (data not shown).

A stalled ternary complex is an imperfect representation of true transcription intermediates, whose normal structures are likely to be kinetically determined[4]. But the recovery of full-length transcripts during a chase in which rolling circle replication is ongoing (Fig. 4*a*) suggests that many transcription intermediates (and not just our stalled ternary complex) can survive the replication fork; further evidence supporting this point will be presented elsewhere.





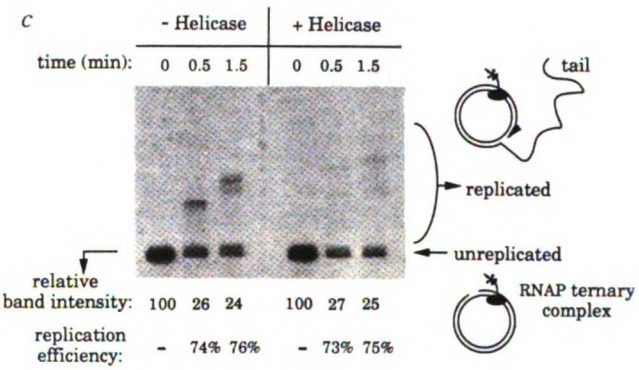


FIG. 4 The ability of a by-passed ternary complex to resume RNA chain elongation. *a*, Chase experiment on a full-length DNA template. Lane 1, (control) nascent 18-nt RNA; lane 2, (control) full-length 427-nt transcript elongated from the 18-nt RNA (no replication); lane 3, replication without helicase, no elongation of the 18-nt RNA; lane 4, replication without helicase, the 18-nt RNA was elongated with non-radioactive rNTPs; lane 5, replication with helicase (gene 41 protein), no elongation of 18-nt RNA; lane 6, replication with helicase, the 18-nt RNA was elongated with non-radioactive rNTPs. *b*, Chase experiment with *AluI*-cut DNA template. Lane 1, 18-nt RNA control; lane 2; control run-off transcript (~33-nt RNA); lane 3, run-off transcript after replication without helicase; lane 4, run-off transcript after replication with helicase. *c*, Determination of replication efficiency. Replication (with or without DNA helicase) proceeded at 37 °C for the time indicated. The replication efficiencies are calculated from the reduction of the radioactive signal (quantified using a PhosphorImager) at the position of the non-replicated molecules.

METHODS. *a*, *In vitro* replication was as described in Fig. 2 (except that the DNA was not linearized) for 5 min (without helicases) or for 2 min (with helicases), followed by the addition of cold rNTPs (0.5 mM rATP, 0.5 mM rGTP, 0.2 mM rCTP and 0.2 mM rUTP) to chase the nascent transcript at 37 °C for 8 min. Samples were then chilled on ice, treated with 2 units of DNase I (with $CaCl_2$ at a final concentration of 0.5 mM), phenol–chloroform extracted and electrophoresed on a 10% denaturing polyacrylamide gel. To rule out the possibility of RNA polymerase reassociation during the rNTP chase, this experiment has been repeated: (1) in the presence of rifampicin (30–50 μg ml^{-1}); (2) with synthetic oligonucleotides containing the promoter sequences in 10–20-fold molar excess over the template; (3) with yeast ribosomal RNA (40–100 mg ml^{-1}). These variations did not change the outcome of the experiment. *b*, As in *a*, except that RNA chains were elongated on templates that had been digested with 10 units of *AluI*. *c*, *In vitro* replication was done on the column-purified complex under the same conditions of the chase experiments described in *a* and *b*. The reaction was stopped by chilling the samples on ice at the indicated times. Heparin and Ficoll were added to concentrations of 100 μg ml^{-1} and 3%, respectively. Samples were then loaded on a 0.8% neutral agarose gel (non-denaturing) in 1 × TBE and electrophoresed at room temperature for 4–5 h at 7 V cm^{-1}. The gel was dried and autoradiographed or exposed to a PhosphorImager screen for quantitative analysis.

It is not obvious how two bulky enzyme complexes can pass one another in a non-destructive way. In particular, because the codirectionally moving DNA and RNA polymerases use the same DNA single strand as template, the replication apparatus almost certainly unwinds the end of the growing RNA transcript that is base-paired to DNA. How difficult is this likely to be? Recent structural studies on transcription complexes[17,18] suggest that growing RNA chains may be relatively loosely held at the 3′ end in a short DNA–RNA hybrid, and periodically transferred to a tighter binding site in *E. coli* RNA polymerase[17,25]. This would imply that: (1) the contribution of the RNA–DNA hybrid to the stability of the ternary complex need not be as great as previously thought[26,27], making its transient unpairing less daunting; (2) the RNA–DNA hybrid need not present an insurmountable barrier to the progression of a replication fork.

Although we cannot rule out the possibility that some property of the T4 DNA replication proteins is important for our observations (for example, tethering the RNA polymerase to the DNA molecule, thereby preventing its release as the DNA polymerase passes), it seems more likely that our results are intrinsic to the *E. coli* RNA polymerase, a multiple-subunit enzyme[28] that can undergo large conformational changes[29]. A schematic model of this type is shown in Fig. 6, where at least two DNA-interacting domains of the polymerase are present within the ternary complex, each individually detachable from the DNA without destroying the complex. When the replication fork invades the interior of the complex, it causes a momentary unpairing of the short RNA–DNA hybrid at the 3′ end of the nascent RNA in the ternary complex, but the other DNA-binding domain keeps the RNA polymerase attached to the daughter DNA helix. The nascent RNA remains bound to RNA polymerase, and it may ensure the maintenance of transcription fidelity by its specific hybridization back to the DNA template.

In contrast to the ternary transcription complex, a promoter-bound RNA polymerase that is not transcribing is displaced from the template after replication[30]. Compared to the very tight ternary complex that enables RNA polymerase to transcribe in a highly processive manner, promoter binding by RNA polymer-

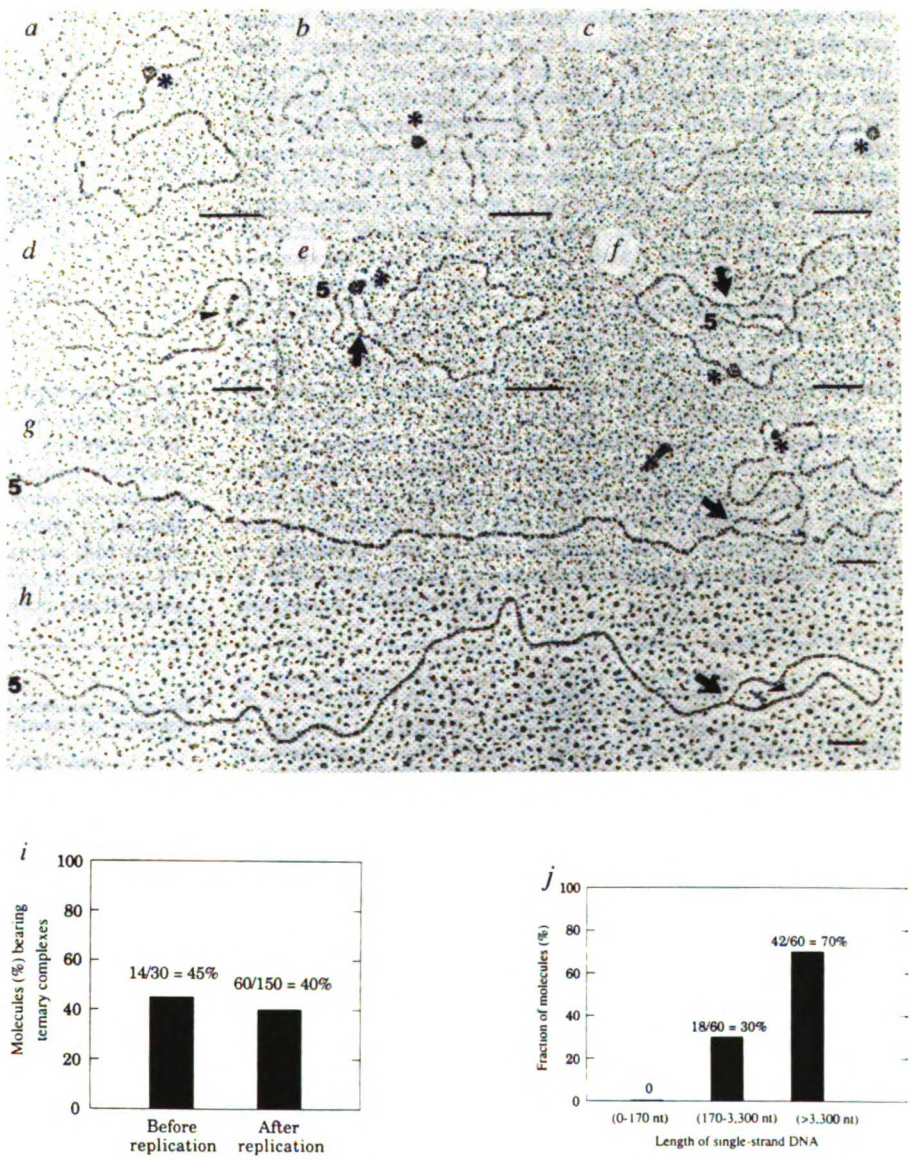


FIG. 5 Electron microscopic examination of replication products. *a*, A non-replicated molecule bearing a globular particle (the putative RNA polymerase ternary complex). *b* and *c*, Mapping the position of the globular particle by restriction enzyme digestion (*b*, *Ssp*I; *c*, *Hin*dIII). The relative distances from the particle to the two ends (long/short): for *Ssp*I, the measured ratio is 1.8 (±0.1) (expected 1.8); for *Hin*dIII, the measured ratio is 23 (±3) (expected 25). *d*, Production of nascent RNA. *e–g*, Various extents of replication take place on templates bearing a ternary complex. The ternary complex remains on the replicated DNA. *h*, Production of RNA on the extensively replicated DNA template. *i*, Comparison of the percentage of molecules bearing a ternary complex before and after replication. Randomly selected samples of 30 non-replicated and 150 replicated molecules were examined. *j*, Distribution of replicated molecules with tails of varying lengths. The distance from the gene 2 nick (replication origin) to the ternary complex is ~170 nt. If the replication fork stalls permanently before the ternary complex, no molecules should bear a tail exceeding this length. Scale bar, 0.1 μm. Arrows, replication forks. Asterisks, RNA polymerase (*a–c*, *e–g*). Arrowheads, nascent RNA (*d* and *h*). '5', the 5′ end of a displaced DNA tail.

METHODS. *In vitro* replication was done on CL-2B-column-purified ternary complex as described in the legend to Fig. 1*b*. The reaction was stopped by chilling the sample on ice, followed by the addition of NaCl to 0.5 M and passage through CL-2B to remove replication proteins. Electron microscopy studies were done on the radioactive fractions as follows: *a–c*, and *e–g*: 2–8 μl samples were applied to glow-discharged carbon grids for 2 min, dehydrated in 100% ethanol and uranyl acetate, and shadowed with platinum at an angle of 8 degrees[45]; *d* and *h*, rNTPs (1 μM each) were added to elongate the nascent 18-nt RNA at 37 °C for 30 s. The reaction was stopped with 20 mM Na_3-EDTA. Transcripts were crosslinked to DNA templates by ultraviolet light (254 nm) irradiation at 25 °C for 10 min at a distance of ~2 cm from a UVGL-25 lamp. Samples were spread with cytochrome *c* as described in ref. 46 before examining them with a Philips EM400 microscope.

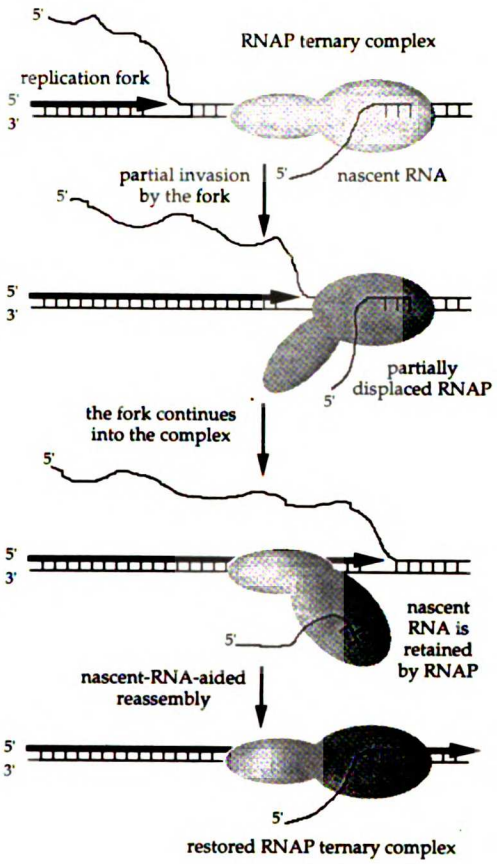


FIG. 6 A schematic model to account for some of the experimental observations. Well-separated DNA-binding domains might allow the *E. coli* RNA polymerase (RNAP) to retain its place as replication passes through. Replication proteins and the transcription bubble are not drawn. As described in the text, the retention of exact transcription register that we observed (Fig. 4*b*) is likely to involve base-pair reformation by the 3′ end of nascent RNA. In addition, the ternary complex contains about 17 base pairs of separated DNA strands[47] in the form of a transcription bubble. Because there is a substantial energetic cost to reforming this bubble[4], the passage of a replication fork through the ternary transcription complex might involve a reaction pathway that never entirely dissipates the DNA strand separation.

ase is a weaker interaction. Because it relies on hydrogen-bonding interactions with specific bases on both DNA strands[4], the separation of the two strands of the double helix during replication would be expected to destabilize the promoter complex. There is little energy investment during promoter binding by RNA polymerase, and its displacement is less costly to the cell.

Our results suggest the existence of a mechanism that allows the RNA polymerase and its attached transcript to survive the collision between the replication and transcription machineries. Because the *E. coli* and the eukaryotic nuclear RNA polymerases have evolved from a common ancestor and have homologous subunits that share amino-acid sequence homology[31,32], our results may also be relevant to the behaviour of these polymerases when a replication fork passes.

Our conclusions are entirely based on *in vitro* experiments done with highly purified proteins. Is there any *in vivo* evidence for or against a mechanism of this type? When the fate of the nascent transcript of a large *Drosophila* gene (Ubx) whose complete transcription takes longer than the time of a cell cycle was observed[33], DNA synthesis *in vivo* did not abort the nascent transcript (in this case, the orientation of the fork relative to the RNA polymerase movement is unknown). In contrast, electron microscopy has been used to show that the nascent transcripts of a rRNA gene of *E. coli* are displaced from the template when a replication fork invades the transcription unit from either direction[34]. But these rRNA transcripts are unusual in at least two aspects: they are attached to closely spaced RNA polymerase molecules, and they are modified by a set of specialized RNA-binding proteins[35]. We would predict that a different result would be obtained with other transcription units. Methods that permit a quantitative analysis, such as simultaneously probing the fork movement and nascent transcript production by nucleic acid hybridization, should be useful for examining this issue.

It is certainly possible that unidentified protein factors exist *in vivo* that modulate the basic mode of interaction between a replication fork and RNA polymerase observed in our experiments. Nevertheless, the fact that a ternary complex can survive a replication fork *in vitro* demonstrates a remarkable ability of RNA polymerase to cope with perturbing events during its elongation of RNA chains. □

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Chapter 3

The fate of a transcribing RNA polymerase during a co-directional collision with T4 DNA polymerase

This chapter is a reprint of the material as it appears in Proc. Natl. Acad. Sci. USA 91, 10660-10664 (1994). I performed all the experiments shown except Fig. 4, which was a collaborative effort between Mei Lie Wong (EM lab, HHMI and Dept. Biochemistry, UCSF) and myself. I drafted the manuscript that was edited carefully by Prof. Alberts.

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A transcribing RNA polymerase molecule survives DNA replication without aborting its growing RNA chain

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Contributed by Bruce Alberts, July 14, 1994

ABSTRACT **We have demonstrated elsewhere that a precisely placed, stalled *Escherichia coli* RNA polymerase ternary transcription complex (polymerase–RNA–DNA) stays on the DNA template after passage of a DNA replication fork. Moreover, the bypassed complex remains competent to resume elongation of its bound RNA chain. But the simplicity of our experimental system left several important questions unresolved: in particular, might the observation be relevant only to the particular ternary complex that we studied, and can the finding be generalized to a transcribing instead of a stalled RNA polymerase? To address these issues, we have created three additional ternary transcription complexes and examined their fates after passage of a replication fork. In addition, we have examined the fate of moving RNA polymerase molecules during DNA replication. The results suggest that our previous finding applies to all transcription intermediates of the *E. coli* RNA polymerase.**

DNA replication and DNA transcription are two fundamental biological processes that take place on a DNA molecule at the same time. In *Escherichia coli*, the chain elongation rate for DNA synthesis is 10–20 times greater than that for RNA synthesis, making collisions between DNA and RNA polymerases inevitable even when these polymerases move in the same direction (1, 2). We have previously examined the consequences of such a collision *in vitro*, by using the highly purified T4 DNA replication proteins to replicate through a stalled *E. coli* RNA polymerase ternary transcription complex (bearing an 18-nt RNA) (3). To our surprise, this ternary complex remains bound to DNA at its original site after passage of the replication fork, and it is fully competent to resume faithful RNA chain elongation. One could argue, however, that our findings reflect the behavior only of one ternary complex or that they are relevant only to a stalled RNA polymerase molecule. These arguments gain force from the evidence indicating that the structure of the ternary complex changes as the enzyme (*i*) moves along the template and (*ii*) encounters different nucleotide sequences (4, 5). In addition, a stalled ternary transcription complex is an incomplete model for the many intermediates in transcription elongation, whose structures are likely to be kinetically determined (4, 6). We have therefore determined the fate of three additional stalled ternary complexes after the passage of a replication fork and have also extended our studies to transcribing RNA polymerase molecules. The results presented in this manuscript suggest that our previous findings are generalizable to all transcription intermediates.

MATERIALS AND METHODS

Enzymes. *E. coli* core RNA polymerase and the purified T4 gene *55* and *33* proteins were gifts from the laboratory of E. P. Geiduschek (University of California, San Diego). DNA replication proteins (the products of T4 genes *43*, *44*, *62*, *45*, *32*, and *41*) were purified in this laboratory as described (7–12). The purification of T4 gene *59* protein will be described elsewhere (30).

The Template for Codirectional Replication and Transcription. The template for *in vitro* replication by T4 replication proteins and codirectional transcription by *E. coli* RNA polymerase is a 3.3-kb circular plasmid (pRT510-C+18). It contains (*i*) the replication origin of bacteriophage M13, which we nick with the filamentous bacteriophage gene *2* endonuclease to provide a unique DNA 3′ end that serves as a starting site for initiation of *in vitro* rolling circle DNA synthesis (13) and (*ii*) an *E. coli* σ^{70} promoter superimposed on a T4 late promoter, as described in ref. 3. The RNA made from these promoters starts with the 18-nt sequence UGAUAUGAAGAGUUGGAU, there being no cytidine until position 19.

Creating Three Additional Ternary Complexes. The ternary complex bearing a radioactively labeled 18-nt nascent RNA was prepared in the presence of UpG to prime chain initiation, [α-^{32}P]UTP, ATP, and GTP (withholding CTP), as described (3). It was purified on a Sepharose Cl-2B gel filtration column that removes the gene *55* protein (a σ factor) and NTPs. Therefore, no new ternary complexes can reform during the subsequent replication reactions (3). By isolating this complex and incubating it at 37°C for 8 min with CTP (5 μM), CTP (5 μM) and ATP (5 μM), or CTP (5 μM), ATP (5 μM), and UTP (5 μM), three other ternary complexes carrying 21-nt, 22-nt, and 25-nt RNAs were prepared, respectively.

DNA Replication Through Three Different Ternary Complexes. *In vitro* replication was carried out on templates bearing the three ternary complexes described above in replication buffer [33 mM Tris acetate, pH 7.8/66 mM potassium acetate/10 mM magnesium acetate/0.5 mM dithiothreitol/nuclease-free bovine serum albumin as protein carrier (100 μg/ml)]. A reaction volume of 20 μl contained gene *43* protein (3 μg/ml), gene *32* protein (80 μg/ml), gene *44/62* protein (40 μg/ml), gene *45* protein (20 μg/ml), gene *41* protein (20 μg/ml), gene *59* protein (1.2 μg/ml), rifampicin (50 μg/ml), 0.5 μM dATP, 0.5 μM dGTP, 0.2 μM dCTP, and 0.2 μM dTTP. Reactions were allowed to proceed for either 5 min (without a helicase) or 2 min (with a helicase), followed by the addition of nonradioactive NTPs (0.5 mM ATP, 0.5 mM GTP, 0.2 mM CTP, and 0.2 mM UTP) to chase the nascent transcript at 37°C for 8 min. Samples were then chilled on ice, treated with 2 units of DNase I (with 0.5 mM $CaCl_2$), phenol/chloroform-extracted, and electrophoresed on denaturing polyacrylamide gels containing 8 M urea. The gels were dried and autoradiographed or exposed to a PhosphorImager screen (Molecular Dynamics) for quantitative analysis.

Concurrent Replication and Transcription. To the purified ternary complex bearing ^{32}P-labeled 18-nt RNA, NTPs were added at low concentrations (20 μM ATP, 20 μM GTP, 10 μM CTP, and 1 μM UTP), along with DNA replication proteins and dNTPs at the concentrations described earlier. Aliquots were taken at the indicated time points, chilled on ice, treated with 2 units of DNase I (with 0.5 mM $CaCl_2$), phenol/

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chloroform-extracted, and then electrophoresed on a 10% denaturing polyacrylamide gel containing 8 M urea.

Measuring the Rate of DNA Chain Elongation. Concurrent replication and transcription were performed as described above, except that 60 μM [α-^{32}P]dTTP (specific activity, 50,000–100,000 cpm/pmol) was used to pulse-label the DNA made at the replication fork for 30 sec before adding 1 mM excess nonradioactive dTTP to stop the labeling. Aliquots were taken at various times, mixed with Na_3EDTA (20 mM, final concentration), loaded on alkaline denaturing 0.6% agarose gel, and electrophoresed in 30 mM NaOH/1 mM Na_3EDTA for 18 hr at 2 V/cm. As a control, all four NTPs (each at 80 μM) were added to the purified ternary complex at 37°C for 30 min to chase all RNA polymerases off the template. The mixture was further heated at 56°C for 10 min to destabilize any remaining RNA polymerase. The resulting DNA, which was essentially devoid of transcription complexes as judged by transcript production and by direct examination under an electron microscope (data not shown), was used as the control template for DNA synthesis in reactions parallel to those on DNA templates bearing ternary complexes.

Electron Microscopic Examination of Reaction Products. Concurrent replication and transcription were performed as described in the previous section. The reaction was allowed to proceed for 2 min at 37°C and stopped by chilling the sample on ice; NaCl was then added to 0.5 M, and the sample was passed through a Sepharose Cl-2B column to remove replication proteins. Radioactive fractions were pooled and concentrated with a Microcon-100 concentrator (Amicon). Transcripts were crosslinked to DNA templates by irradiation at 254 nm at 25°C for 10 min at a distance of ≈2 cm from a UVGL-25 lamp (Ultraviolet Products). Samples were spread with cytochrome *c* (3, 14) before examining them with a Philips EM400 microscope. In some cases, the samples were simply treated with NaCl (0.3–0.4 M) before UV-crosslinking, and the results were found to be unaffected by the failure to remove the replication proteins under our conditions.

RESULTS

Three Additional Ternary Complexes Can Survive the Passage of a Replication Fork. We have previously used a uniquely nicked circular DNA molecule containing a specially designed promoter and downstream sequence as a DNA template that supports codirectional replication and transcription. By withholding CTP, we stalled the RNA polymerase at a specific downstream site, creating a stable ternary transcription complex composed of RNA polymerase, a ^{32}P-labeled 18-nt nascent RNA transcript, and the DNA template. The behavior of this ternary complex upon passage of a replication fork was studied extensively (3).

By isolating the above molecule and incubating it further with CTP, with CTP and ATP, or with CTP, ATP, and UTP, the original ternary complex was converted into three additional ternary complexes bearing 21-nt, 22-nt, and 25-nt RNAs, respectively. To examine the abilities of these three complexes to resume RNA chain elongation after the passage

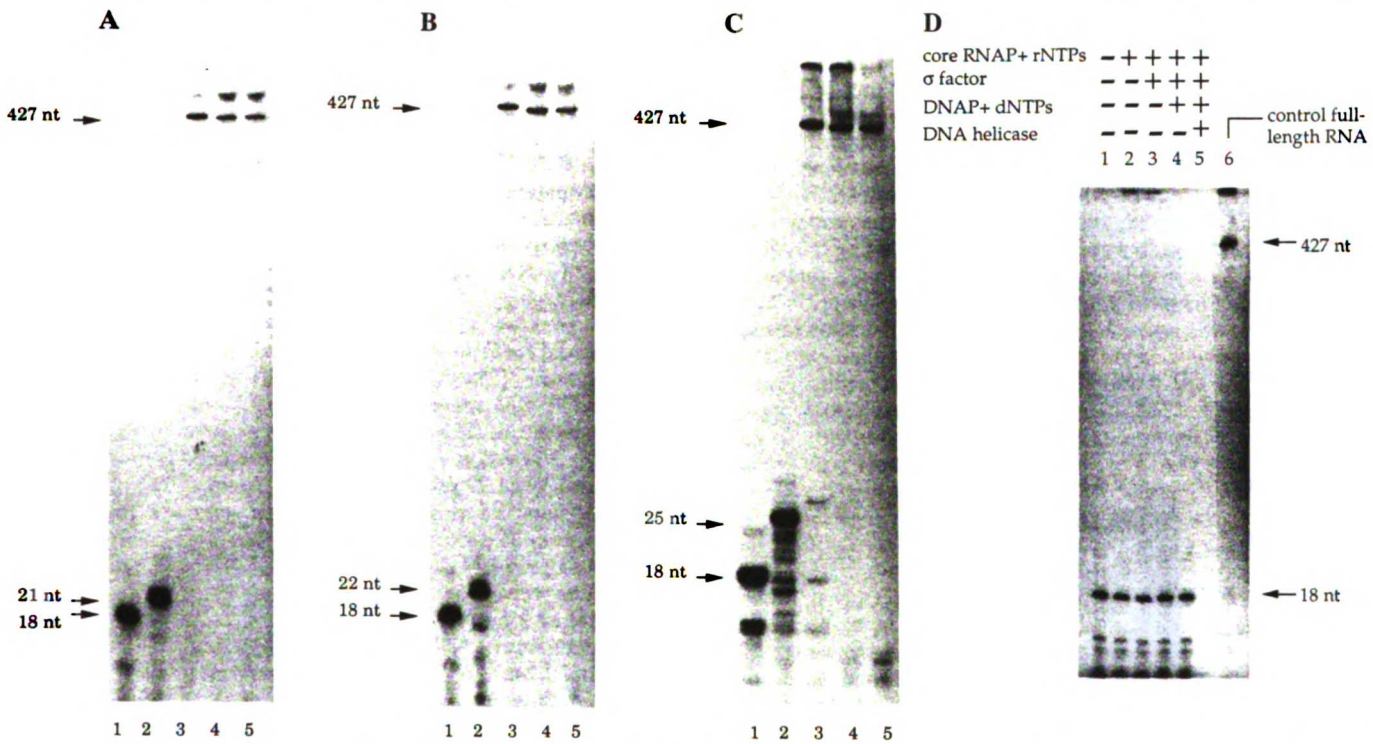

FIG. 1. Abilities of the three additional ternary complexes to survive a replication fork and to resume RNA chain elongation. (*A*) Chase experiment with a ternary complex bearing 21-nt RNA. Lanes: 1, parental 18-nt RNA; 2, 21-nt RNA; 3, full-length 427-nt transcript elongated from the 21-nt RNA (no replication); 4, after replication without DNA helicase, the labeled nascent RNA (21 nt) was elongated with nonradioactive NTPs; 5, after replication with helicase (gene *41* protein), the labeled nascent RNA was elongated with nonradioactive NTPs. (*B*) Chase experiment with a ternary complex bearing 22-nt RNA. Lanes are as in *A*, except that the nascent RNA is 22 nt. (*C*) Chase experiment with a ternary complex bearing a 25-nt RNA. Lanes are as in *A*, except that the nascent RNA is 25 nt. The samples were analyzed on a denaturing 10% (*A* and *B*) or 12% (*C*) polyacrylamide gel. (*D*) Control experiment to test for the possible effects of incubations on free 18-nt RNA molecules. Lanes: 1, purified 18-nt RNA control; 2–5, purified 18-nt RNA after incubation, as indicated (−, not added; +, added), with core RNA polymerase (RNAP) and all four NTPs, σ factor (gene *55* protein) plus gene *33* protein, DNA replication proteins (DNAP, products of genes *43*, *44*, *62*, *45*, and *32*) and all four dNTPs, and DNA helicase (gene *41* protein) plus gene *59* protein; 6, control 427-nt full-length transcript. For these experiments, 0.02 pmol of ^{32}P-labeled 18-nt RNA (purified from deproteinated ternary complexes) was incubated at 37°C for 5 min with 0.02 pmol of DNA template, 0.08 pmol of core *E. coli* RNA polymerase, 0.25 mM ATP, 0.25 mM GTP, 0.1 mM CTP, and 0.1 mM UTP, yeast rRNA at 40 μg/ml (to neutralize potential RNase contamination), and the replication proteins and dNTPs, where indicated. For analysis, samples were treated with DNase I followed by proteinase K and electrophoresed through a denaturing 12% polyacrylamide gel.

of a replication fork, rolling circle DNA replication was begun and allowed to proceed until the fork had traveled several times around the circular DNA template (an incubation of 2 min at 37°C when the gene *41* DNA helicase was present, or 5 min when it was absent). All four nonradioactive NTPs were then added to elongate any nascent RNA that has survived DNA replication and the size of the ^{32}P-labeled RNA was examined by polyacrylamide gel electrophoresis followed by autoradiography. The results are shown in Fig. 1. We know that 65–70% of the template molecules bearing a ternary complex have been replicated (ref. 3, this study, and data not shown). Nevertheless, as shown by the elongation of all of the nascent RNAs and the near equal recovery of the full-length transcript with or without DNA replication in Fig. 1 *A–C*, all three ternary complexes survived the passage of a replication fork, remaining competent to resume subsequent RNA chain elongation.

To test for the possibility that a nascent transcript that has been released into solution can reassociate with the DNA so as to be further elongated under our conditions, we added purified 18-nt ^{32}P-labeled RNA molecules to a reaction mixture containing all of the components present during the chase with all four nonradioactive NTPs in the experiment in Fig. 1 *A–C*: RNA polymerase core (with or without the σ factor gene *55* protein), all four unlabeled NTPs, DNA replication proteins, and all four dNTPs. As shown in Fig. 1*D*, none of the 18-nt RNA molecules were elongated in this experiment. We conclude that a RNA molecule is unable to reassociate with a DNA template molecule and be elongated once it has been released from a ternary complex.

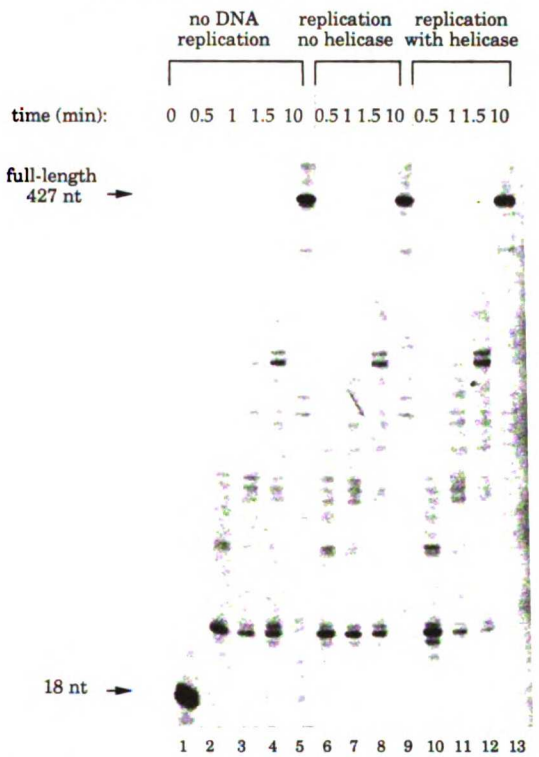

FIG. 2. Concurrent DNA replication does not abolish the production of full-length transcripts. Lanes: 1, 18-nt RNA before elongation; 2–5, control time course showing the rate of transcript production in the absence of DNA replication; 6–9, time course showing the rate of transcript production during concurrent replication without a DNA helicase; 10–13, time course showing the rate of transcript production during concurrent replication in the presence of the gene *41* DNA helicase. Analysis of the radioactivity at the position of 427-nt RNA with a PhosphorImager shows an equal recovery (<5% difference) of the full-length transcript in lanes 5, 9, and 13.

Replication Through Moving RNA Polymerase Molecules. To generalize our finding to the transcription intermediates formed by a moving RNA polymerase molecule, a very low concentration of all four NTPs was added to the purified ternary complex, allowing the RNA polymerase to elongate at a slow rate that completes the full-length transcript of 427 nt in 8–10 min. Concurrently, DNA replication proteins and all four dNTPs were also added to allow rapid DNA replication on the templates being transcribed. If an elongating molecule of RNA polymerase behaves differently from a stalled ternary complex and is unable to survive the passage of a replication fork, no more than 30–35% full-length transcripts should be recovered in this experiment, since 65–70% of the DNA molecules are replicated under our experimental conditions. As shown in Fig. 2, we obtained nearly complete recovery of full-length transcripts, suggesting that a transcribing RNA polymerase is also not displaced by the repli-

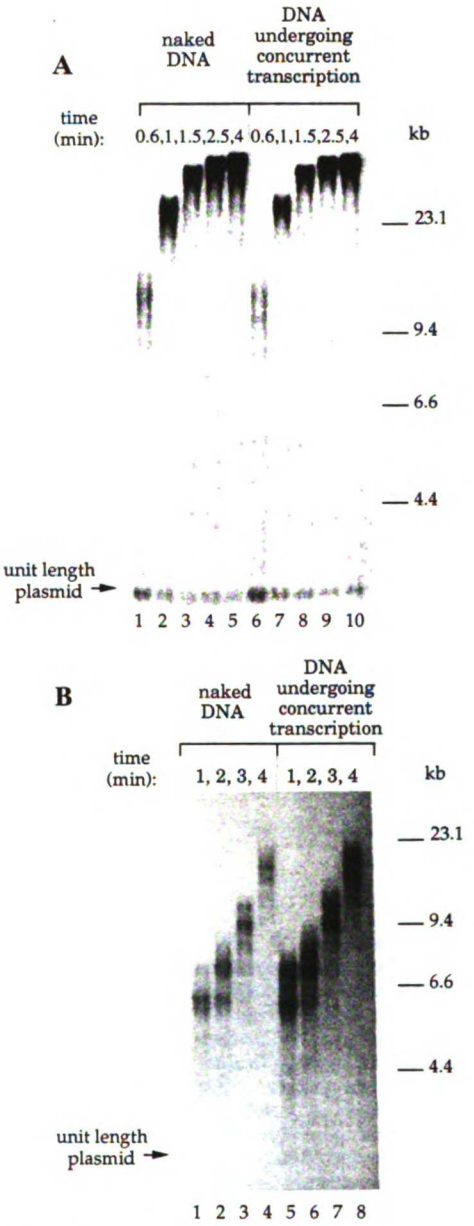

FIG. 3. Rate of DNA synthesis on templates undergoing concurrent transcription. (*A*) Replication with a DNA helicase. Lanes: 1–5, time course (as indicated) of replication on templates that lack a transcription complex (naked DNA); 6–10, time course of replication on templates bearing a slow-moving RNA polymerase. (*B*) Replication without the gene *41* DNA helicase. Lanes are as in *A*.

Table 1. Quantification of electron microscropic studies on products of concurrent DNA replication and transcription

Replication status	Molecules bearing a visible RNA, no. found/ no. examined	Molecules bearing a visible RNA with indicated tail, no. found/no. examined		
		0–600 nt	600–3300 nt	>3300 nt
Nonreplicated	22/30 (74)	0	0	0
Replicated	25/35 (71)	3/25 (12)	6/25 (24)	16/25 (64)

Randomly selected samples of 30 nonreplicated and 35 replicated molecules were examined. Column 2 shows that concurrent DNA replication does not reduce significantly the fraction of templates bearing a nascent RNA. Columns 3–5 determine the extent of DNA replication on those RNA-bound templates. The distance from the gene *2* nick (replication origin) to the end of the transcription unit is ≈600 nt. If the replication fork can only follow behind the RNA polymerase, no molecules with a RNA transcript should bear a tail exceeding this length. Samples were replicated at 37°C for 2 min, UV-irradiated, and spread with cytochrome *c*. Although nearly 100% of the DNA templates bear a visible RNA polymerase ternary transcription complex viewed on glow-discharged carbon grids (15), only 74% of these templates bear a visible RNA transcript using the cytochrome *c* spreading method. This discrepancy is likely to be due to (*i*) not all of the RNA being crosslinked to the DNA template by UV irradiation, (*ii*) some transcripts (e.g., those <30 nt) being too short to be visible, or (*iii*) some molecules of RNA polymerase having finished transcription, so that the RNA has fallen off the template. Numbers in parentheses are percent of total.

cation fork. When analogous experiments were performed with the RNA polymerase elongating at faster rates (≈1.4 nt per sec and ≈40 nt per sec), similar results were obtained (data not shown).

To test whether each replication fork was simply following behind a transcribing RNA polymerase molecule, we used alkaline gel electrophoresis to examine the rate of DNA synthesis on templates undergoing concurrent transcription. As shown in Fig. 3, the rate of replication was not significantly reduced by the presence of a slow-moving RNA polymerase molecule. For example, after a 4-min incubation, when the RNA polymerase was still in the middle of the transcription unit (8–10 min was required to complete transcription, see Fig. 2), the replication fork had traveled several times around the circular template (three or four times in the absence of a DNA helicase and >10 times in the presence of the gene *41* DNA helicase). As essentially all of the DNA templates bear a RNA polymerase ternary complex (as determined by electron microscopic examination), the replication fork must pass rather than follow behind the transcribing RNA polymerase molecules.

Electron Microscopic Studies on the Replication Products. We further examined the products of the concurrent replication and transcription reaction by using electron microscopy. In these experiments, purified ternary complexes were prepared and then provided with a low concentration of all four NTPs to allow DNA transcription to proceed at a rate of ≈50 nt per min. At the same time, DNA replication was begun. For each DNA molecule that undergoes rolling circle replication, the extent of replication can be determined from the length of its protruding single-stranded DNA tail. In principle, the replication fork must have passed the tran-

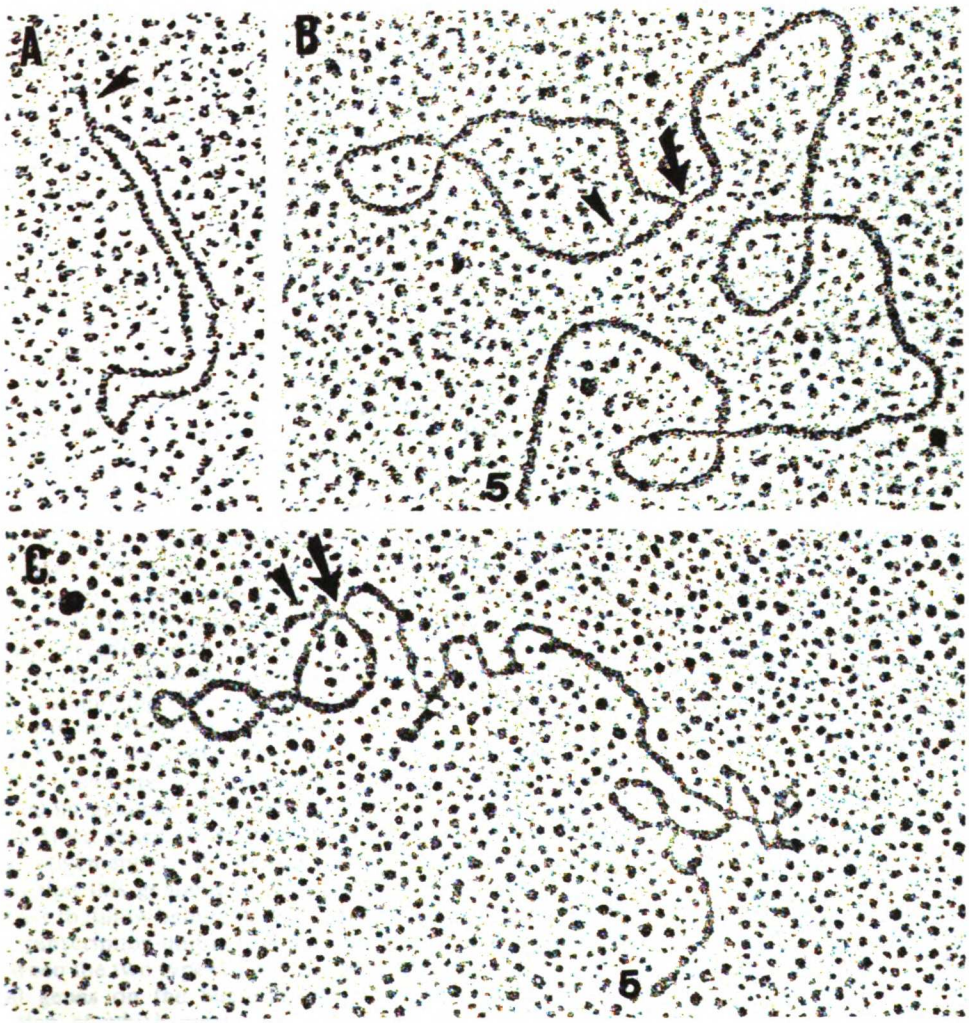

FIG. 4. Electron microscopic examination of products of concurrent replication and transcription. (*A*) Control nonreplicated molecule bearing a nascent RNA. (*B* and *C*) Various extents of replication take place on templates undergoing concurrent transcription. The nascent RNA remains on the replicated DNA. The relatively close spacing between the replication fork and the nascent RNA seen in *B* and *C* is coincidental, as the overall pattern of spacing is essentially random among molecules examined. The arrow indicates the replication fork. The arrowhead indicates the nascent RNA. 5, 5′ end of a displaced DNA tail. (*A*, ×99,120; *B*, ×110,880; *C*, ×72,240.)

scribing RNA polymerase molecule without displacing it from the template if a DNA molecule bears both a visible RNA transcript and a single-stranded DNA tail that is >600 nt (the distance from the gene *2* nick to the end of the transcription unit). An analysis of randomly selected electron micrographs shows that the fraction of templates bearing a nascent transcript does not change after replication (Table 1). Moreover, the length of the single-stranded DNA tail on many of the DNA molecules with transcripts is longer than the length of the plasmid (Fig. 4 and Table 1). We conclude that the replication fork can pass a moving RNA polymerase molecule without displacing it from the template.

DISCUSSION

Many cellular enzymes perform their functions on the same double-helical DNA molecule. Little is known about how these trafficking events are coordinated inside the cell. In particular, when DNA replication and DNA transcription take place concurrently and progress in the same direction, the respective polymerases must use a common DNA strand as their template. Given the large dimensions of the two enzyme complexes involved (16–19), one might predict that a transcription complex could not survive the passage of a replication fork without disassembling. By using the highly purified T4 DNA replication apparatus to replicate through a uniquely stalled ternary transcription complex, we (3) showed recently not only that the RNA polymerase can survive its collision with a DNA replication fork but also that it does so without loss of its transcript. However, an extraordinary heterogeneity (both structural and functional) is displayed by ternary transcription complexes (4, 5, 20, 21), and we needed to determine whether our finding, obtained from one particular ternary complex, is generalizable to other such complexes. Moreover, because a stalled transcription complex may display properties that are not seen in a moving complex (4, 22), one cannot automatically extrapolate findings made with a stalled RNA polymerase to the actively transcribing enzyme.

In this report, we have now extended our previous studies by demonstrating that the ability to survive DNA replication is not peculiar to one particular stalled ternary transcription complex (Fig. 1). In addition, we find that a moving RNA polymerase can survive the passage of a replication fork without aborting its nascent transcript (Figs. 2–4 and Table 1).

We have used a set of well-characterized highly purified replication proteins encoded by bacteriophage T4 for our *in vitro* studies. In previous studies from this laboratory (23), a T4-encoded DNA helicase, the Dda protein, was required for the replication fork to pass a promoter-bound RNA polymerase. More recently, this requirement has been eliminated by the addition of the T4 gene *59* protein, an accessory protein to the DNA helicase of the T4 primosome, the gene *41* protein (30). We have omitted the Dda protein in the experiments described here, since we find that it destabilizes ternary transcription complexes, independently of DNA replication and any of the replication proteins. The degree of destabilization increases with an increasing concentration of Dda protein (B.L., data not shown). Further characterization of this destabilizing effect of Dda protein on the ternary complex will be presented elsewhere.

In *E. coli*, the replication fork is initiated bidirectionally from a single site, *oriC*, and it travels around each half of the ≈4700-kb genome in 40 min (24, 25). Most of the heavily transcribed genes are oriented in the direction of replication fork movement (2). Brewer and Dennis (2) originally speculated that this genomic organization allows the DNA polymerase to follow behind the RNA polymerase until the end of the transcription unit is reached, hence, avoiding a collision between the two types of polymerases. But there are at least 3000 genes on the bacterial chromosome (26), a sizable fraction of which must be transcribed throughout the cell cycle (27). To make the replication fork wait for transcription would make DNA replication a slave of gene expression. Although the available physiological evidence is scanty and conflicting (28, 29), in our *in vitro* studies the replication fork does not follow behind a transcribing RNA polymerase; instead, it is able to pass the RNA polymerase rapidly without destroying ongoing transcription. In our view, the genomic organization of *E. coli* described in ref. 2 is best interpreted as an indication that the bypass reaction that we observe occurs most readily when both the DNA and the RNA polymerase molecule are moving in the same direction. Further experiments are needed to test this hypothesis.

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Chapter 4

Consequences of a head-on polymerase collision

This chapter is a preprint of the material that will appear in Science (in press). I performed all the experiments shown and drafted the manuscript, which was edited carefully by Prof. Alberts.

Science, in press

Head-on collision between DNA replication apparatus and RNA polymerase transcription complex

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Abstract:

Using an *in vitro* system reconstituted from purified proteins, we have examined what happens when the DNA replication apparatus of bacteriophage T4 collides with an *E. coli* RNA polymerase ternary transcription complex that is poised to move in the direction opposite to that of the moving replication fork. In the absence of a DNA helicase, the replication fork stalls for a long time after the encounter with the RNA polymerase. However, when the T4 gene 41 DNA helicase is present, the replication fork passes the RNA polymerase after a pause. This pause is longer than the pause observed for a co-directional collision between the same two polymerases, suggesting that there is an inherent disadvantage to having replication and transcription directions oriented head-to-head. After the DNA replication machinery passes, the RNA polymerase not only remains competent to resume faithful RNA chain elongation (as for a co-directional collision), but it switches from its original template strand to use the newly synthesized daughter DNA strand as template.

Introduction:

The *E. coli* genome is arranged in a curious way: most of the heavily transcribed genes are oriented in the direction of the leading strand of the DNA replication fork (1, 2). A similar non-random gene organization is also seen in other bacteria (3), plasmids, and bacteriophages (1). These observations suggest that a co-directional collision between RNA and DNA polymerases is less disadvantageous to an organism than an oppositely-

oriented (head-on) collision.

We have previously examined the consequences of a collision between a DNA replication fork and co-directionally moving RNA polymerase (4, 5). We find that the replication fork can pass the RNA polymerase ternary complex even in the absence of a DNA helicase; surprisingly, the bypassed RNA polymerase ternary complex remains bound at its original place on the DNA template, and is fully competent to resume RNA chain elongation.

We now examine the consequences of an oppositely-oriented collision between a replication fork and a RNA polymerase ternary complex *in vitro*. We find that the replication fork stalls for a long time during such a head-on collision with RNA polymerase if there is no DNA helicase present. However, when the DNA helicase is added, the replication fork passes the RNA polymerase after a brief pause. We also investigate the consequences of this bypass reaction and find that the RNA polymerase switches its template strand, requiring that its RNA-DNA helix break up and reform with a new DNA partner.

A template for investigating the head-on collision of a replication fork with a RNA polymerase molecule. A uniquely nicked circular DNA molecule containing an appropriately oriented *E. coli* σ^{-70} promoter has been used as a DNA template that supports oppositely-directed DNA replication and DNA transcription (the nick that primes leading-strand DNA synthesis is located in the DNA strand that serves both as the

template for transcription and as the template for lagging-strand DNA synthesis). We begin our reaction by adding purified *E. coli* RNA polymerase and ribonucleoside triphosphates (NTPs) to this DNA; because we omit CTP, the RNA polymerase begins synthesis at the promoter but stops at the first G nucleotide on the template. This creates a stable ternary transcription complex composed of RNA polymerase, an 18-nt nascent RNA transcript, and the DNA template (6). After purifying this ternary complex on Sepharose Cl-2B to remove promoter-bound RNA polymerase and a few other, less stable ternary complexes (4), we add the proteins and nucleotides required to start DNA synthesis. Because the σ factor and NTPs are removed by the Cl-2B treatment, new RNA chains cannot be initiated during the DNA replication reaction (4).

During a head-on collision, the T4 DNA helicase is required for the replication fork to pass the ternary complex. Seven highly purified bacteriophage T4-encoded proteins reconstitute an *in vitro* replication system that catalyzes efficient leading-strand DNA synthesis. The proteins involved are the T4 DNA polymerase holoenzyme (consisting of the products of T4 genes 43, 44, 62, and 45), a helix-destabilizing single-stranded DNA-binding protein (gene 32 protein), the highly processive DNA helicase (gene 41 protein), and the gene 59 protein that greatly facilitates the loading of the gene 41 protein onto DNA at a replication fork (Barry, J. & B.M.A., manuscript in press). Gene 61 protein (primase) along with gene 41 protein

constitutes the primosome, making primers for lagging-strand DNA (Okazaki fragment) synthesis. Altogether, those eight proteins constitute the complete T4 replication apparatus, catalyzing coupled leading- and lagging-strand DNA synthesis at a rate comparable to that observed *in vivo* (reviewed in ref. 7)

We determined the effect of stalled RNA polymerase ternary complexes on the movement of oppositely-oriented replication forks by analyzing the rate of increase in DNA strand lengths during replication, using alkaline agarose gel electrophoresis (8). As the DNA template, we used either naked DNA or Cl-2B purified ternary complexes. In the absence of the gene 41 DNA helicase, the replication fork experiences a prolonged stall when the collision is head-on (Fig. 1A, compare lanes 1-3 with lanes 7-9). In marked contrast, this DNA helicase is not required for the replication fork to pass the ternary transcription complex when they move in the same direction (4).

When the DNA helicase is added to the reaction, the replication fork quickly overcomes the ternary complex roadblock, but it advances at a net rate that is notably reduced from that observed for a template lacking RNA polymerase (compare lane 4 with lane 10 in Fig. 1A), suggesting that the fork pauses transiently before passing the ternary complex. From such data, the pause time can be estimated at 1.7 sec for a head-on collision, which is about twice as long as the estimated pause time observed when the colliding polymerases are oriented in the same direction (4).

We next allowed coupled leading- and lagging-strand DNA synthesis to

occur by including gene 61 primase (along with gene 41 protein) in the reaction (8). (In these reactions, ATP and CTP were also added because they are required to initiate Okazaki fragment synthesis (9), causing the 18-nt RNA to be elongated to a 22-nt RNA as an incidental consequence (5)). As shown in Fig. 1B, the complete replication fork is again able to pass the ternary complex after a pause (again estimated at 1.7 sec), and the synthesis of Okazaki fragments is not affected by the presence of the RNA polymerase.

The ternary complex remains bound to the duplex DNA after the replication fork passes. The experiment in Fig. 1 shows that, when a DNA helicase is present, the DNA replication fork passes a DNA template-bound RNA polymerase molecule that carries a nascent transcript. What is the fate of this bypassed ternary complex? In principle, there are three possible outcomes (illustrated in Fig. 2A): the ternary complex can fall off the DNA template, it can remain attached to its original template strand (the displaced, single-stranded DNA tail in this case), or it can be re-established on the replicated, duplex DNA (which requires a switch of template strands). To distinguish between these possibilities, ternary complexes, labeled on their 18-nt RNA, were used as templates for replication in the presence of the gene 41 DNA helicase. After extensive DNA replication, the DNA was cut with restriction enzymes SmaI and HaeIII, and the amount of ternary-complex-labeled SmaI - HaeIII fragment remaining was determined by electrophoresis through a neutral polyacrylamide gel (10).

As shown in Fig. 2B, this labeled restriction fragment, carrying RNA polymerase and its nascent RNA, is almost completely recovered after replication (quantification of the radioactive signal typically shows < 8% difference before and after replication), indicating that the ternary complex stays bound to duplex DNA rather than being displaced into solution or onto a DNA single strand.

The above gel shift experiment is significant only if a major fraction of the DNA molecules bearing ternary complexes have been replicated. To determine this fraction, we analyzed the mobility of the RNA-label bound to intact replicated DNA molecules by neutral agarose gel electrophoresis (11). The non-replicated circular DNA templates run as a defined band during such electrophoresis, but replication converts these molecules to circular molecules with long single-stranded tails, which migrate more slowly. Since only the RNA is labeled, the changing distribution of radioactive signals in the gel reflects the efficiency of replication on templates bearing ternary complexes. The results are shown in Fig. 2C: quantitation of radioactivity at the position corresponding to the non-replicated template reveals that 70% - 75% of the templates with a ternary complex have been replicated. We conclude that the majority of our DNA templates have undergone extensive DNA synthesis, and therefore that the experiment in Fig. 2B shows that the bypassed ternary transcription complexes remain bound to duplex DNA.

The bypassed RNA polymerase ternary complex remains fully functional.

We next assess the functional competence of ternary complexes after replication forks have passed through them (12). As before, ternary complexes bearing labeled nascent transcripts were purified through Cl-2B. Replication proteins (including gene 41 protein) were added and replication was allowed to proceed until the fork had traveled several times around the circular DNA template. Non-radioactive NTPs were then added to permit the elongation of any nascent transcripts. If the ternary complexes are inactivated by the passage of the replication fork, the pre-labeled, radioactive 18-nt nascent transcripts should fail to be elongated into full-length RNA. It is also important to note that no new ternary complexes should form under our experimental conditions (no NTPs or σ factor present during replication, no σ factor present during the chase); moreover, any newly initiated transcripts would not be radioactively labeled.

The results of the above experiment are shown in Fig. 3A. Lane 1 shows the expected 18-nt nascent transcript prior to a chase. Lane 2 is a control without replication: as expected, the nascent transcripts on column-purified ternary complexes chase into 427-nt full-length RNA. Lane 3 is the actual experiment, which shows that the 18-nt transcripts are also nearly completely converted to full-length transcripts following replication with DNA helicase.

To assess the fidelity of RNA synthesis after replication, we repeated the experiment in Fig. 3A on a DNA template cut with AluI where we expect only a 33-nt run-off transcript. As shown in Fig. 3B, identical yields of run-

off transcripts were obtained before and after replication (< 5% difference), demonstrating the precise retention of position by the functional ternary complex.

There was no lagging-strand DNA synthesis in the above experiments, because the DNA primase (gene 61 protein) was omitted. We have also performed the experiment in Fig. 3A with the primase present, so that the T4 replication apparatus catalyzes both leading- and lagging-strand DNA synthesis. In this further experiment, the original ternary complex bearing an 18-nt RNA is converted into one bearing a 22-nt RNA due to the presence of ATP and CTP in the reaction (5). As shown in Fig. 3C, this 22-nt RNA remains fully functional and can be elongated into a 427-nt full-length RNA after replication. This result also extends our observation to a second ternary complex, located 4 nucleotides downstream from the original one.

For all of the Fig. 3 experiments, the replication efficiency was estimated as 70% -75% (the assay used is described in Fig. 2C). The resumed RNA transcript terminates at the normal termination site (Fig. 3A & 3C), and its termination occurs prematurely after restriction enzyme digestion of the DNA duplex (Fig. 3B), confirming the observation made in Fig. 2B that the bypassed ternary complex remains on double-stranded DNA. This outcome is very surprising. Because of the 5' to 3' direction of polymerization of both DNA and RNA polymerase, a direct head-on collision between these two polymerases does not occur on DNA: that is, two polymerase molecules that are moving in opposite directions on the same DNA duplex are using complementary DNA strands as the template. At first glance, a head-on

collision could be most easily resolved if each respective polymerase molecule held onto its original DNA template strand. In this case, the DNA polymerase would travel along the leading-strand template, while the RNA polymerase would remain bound to its displaced template strand (the single-stranded DNA in our experiment, which is also the template for lagging-strand DNA synthesis; see illustrations in Fig. 2A). However, our results indicate that this conceptually simple outcome of a polymerase head-on collision is not realized. Rather, a template switch by the bypassed RNA polymerase accompanies DNA replication.

Replication through moving RNA polymerase molecules. A stalled ternary transcription complex is an incomplete model for the many intermediates in transcription elongation, whose structures are likely to be kinetically determined (13). Could our findings reflect the behavior only of a stalled RNA polymerase molecule? As done previously for the analogous co-directional collision (5), we performed concurrent DNA replication and DNA transcription reactions to address this issue (14).

In order to maintain transcription for a prolonged period, a low concentration of the four NTPs was added to the purified ternary complex, causing the RNA polymerase to elongate at a slow rate that completes the full-length transcript of 427 nt in 2 to 5 minutes. Concurrently, DNA replication proteins and dNTPs were also added to allow leading- and lagging-strand DNA replication on the templates being transcribed. Alkaline gel electrophoresis followed by autoradiography of the radioactive

DNA was used to examine the rate of DNA synthesis on these transcribing, circular DNA molecules. As shown in Fig. 4, the rapidly-moving replication fork is able to pass the moving RNA polymerase molecule approaching head-on, although there is a reduction of strand elongation rate that indicates a brief pause, analogous to the result obtained for the stalled ternary complex. After a 1 min incubation, when the RNA polymerase should still be in the middle of the transcription unit, the replication fork had travelled ~ 3 - 4 times around the circular template as judged by the length of the DNA product strand.

We next examined the fate of the elongating RNA polymerase molecules during DNA replication. If an elongating molecule of RNA polymerase behaves differently from a stalled ternary complex and is unable to survive the passage of a replication fork, no more than 25% - 30% of the control amount of full-length ^{32}P-labeled RNA transcripts should be recovered in this experiment, since 70% - 75% of the DNA molecules are being replicated under our experimental conditions. However, as shown in Fig. 5, we obtained nearly complete recovery of full-length transcripts (compare lane 5 with lane 10), indicating that a transcribing RNA polymerase is not displaced when it encounters the replication fork approaching head-on.

Discussion:

We have studied the consequences of a head-on collision between the T4 DNA replication machinery and a transcribing *E. coli* RNA polymerase molecule and obtained unexpected results: first, it is more difficult for the replication fork to pass a oppositely oriented transcription complex than one

that is co-directionally oriented, although the fork readily passes either transcribing polymerase molecule when the T4 DNA helicase is present; second, the bypassed RNA polymerase molecule switches to the newly synthesized daughter DNA strand as its template, and it thereby remains on the double-stranded DNA where it is competent to resume faithful RNA chain elongation.

Why does a head-on collision retard replication fork movement more than a co-directional one? The current model for DNA replication involves a coupled leading-and lagging-strand DNA polymerase complex with additional proteins (helicase and primase, etc) assembled at the replication fork (7). The blockage of any one component in this complex, especially the DNA helicase, could impede the movement of the entire replication complex (15). However, we observed the most interference with fork movement with a minimal replication system that allows only leading-strand DNA synthesis in the absence of a DNA helicase, suggesting that the blockage acts directly on the leading-strand DNA polymerase holoenzyme rather than indirectly on other components. Although other possibilities could be entertained (16), we favor the hypothesis that the RNA polymerase is intrinsically polar in regard to the passage of a replication complex.

Perhaps the most surprising observation is the RNA polymerase template switch during passage of the replication fork (see Results). At present, there are two competing models that describe the structure of a transcription intermediate: one invokes a relatively long RNA-DNA hybrid (~ 12 base pairs) that plays a role in stabilizing the ternary complex (17); the

other suggests that the RNA-DNA hybrid is very short (~ 2- 3 base pairs) and unlikely to be a major contributor to the stability of the ternary complex (18). Our results are most easily explained by the latter model, inasmuch as it would seem to make template switching a less daunting task. Alternatively, if a long RNA-DNA hybrid exists, it does not play a major role in stabilizing the ternary complex. (Note that unlike the case of a co-directional collision, the RNA-DNA hybrid would not be destined for destruction in a head-on collision if the RNA polymerase remained on its original template DNA strand.)

Switching templates and holding onto DNA in the midst of traffic undoubtedly requires some acrobatic movements by the RNA polymerase. This remarkable flexibility may originate in part from the complexity of this enzyme. The *E. coli* RNA polymerase is a very large, multi-subunit protein complex, wrapping around at least four turns of double helical DNA during RNA elongation (19). Partial detachment of the enzyme from DNA, an almost inevitable step to accommodate a passing DNA polymerase, is presumably tolerated (4). In contrast, the bacteriophage-encoded RNA polymerases are much simpler: a single polypeptide chain of ~ 110 kd executes all the functions of promoter recognition and RNA chain elongation (20). It would therefore be interesting to determine whether these simpler RNA polymerases are displaced by the passage of the DNA replication machinery.

Is our conclusion consistent with known *in vivo* observations? The available physiological studies on this subject have yielded controversial

results. On the one hand, French has used electron microscopy to examine the fate of a replication fork travelling through an *E. coli* ribosomal RNA operon (21). She observed that the movement of the replication fork was hardly affected by co-directionally transcribing RNA polymerase but was retarded significantly by RNA polymerase approaching from the opposing direction, consistent with our *in vitro* observations (4, 5 and this study). However, French suggested that RNA polymerase is dislodged from the template when the replication fork approaches from either direction. Possible causes of this discrepancy have been discussed in ref. 4. Gene units other than the ribosomal RNA operon should be studied to help resolve this issue.

Unlike *E. coli*, where DNA replication starts from a single OriC site, eukaryotes initiate DNA synthesis from numerous discrete sites along their large chromosomes. They also have large genes that measure up to several megabases and require several hours to be completely transcribed (22). Aborting such large transcripts during a collision with the DNA replication apparatus is clearly inefficient. When the fate of a very large *Drosophila* gene (Ubx) was studied, it was found that DNA synthesis did not abolish the ongoing transcription, although the origin of replication has not yet been mapped and the orientation of replication fork movement is unknown (23). Given the results of our *in vitro* studies showing that the *E. coli* RNA polymerase can stay on a DNA duplex regardless of the orientation of the collision (4, 5 and this study), and considering that eukaryotic DNA and RNA polymerases share common structural

organization with their prokaryotic counterparts (24), it is tempting to speculate that the eukaryotic transcription apparatus may survive DNA replication as well.

So far our study has revealed only one clear disadvantage for a polymerase head-on collision: the notably obstructed movement of the replication fork. However, this may not be the only problem generated by this type of collision. Our suspicion in this regard is raised upon examining the ribosomal DNA locus in yeast and other eukaryotic cells (25). The yeast ribosomal DNA locus consist of tandemly repeated transcription units (genes) with replication origins situated in the nontranscribed spacers. The two forks initiating at each of these origins experience unequal fates. The fork moving in the direction of transcription proceeds unimpeded through multiple gene repeats, while the oppositely-directed fork arrests at a polar barrier just before it encounters the transcription terminator for the adjacent, upstream transcription unit (26). The arrest is mediated by proteins binding to specific DNA sequences, independent of transcription (27). Polar replication fork barriers permanently block rather than reduce the rate of movement of the replication fork (26, 27). We speculate that, in addition to retarding the fork movement, a head-on collision may expose the RNA polymerase to other potential problems, the nature of which has not yet been revealed by our experiments.

Has a selective pressure against a head-on collision between RNA and DNA polymerase been maintained through out the evolution of all cells? We should soon have the information we need to catalogue the gene

organization around each local replication origin in eukaryotes such as yeast, allowing us to determine whether the relative directions of DNA replication and transcription are non-randomly arranged, as in *E. coli*.

Figure legends:

FIG. 1 Effect of the ternary complex on DNA replication. (**A**) Leading-strand DNA synthesis only. The products of *in vitro* DNA synthesis with or without gene 41 helicase, sampled at the indicated time points using either naked DNA (as control) or column-purified ternary complexes as the template, were analyzed by alkaline agarose gel electrophoresis followed by autoradiography. Arrows at right indicate band positions caused by the indicated blockages. (**B**) Complete replication fork catalyzing coupled leading- and lagging-strand DNA synthesis. As only ATP and CTP are present as ribonucleoside triphosphates, the priming efficiency is lower than normal and the average size of Okazaki fragment is ~ 6 - 7 kb instead of the usual 2 - 3 kb (28).

FIG. 2 Replication past a ternary complex with a head-on orientation to the approaching replication fork. Replication was carried out in the presence of the gene 41 DNA helicase, but without the gene 61 DNA primase, so that no Okazaki fragments are made. (**A**) Possible fates of the bypassed ternary complex for reactions in which only the leading strand DNA is synthesized.

Since the displaced DNA single strand is the original template for RNA synthesis, if the RNA polymerase is to remain on duplex DNA, it must switch its template to the newly synthesized daughter DNA strand. The possibilities shown can be distinguished by monitoring the mobility status of the SmaI - HaeIII fragment carrying the RNA-labeled ternary complex: this DNA fragment remains unchanged after replication only if the bypassed RNA polymerase resides on duplex DNA. (**B**) Gel autoradiograph of a test for retention of the RNA polymerase ternary complex after replication under the Fig. 1A conditions. The DNA is cut with SmaI and HaeIII after replication for ~ 30 sec at 37 ^{0}C, and the SmaI - HaeIII fragment bearing the ternary complex (arrow) is monitored for its mobility change. Lane 1: control, mobility before replication. The ternary complex is identified by its radioactive nascent transcript. Lane 2: mobility after replication. No significant reduction of the radioactive signal is seen, indicating that the bypassed ternary complex stays on duplex DNA. (**C**) Determination of the fraction of DNA molecules carrying a ternary complex that are replicated (replication efficiency). In this experiment, all of the DNAs are left intact (no restriction nuclease digestion), so that replicated molecules move as a heterogeneous smear near the top of the gel. Lane 1: non-replicated molecules carrying the labeled ternary complex. Lane 2: replication without the gene 41 DNA helicase for 1 min at 37 ^{0}C. The fork stalls, generating a unique, branched DNA structure that appears as a band that moves more slowly than the original DNA band. Lane 3: replication with the gene 41 DNA helicase for 30 sec at 37 ^{0}C. The

replication efficiencies are calculated from the reduction of the radioactive RNA signal (quantified using a PhosphorImager) at the position of the non-replicated molecules.

FIG. 3 The ability of a bypassed ternary complex to resume RNA chain elongation. (**A**), Chase experiment on a full-length DNA template after leading-strand DNA synthesis. Lane 1: (control) nascent ^{32}P-labeled 18-nt RNA; lane 2: (control) full-length 427-nt transcript elongated from the 18-nt RNA (no replication); lane 3: replication with gene 41 helicase, the ^{32}P-labeled 18-nt RNA was elongated with non-radioactive NTPs. (**B**), Chase experiment with AluI-cut DNA template after leading-strand DNA synthesis. Lane 1: 18-nt RNA control; Lane 2: control run-off transcript (33-nt RNA). Lane 3: run-off transcript after replication with helicase. The "*" indicates a cleavage product of the 18-nt RNA that is carried by non-chasable ("dead-end") ternary complex (29). (**C**), Chase experiment on a full-length DNA template after coupled leading- and lagging-strand replication. Lane 1: (control) 22-nt RNA; lane 2: (control) full-length 427-nt transcript elongated from the 22-nt RNA (no replication); lane 3: after coupled leading- and lagging-strand replication, the ^{32}P-labeled 22-nt RNA was elongated with non-radioactive NTPs.

FIG. 4 Increase in DNA strand lengths with time during DNA replication on a DNA template undergoing concurrent transcription (14). Low concentrations of NTPs (0.1 mM for ATP and GTP, 0.05 mM for CTP and

0.02 mM for UTP) were added to a purified ternary complex in order to allow elongation of the RNA polymerase at a slow rate (~ 3 nt / sec), along with a complete set of DNA replication proteins and dNTPs to start coupled leading- and lagging-strand DNA synthesis. Samples were taken at the indicated time points and DNA strand lengths were analyzed on a 0.6% alkaline denaturing gel.

FIG. 5 Increase in RNA size caused by RNA synthesis during concurrent head-on DNA replication. Concurrent replication and transcription reactions were performed as described (14). Samples were taken at the indicated time points and analyzed on a 10% denaturing polyacrylamide gel containing 8 M urea. The "*" indicates a cleavage product from the "dead-end" ternary complex described in the legend to Fig. 3B (29).

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6. The 3.3 kilobase pair (kb) circular plasmid is derived from pRT510-C+18 (4) by inverting a 116 bp EcoRI - EcoRI fragment containing the replication origin of bacteriophage M13, located ~ 190 bp away from the stalled RNA polymerase. It contains an *E. coli* σ^{70} promoter superimposed with a T4 late promoter. In the presence of the dinucleotide UpG, the RNA made from this promoter starts with the 18-nt sequence UGAUAUGAAGAGUUGGAU, there being no C nucleotide until position 19. To initiate DNA synthesis on this circular plasmid, the DNA was specifically nicked at the M13 bacteriophage gene 2 protein recognition site. The ternary complex was prepared on Cl-2B as described in ref. 4; it carries a 32P-labeled 18-nt RNA molecule. As examined by electron microscopy, all the template DNA is occupied by the desired ternary complex. About 50% of the DNA molecules also bear an additional ternary complex initiated at a cryptic promoter (located ~ 2 kb away from the desired ternary complex). The degree of occupancy by the cryptic complex decreases when increased salt concentrations are used prior to loading the ternary complex on the Cl-2B column. Elimination of this cryptic complex by high salt treatment (> 0.5 M NaCl) is possible, but this salt concentration also decreases the recovery of the desired ternary complex bearing an 18-nt RNA. Although we have not further examined the nature of this cryptic complex, it does not affect the outcome of our studies with the desired ternary complex.

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added to 100 μg/ml and 3%, respectively. Samples were loaded on a 4% non-denaturing polyacrylamide gel (37.5: 1 acrylamide: bisacrylamide in 1 X TBE (89 mM Tris base, 89 mM boric acid, 2.5 mM Na_3EDTA)) for electrophoresis at room temperature for ~ 5 hours at 11 V/cm.

11. After 30 sec or 1 min of replication with or without gene 41 helicase respectively, the reaction was stopped by chilling the sample on ice. Heparin and ficoll were added to concentrations of 100 μg/ml and 3%, respectively. Samples were then loaded on a 0.8% neutral agarose gel in 1 X TBE and electrophoresed at room temperature for 4-5 hr at 7 V/cm. The gel was dried and autoradiographed or exposed to a PhosphorImager screen (Molecular Dynamics, CA) for quantitative analysis.

12. For the experiment shown in Fig 3A, leading-strand replication only was carried out for 1 min, followed by the addition of cold NTPs (0.5 mM ATP, 0.5 mM GTP, 0.2 mM CTP and 0.2 mM UTP) to elongate the nascent transcript at 37 ^{0}C for 8 min. Samples were then chilled on ice, treated with 2 units of DNaseI (with $CaCl_2$ at a final concentration of 0.5 mM), phenol-chloroform extracted and electrophoresed on a 10% denaturing polyacrylamide gel. The experiment shown in Fig. 3B differed only in that RNA chains were elongated on templates that had been digested with 10 units of AluI. For the experiment shown in Fig. 3C, coupled leading- and lagging-strand replication was carried out for 1 min, followed by the addition of cold NTPs (0.5 mM ATP, 0.5 mM GTP, 0.2 mM CTP and 0.2 mM UTP) to elongate the nascent transcript at 37 ^{0}C for 8 min.

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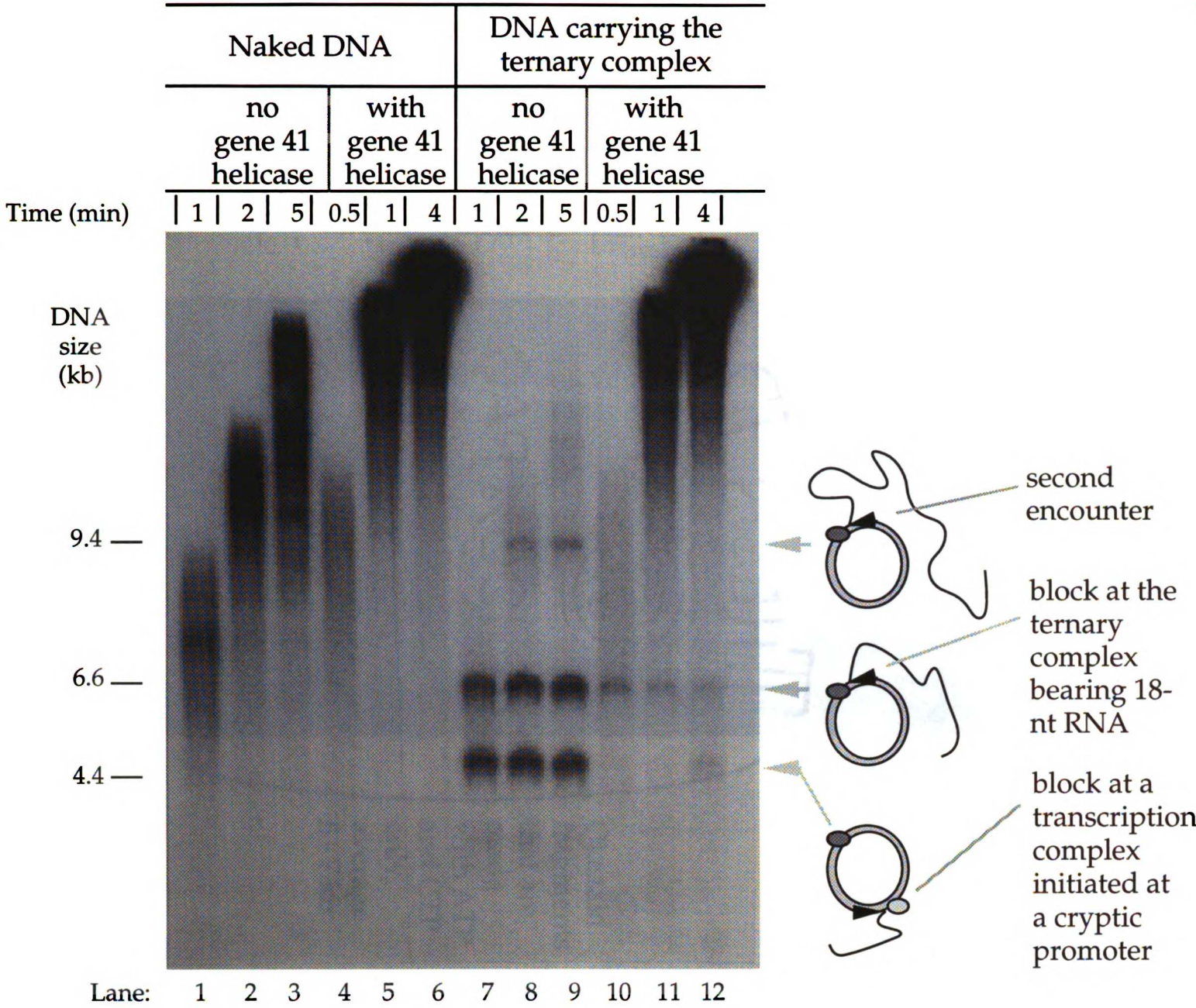
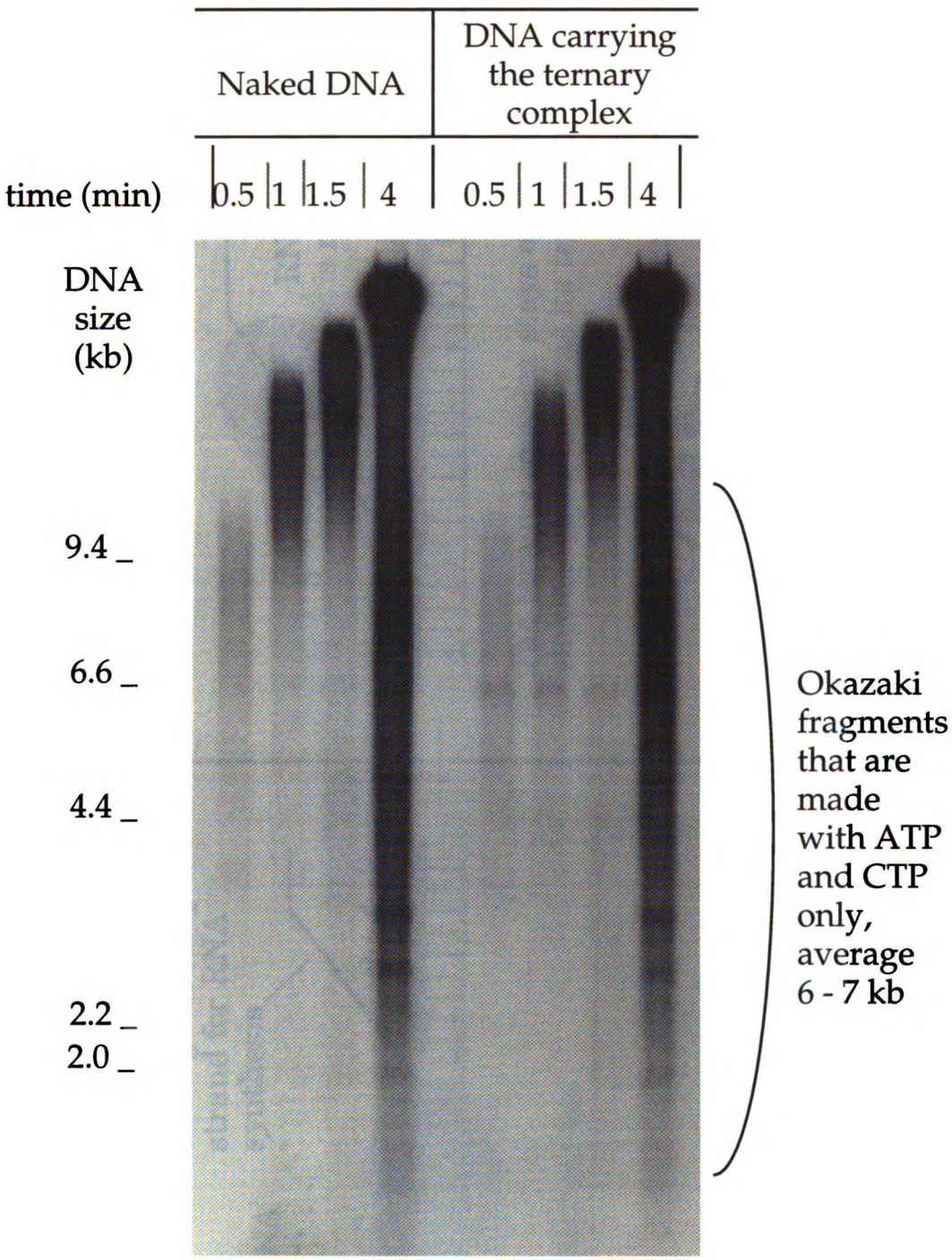
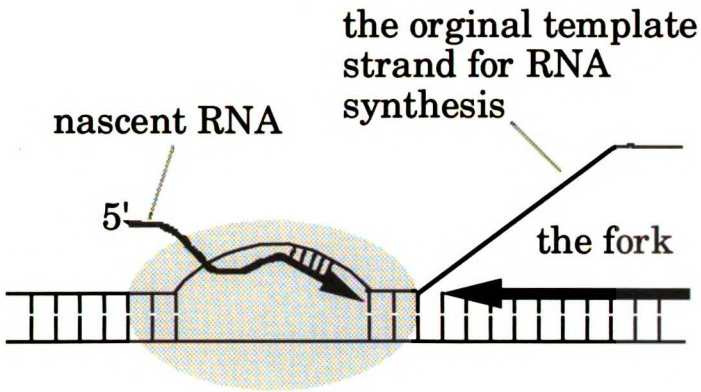

Naked DNA
DNA carrying the ternary complex
no gene 41 helicase
with gene 41 helicase
no gene 41 helicase
with gene 41 helicase
Time (min)
1 2 5 0.5 1 4 1 2 5 0.5 1 4
DNA size (kb)
9.4
6.6
4.4
Lane: 1 2 3 4 5 6 7 8 9 10 11 12
second encounter
block at the ternary complex bearing 18-nt RNA
block at a transcription complex initiated at a cryptic promoter

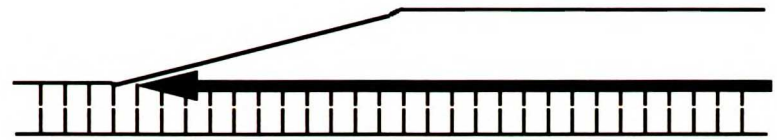

Naked DNA
DNA carrying the ternary complex
time (min)
0.5
1
1.5
4
0.5
1
1.5
4
DNA size (kb)
9.4
6.6
4.4
2.2
2.0
Okazaki fragments that are made with ATP and CTP only, average 6 - 7 kb

A head-on collision between the DNA replication fork and RNA polymerase:

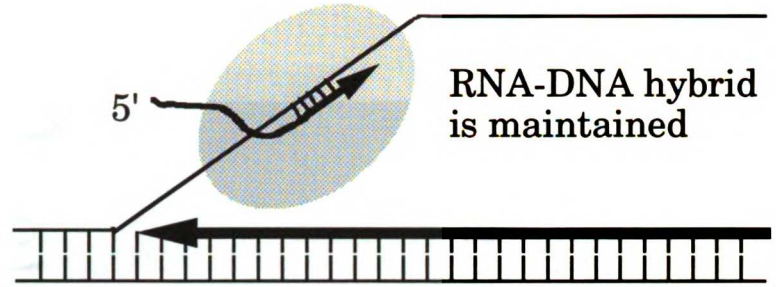


RNA polymerase

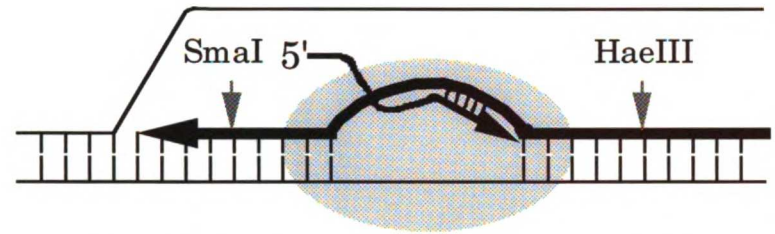
Possible outcomes:



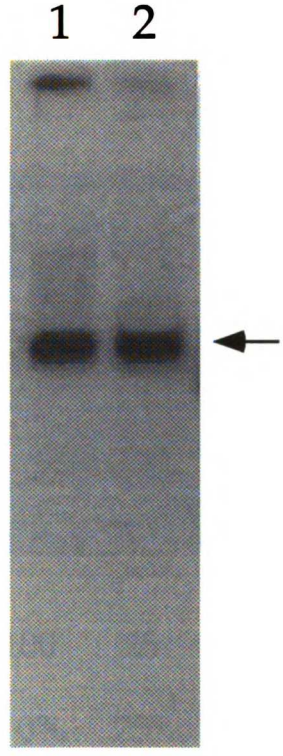
The ternary complex is destroyed

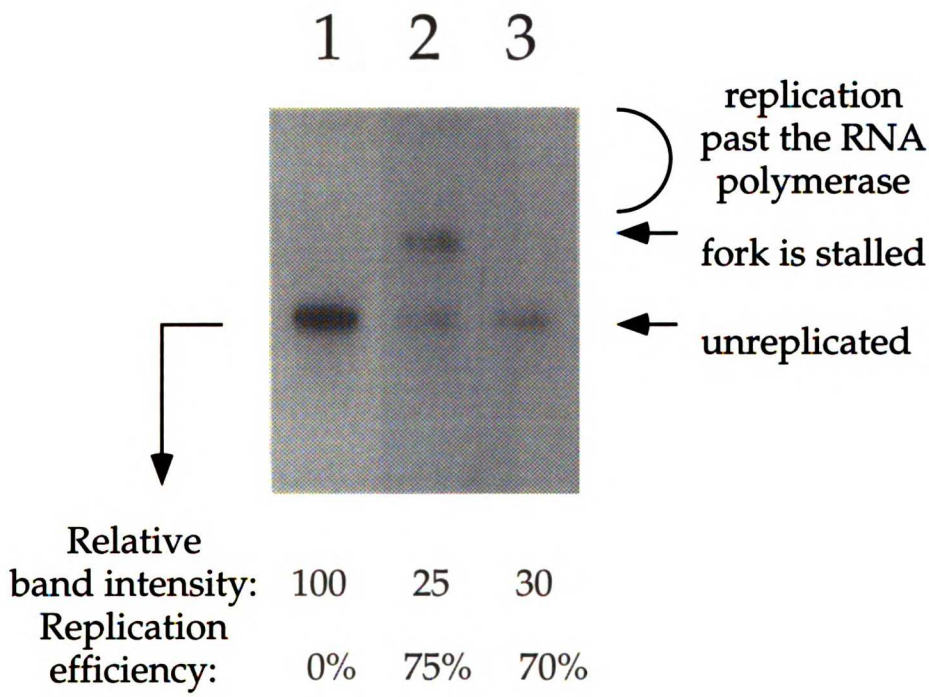


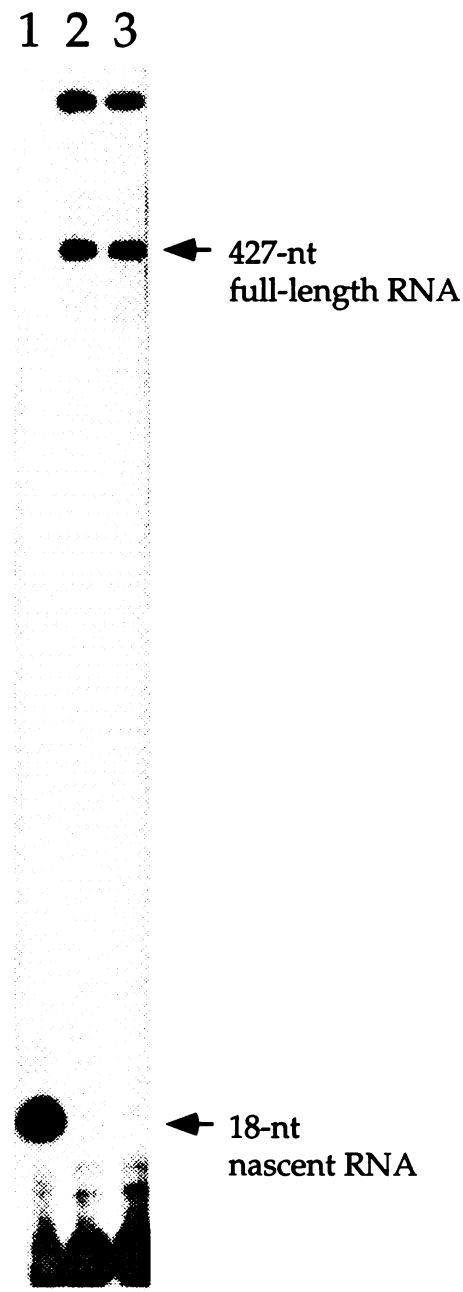
The ternary complex stays on the DNA single strand that was its template

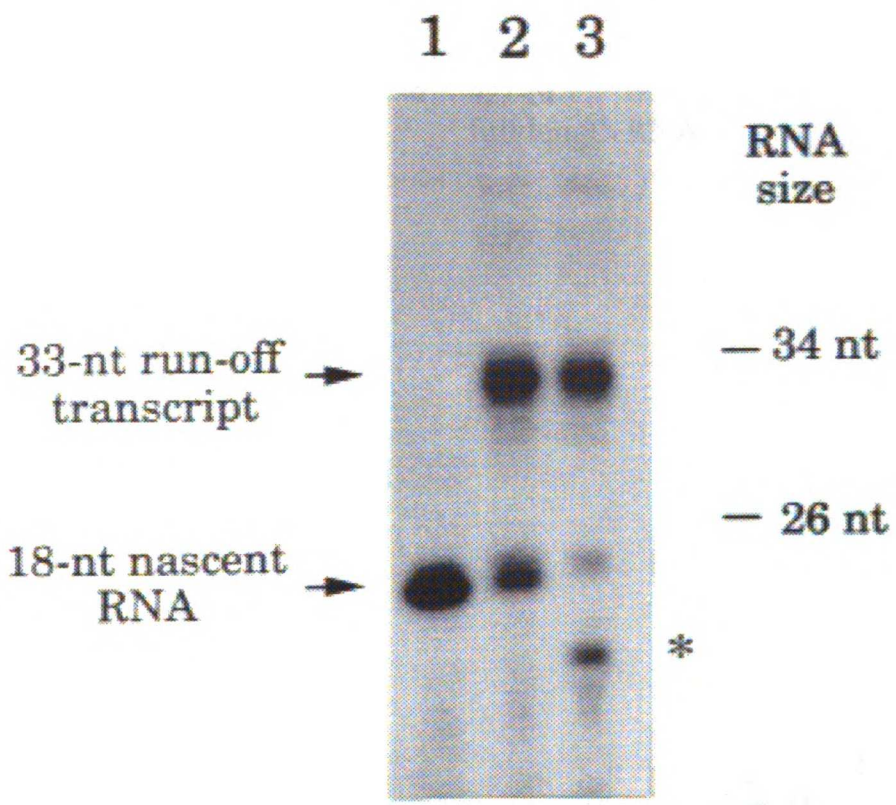


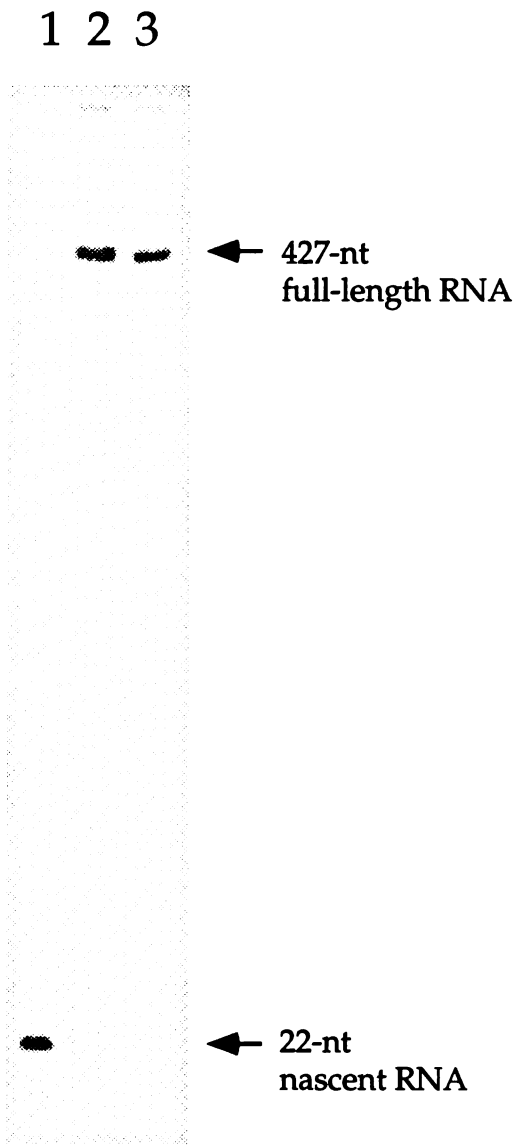
The nascent RNA switches its template strand as the ternary complex is re-established on the replicated duplex DNA

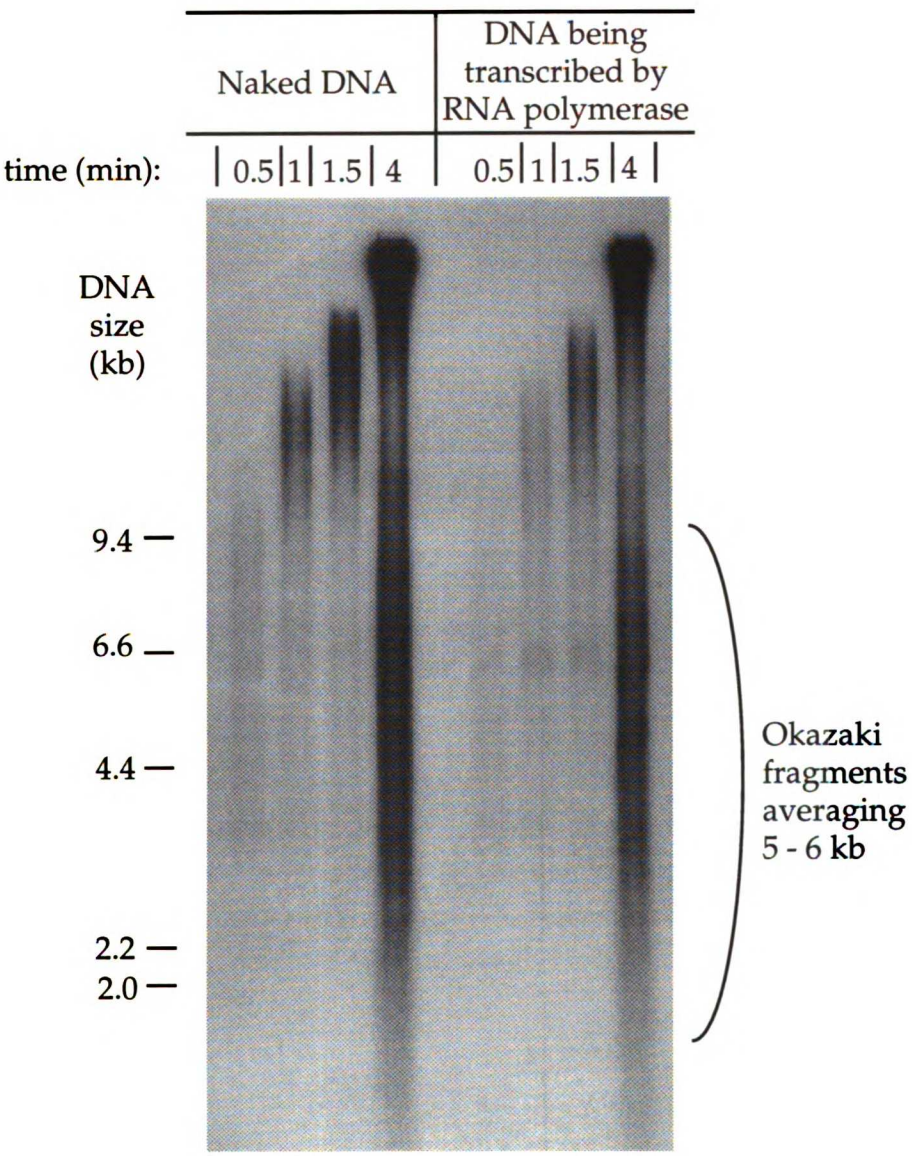

1
2

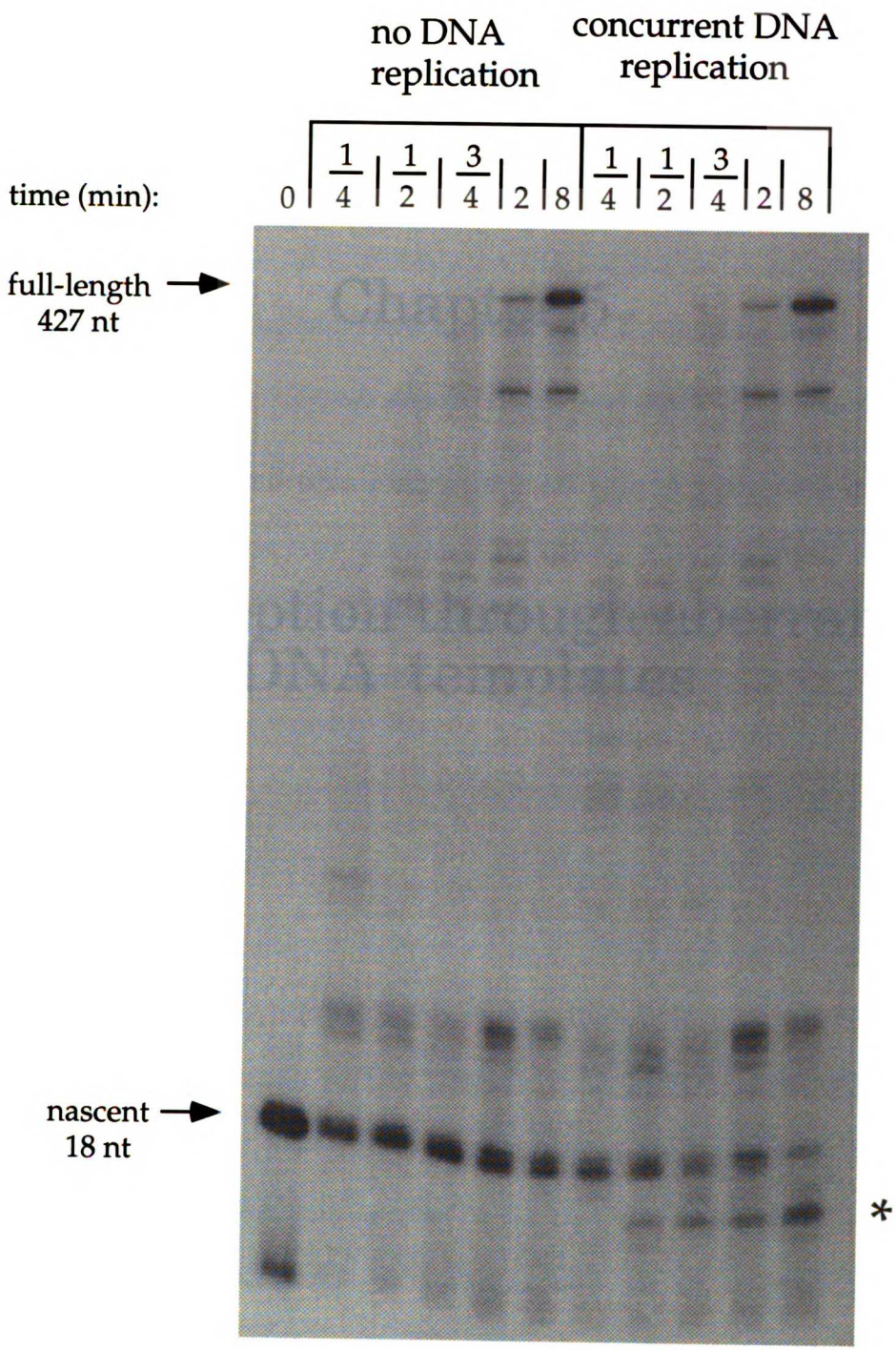

1 2 3
replication past the RNA polymerase
fork is stalled
unreplicated
Relative band intensity: 100 25 30
Replication efficiency: 0% 75% 70%


1 2 3
427-nt
full-length RNA
18-nt
nascent RNA


1 2 3
RNA size
33-nt run-off transcript
18-nt nascent RNA
— 34 nt
— 26 nt
*


1 2 3
427-nt
full-length RNA
22-nt
nascent RNA





Chapter 5

Transcription through aberrant DNA templates

Abstract:

When *E. coli* RNA polymerase moves along a single-stranded DNA template, it displays poor fidelity and fails to stop at the normal termination sites. When an RNA polymerase molecule that is moving along duplex DNA encounters a single-stranded DNA branch, it is able to switch its template DNA strand so as to remain on the duplex DNA for continued RNA synthesis. The template switch reaction is favored by high nucleotide concentrations and is inhibited when ATPγS and AMP-PNP are used as substrates for RNA synthesis, suggesting an energy-requiring mechanism. A template switch of this type may be a relatively common event that accompanies the elongation of RNA chains, preventing the entry of the RNA polymerase onto a single-stranded template.

Introduction:

Our studies on the collision between the T4 DNA replication apparatus and the *E. coli* RNA polymerase have shown that RNA polymerase is capable of performing some sort of "molecular acrobatics" that allows it to survive the collision. In particular, during a head-on collision with a replication fork, the RNA polymerase switches template to the daughter DNA strand on the leading side of the fork, where it remains competent for subsequent RNA chain elongation (Chapter 4). Is this template switch only

relevant to the behavior of RNA polymerase under unusual circumstances (i.e., a collision with the DNA polymerase), or can it be a more common event during the elongation of RNA chains? By specifically studying the behavior of RNA polymerase encountering branched and nicked DNA templates, we find that it is able to pass those aberrant DNA structures and can more generally switch templates.

Results:

In the absence of the T4 gene 41 DNA helicase, a head-on collision between a replication fork and an *E. coli* RNA polymerase ternary complex results in a stalled DNA replication fork, creating a branched DNA structure with a long single-stranded tail (Fig. 1a; about 60-70% of the template molecules are replicated as determined by the gel shift assay, Chapter 4, Fig. 2c). Surprisingly, although the original DNA template strand for RNA polymerase is now single-stranded behind the fork, we find that the RNA polymerase does not stop at the branch site when all four NTPs are added; instead, a 427-nt RNA (identical to the control template without the branch) is generated. Therefore, the RNA polymerase has terminated at the normal termination site, even though this site is located behind the fork. The size of the RNA transcript is sensitive to restriction enzyme digestion of the double-stranded DNA (Fig. 1b, lane 4), indicating that the RNA polymerase has transcribed the duplex DNA behind the fork, rather than the single-stranded DNA tail. The RNA polymerase must

therefore have switched its template strand.

The T4 DNA polymerase holoenzyme is a very dynamic protein complex that undergoes rapid dissociation upon encountering insurmountable roadblocks (Hacker and Alberts, 1994a, b; Mirzayan and Alberts, manuscript in preparation). We believe that the T4 DNA polymerase that stalls during this encounter with the RNA polymerase has dissociated from the DNA because it does not impede the progression of the transcription complex.

In contrast, a mutant T7 DNA polymerase (Sequenase 2.0, USB) is a very processive enzyme that is likely to bind its 3' OH end much more tightly than the T4 DNA polymerase (Tabor and Richardson, 1989). This T7 DNA polymerase (added at > 15 U /10 µl reaction volume containing ~ 0.1 pmol DNA) also stalls upon encountering the RNA polymerase head-on, generating a branched DNA structure similar to that generated by the T4 DNA polymerase in the absence of the gene 41 helicase (data not shown). Repeat of the experiment in which all four NTPs are added, however, yields very different outcomes: no discrete 427-nt RNA is made; rather, a mixture of long RNAs are evident, indicating readthrough products that result from failed termination events (Fig. 1c, lane 2). Examination of the length of RNA synthesized as a function of time reveals that the RNA polymerase moves unusually fast and pauses only briefly at the normal termination site before elongating further to make much longer products. These are very different kinetics from those observed when RNA polymerase travels on

duplex DNA, indicating that the RNA polymerase keeps copying its original template strand and moves onto the single-stranded DNA tail (Fig. 2a). It thus seems that RNA polymerase can be forced onto the single-stranded DNA by a tightly bound protein roadblock.

When high salt is used to disassociate the T7 DNA polymerase from DNA (from 0.27 M and up to 0.57 M potassium acetate), the RNA polymerase starts to transcribe the double-stranded DNA behind the fork and yields the corresponding 427 nt products (Fig. 2b, lane 3); this result supports the notion that the tight binding of the T7 DNA polymerase to the 3' OH end blocks entry on the duplex DNA and forces the RNA polymerase to continue onto the single-stranded DNA without a template switch.

A trivial explanation for the above results would be that the single-stranded DNA tail rehybridizes with its original DNA partner through "branch migration", a reaction propelled by the elongating RNA polymerase. In this case, there would be no need for a template switch. To simplify this experiment, we created a special branched DNA template molecule from three synthetic oligonucleotides (of lengths 91, 81 and 21 nucleotides). In this branched DNA structure, the tail is formed from nucleotide sequences that cannot pair with the double-stranded DNA, so that there is no possibility for branch migration (Fig. 3). As a control for transcription on the single-stranded tail, we also made a construct by hybridizing only the 91 and the 81 nucleotide molecules (called the "91-81" construct). In addition, as a control for a perfect duplex template, the 21 nucleotide fragment was annealed to the 91 nucleotide molecule, extended

by T7 Sequenase, and the product double-helix purified by phenol/chloroform extraction.

RNA synthesis on these three templates was initiated in the presence of the dinucleotide UpG plus ATP, GTP, UTP (no CTP). RNA polymerase is the only protein present in these reactions. A ternary complex bearing an 18-nt RNA was formed and purified on a Sepharose Cl-4B after a high salt treatment to reduce non-specific binding by RNA polymerase, especially to the branch site. The single ribonucleotide CTP was then added to the purified ternary complex. The active polymerase molecule moves three nucleotides downstream, forming a new complex bearing 21-nt RNA, which is the starting material for all of the experiments described below.

On the branched DNA construct, the RNA polymerase molecule described above displays an ability to switch its template when all four NTPs are added (Fig. 4a). The efficiency of this template switching depends in part on the concentration of NTPs: at a high NTP concentration, RNA polymerase prefers the duplex DNA and switches DNA template strands readily, while at a low NTP concentration it favors retaining its original strand, moving to the single strand template after the branch site (Fig. 4b).

When ATPγS and AMP-PNP were used as substrates instead of ATP for RNA synthesis (ATPγS and AMP-PNP are substrates for RNA polymerase on linear but not supercoiled DNA, see Appendix 3), the majority of the RNA polymerase molecules failed to switch template even at high NTP concentrations and transcribed mainly the single-stranded DNA (Fig. 4c,

lanes 3 and 8). Substituting GTP with GTPγS (also a substrate for RNA synthesis on a linear template) had little effect on template choice by RNA polymerase (Fig. 4c, lane 5). Thus, ATP hydrolysis may be required for a template switch. Alternatively, the two ATP analogs could induce certain kinetic states of RNA polymerase that cause it to prefer its original template (the single-stranded DNA). Elongation in high salt buffer (up to 0.57 M potassium acetate) slightly increases the fraction of RNA polymerase molecules that favor the duplex DNA (Fig. 4d).

To measure the fidelity of the RNA synthesis that occurs through the branched DNA structure, we performed sequential RNA elongation by providing only a subset of NTPs. On the control duplex DNA, limited nucleotide addition resulted in the expected, stepwise elongation of the RNA chain (Fig. 5a). On the "91-81" construct where the only option is to enter the DNA single strand, ATP plus UTP at low concentrations failed to allow the RNA polymerase to elongate its 21-nt RNA, even though UTP is the nucleotide that should be added next (Fig. 5b). Curiously, when GTP is added at low concentration, the RNA polymerase moves one nucleotide forward, suggesting some form of A-G base pairing (Fig. 5b, lanes 5, 6 and 8). However, G is not incorporated opposite to A elsewhere on the single-stranded template, since RNA polymerase stops where U should be incorporated if UTP is omitted (see lane 6 in Fig. 5b). However, high concentrations of ATP and CTP alone allow RNA synthesis to the end of the single-stranded template (Fig. 5c), suggesting a high frequency of

misincorporation (perhaps 7 mistakes in total of 11 nucleotides).

When we repeated the limited nucleotide addition experiment on the branched DNA template at low nucleotide concentrations, the pattern of RNA elongation resembled that of the "91-81" construct: the RNA polymerase does not extend the 21-nt RNA in the presence of ATP and UTP (Fig. 6a), but it moves onto the single-stranded DNA when GTP is present (Fig. 6b). At higher concentrations of limited nucleotides, there are elongated RNAs, but they do not show the stepwise increase in size seen on a normal duplex DNA template. It seems that RNA polymerase requires high nucleotide concentrations with a complete set of NTPs to switch template after the branch site. Curiously, when GTP or GTP plus ATP were added first to "prime" RNA polymerase to transcribe the single-stranded tail, followed by the addition of an excess of all four NTPs, products corresponding to the template switch appeared (Fig. 6c). Moreover, there was no detectable size difference between the RNA products obtained from a ternary complex primed with GTP or GTP plus ATP compared with the unprimed one. Some form of RNA cleavage may therefore have occurred just prior to the template switch. I am currently sequencing these RNAs and examining in detail the nature of this curious reaction.

We next studied the behavior of RNA polymerase transcribing on nicked templates, where the break is placed on the template strand. One would expect the RNA polymerase to stall at such a nick. However, to our surprise, the RNA polymerase readily passed the nick and produced normal full-length products (Fig. 7). Limited elongation with subsets of NTPs shows

that RNA synthesis is faithful through the nick (Fig. 7). Because our synthetic oligonucleotides are not 5' phosphorylated, the nick is more than a simple break in the phosphodiester backbone, and it cannot be sealed by DNA ligase.

We have also examined the behavior of an RNA polymerase that encounters a gap in its template DNA strand. As shown in Fig. 8a, even a one nucleotide gap is able to stop the RNA polymerase from progressing along duplex DNA. On a branched template, a one nucleotide gap leads to exclusive engagement of the single-stranded DNA by the RNA polymerase, there being no jumping over the gap to switch template (Fig. 8b). By refusing to jump over a gap, the RNA polymerase avoids producing mutant RNA transcripts.

Discussion:

We have examined the behavior of the RNA polymerase when it is forced to move along an aberrant DNA template (a branched, nicked or gapped template).

Upon encountering single-stranded branch in the DNA, the *E. coli* RNA polymerase can switch its template strand so as to remain on duplex DNA beyond the branch. However, it can be forced onto the single strand at the branch by a protein that is tightly bound to the 3' OH at the branch site. On single-stranded DNA, this RNA polymerase shows poor fidelity and fails to terminate at the normal termination site. Therefore, the cell benefits if the RNA polymerase remains on the duplex DNA after the branch. Similarly,

RNA polymerase elongates faithfully through a nick in its template DNA, but does not move past a gap of one or more nucleotides, thereby avoiding the production of mutant RNAs.

The behavior of the RNA polymerase upon encountering an apurine/apyrimidine (AP) site been examined previously (Zhou et al.,1993). It was found that *E. coli* RNA polymerase passed the abasic site after a pause but stopped at the break generated by specific endonucleases at the abasic site. This indicates that proper base-pairing at the break of the phosphodiester backbone is required for a continuous elongation of an RNA chain. Consistent with our study, a one nucleotide gap (created by endonuclease digestion at the AP site) stopped the *E. coli* RNA polymerase completely (Zhou et al., 1993).

Our study does not directly address the nature of the RNA-DNA hybrid inside the ternary complex. It seems that a long RNA-DNA hybrid would create a much larger energy barrier for a switching event than a short hybrid. The nucleotide concentration dependence may reflect some form of energy requirement. Alternatively, it may indicate that different kinetic states of RNA polymerase (Erie et al., 1993) show a different preference for duplex DNA, or that the length of the RNA-DNA hybrid depends on the polymerization rates during RNA chain elongation (Nudler et al., 1994). The endonuclease activity (transcript cleavage) displayed by RNA polymerase (Surratt et al., 1991) could reduce the length of the RNA-DNA hybrid under special circumstances and could be involved in our experiments. In particular, the mis-incorporated G at the branch site (Fig. 6b and 6c) may

serve as a signal for a precise cleavage of the nascent RNA transcribed from the single-stranded DNA, prior to a template switching event.

Does *E. coli* RNA polymerase possess an ATPase activity during elongation of RNA chain? Because ATPγS and AMP-PNP inhibit both elongation on supercoiled DNA and the template switch at a DNA branch, we suspect that *E. coli* RNA polymerase may be equipped with a previously unidentified ATPase activity that enables this enzyme to deal with unusual circumstances during elongation of RNA chains. In this regard, the α subunit of *E. coli* RNA polymerase shares significant sequence homologies with the α subunit of F1-ATPase (Ohnishi, 1985).

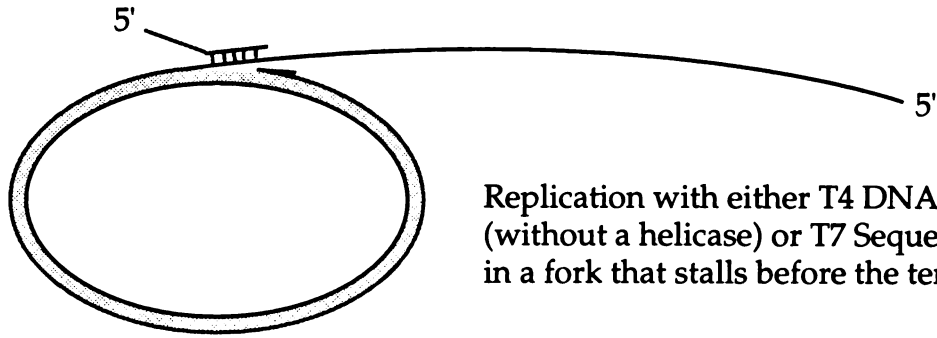
The synthetic, branched DNA structure mimics the passing intermediates that arise during a polymerase head-on collision, when the fork invades the ternary complex. The mechanism used by the RNA polymerase to transverse the branch may therefore also be used to move it to the leading strand product at the replication fork (see Chapter 4). However, during a head-on polymerase collision, especially on tandemly transcribed gene units, our results suggest that the RNA polymerase will sometimes be forced onto the single-stranded DNA, where it cannot maintain faithful and controlled RNA synthesis (Fig. 2a). The biased gene organization seen in *E. coli* (Brewer, 1988) and at the rDNA locus in eukaryotes (Brewer and Fangman, 1988) can perhaps be partially explained in this way.

Figures

(Chapter 5)

Figure 1a. The branched DNA structure generated by replication without a DNA helicase. The displaced, single-stranded DNA tail is the continuation of the original template strand for RNA synthesis, and its transcription after the branch point would result in long RNA products. If the RNA polymerase instead switches its template strand and transcribes the duplex DNA, the size of the RNA product will be 427 nt and this length should be reduced by restriction enzyme digestion of the double-stranded DNA template. The nascent RNA is drawn so as to emphasize the relationship between product RNA and template DNA.

Methods: The ternary complex was formed and purified on CL-2B as described (Chapter 4). T4 DNA replication without gene 41 helicase was performed as in Fig. 1a of Chapter 4. For replication with T7 DNA polymerase: 15 units T7 Sequenase 2.0 was added to ~ 0.1 pmol ternary complex along with all four dNTPs (0.5 mM each) in the T4 replication buffer (33 mM Tris-acetate (pH 7.8), 66 mM potassium acetate, 10 mM magnesium acetate, 100 μg/ml BSA and 0.5 mM DTT) and 20 μg/ml *E. coli* SSB. The mixture was incubated at 37 ^{0}C for 3 min and then chilled on ice. In general, over 70% of all template molecules were replicated as determined by electrophoresis on a 0.8% neutral agarose gel (see Fig. 2c in chapter 4).

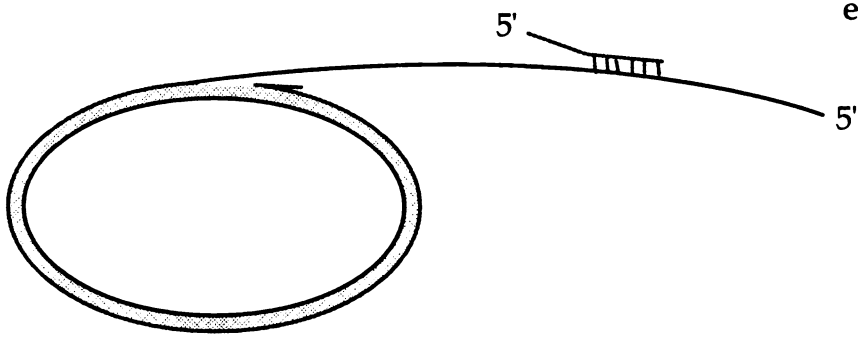


Replication with either T4 DNA polymerase (without a helicase) or T7 Sequenase results in a fork that stalls before the ternary complex

addition of all four NTPs

possible outcomes:

RNA polymerase retains its original template and travels on the single-stranded DNA



RNA polymerase switches template and transcribes duplex DNA. In this case, RNA chain should be terminated at the normal termination site unless shortened by restriction enzyme cutting of duplex DNA

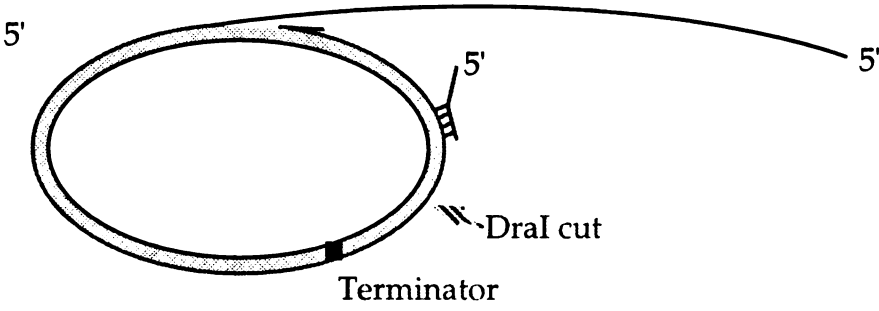


Figure 1b. RNA polymerase switches template and passes through the branched DNA structure generated when the T4 DNA replication proteins meet an RNA polymerase head-on in the absence of a DNA helicase. Lane 1: control, unelongated 18-nt RNA; lane 2: control, elongated RNA (427 nt) on non-replicated template; lane 3: elongation of RNA on branched template; lane 4: sensitivity of product RNA size to DraI digestion of the duplex DNA template.

Methods: 0.4 mM ATP, 0.4 mM GTP, 0.15 mM CTP and 0.15 mM UTP were added to the branched templates to elongate the nascent RNA at 37 ^{0}C for 8 min. Samples of 15 μl were digested with DNase I (2 units) and proteinase K and electrophoresed on a 10% denaturing polyacrylamide gel (containing 8 M urea).

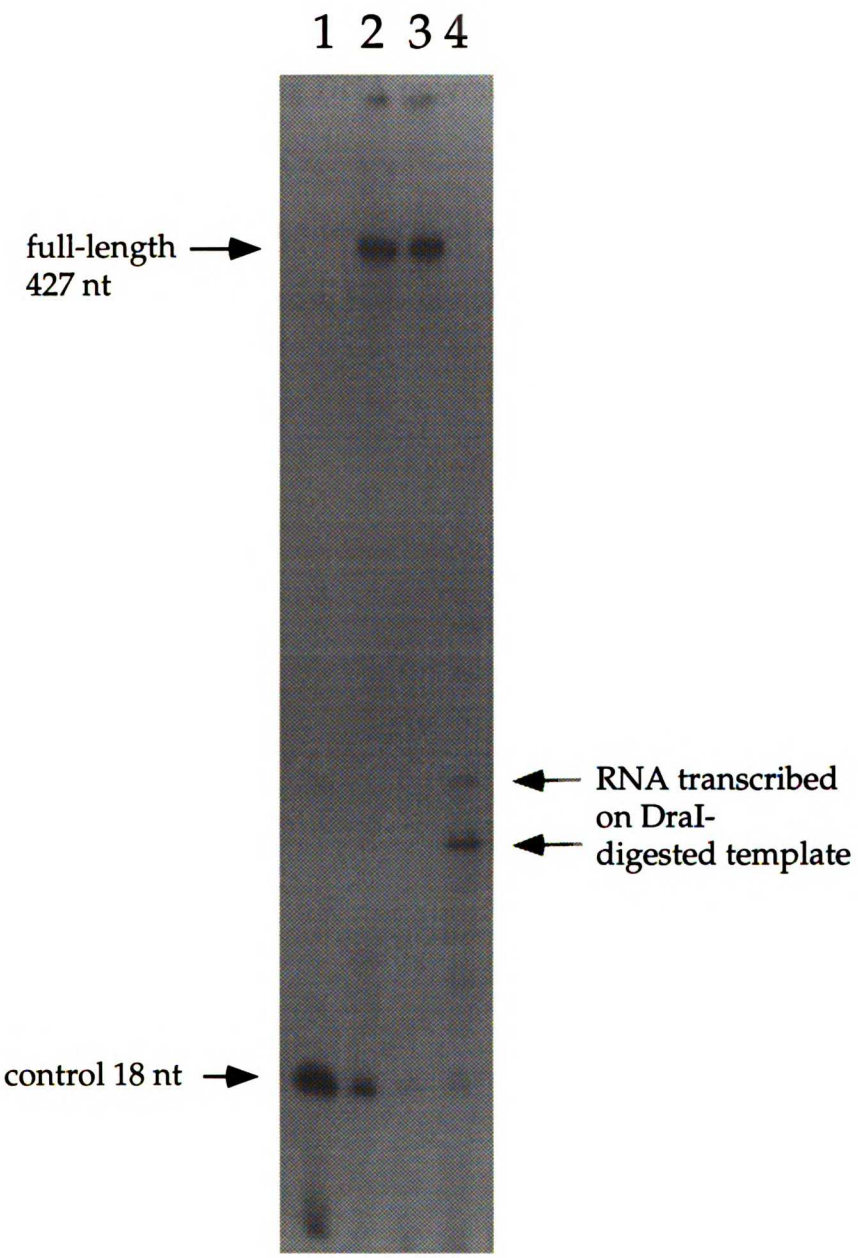

1 2 3 4
full-length
427 nt
RNA transcribed
on DraI-
digested template
control 18 nt

Figure 1c. RNA polymerase can be forced onto the single-stranded DNA by a protein roadblock. Lane 1: RNA elongation on the branched template generated by T4 DNA replication proteins (without a helicase) results in the normal 427-nt RNA; lane 2: elongation on the same branched template generated by the tight binding DNA polymerase T7 Sequenase 2.0, results in very long RNA products.

Methods: RNA elongation was performed as described in the legend to Fig. 1b. Samples were analyzed on a 10% denaturing polyacrylamide gel containing 8 M urea.

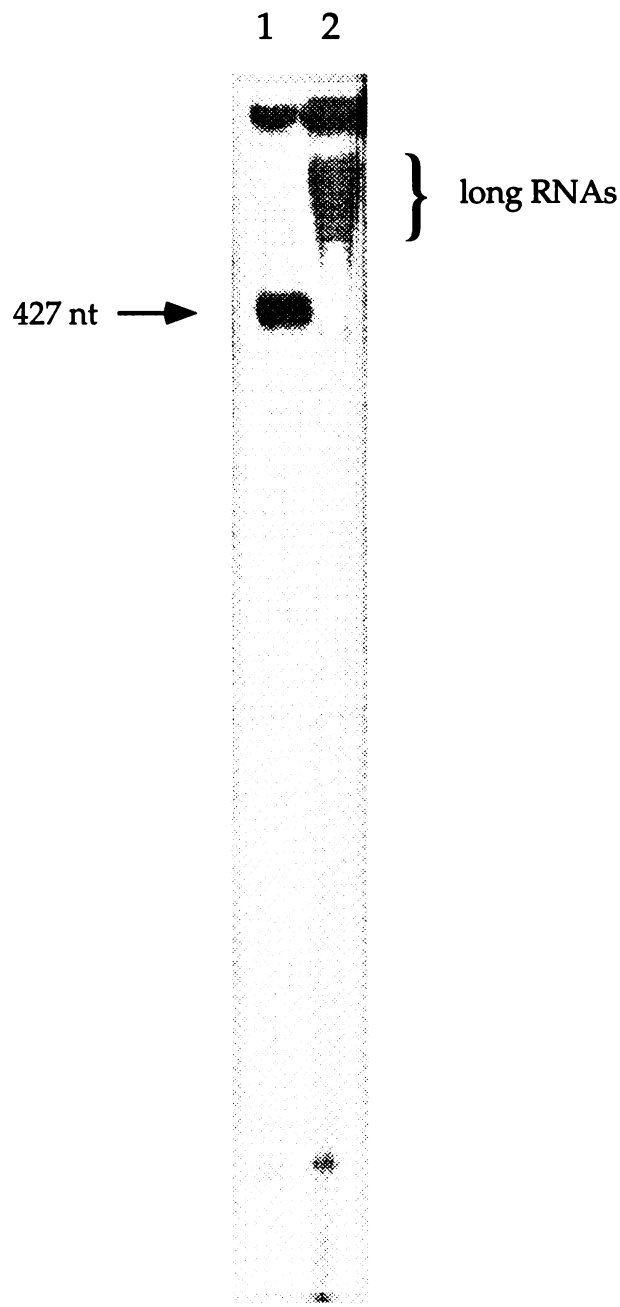

1
2
long RNAs
427 nt

Figure 2a. Comparison of RNA synthesis on the branched DNAs formed by two different DNA polymerase. Lane 1: control 18-nt RNA. Lanes 2-6: RNA elongation for the indicated periods of time on the branched DNA structure generated by a mutant T7 DNA polymerase (Sequenase 2.0). RNA polymerase pauses only briefly at the normal termination site on single stranded DNA (lane 3). The tiny amount of 427-nt RNA seen in lane 6 is most likely due to transcription on a fraction of non-replicated templates. Lanes 7-11: RNA elongation for the indicated period of time on the branched DNA structure generated by T4 DNA replication proteins. For this particular preparation, 20% of the ternary complexes failed to elongate, leaving some 18 nt RNA in lane 10.

Methods: The nucleotides 0.6 mM ATP, 0.6 mM GTP, 0.2 mM CTP and 0.2 mM UTP were added to 30 µl of the branched DNA templates. The mixture was incubated at 37 ^{0}C, and 7 µl of aliquots were taken at the indicated time intervals. Samples were chilled on ice, digested with proteinase K and electrophoresed on a 10% denaturing polyacrylamide gel (containing 8 M urea).

Time course of RNA elongation on branched DNA generated by:

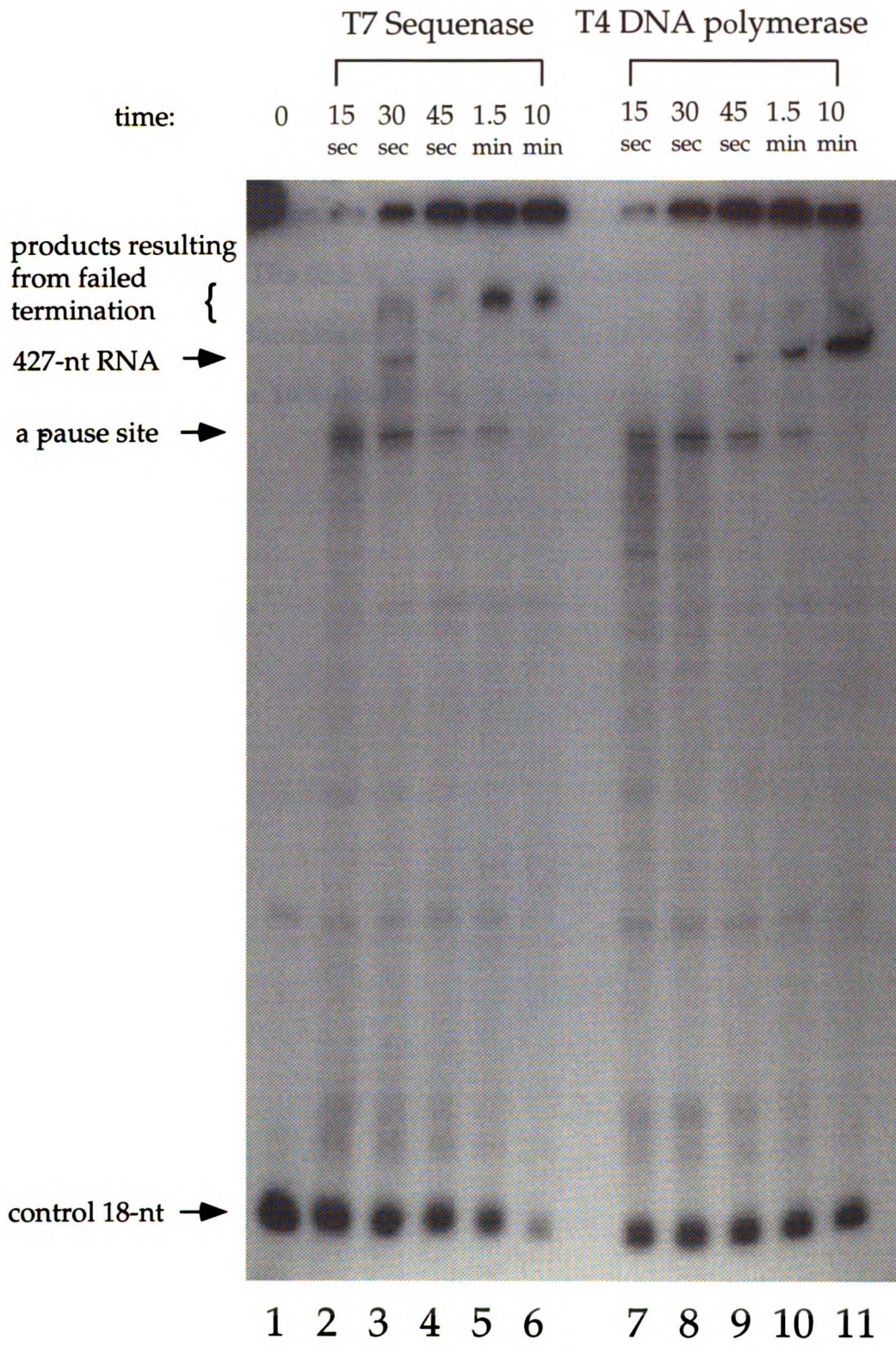


Figure 2b. Treatment of the branched template generated by T7 Sequenase with high salt allows the RNA polymerase to switch template. Lane 1: control 18-nt RNA. Lane 2: control 427-nt RNA. Lane 3: RNA elongation on a branched template that had been treated with 0.27 M potassium acetate. Methods: Potassium acetate was added to 10 µl of T7 Sequenase-replicated ternary complex to produce a final concentration of 0.27 M and incubated on ice for 2 min. The NTPs (0.5 M each) were then added to elongate the RNA at 37 ^{0}C for 8 min. Samples were digested with proteinase K and electrophoresed on a 10% denaturing polyacrylamide gel containing 8 M urea.

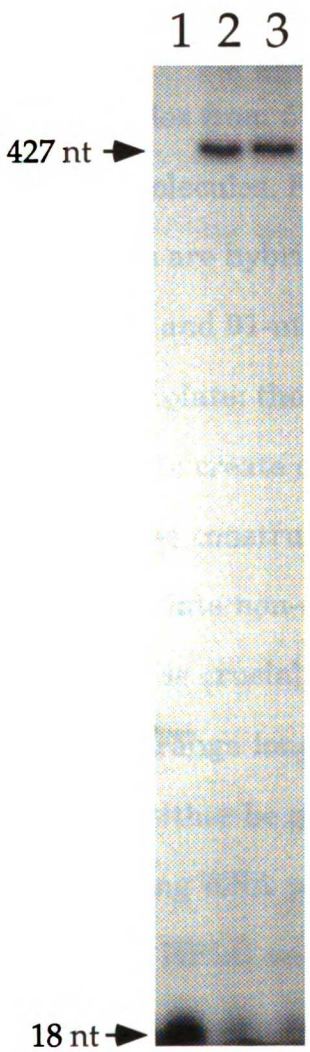

1 2 3
427 nt
18 nt

Figure 3. The synthetic DNA constructs used at templates for RNA synthesis: branched, nicked, gapped and perfect double-stranded DNA. For branched DNA: oligonucleotides of lengths 91, 81 and 21 nucleotides are hybridized together. An eleven-nucleotide "tail" sticks out from the double helix, whose sequences are so designed to avoid base-pairing with the 91 nt partner (the tail thus is unable to branch-migrate). For the nicked DNA: a 70-nt molecule, lacking 11 nucleotides from 5' end of the 81-nt molecule, is hybridized to the 91-nt and 21-nt molecules. For the gapped, linear template: the 20-nt, 70-nt and 91-nt molecules are hybridized together; for the gapped, branched template: the 20-nt, 81-nt and 91-nt molecules are hybridized together. For the perfect duplex template: the 21-nt molecule was hybridized to the 91-nt molecule and extended to create duplex DNA. A ternary complex was formed on each of these constructs and purified on CL-4B after a high salt treatment to dissociate non-specifically bound RNA polymerases. This purification step is crucial for this study because unpurified ternary complex gave strange long products upon the addition of all four NTPs (those products may either be generated directly by or somehow affected by the nick-binding RNA polymerase).

Methods: All oligonucleotides were HPLC purified. They were mixed in water and heated at 70 ^{0}C, followed by slow cooling to room temperature in a water bath over a period of more than one hour. Proper hybridization was checked through restriction enzyme digestion. For the perfect duplex DNA: the 21-nt molecules was hybridized to the 91-nt molecule and elongated by T7 Sequenase at 37 ^{0}C for 3 min, followed by phenol/chloroform extraction

and passage through Bio-spin 6 column. For purification of the ternary complexes: 10 pmol DNA was incubated with 4.6 pmol *E. coli* RNA polymerase, 120 mM UpG, 10 μM ATP, 10 μM GTP and 5 μM [α-^{32}P] UTP (specific activity 100,000 c.p.m./pmol) at 37 ^{0}C for 20 min in a buffer containing 33 mM Tris-acetate (pH 7.8), 250 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT), 100 μg/ml nuclease-free BSA as carrier. NaCl was added to a final concentration of 0.45 M and samples were chilled on ice for 10 - 15 min before loading them on to a Sepharose Cl-4B column.

91 nt

5' GGA TTA TTC AGA TCT TGA CAT GCA CTA TTA CTG AGA GTA TAA ATA CTC CTG ATA TGA AGA GTT GGA TCC CAT TTC GTC ACT GAA GCT GTA G

81 nt

5' CTG TCA ACT CAG GGA TCC AAC TCT TCA TAT CAG GAG TAT TTA TAC TCT CAG TAA TAG TGC ATG TCA AGA TCT GAA TAA TCC

70 nt

5' G GGA TCC AAC TCT TCA TAT CAG GAG TAT TTA TAC TCT CAG TAA TAG TGC ATG TCA AGA TCT GAA TAA TCC

21 nt

5' CTA CAG CTT CAG TGA CGA AAT

20 nt

5' CTA CAG CTT CAG TGA CGA AA

BRANCHED TEMPLATE

RNA synthesis

81 nt (the template strand)

ACTCAACTGCT 5'

21 nt

CCT AAT AAG TCT AGA ACT GTA CGT GAT AATGAC TCT CA T ATT TAT GAG GAC TAT ACT TCT CAA CCT AGGG *TAA AGC AGT GAC TTC GAC ATC* 5'

5' GGA TTA TTC AGA TCT TGA CAT GCA CTA TTA CTG AGA GTA TAA ATA CTC CTG ATA TGA AGA GTT GGA TCCCAT TTC GTC ACT GAA GCT GTA G

91 nt

(Substituting 21 nt with 20 nt creates gaped, branched template; hybridizing 81 nt with 91 nt only creates the "91-81" control construct)

NICKED TEMPLATE

RNA synthesis

NICK

70 nt (the template strand)

5'

21 nt

CCT AAT AAG TCT AGA ACT GTA CGT GAT AATGAC TCT CA T ATT TAT GAG GAC TAT ACT TCT CAA CCT AGGG *TAA AGC AGT GAC TTC GAC ATC* 5'

5' GGA TTA TTC AGA TCT TGA CAT GCA CTA TTA CTG AGA GTA TAA ATA CTC CTG ATA TGA AGA GTT GGA TCCCAT TTC GTC ACT GAA GCT GTA G

91 nt

(Substituting 21 nt with 20 nt creates gaped template)

Figure 4a: Transcription through a branched template as a function of time. The products of transcription on ds DNA can only be obtained by a template switch (see Figure 3). At long incubation times, some of the products transcribed on ss DNA are converted to long RNAs. This secondary reaction can be reduced by addition of either heparin at 80 μg/ml or ATPγS (data not shown).

Methods: The nucleotide CTP was added to 80 μl of the CL-4B purified ternary complex to elongate the active ternary complexes -so as to form a 21-nt RNA (incubation at 37 ^{0}C for 5 min). The NTPs (0.1 mM each) were then added and samples were taken at the indicated time intervals, treated with 2 unit DNaseI followed by proteinase K digestion, and electrophoresed on a 10% denaturing polyacrylamide gel containing 8 M urea.

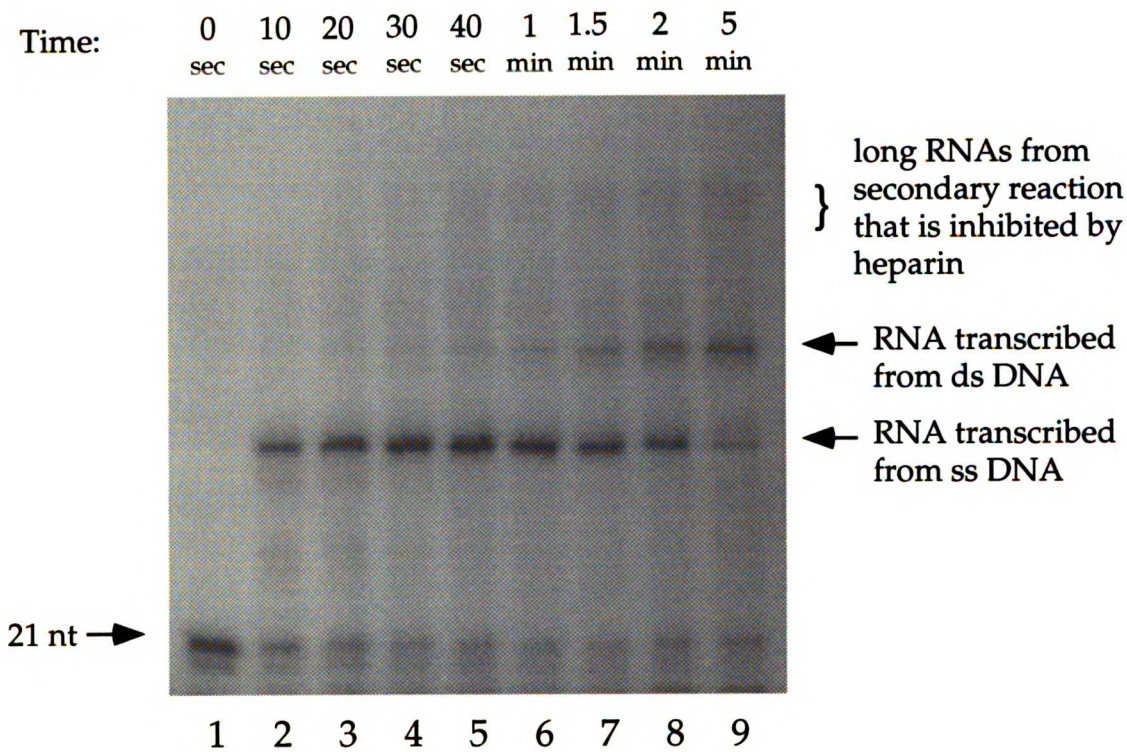

Time:
0 sec
10 sec
20 sec
30 sec
40 sec
1 min
1.5 min
2 min
5 min
long RNAs from secondary reaction that is inhibited by heparin
RNA transcribed from ds DNA
RNA transcribed from ss DNA
21 nt
1 2 3 4 5 6 7 8 9

Figure 4b. The efficiency of a template switch increases with an increase in the concentration of the four NTPs. Lane 1: control 21-nt. Lane 2: elongation with 0.15 mM NTPs. Lane 3: elongation with 0.05 mM NTPs. Lane 4: elongation with 0.025 mM NTPs.

Methods: RNA elongation was performed at 37 ^{0}C for 3 - 5 min at the indicated concentrations of CTP, ATP, UTP and GTP. Samples were analyzed on a 10% denaturing polyacrylamide gel containing 8 M urea.

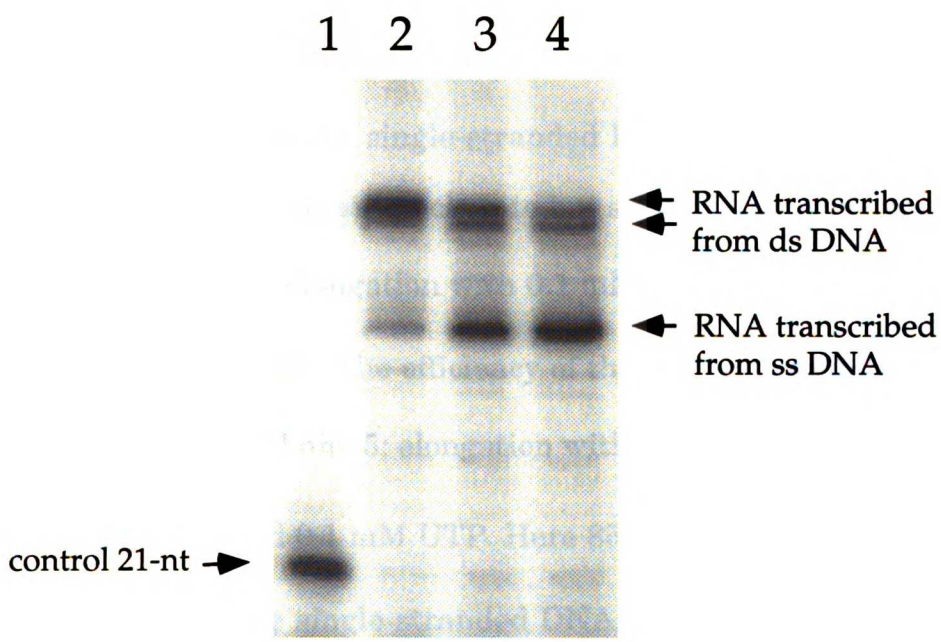

1 2 3 4
RNA transcribed from ds DNA
RNA transcribed from ss DNA
control 21-nt

Figure 4c. Effect of non-hydrolyzable nucleotide analogs on the efficiency of a template switch. Left panel: lane 1: control 21-nt RNA . lane 2: control elongation with all four NTPs (0.1 mM each). For this particular ternary complex preparation, ~ 42% of the RNA polymerase molecules switched template (RNA polymerase taken freshly from a - 80 ^{0}C stock gave a 60-85% efficiency of template switch). Lane 3: elongation with 0.1 mM ATPγS, 0.1 mM GTP, 0.1 mM CTP and 0.1 mM UTP. Nearly all of the RNA polymerase molecules now transcribe the single-stranded DNA (~ 80%); there are some longer RNAs (~ 20%) at a position close to but shorter than the expected switch products. Lane 4: elongation with 0.1 mM GTPγS, 0.1 mM ATP, 0.1 mM CTP and 0.1 mM UTP. The efficiency of the template switch is only slightly reduced to 38%. Lane 5: elongation with 0.1 mM ATPγS, 0.1 mM GTPγS, 0.1 mM CTP and 0.1 mM UTP. Here 85% of RNA polymerase molecules transcribe the single-stranded DNA, failing to switch. Right panel: lane 6: control 21-nt RNA. Lane 7: elongation with 0.1 mM NTPs. Lane 8: elongation with 0.1 mM AMP-PNP, 0.1 mM GTP, 0.1 mM CTP and 0.1 mM UTP. The efficiency of a template switch is reduced from 35% to about 15%.

Methods: The elongation of the ternary complex was performed at 37 ^{0}C for 3 min. The samples were treated with proteinase K and analyzed on a 12%

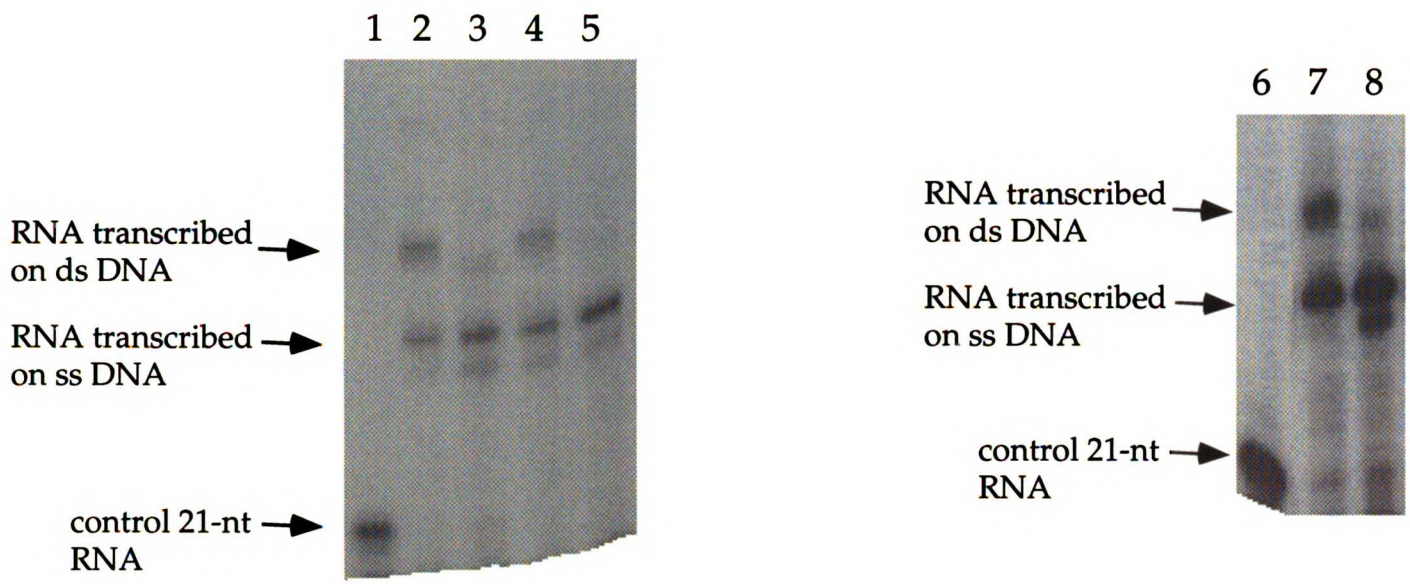

1 2 3 4 5
RNA transcribed on ds DNA
RNA transcribed on ss DNA
control 21-nt RNA
6 7 8
RNA transcribed on ds DNA
RNA transcribed on ss DNA
control 21-nt RNA

Figure 4d. Effect of monovalent salt concentration on the efficiency of a template switch by RNA polymerase. Lane 1: control 21-nt RNA at 0.066 M potassium acetate. Lane 2: elongation with 0.1 mM NTPs in 0.066 M potassium acetate. About 48% of RNA polymerase molecules switch template. Lane 3: control 21-nt RNA at 0.27 M potassium acetate. Lane 4: elongation with 0.1 mM NTPs in 0.27 M potassium acetate. About 50% of the RNA polymerase molecules switch template. Lane 5: control 21-nt RNA at 0.57 M potassium acetate. Lane 6: elongation with 0.1 mM NTPs in 0.57 M potassium acetate. About 60% of the RNA polymerase molecules switch template.

Methods: RNA elongation at the indicated salt concentrations was performed at 37 ^{0}C for 3 min. Samples were analyzed on a 20% polyacrylamide gel with 8 M urea.

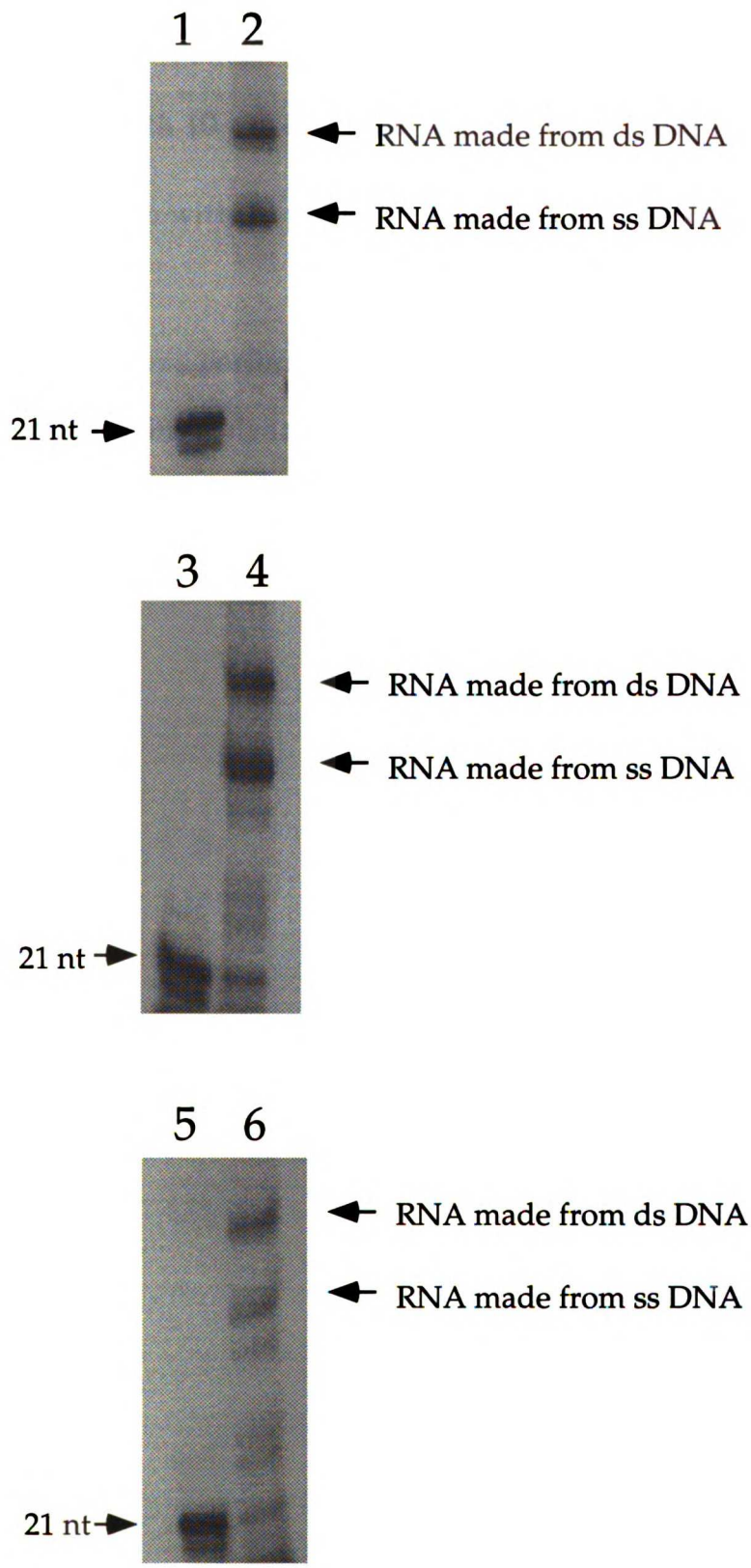

1 2
RNA made from ds DNA
RNA made from ss DNA
21 nt
3 4
RNA made from ds DNA
RNA made from ss DNA
21 nt
5 6
RNA made from ds DNA
RNA made from ss DNA
21 nt

Figure 5a: The effects of limited nucleotide addition on RNA elongation on perfect duplex DNA. Lane 1: control 21-nt RNA (elongated with 10 μM CTP only). Lane 2: elongation with 10 μM CTP and 10 μM ATP. a 23-nt RNA is produced. Lane 3: elongation with 10 μM CTP, 10 μM ATP and 10 μM UTP; a 25-nt RNA is made.

Methods: RNA elongation was performed at 37 ^{0}C for 5 min. Samples were analyzed on a 12% denaturing polyacrylamide gel containing 8 M urea.

Elongation with subsets of
NTPs on duplex DNA

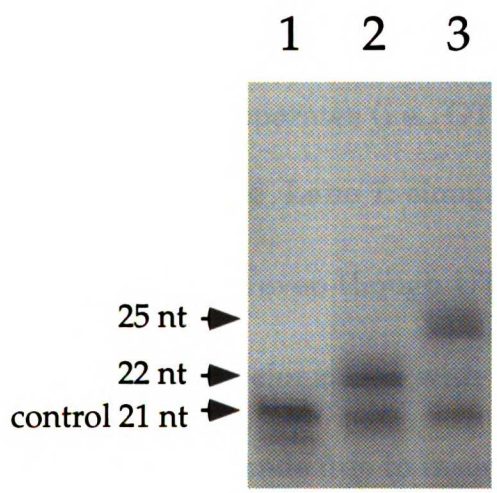


Figure 5b. The effects of limited nucleotide addition on RNA elongation with subsets of nucleotides on the "91-81" template. Lane 1: control 21-nt (elongated with 10 μM CTP only). Lane 2: elongation with 10 μM CTP and 10 μM ATP. Lane 3: elongation with 10 μM CTP, 10 μM ATP and 10 μM UTP. Lane 4: elongation with 0.1 mM of all four NTPs. Lane 5: elongation with 10 μM CTP and 10 μM GTP; a 23-nt RNA is produced even though UTP rather than GTP should be the nucleotide incorporated according to Watson-Crick base pairing (some kind of A-G base pair may have formed). Lane 6: elongation with 10 μM CTP, 10 μM GTP and 10 μM ATP; at the next A in the template, the Watson-Crick rule operates (i.e., GTP will not substitute for UTP) and a 25-nt RNA is produced. Lane 7: elongation with 10 μM CTP and 10 μM UTP; no elongation is seen even though UTP should be incorporated under normal conditions. Lane 8: elongation with 10 μM CTP, 10 μM UTP and 10 μM GTP; a 23-nt RNA is made due to incorporation of GTP at the branch site, but Watson-Crick base pairing holds, so there is no further elongation (ATP, which is omitted, should be incorporated next). All reactions were performed in the presence of ~ 80 μg/ml heparin. This causes a fraction of RNA polymerase to stop one nucleotide before the end of the single-stranded template even with all NTPs present (see lane 4). Methods: RNA elongation was performed at 37 ^{0}C for 3 min. Samples were analyzed on a 12% denaturing polyacrylamide gel containing 8 M urea.

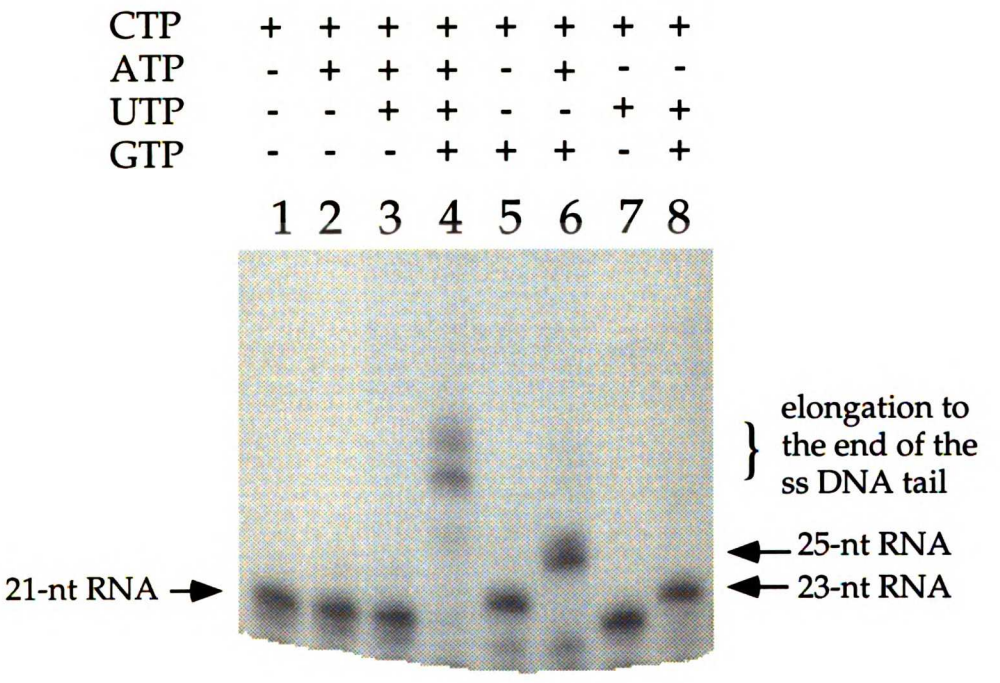

CTP + + + + + + + +
ATP - + + + - + - -
UTP - - + + - - + +
GTP - - - + + + - +
1 2 3 4 5 6 7 8
elongation to the end of the ss DNA tail
25-nt RNA
23-nt RNA
21-nt RNA

Figure 5c. RNA elongation at high nucleotide concentrations on the "91-81" construct. Lane 1: control 21-nt RNA. Lane 2: elongation with 0.01 mM CTP and 0.1 mM ATP. RNA polymerase has reached the end of the template despite the lack of GTP and UTP as substrates. Even though contaminating GTP and UTP could be an alternative explanation, we notice that on perfect duplex DNA template, this concentration of ATP and CTP only allow limited elongation of the RNA polymerase (data not shown).

Methods: RNA elongation was performed at 37 ^{0}C for 3 min. Samples were analyzed on a 14% denaturing polyacrylamide gel containing 8 M urea.

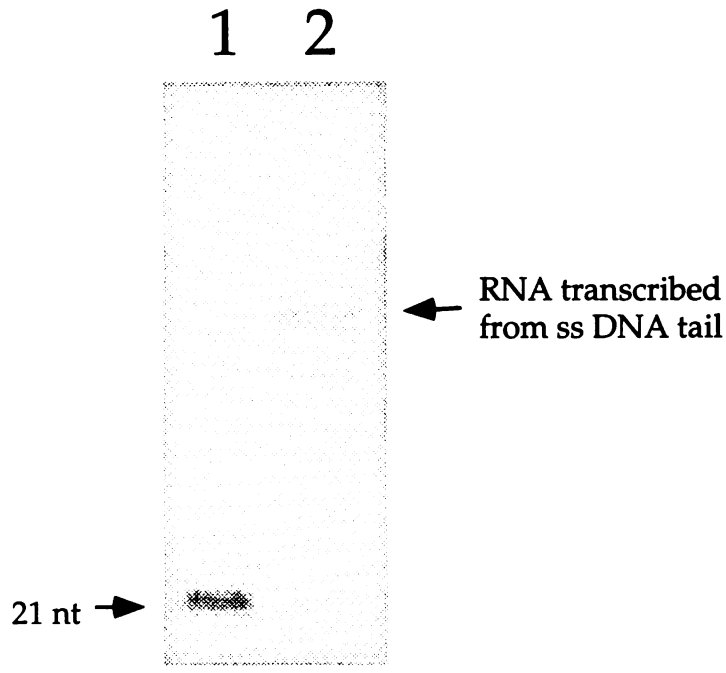

1 2
RNA transcribed
from ss DNA tail
21 nt

Figure 6a. Elongation with subsets of NTPs on the branched template. Lane 1: control 21-nt RNA (active complex elongated with 10 μM CTP only). Lane 2: elongation with 10 μM of CTP and ATP. No 23-nt (the product expected for a template switch) is made. Lane 3: elongation with 10 μM of CTP, ATP and UTP. Again no product expected for a template switch (25-nt RNA) is seen. Lane 4: elongation with all four NTPs (0.1 mM each). About 40% of the RNA polymerase has switched template.

Methods: Elongation was performed at 37 ^{0}C for 3 - 5 min. Samples were analyzed on a 12% denaturing polyacrylamide gel containing 8 M urea.

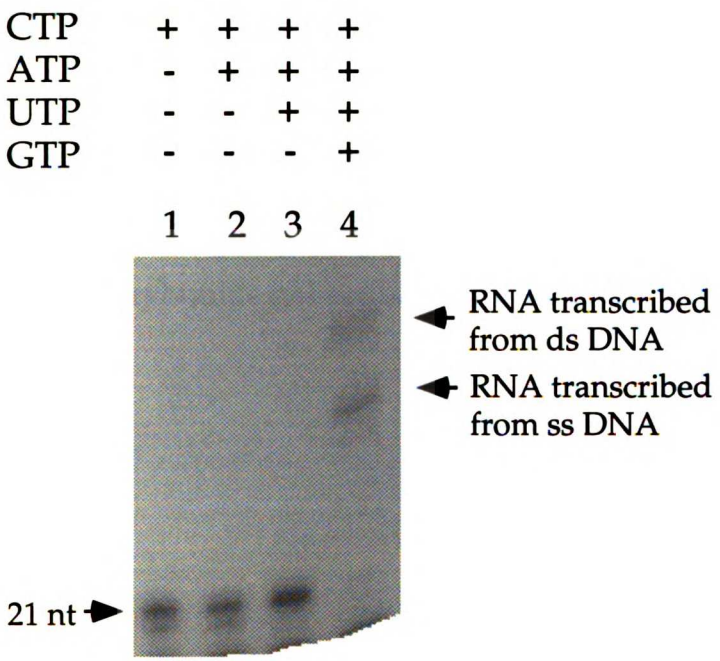

CTP + + + +
ATP - + + +
UTP - - + +
GTP - - - +
1 2 3 4
RNA transcribed from ds DNA
RNA transcribed from ss DNA
21 nt

Figure 6b. Effect of GTP on elongation on the branched template. Lane 1: control 21-nt RNA. Lane 2: elongation with 10 μM CTP and 10 μM GTP; a 23-nt RNA is produced. Lane 3: elongation with 10 μM of CTP, GTP and ATP; a 25-nt RNA is made. Lane 4: elongation with 10 μM of CTP, GTP and UTP; only the 23-nt RNA is made. Lane 5: elongation with 10 μM CTP and 10 μM UTP; no elongation at all. The pattern resembles transcription on "91- 81" construct, indicating that RNA polymerase is transcribing the single-stranded DNA exclusively under those conditions.

Methods: All reactions were done at 37 ^{0}C for 3 min. Samples were analyzed on a 12% denaturing polyacrylamide gel containing 8 M urea.

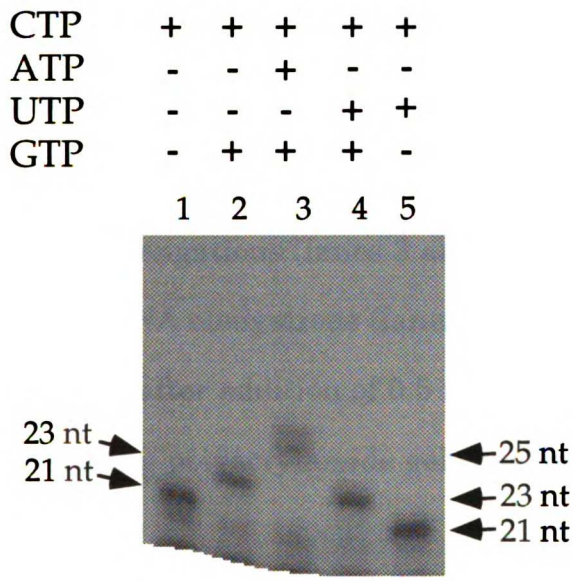

CTP + + + + +
ATP - - + - -
UTP - - - + +
GTP - + + + -
1 2 3 4 5
23 nt
21 nt
25 nt
23 nt
21 nt

Figure 6c. RNA polymerase that has moved onto the single-stranded DNA can still switch template when a high concentrations of NTPs are added. Lane 1: control 21-nt RNA. Lane 2: control elongation with all four NTPs (0.5 mM each). Lane 3: elongation with 10 μM CTP and 10 μM GTP. Lane 4: elongation with 10 μM CTP and 10 μM GTP first, followed by addition of excess NTPs (0.5 mM each). Lane 5: elongation with 10 μM of CTP, GTP and ATP. Lane 6: elongation with 10 μM of CTP, GTP and ATP, followed by the addition of excess NTPs (0.5 mM each).

Methods: The limited RNA elongations (lanes 3 and 5) were performed at 37 ^{0}C for 3 min; the extensive RNA elongations (lanes 2, 3 and 6) were performed at 37 ^{0}C for 3 min after addition of 0.5 mM NTPs. Samples were analyzed on a 20% denaturing polyacrylamide gel containing 8 M urea.

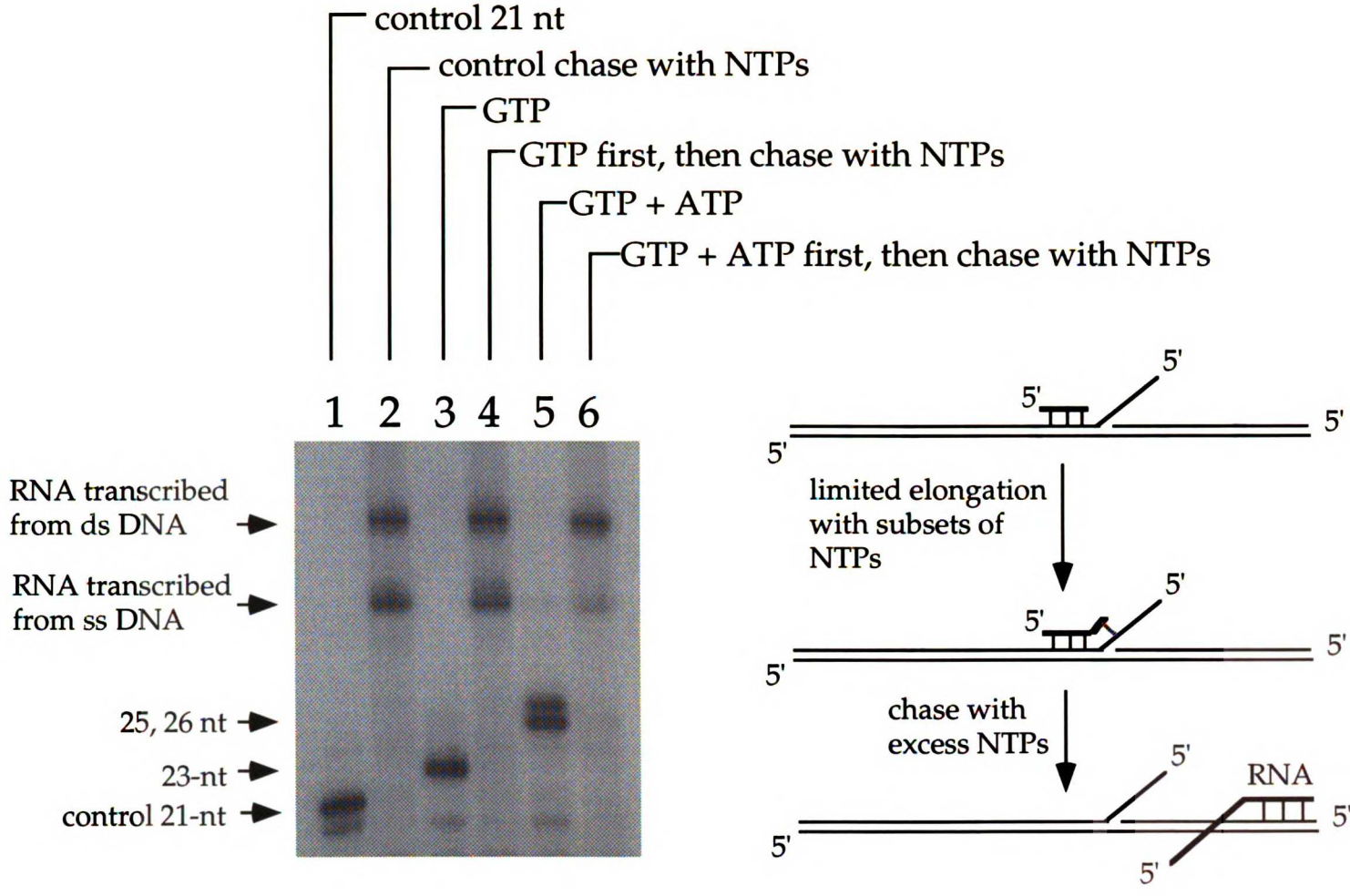

control 21 nt
control chase with NTPs
GTP
GTP first, then chase with NTPs
GTP + ATP
GTP + ATP first, then chase with NTPs
1 2 3 4 5 6
RNA transcribed from ds DNA
RNA transcribed from ss DNA
25, 26 nt
23-nt
control 21-nt
5'
5'
5'
limited elongation with subsets of NTPs
chase with excess NTPs
RNA

Figure 7. Transcription on a nicked template. (A) Polyacrylamide gel. Lane 1: control 21-nt RNA (elongated with 10 μM CTP only). Lane 2: elongation through the nick with 10 μM CTP and 10 μM ATP; a 22-nt RNA is made. Lane 3: elongation through the nick with 10 μM CTP, 10 μM ATP and 10 μM UTP; a 25-nt RNA is made. Lane 4: elongation with all four NTPs (0.1 mM each); some of the RNA polymerase stops one nucleotide before the end of the template. (B) Schematic showing sequence at nick.
Methods: RNA elongations were performed at 37 ^{0}C for 3 min. Samples were treated with proteinase K and analyzed on a 12% denaturing polyacrylamide gel containing 8 M urea.

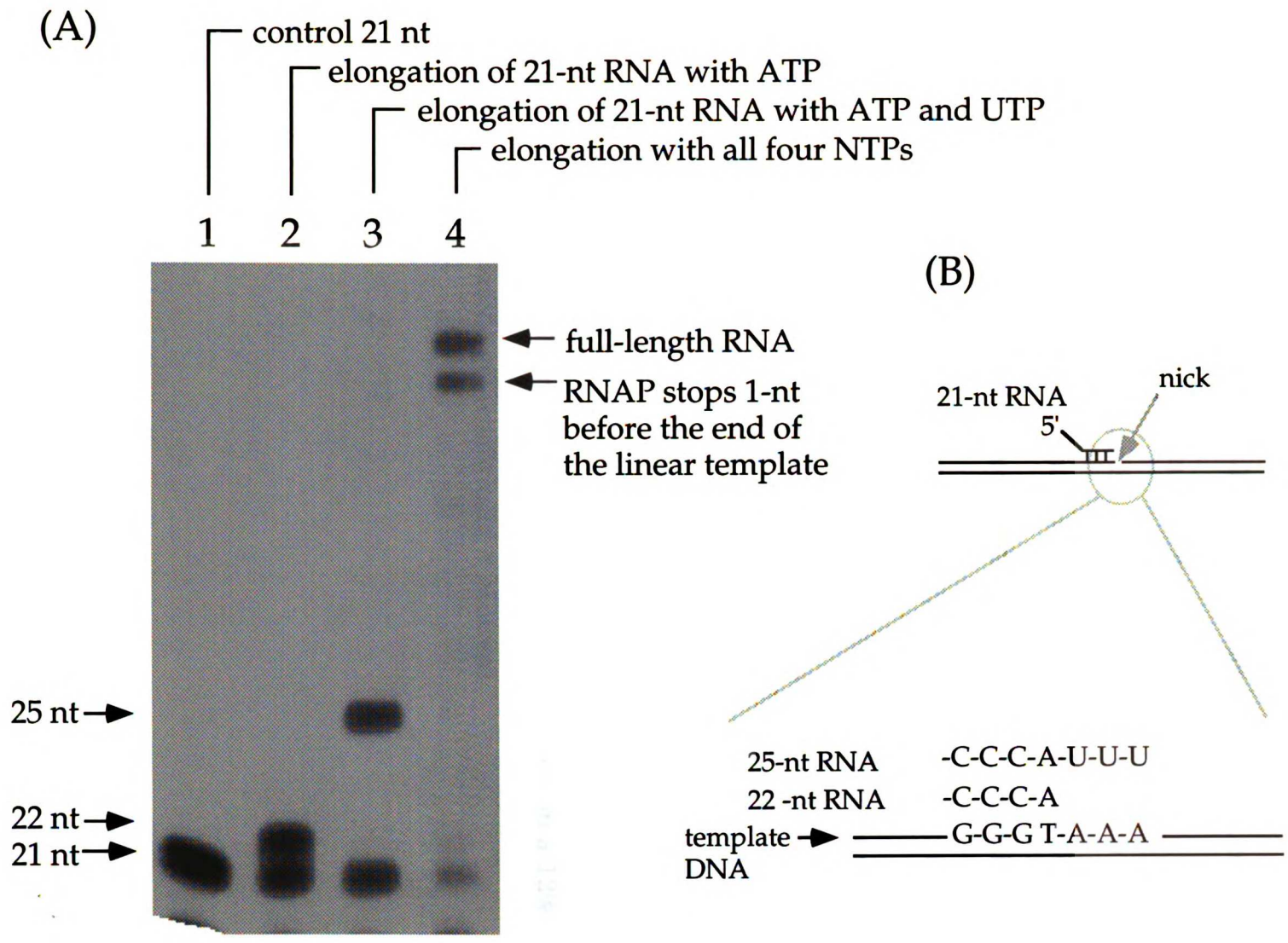

(A)
control 21 nt
elongation of 21-nt RNA with ATP
elongation of 21-nt RNA with ATP and UTP
elongation with all four NTPs
1
2
3
4
full-length RNA
RNAP stops 1-nt before the end of the linear template
25 nt
22 nt
21 nt
(B)
21-nt RNA
5'
nick
25-nt RNA -C-C-C-A-U-U-U
22 -nt RNA -C-C-C-A
template DNA G-G-G T-A-A-A

Figure 8a. Elongation on templates with a one nucleotide gap as a function of time. Lane 1: control 21-nt (elongated with CTP only). some of the RNA polymerase molecules have incorporated one extra nucleotide. Lanes 2-8: elongation for the indicated times at 37 ^{0}C with all four NTPs (0.1 mM each). No full-length products are seen even after 5 min incubation.

Methods: Oligonucleotides of lengths 20 nt, 71 nt, and 91 nt were hybridized together, creating a one nucleotide gap in the template strand for RNA synthesis (see Fig. 3). This DNA construct (10 pmol) was mixed with 4.6 pmol RNA polymerase, 100 μM UpG, 10 μM ATP, 10 μM GTP and 10 μM [α-^{32}P]-UTP, incubated at 37 ^{0}C for 20 min. The product were then treated with 0.45 M NaCl at 4 ^{0}C for 10 min and purified on Sepharose Cl-4B. Active ternary complexes were identified by elongation with 10 μM CTP (an active complex will incorporate CMP, yielding a 21-nt nascent RNA). After elongation with all four NTPs at 37 ^{0}C for the indicated amounts of time, samples were digested with proteinase K and electrophoresed on a 12% denaturing polyacrylamide gel (containing 8 M urea).

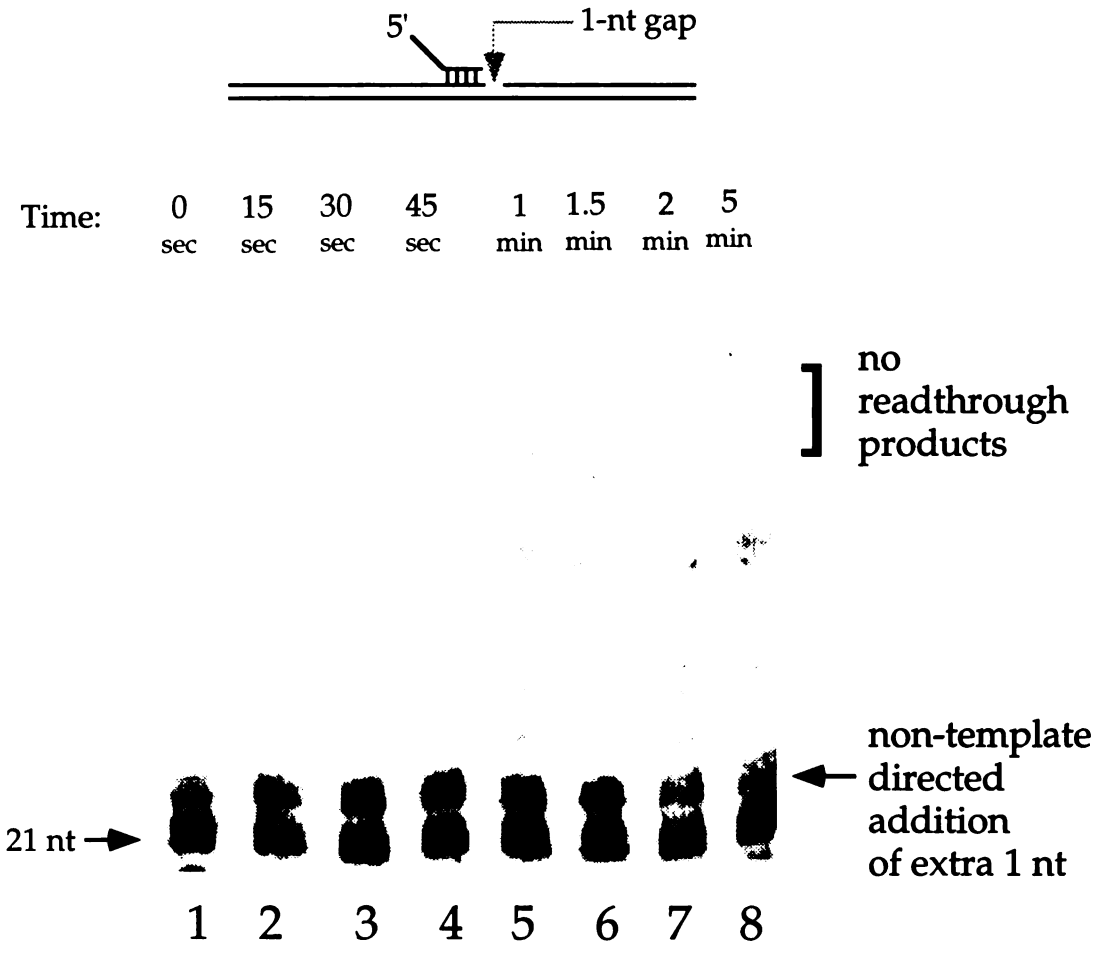
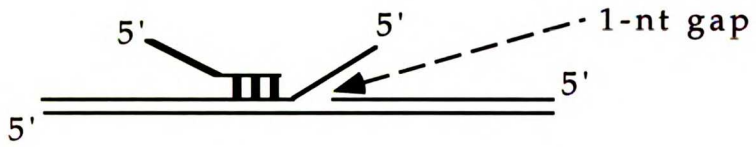
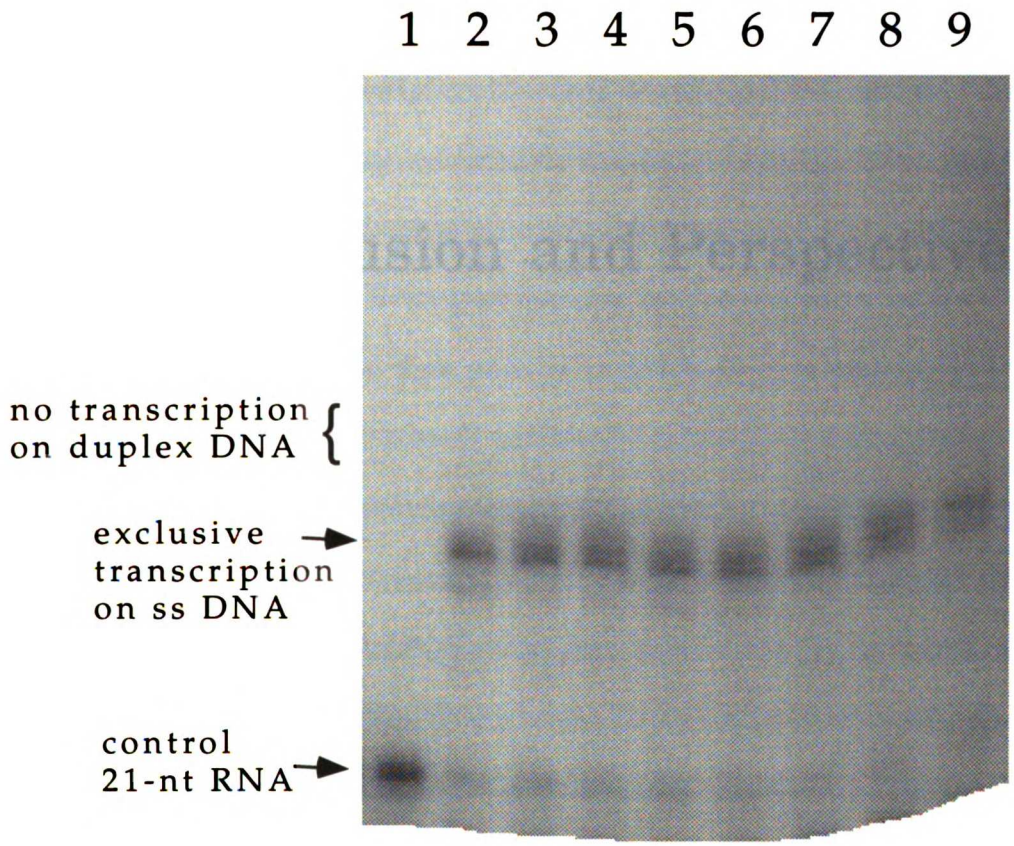

5'
1-nt gap
Time:
0 sec
15 sec
30 sec
45 sec
1 min
1.5 min
2 min
5 min
no readthrough products
non-template directed addition of extra 1 nt
21 nt
1 2 3 4 5 6 7 8

Figure 8b. Transcription on the gapped, branched DNA. Lane 1: control 21-nt RNA. Lanes 2-9: elongation with all four NTPs (0.1 mM each) at 37 ^{0}C for the indicated amount of time. RNA polymerase transcribes exclusively the single-stranded DNA, there being no template switch at all. (At late time points, some of the RNA polymerase molecules seem to have incorporated one or two additional nucleotides after reaching the end of the single-stranded DNA.)

Methods: The 20 nt, 81 nt and 91 nt molecules were mixed together, heated to 65 ^{0}C and slowly cooled to room temperature over a period of one hour, creating a gapped, branched DNA template. The ternary complex was formed and purified as described in the legend to Fig. 1a. Elongation with all four NTPs (0.1 mM each) at 37 ^{0}C for the indicated amount of time was followed by proteinase K digestion and analysis on a 12% denaturing polyacrylamide gel containing 8 M urea.

Elongation through gaped, branched template





Chapter 6

Conclusion and Perspectives

Summary of observations.

We have studied the outcome of a polymerase collision, using the highly purified T4 DNA replication proteins to replicate through an *E. coli* RNA polymerase transcription complex from either direction, and obtained surprising findings: (1) regardless of the orientation of the collision, the RNA polymerase is able to stay bound to the replicated, double-stranded DNA and remain competent to resume subsequent RNA chain elongation. In so doing the RNA polymerase must have given up its original template and switched to the newly synthesized daughter DNA strand (Chapters 2, 3 & 4). (2): a DNA helicase encoded by the bacteriophage T4 is required for the DNA replication fork to pass an opposingly oriented but not a co-directionally oriented RNA polymerase. Moreover, even with this helicase present, the replication fork pauses longer before an opposingly oriented RNA polymerase than a co-directionally oriented one, implying an inherent disadvantage to have polymerases collide head to head (Chapter 4). (3): the RNA polymerase is intrinsically capable of switching templates upon encountering aberrant DNA structures and is biased towards residing on duplex DNA (Chapter 5). When being forced onto single-stranded DNA under certain conditions, it fails to undergo faithful and controlled RNA synthesis (Chapter 5).

Implication for prokaryote genome organization:

In *E. coli*, the replication fork is initiated bidirectionally from a single site, *ori*C, and it travels around each half of the ~ 4,700 kb genome in 40 minutes (Helmstetter, 1968). It has been observed that the *E. coli* genome is arranged in a curious way: most of the heavily transcribed genes are oriented in the direction of the leading strand of DNA replication fork (Brewer, 1988; Medigue et al., 1990). A similar non-random gene organization is also seen in other bacteria (Sanderson and Roth, 1988; Zeigler and Dean, 1990), plasmids, and bacteriophages (Brewer, 1988). *Brewer* originally speculated that this genomic organization allows the DNA polymerase to follow behind the RNA polymerase until the end of the transcription unit is reached, hence avoiding a collision between the two types of polymerases (Brewer, 1988). But there are at least 3000 genes on the bacterial chromosome (Kohara, 1990), a sizable fraction of which must be transcribed throughout the cell cycle (Ingraham, 1983). To make the replication fork wait for transcription would make DNA replication a slave of gene expression. At least in our *in vitro* studies the replication fork does not follow behind a transcribing RNA polymerase; instead, it is able to pass the RNA polymerase rapidly without destroying ongoing transcription. We suggest an alternative explanation for the non-random genome organization in *E. coli*: because a head-on collision retards the DNA replication fork more severely than a co-directional collision, and because this retardation will be most severe on heavily transcribed gene units, it is

advantageous for cell to orient most of its heavily transcribed genes in the same direction as that of the DNA replication fork.

Implication for eukaryote gene organization:

Unlike *E. coli*, where DNA replication starts from a single OriC site, eukaryotes initiate DNA synthesis from numerous discrete sites along their large chromosomes. They also have large genes that measure up to several megabases and require several hours to be completely transcribed. Aborting such large transcripts during a collision with the DNA replication apparatus is clearly inefficient. Given the results of our *in vitro* studies showing that the *E. coli* RNA polymerase can stay on a DNA duplex regardless of the orientation of the collision (Liu et al., 1993; Liu et al., 1994a; Liu et al., 1994b), and considering that eukaryotic DNA and RNA polymerases share common structural organization with their prokaryotic counterparts (Sentenac, 1985; Young, 1991; Waga and Stillman, 1994), it is tempting to speculate that the eukaryotic transcription apparatus may survive DNA replication as well.

The studies presented in Chapters 2-4 have revealed only one clear disadvantage for a polymerase head-on collision: the notably obstructed movement of the replication fork. However, this may not be the only problem generated by this type of collision. In particular, eukaryotic cells have evolved new features of gene organization, which requires further explanation. For example, the yeast ribosomal DNA locus consist of

tandemly repeated transcription units (genes) with replication origins situated in the nontranscribed spacers. The two forks initiating at each of these origins experience unequal fates. The fork moving in the direction of transcription proceeds unimpeded through multiple gene repeats, while the oppositely-directed fork arrests at a polar barrier just before it encounters the transcription terminator for the adjacent, upstream transcription unit (Brewer and Fangman, 1988; Kobayashi et al., 1992). The arrest is mediated by proteins binding to specific DNA sequences, independent of transcription (Brewer et al., 1992; MacAllister et al., 1989; Hidaka et al., 1992; Lee and Kornberg, 1992). Polar replication fork barriers permanently block rather than reduce the rate of movement of the replication fork.

We speculate that, in addition to retarding the fork movement, a head-on collision may expose the RNA polymerase to other potential problems, the nature of which may have been revealed by studies presented in Chapter 5: the RNA polymerase may be derailed by a DNA polymerase stably-bound to the 3' OH end (see Fig. 2a in Chapter 5). The protein-protein and protein-DNA interaction within a cellular DNA polymerase holoenzyme is likely to be much stronger than that of T4. For example, while the entire *E. coli* holoenzyme, composed of at least 10 subunits, as well as holoenzyme subassemblies can be isolated by gel filtration (Wickner, 1976, 1978; Burgers and Kornberg, 1982; O'Donnell, 1987), a T4 DNA polymerase-DNA, or DNA polymerase-accessory protein complex, has never been physically isolated. Because the RNA polymerase on heavily transcribed gene units will

severely retard the DNA polymerase, generating a branched DNA structure inside the transcription unit, the stable binding of the cellular DNA polymerase to the 3' OH end may force the RNA polymerase onto the single-stranded DNA, where it would show poor fidelity and incorrect chain termination (Figs 2a and 5c in Chapter 5).

Mechanism of the bypass reaction:

Given the large dimensions of the two polymerases involved, it is not obvious how they manage to pass one another in a non-destructive way. In particular, because the RNA polymerase cannot continue RNA chain elongation once it is completely released into solution, the ternary complex must remain bound to the DNA at its original place during passage of the replication fork. How is this high stability and high fidelity achieved?

Because the co-directionally moving DNA and RNA polymerases use the same DNA single strand as template, the replication apparatus almost certainly unwinds the end of the growing RNA transcript that is base-paired to DNA. Because after a head-on collision the RNA polymerase stayed on double-stranded DNA, the original RNA-DNA hybrid must also have been broken up. Considering that the E. coli RNA polymerase is a large enzyme with multiple DNA binding protein subunits whose individual detachment from DNA may be tolerated, we propose that the stability of the ternary complex is contributed mainly by the protein-DNA rather than RNA-DNA interaction during the passing event. This

hypothesis can be tested by cutting the nascent RNA to a shorter length (e.g., 2-3 nt) by RNase digestion (Rice et al., 1991), and observing the fate of this new ternary complex after DNA replication.

Because the nascent RNA is the only component in the ternary complex that is capable of sequence-specific recognition of the DNA template, we speculate that the faithful reassembly of a bypassed ternary complex is achieved through a protein-based rapid sequence scanning via the formation of a short RNA-DNA hybrid. This hypothesis can be tested by observing the behavior of the ternary complex on template bearing repeated DNA sequences. Errors in RNA synthesis may occur on such templates during DNA replication.

In contrast to the large cellular RNA polymerase, a bacteriophage-encoded RNA polymerase is much simpler: within a single polypeptide chain of ~ 110 kd, it contains all the functions of promoter recognition and RNA chain elongation (Chamberlin & Ring, 1973). It would be interesting to determine whether these simpler RNA polymerases are more susceptible to being destroyed by the passage of the DNA replication machinery. I have performed some preliminary experiments along this line, but more control experiments are needed in order to reach a conclusion.

Behavior of the passing T4 DNA replication apparatus:

Although DNA synthesis seems to be continuous, especially in the presence of the gene 41 DNA helicase, transient dissociation of the T4 DNA

polymerase may nevertheless occur during passage through the ternary complex. Dilution of specific subunits (including the gene 43 DNA polymerase) as well as depletion of energy source (ATP) (a treatment that prevents reassembly of the DNA polymerase holoenzyme) should help resolve this matter (I have performed some of these experiments but the results were too preliminary to be conclusive).

Comparison with fates of other DNA-binding proteins after DNA replication.

In contrast to the ternary transcription complex, a promoter-bound RNA polymerase that is not transcribing has previously been shown to be displaced from the template after replication (Bedinger et al., 1987). Certain regulatory protein complexes (activator and repressor) have also been shown to be displaced from DNA after replication (Wolffe and Brown, 1986). Compared to the very tight ternary complex that enables RNA polymerase to transcribe in a highly processive manner, promoter binding by RNA polymerase and other sequence-specific DNA binding by regulatory proteins are weaker interactions. Because they rely on hydrogen-bonding interactions with specific bases on both DNA strands (von Hippel et al., 1984), the separation of the two strands of the double helix during replication would be expected to destabilize those complexes. However, it is possible that a large, sequence-specific DNA binding protein complex with two or more well-separated DNA attachment sites may nevertheless

survive DNA replication, at least occasionally.

There are indications that some non sequence-specific DNA binding proteins are not displaced into solution by the passage of either a DNA or RNA polymerase. For example, the nucleosome remains attached to the DNA after the passage of either a DNA replication fork or a RNA polymerase (Bonne-Andrea et al., 1990; Clark and Felsenfeld, 1992; O'Neill et al., 1993; Studitsky et al., 1994). However, the bypassed nucleosome slides along the template, losing its original register on DNA. It should be noted that in contrast to a transcription complex carrying a nascent RNA, nucleosome lacks a component that would allow it to recognize its cognate DNA template through base pairing and therefore is unable to reassemble faithfully.

Comparison with *in vivo* observations:

Is our conclusion consistent with known *in vivo* observations? The available physiological studies on this subject have yielded controversial results. On the one hand, French has used electron microscopy to examine the fate of a replication fork travelling through an *E. coli* ribosomal RNA operon (French, 1992). She observed that the movement of the replication fork was hardly affected by co-directionally transcribing RNA polymerase but was retarded significantly by RNA polymerase approaching from the opposing direction, consistent with our *in vitro* observations (Liu et al., 1993; Liu et al., 1994; Liu and Alberts, 1994). However, French suggested that RNA

polymerase is dislodged from the template when the replication fork approaches from either direction. These rRNA transcripts are unusual in at least two aspects: they are attached to closely spaced RNA polymerase molecules, and they are modified by a set of specialized RNA-binding proteins (Richardson, 1993). We would predict that a different result would be obtained with other transcription units. Methods that permit a quantitative analysis should be useful for examining this issue, such as simultaneously probing the fork movement and nascent transcript production by nucleic acid hybridization.

Shermoen and O'Farrell observed the fate of the nascent transcript of a large *Drosophila* gene (Ubx) whose complete transcription takes longer than the time of one cell cycle. They found that DNA synthesis *in vivo* did not abolish the ongoing transcription, although the origin of replication has not yet been mapped and the orientation of replication fork movement is unknown (Shermoen and O'Farrell, 1991). Clearly, more studies are needed to assess the outcome of a polymerase collision *in vivo*.

Factors modulating outcomes of a polymerase collision: effects of the T4 Dda protein.

It is certainly possible that yet unidentified protein factors exist *in vivo* that modulate the basic mode of interaction between a replication fork and RNA polymerase observed in our experiments. But one should carefully differentiate them from factors that affect the stability of the ternary

complex in a manner independent of DNA replication. The T4-encoded Dda DNA helicase is one of those factors. It inactivates the ternary complex and prevents RNA chain elongation, independent of any of the DNA replication proteins and indepedent of ATP hydrolysis (helicase activity, see Appendix 1 for details).

Appendix 1

T4 *Dda* DNA helicase inactivates but does not disassemble RNA polymerase ternary complex

We have used a set of well-characterized, highly purified replication proteins encoded by bacteriophage T4 for our *in vitro* studies on a polymerase collision. In previous studies from this laboratory (Bedinger et al., 1987), a T4-encoded DNA helicase, the *dda* protein, was required for the replication fork to pass a promoter-bound RNA polymerase. More recently, this requirement has been eliminated by the addition of the T4 gene 59 protein, an accessory protein to the DNA helicase of the T4 primosome, the gene 41 protein (Barry, J & Alberts, B. M., 1994b). I found that *dda* by itself inactivates ternary transcription complexes, independently of DNA replication and any of the replication proteins. The degree of inactivation increases with an increasing concentration of *dda* protein (Fig. 1, lanes 3-5). The RNA polymerase stops at discrete sites on the template, most of which overlap with natural pausing sites. The template DNA is supercoiled (or linear DNA duplex, data not shown) rather than nicked, there being no entry point for a DNA-dependent helicase like *dda*. It is likely that functions other than the DNA helicase activity are responsible for this transcription inactivation by *dda*. Indeed, ATPγS, AMP-PNP or GTPγS does not prevent *dda* from inactivating the ternary complex (data not shown).

Has the ternary complex been displaced from the template by the *dda* protein? We analyzed the fate of the ternary complex after elongation with NTPs with or without the presence of *dda* by monitoring the mobility of an AvaI - DraIII restriction fragment bearing the ternary complex on a non-denaturing polyacrylamide gel. The result is shown in Fig. 2. Lane 1 is a

control, showing the mobility of the ternary complex before elongation with NTPs. After elongation, most of the ternary complexes have moved off the AvaI-DraIII fragment, as shown in lane 2. Lane 3, 4 and 5 shows elongation in the presence of increasing concentrations of *dda* (0.28 μg/ml, 4 μg/ml and 62 μg/ml respectively). Most of the ternary complex are able to move off the template at 0.28 μg/ml of *dda* (lane 3), an expected result because this low concentration of *dda* does not inactivate the ternary complex (see Fig. 1, lane 3). At higher concentrations (lanes 4 and 5) where elongation is inhibited (see Fig. 1, lanes 4 and 5), however, increasing amount of ternary complexes stay on the AvaI - DraIII fragment. They are not disassembled by *dda;* rather, *dda* protein seems to block RNA chain elongation. Further experiments are needed to determine whether *dda* binds to the nascent RNA directly or binds to the core RNA polymerase to achieve its inhibitory effect.

Curiously, *E. coli* GreA and GreB proteins can reactivate the ternary complex that is inactivated by the *dda* protein (data not shown).

Figure legend:

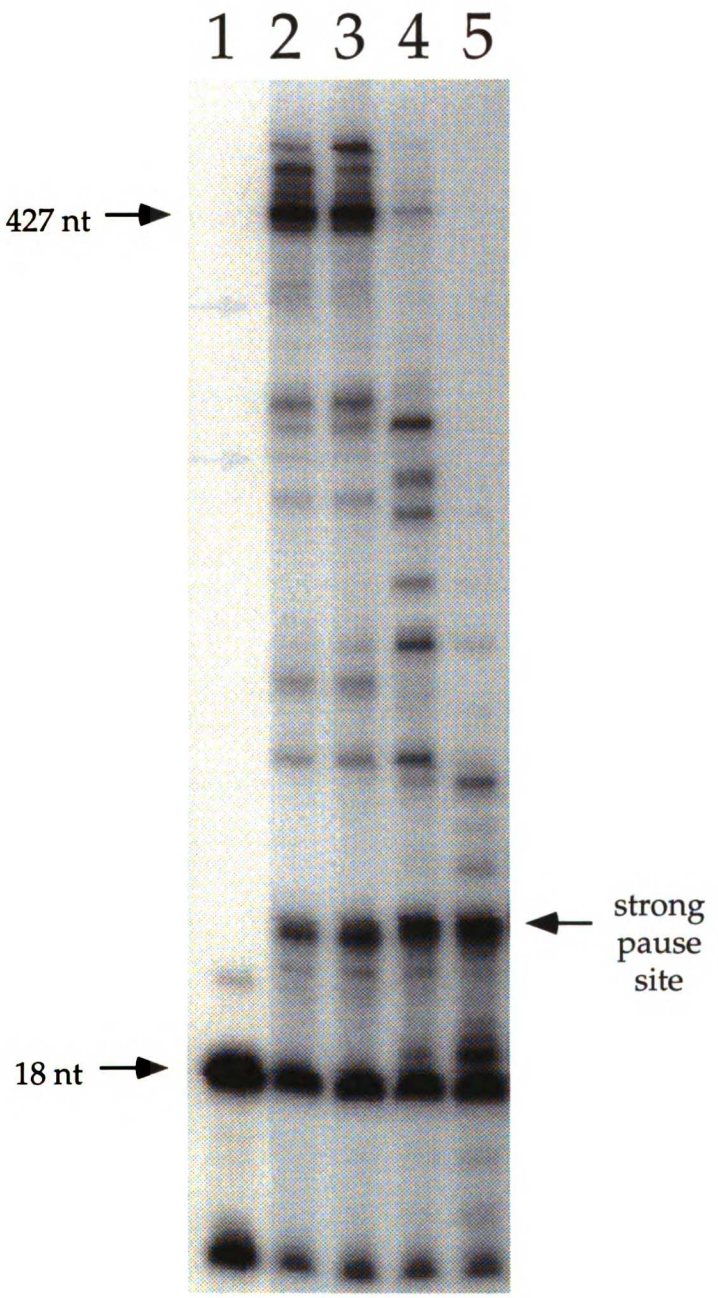
Fig. 1. A *dda* concentration dependent inactivation of ternary complexes formed on supercoiled DNA. Lane 1: control 18-nt RNA. Lane 2: control elongation without *dda*. Lane 3: elongation in the presence of 0.28 μg/ml *dda*. Lane 4: elongation in the presence of 4 μg/ml *dda*. Lane 5: elongation in the presence of 62 μg/ml *dda*.

Fig. 2. *dda* inactivates but does not disassemble the ternary complex. Lane 1: control AvaI - DraIII fragment bearing the ternary complex. Lane 2: elongation with NTPs causes the RNA polymerase to move off the AvaI - DraIII fragment. Lane 3: elongation in the presence of 0.28 μg/ml *dda*. Lane 4: elongation in the presence of 4 μg/ml *dda*. Lane 5: elongation in the presence of 62 μg/ml *dda*.

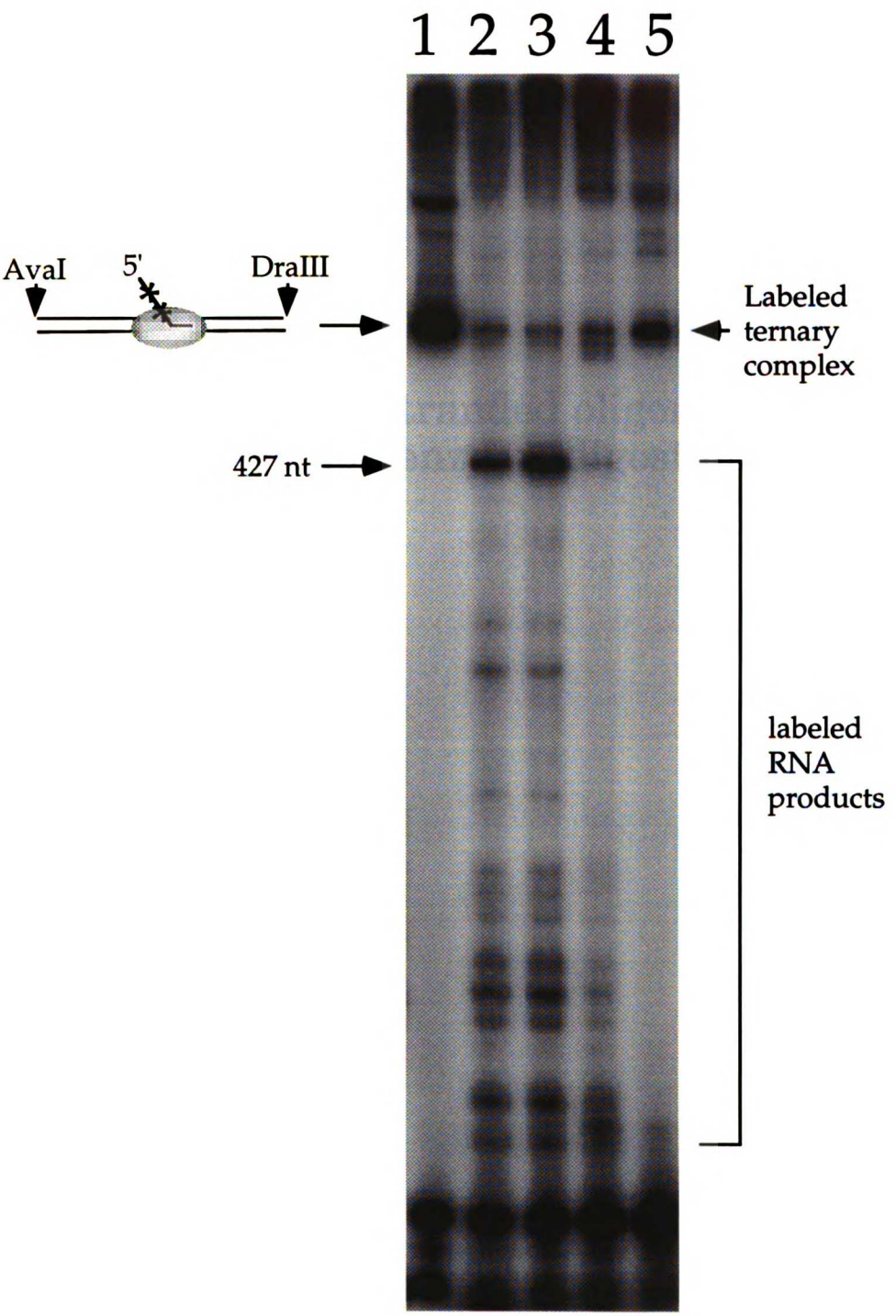
Methods: about 0.07 pmol Cl-2B purified ternary complex (labeled with ^{32}P-UTP) formed on supercoiled plasmid pRT-510C+18 were incubated with various amount of *dda* proteins (concentration as indicated) at 37 ^{0}C for 30 seconds, followed by elongation with 0.5 mM ATP, 0.5 mM GTP, 0.24 mM CTP and 0.24 mM UTP at 37 ^{0}C for 5 min. 2/3 of the samples were digested with proteinase K and analyzed on a 10% denaturing polyacrylamide gel containing 8 M urea (Fig. 1). The remaining samples were digested with 10

units of AvaI and DraIII at 37 ^{0}C for 5 min. Heparin and ficoll were added to 80 μg/ml and 5% respectively, and samples were electrophoresed on a 4% non-denaturing polyacrylamide gel (Fig. 2).

High concentrations of Dda inactiviate the ternary complex



dda inactivates but does not displace ternary complexes from DNA



Appendix 2

Sensitivity of single-stranded oligonucleotides to restriction enzyme digestion

Sensitivity of single-stranded DNA to restriction enzyme cutting:

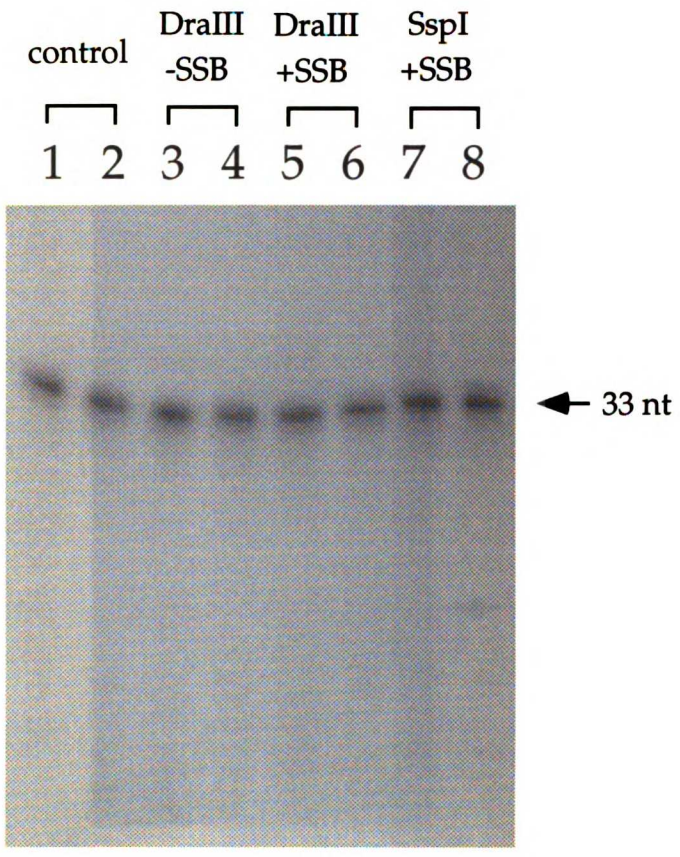
5' phosphorylated synthetic oligonucleotides (either 33 mer, 66 mer or 100 mer) are incubated with various restriction endonucleases at 37 ^{0}C for 1 min and 5 min. In most cases (as indicated), the *E. coli* SSB protein was added to cover the single-stranded DNA prior to a digestion. It seems that the SSB-covered oligonucleotide is more resistant to cutting than the uncovered one (compare lanes 3 and 4 in Fig. 1a). All except HaeIII are ineffective in cutting single-stranded DNA covered with SSB (Fig. 1b, lanes 4 and 5).

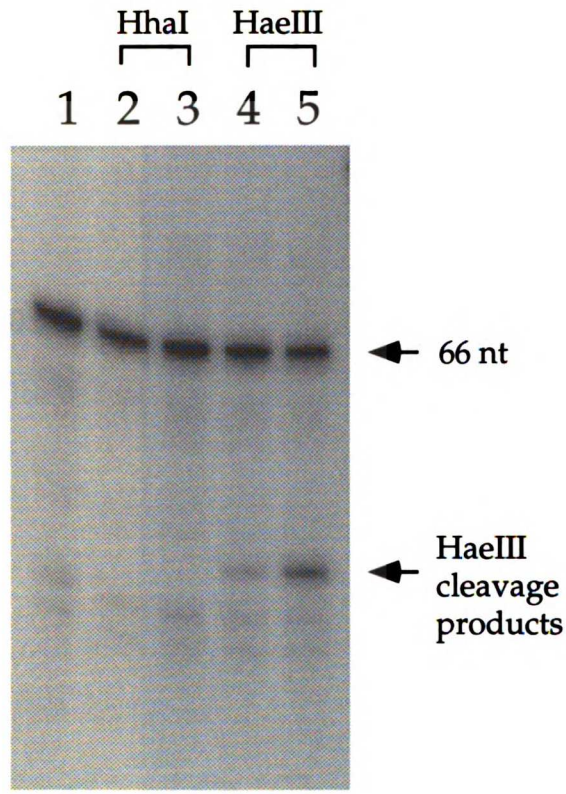
Figure legend:

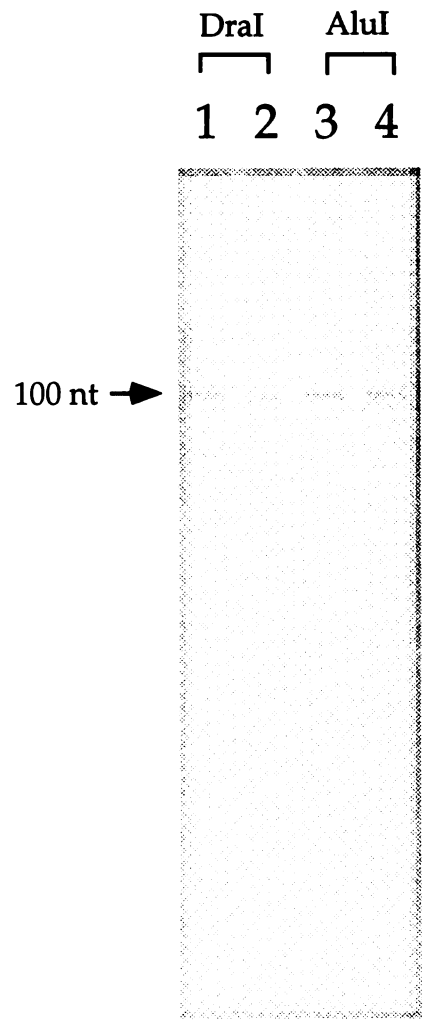
Fig. 1: Sensitivity of single-stranded DNA to restriction enzyme cutting. *a*, restriction enzyme cutting of the 33 mer. Lanes 1 and 2: controls, incubation of the 33 mer at 37 ^{0}C for 1 min and 5 min respectively. Lanes 3 and 4: cutting of uncovered 33 mer by DraIII for 1 min and 5 min respectively. There is about 30% reduction of the full-length 33 mer after 5 min incubation. Lanes 5 and 6: cutting of SSB-covered 33 mer by DraIII for 1 min and 5 min respectively, there being no significant reduction of the full-length 33 mer even after 5 min incubation. Lanes 7 and 8: cutting of SSB-covered 33 mer by SspI at 37 ^{0}C for 1 min and 5 min respectively. *b*, restriction enzyme cutting of the 66 mer covered by SSB. Lane 1: control 66 mer prior to a digestion. Lanes 2 and 3: HhaI digestion at 37 ^{0}C for 1 min and 5 min respectively. Lanes 4 and 5: HaeIII digestion at 37 ^{0}C for 1 min and 5 min respectively. HaeIII differs from other tested enzymes in its ability to cut single-stranded DNA covered with SSB. *c*, restriction digestion of SSB-covered 100 mer. Lanes 1 and 2, DraI digestion at 37 ^{0}C for 1 min and 5 min respectively. Lanes 3 and 4: AluI digestion at 37 ^{0}C for 1 min and 5 min respectively.

Methods: oligonucleotides were purchased from Operon (100 mer) and Oligo *etc.* (33 mer and 66 mer). They were 5' phosphorylated with T4 polynucleotide kinase, deproteinated and purified through Bio-spin column. All digestions were performed in the T4 DNA replication buffers. Samples

were incubated at 37 ^{0}C for 1 min and 5 min, treated with proteinase K and electrophoresed on a 15% denaturing polyacrylamide gel containing 8 M urea.


control
DraIII -SSB
DraIII +SSB
SspI +SSB
1 2 3 4 5 6 7 8
33 nt


HhaI
HaeIII
1 2 3 4 5
66 nt
HaeIII
cleavage
products


DraI
AluI
1 2 3 4
100 nt

Appendix 3

Non-hydrolyzable ATP analogs inhibit transcription on supercoiled DNA

We purified ternary complex formed on supercoiled plasmid pRT-510C+18 on Cl-2B and elongated the 18-nt nascent RNA with ATPγS, GTPγS, CTP and UTP. To our surprise, elongation stalls (and most frequently at natural pause sites) under this condition (Fig. 1a). AMP-PNP, GTP, CTP and UTP also failed to elongate the 18-nt RNA into full-length product (Fig. 1b). It therefore seems that non-hydrolyzable ATP analogs prevent RNA chain elongation on supercoiled DNA. (Curiously, RNA polymerase taken freshly from a - 80 ^{0}C stock seems to be less sensitive to this inhibitory effect caused by ATPγS and AMP-PNP (data not shown)).

However, when the supercoiled template was cut with a restriction enzyme (DraI), even ATPγS and GTPγS together do not prevent elongation on such linearized template (Fig. 2). Elongation on supercoiled DNA may require hydrolysis of ATP. It remains to be seen whether dATP can release the inhibition caused by non-hydrolyzable ATP analogs. It would also be interesting to determine whether the addition of topoisomerases can bypass the requirement of ATP hydrolysis during elongation on supercoiled DNA.

We also noticed that *E. coli* GreA and GreB proteins can release this inhibition caused by ATPγS and AMP-PNP (data not shown).

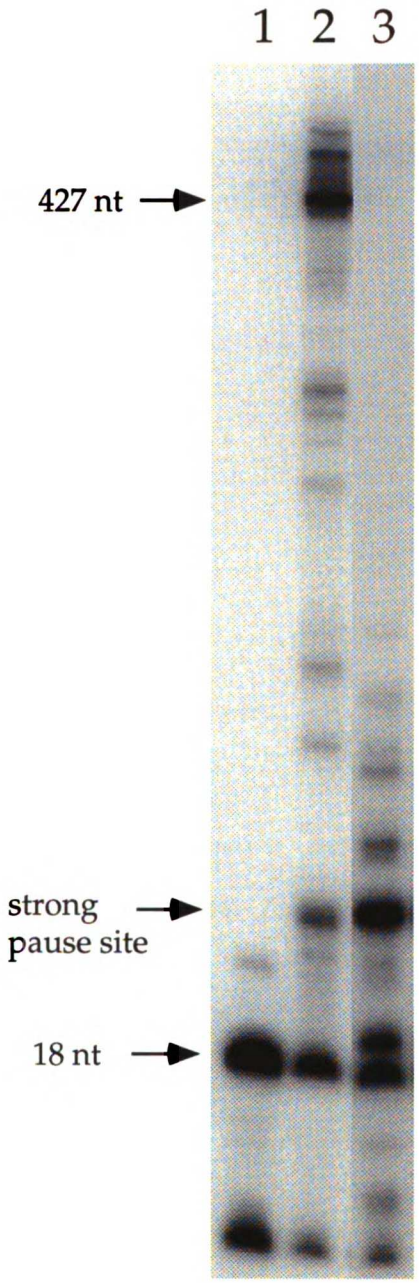
Figure legend:

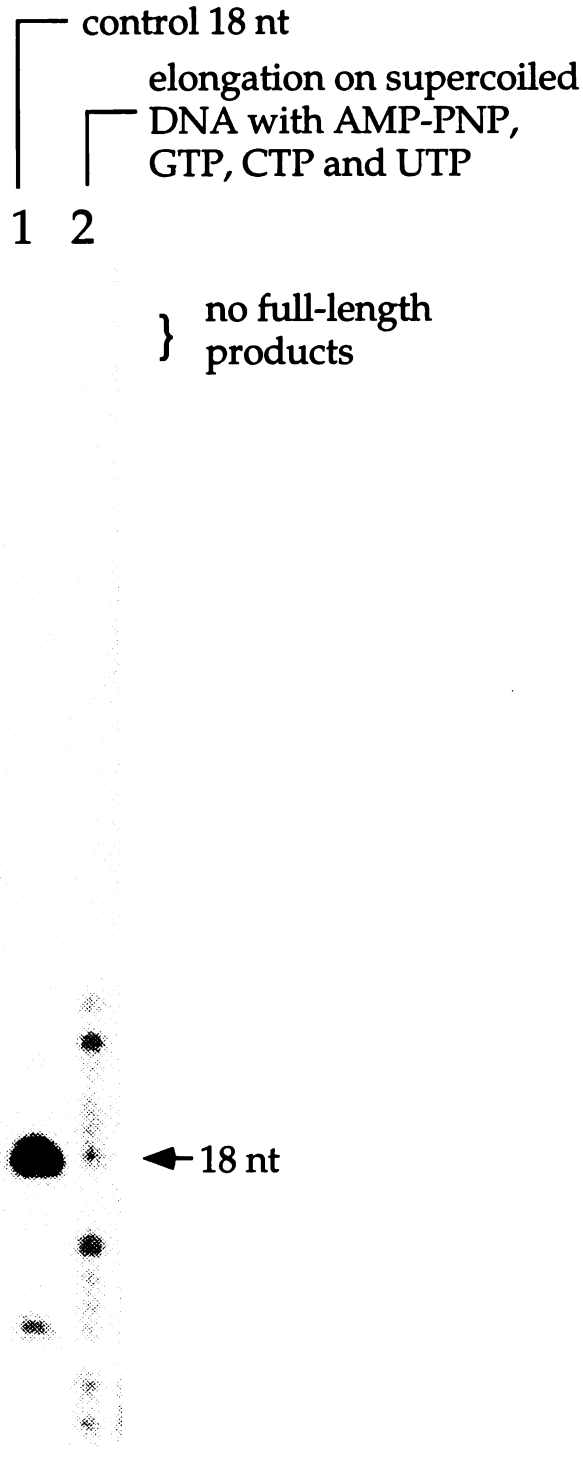
Fig. 1 Inhibitory effect of non-hydrolyzable ATP analogs on RNA elongation on supercoiled DNA. *a*, Lane 1: control 18 nt before elongation. Lane 2: elongation with NTPs. Lane 3: elongation with ATPγS, GTPγS, CTP and UTP. *b*, Lane 1: control 18-nt RNA. Lane 2: elongation with AMP-PNP, GTP, CTP and UTP.

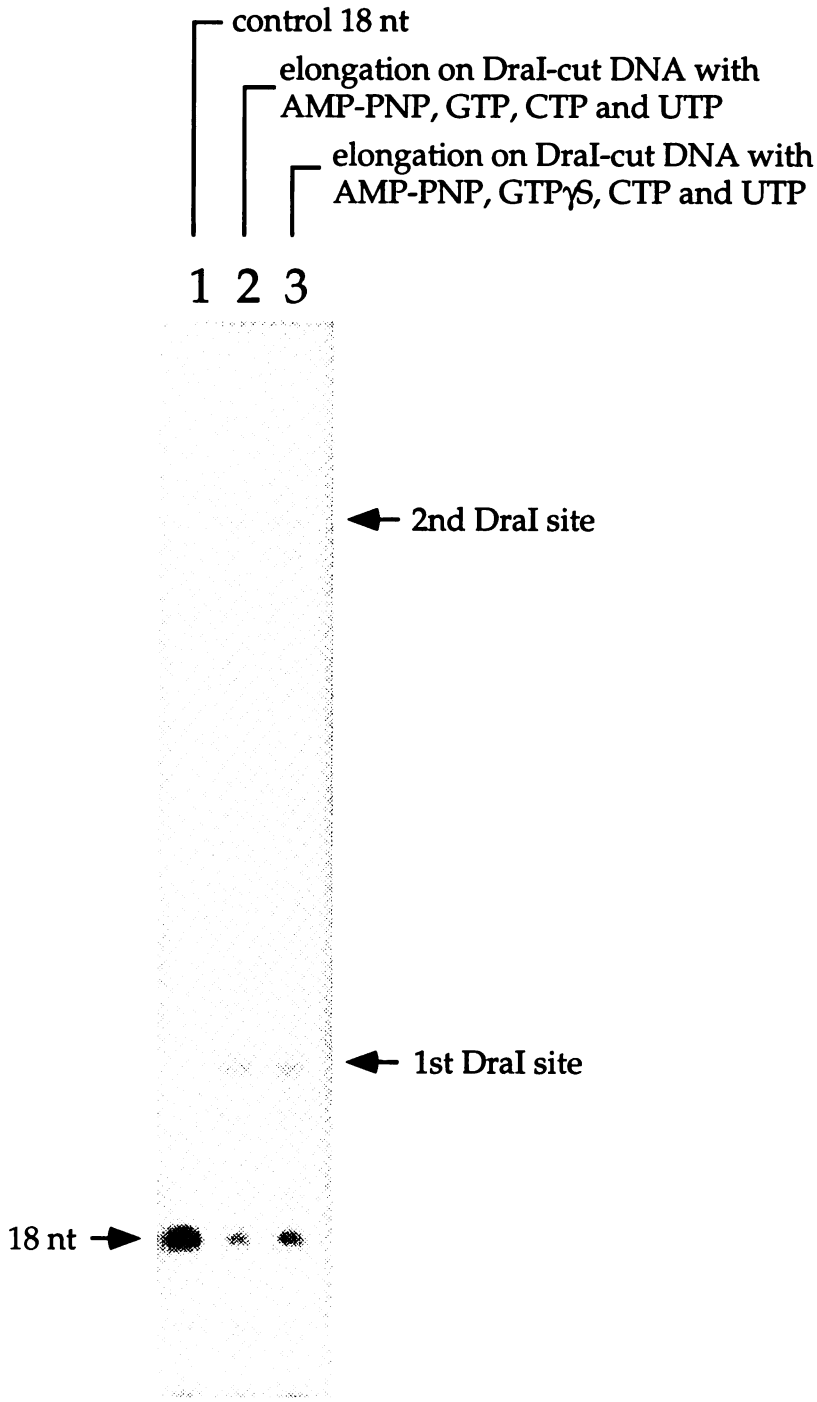
Fig. 2 Non-hydrolyzable ATP and GTP analogs are substrates for RNA synthesis on linear DNA. Lane 1: control 18-nt RNA. Lane 2: elongation on DraI-cut DNA with AMP-PNP, GTP, CTP and UTP. Lane 3: elongation on DraI-cut DNA with AMP-PNP, GTPγS, CTP and UTP.

Methods: 0.5 mM ATPγS or AMP-PNP, 0.5 mM GTPγS or GTP, 0.25 mM CTP and 0.25 mM UTP were added to the purified ternary complex formed on supercoiled DNA as indicated to elongate the 18-nt RNA at 37 ^{0}C for 5 min (Fig. 1). For the experiment shown in Fig. 2, supercoiled DNA was cut with 10 units of DraI. Then 0.5 mM AMP-PNP, 0.5 mM GTP or GTPγS (as indicated), 0.25 mM CTP and 0.25 mM UTP were added to elongate the nascent RNA at 37 ^{0}C for 5 min. All samples were digested with proteinase K and electrophoresed on a 10% denaturing polyacrylamide gel containing 8 M urea.

ATPγS and GTPγS inhibit elongation on supercoiled DNA




control 18 nt
elongation on supercoiled DNA with AMP-PNP, GTP, CTP and UTP
1 2
no full-length products
18 nt


control 18 nt
elongation on DraI-cut DNA with AMP-PNP, GTP, CTP and UTP
elongation on DraI-cut DNA with AMP-PNP, GTPγS, CTP and UTP
1 2 3
2nd DraI site
1st DraI site
18 nt

Reference

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