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# Transcription arrest caused by long nascent RNA chains

Thomas Bentin[a,*], Dmitry Cherny[b,1], H. Jakob Larsen[a,2], Peter E. Nielsen[a]

[a]*Department of Medical Biochemistry and Genetics, The Panum Institute, University of Copenhagen, Blegdamsvej 3c, 2200 Copenhagen N, Denmark*
[b]*Max Plank Institute for Biophysical Chemistry, am Fassberg 11, D-37077 Göttingen, Germany*

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**Abstract**

The transcription process is highly processive. However, specific sequence elements encoded in the nascent RNA may signal transcription pausing and/or termination. We find that under certain conditions nascent RNA chains can have a strong and apparently sequence-independent inhibitory effect on transcription. Using phage T3 RNA polymerase (T3 RNAP) and covalently closed circular (cccDNA) DNA templates that did not contain any strong termination signal, transcription was severely inhibited after a short period of time. Less than ~10% residual transcriptional activity remained after 10 min of incubation. The addition of RNase A almost fully restored transcription in a dose dependent manner. Throughout RNase A rescue, an elongation rate of ~170 nt/s was maintained and this velocity was independent of RNA transcript length, at least up to 6 kb. Instead, RNase A rescue increased the number of active elongation complexes. Thus transcription behaved as an all-or-none process. The mechanism of transcription inhibition was explored using electron microscopy and further biochemical experiments. The data suggest that multiple mechanisms may contribute to the observed effects. Part of the inhibition can be ascribed to the formation of R-loops between the nascent RNA and the DNA template, which provides "roadblocks" to trailing T3 RNAPs. Based on available literature we discuss possible in vivo implications of the results.


*Keywords:* RNA polymerase; RNase; DNA topology; R-loop

## 1. Introduction

The transcription cycle consists of (i) initiation that includes promoter recognition, activation of RNA synthesis and promoter escape, (ii) RNA chain elongation, and (iii) termination. Once the newly synthesized, nascent RNA chain has been released from the ternary RNAP–RNA–DNA elongation complex (EC), it can no longer be elongated. Thus to ensure faithful RNA copying of genes, transcription must be carried out with high processivity. To this end, RNA polymerases (RNAPs) have evolved as "clamp-like" structures that almost encircle the DNA template. This general architecture is observed in enzymes ranging from the single-subunit phage T7 RNAP [1,2] over bacterial RNAP [3] to eukaryotic RNAP II [4,5]. A number of nucleic acids binding sites have been mapped for example in T7 RNAP [6] and *Escherichia coli* RNAP [7–9]. Together these features form the basis for our current model of RNAPs as "sliding clamps", i.e., enzymes that combine swift translocation along the template with tight association to the DNA (reviewed in [10] and [11]).

Intrinsic transcription termination is signaled by specific nucleic acids sequences. For example, a class I terminator, typified by the Tϕ-termination signal in the late phage T7 DNA, encodes an RNA that can form a hairpin structure

*Abbreviations:* RNAP, RNA polymerase; EC, elongation complex; NTPs, ribonucleotide triphosphates; cccDNA, covalently closed circular DNA

* Corresponding author. Tel.: +45 35327771; fax: +45 35396042.
*E-mail address:* thomas@imbg.ku.dk (T. Bentin).
[1] Permanent address: Institute of Molecular Genetics, Russian Academy of Sciences, Kurchatov's sq. 123182 Moscow, Russia.
[2] Present address: Department of Forensic Genetics, Institute of Forensic Medicine, University of Copenhagen, Frederik V's Vej 11, 2100 Copenhagen Ø, Denmark.

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followed by a stretch of uridines [12,13]. This is reminiscent of intrinsic termination by *E. coli* RNAP [14–16]. In contrast, a phage T7 class II terminator, originally unveiled in a cloned human preproparathyroid hormone gene [17], encodes a short consensus sequence that does not appear to support the formation of RNA secondary structure [18–20]. Thus phage T7 RNAP class I and class II terminators exemplify two types of sequence specific regulatory elements: those that modulate transcription indirectly by encoding RNA sequences that can form secondary structures and those that function directly through sequence. Here we report that nascent RNA chains can also have a general transcription inhibitory effect, when growing nascent RNAs cannot be released and therefore accumulate on the template. This effect is almost completely reversed by the administration of RNase A and partly by RNase H. The results are discussed mechanistically in relation to contemporary models for transcription elongation.

## 2. Experimental

### 2.1. DNA preparation

The pT3T7 construct was described previously [21]. DNA was propagated in Xl-1 Blue and purified using the JetStar kit (Genomed). Preparation of relaxed circular DNA was done as follows. Supercoiled pT3T7 (1.5 μg) was incubated with 24 U Calf thymus topoisomerase I for 60 min and otherwise as recommended (Life Technologies). The reactions were quenched by the addition of NaCl to a final concentration of 1 M and followed by incubation for 5 min at 37 °C. EDTA was added to a final concentration of 25 mM and the reaction was incubated at 65 °C for 10 min, ethanol precipitated, and dissolved in 10 mM Tris pH 7.6, 1 mM EDTA. Linear DNA was prepared by *Sca*I digestion of supercoiled pT3T7. To ensure that the template preparations were identical in all respects except the DNA topology, each reaction was supplemented with restriction enzyme and topoisomerase in a sequential scheme. When appropriate, the enzymes were heat-inactivated before combination with the DNA. The DNA preparations were analysed by agarose gel-electrophoresis. Visualization of DNA was done by ethidium staining and gels were photographed using a Pharmacia Image Master system.

### 2.2. Transcription reactions

Unless otherwise stated, the transcription reactions were initiated by the addition of 0.5 mM (final concentration) each of ATP, CTP, GTP and UTP ribonucleotides (NTPs) to a reaction containing the indicated amount of T3 RNAP and DNA in a volume of 10–50 μl TB [40 mM Tris (pH 8), 8 mM MgCl2, 25 mM NaCl, 2 mM spermidine, 10 mM DTT, and 0.1 μg BSA/μl]. Pulse labelling was carried out by the addition of the indicated amount of pulse-mix containing 0.1–1 MBq of [α-$^{32}$P]UTP (Amersham-Pharmacia) in TB supplemented with the relevant NTP. When required, RNasin (Promega) was included at a concentration of 1 U/μl. RNase A (50–100 U/mg) and RNase H (Boehringer Mannheim) were as indicated. Whenever required, T3 RNAP was diluted into the enzyme storage buffer (20 mM potassium phosphate, pH 7, 0.1 M NaCl, 0.1 mM EDTA, 1 mM DTT, 50% (v/v) glycerol) and kept on ice until use. T3 RNAP concentrations were measured by Coomassie blue staining of 12% SDS-PAGE gels and using BSA as a standard. One pmol of enzyme corresponded to 10–20 U of activity as defined by the manufacturer (Life Technologies). RNase A stocks were prepared at 10 μg/μl as described [22]. The RNase H experiments were conducted with RNase H storage buffer [25 mM Hepes, 50 mM KCl, 1 mM DTT, 50% (v/v) glycerol, pH 8] added to 20% of the total volume to avoid differences caused by buffer variations.

### 2.3. RNA analysis

RNA analysis was done essentially as described [22] Briefly, transcription reactions for gel-electrophoretic analysis were quenched into a large volume of 20 mM ice cold EDTA (EDTA quenched), immediately phenol:-chloroform extracted, precipitated, resuspended in a formaldehyde/MOPS buffer [20 mM MOPS, 8 mM Na-acetate, 1 mM EDTA, pH 7 and 1/50 vol. of a 37% formaldehyde stock] supplemented with 75% (v/v) formamide, and incubated for 5–10 min at 65 °C. The RNA was chilled on ice and electrophoresed on 1.4% denaturing agarose gels containing formaldehyde/MOPS buffer at ~4 V/cm at room temperature. The gels were dried onto DE81 chromatography paper (Whatmann) or subjected to Northern transfer using a buffer containing 0.6 M NaCl, 0.12 M Tris, 4 mM EDTA, pH 7 and Hybond+ nylon membranes. Northern transfer eliminated background problems arising from the unincorporated [α-$^{32}$P]UTP. Transcription reactions aimed for dot blot analysis were EDTA quenched and aliquots were transferred to Hybond N+ using a vacuum blot apparatus. The nylon membranes were washed extensively using 0.5 M sodium phosphate, pH 7. Quantification of the results was done by phosphor-imaging or by densitometric scanning of autoradiographs and using ImageQuant software.

### 2.4. Electron microscopy

Transcription reactions for electron microscopy analysis contained 200 fmol of supercoiled or relaxed circular pT3T7, ~2–8 pmol T3 RNAP, 0.5 mM each of ATP, CTP, GTP, and UTP in TB modified to 1 mM DTT and without BSA in a volume of 10–30 μl. The reactions were incubated for 5 min at 37 °C, omitting CTP. Then CTP was added to

start the reaction and 1 μl aliquots were withdrawn at the relevant lengths of incubation and diluted 100–300 times in adsorption buffer (10 mM Tris pH 7.5, 50 mM NaCl, 0.1 mM EDTA). Alternatively the samples were purified using a SMART system (Pharmacia Biosystems) equipped with a Superose 6 gel-filtration column that was pre-equilibrated with adsorption buffer. The samples were adsorbed to a carbon film glow-discharged in the presence of pentylamine vapors as described [23] for 1–5 min depending on the required final DNA concentration. Subsequently the samples were stained with 2% (w/v) uranyl acetate for 10 s and blotted with a filter paper. The samples were examined in a dark-field mode (Zeiss/LEO CEM-902 or Philips CM12 electron microscope).

## 3. Results

### 3.1. Rapid cessation of transcription using cccDNA as a template

We first compared the overall RNA synthesis kinetics by phage T3 RNAP on linear, supercoiled and relaxed plasmid DNA templates by monitoring the accumulation of $^{32}$P-labelled RNA over time (Fig. 1). Using a molar ratio of RNAP to DNA of ~100:1 and circular templates, the amount of RNA synthesized rapidly reached a plateau, and the overall RNA synthesis rate decreased steeply to less than 10% of the initial activity within 10 min of incubation. In contrast, when using the corresponding linear template the overall transcription rate was constant throughout the experiment. Approximately 15 pmol and 40 pmol of ribonucleotides were polymerized into RNA during the experiment (20 min) employing the circular and linear templates, respectively. Thus, the transcription inhibition observed when employing circular templates was not due to NTP depletion — nor was it due to the general inactivation of the enzyme (Fig. 1). Because RNA synthesis during run-off transcription with the linear DNA template was not subject to inhibition, this argues that a physical link between the RNA and the EC is required to establish the observed inhibitory effects, and that the presence of increasing amounts of liberated nascent RNA cannot produce this result.

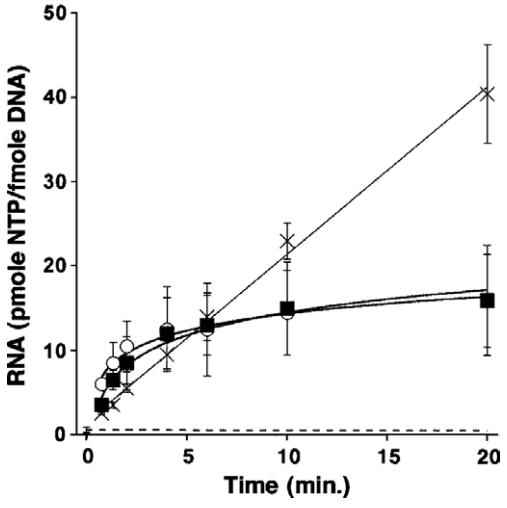



Fig. 1. Transcription inhibition with circular DNA templates. Overall RNA synthesis as a function of time and template topology as measured by dot blot analysis. RNA synthesized as a function of time using a molar RNAP/DNA ratio of 100:1. The reactions contained ~5 fmol (10 ng) of pT3T7 and ~500 fmol of T3 RNAP, ~0.7 MBq [α-$^{32}$P]-UTP, 0.5 mM each of ATP, GTP, CTP and UTP in a final volume of 50 μl TB. At the indicated length of incubation, 5 μl aliquots were removed, EDTA quenched and stored on ice. Aliquots of the samples were subjected to vacuum blotting and analysed as described (Experimental procedures). Symbols: supercoiled DNA (○), relaxed circular DNA (■), and linear DNA (×). The background is shown with broken lines (– – –). Capped bars signify the standard deviation using 2–3 experiment repetitions.

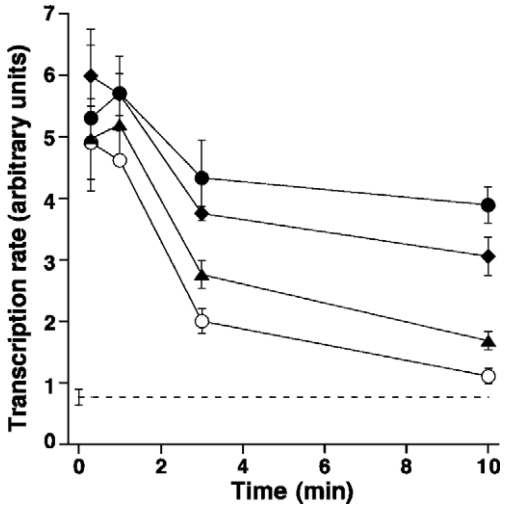



Fig. 2. RNase A mediated transcription rescue. RNA produced during RNase A mediated transcription rescue as measured by dot blot analysis. Two-step reactions were conducted. In the first step, 1 pmol of T3 RNAP and 50 fmol of supercoiled pT3T7 DNA (molar RNAP/DNA ratio ~20) were incubated for 30 s–10 min in the presence of 0–0.16 μg/ml RNase A and 0.5 mM each of ATP, CTP, GTP, and UTP in a volume of 50 μl TB. In the second step, 5 μl aliquots were withdrawn at the indicated times and pulse-labelled for 15 s in TB containing 0.5 mM each of GTP, ATP, CTP, and ~0.1 MBq of [α-$^{32}$]P-UTP, 1 U/μl RNasin, and 1 μg tRNA, in a final volume of 25 μl. The pulse reactions were EDTA quenched and analysed by vacuum blotting (Experimental procedures). The concentration of RNase A was as follows: w/o (○), 0.04 μg/ml (▲), 0.08 μg/ml (◆), and 0.16 μg/ml (●). The background and the standard deviation are as in Fig. 1. Three experiment repetitions are compiled.

### 3.2. Transcription rescue by RNase A

One main difference between using the linear and circular DNA templates was also the length of the resulting RNA transcripts. On the linear template, run-off products of ~0.9 kb were produced. Using the covalently closed circular DNA templates, very large RNA molecules could be formed, because transcription elongation may continue for several rounds on a circular template in the absence of an efficient terminator (data not shown and Fig. 3). We

therefore decided to further examine the importance of RNA integrity for the inhibition process.

A two-step reaction was employed. In the first step, transcription reactions were incubated using supercoiled DNA as a template and varying amounts of RNase A to obtain simultaneous RNA degradation and de novo RNA synthesis. In the second step, residual transcription activity was monitored at various lengths of incubation with a short $^{32}$P-UTP pulse in the presence of RNase inhibitor. The results presented in Fig. 2 show that RNase A efficiently relieved the inhibition of transcription in a dose-dependent manner. A control experiment was designed to evaluate the degree of RNA degradation. At the highest RNase A concentration, the RNA products were severely degraded and with a size of less than 50 nucleotides (data not shown).

### 3.3. All-or-none transcription depends on the nascent RNA

To study this RNA mediated transcription inhibition further we measured the elongation rate as well as the relative number of active ECs per template using the two-step protocol. In the first step, transcription reactions were incubated for a length of time expected to result in full inhibition of transcription (in the absence of RNase A) using supercoiled DNA as a template and including varying amounts of RNase A. In the second step, transcription activity was monitored by $^{32}$P-UTP pulse labelling for 7, 15 and 30 s in the presence of RNase inhibitor. The resulting RNA was analysed by denaturing agarose gel-electrophoresis [Fig. 3 (A)]. To ensure that RNase A inactivation was achieved, a gel-purified run-off transcript was included in the pulse-reaction as an internal standard. This control confirmed that the RNase had been inactivated. Furthermore, a DNA fragment was included in the pre-pulse reaction to monitor differences arising during experimental handling. Only minor differences of the internal standards were observed between samples.

To measure the transcription elongation rate, the RNA distribution generated in each pulse reaction was calculated [Fig. 3 (B)]. These results show that RNase A mediated transcription rescue occurred without significant alterations of the elongation rate (~170 nt/s), at least up to RNA lengths of ~6 kb. In contrast, overall RNA synthesis was strongly potentiated by RNase A. Therefore transcription rescue worked by increasing the number of active ECs per template. This also indicates that transcription is an all-or-none process and suggests that the strong inhibitory effects observed were due to transcription arrest and/or termination and not to a decrease in the elongation rate. Furthermore, with an elongation rate of 170 nt/s, transcription proceeds several rounds on the template before inhibition (cf. Figs. 1 and 3).

### 3.4. Transcription inhibition may be due to EC–EC collisions

In order to obtain structural information that could reveal possible physical causes of the observed inhibition, transcription reactions were monitored over time using electron microscopy (Fig. 4, Table 1). To minimize manipulation and thus be able to visualize the transcription complexes in as closely as possible to the native state, the samples were merely diluted before adsorption to the grid. The distribution of transcription complexes was estimated from the appearance of a nascent RNA projecting from the DNA template. In other experiments we purified transcription complexes using gel-filtration. The distribution of ECs was similar to that of the simply diluted samples implying that they were in the native state.

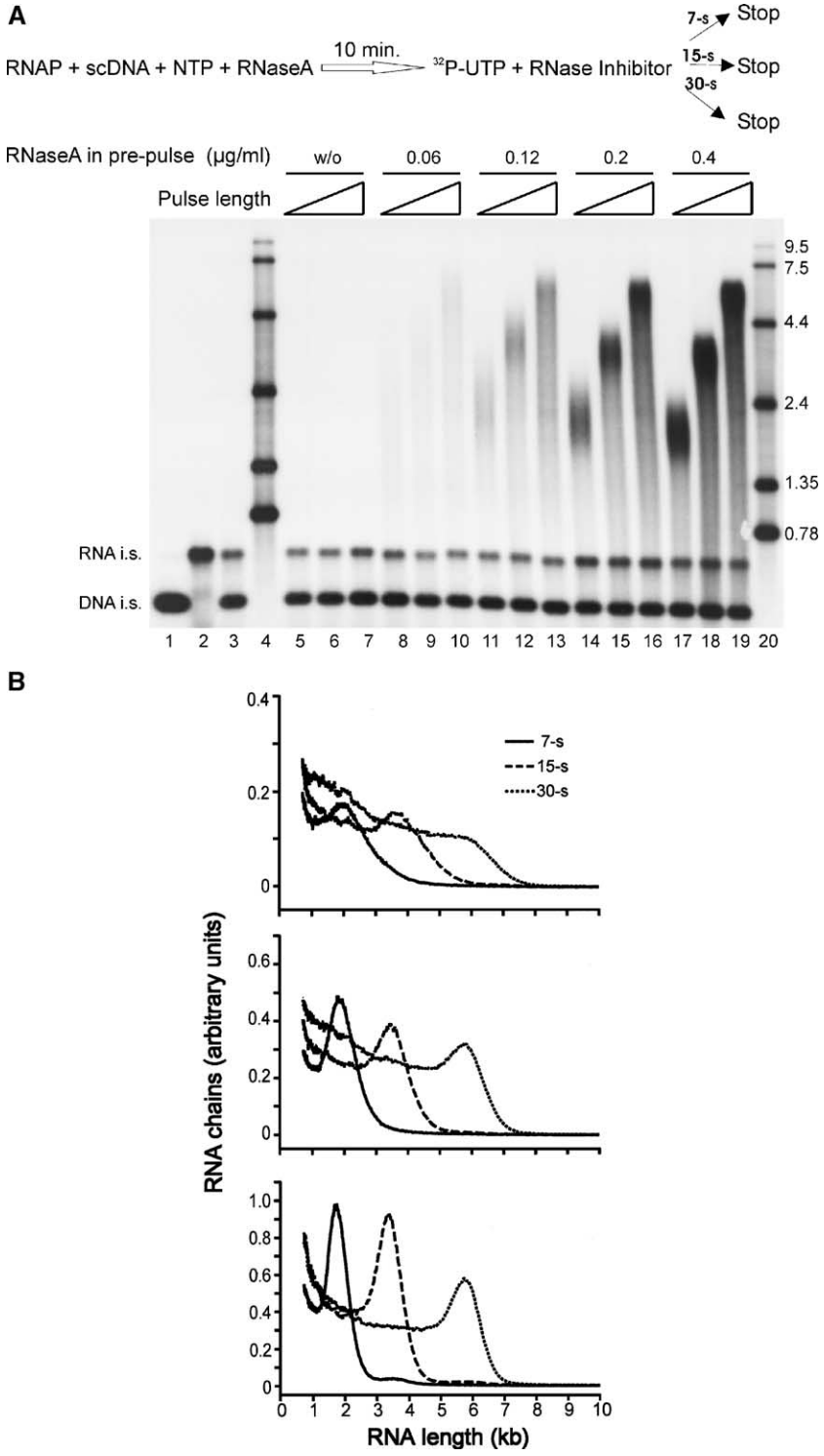
Aliquots were withdrawn at the indicated times and analysed by EM. At short incubation times (30 s, 1 min, and 3 min) the template occupancy ranged from ~1 to ~5 ECs per DNA molecule [Fig. 4(A), Table 1]. After 10 min of incubation, however, the predominant appearance was that of a single or a few confined “RNA aggregate(s)” per template [Fig. 4(B) and (C), Table 1], and we speculate that these RNA aggregates contained several collided and thereby inactivated ECs. These results also suggest that, at least on a fraction of the templates, transcription arrest, and not termination, had taken place.

### 3.5. Possible involvement of R-loops

One possible cause of transcription inhibition could be the formation of R-loops between the template and the

Fig. 3. Transcription elongation rate and kinetics of overall RNA synthesis during RNase A rescue. (A) Autoradiograph showing nascent RNA produced during RNase A mediated transcription rescue as analysed by denaturing agarose gel-electrophoresis. Two-step reactions were employed. In the first step, ~1.5 pmol of T3 RNAP was incubated with 50 fmol of supercoiled pT3T7 (molar RNAP/DNA ratio ~30:1) in TB supplemented with 0.5 mM each of ATP, CTP, GTP, and UTP and the indicated amount of RNase A in a final volume of 50 μl. A 332 bp $^{32}$P-end labelled DNA fragment (see lane 1) was included as an internal standard. After 11 min of incubation, 20 μl of this reaction was combined with 80 μl of a pre-warmed pulse mix. The pulse mix contained ~1 MBq [α-$^{32}$P]-UTP, 0.5 mM each of ATP, CTP, GTP and UTP, 10 μg yeast RNA and 1.25 U/μl RNasin. A $^{32}$P-labelled 465 nt RNA run-off transcript (see lane 2) was included in the pulse mix to ensure that RNase A was inactivated. Aliquots of 30 μl were withdrawn after 7 s (lanes 5, 8, 11, 14, and 17), 15 s (lane 6, 9, 12, 15, 18), and 30 s (lane 7, 10, 13, 16, and 19), EDTA quenched and analysed by denaturing agarose gel-electrophoresis (Experimental procedures). The RNA molecular markers (lanes 4 and 20) are given in kb. DNA and RNA standards combined (lane 3). (B) RNA chain distribution as a function of pulse time. In order to transform the data in (A) to RNA chains of a given length, the results were first digitalized using a phosphorimager. An expression relating RNA size to gel-migration was derived using the RNA molecular markers. The $^{32}$P-intensity in each data point along the contour of each lane was divided by the size of an RNA corresponding to its position on the gel. The figure thus shows the actual distribution of RNA chains for each pulse time. The elongation rate (~170 nt/s) was estimated from the position of the apex of each RNA distribution. RNase A concentrations: upper (0.06 μg/ml), middle (0.2 μg/ml), lower (0.4 μg/ml).

nascent RNA in the wake of elongating RNAP as suggested by the in vivo data of Hraiky et al. 2000 [24]. To address whether R-loops might be generated in the present system, we employed a two-step reaction substituting RNase H for RNase A and monitored by dot blot analysis the propensity of this enzyme to rescue transcription (Fig. 5). The transcription activity was measured after 30 s and 10 min of incubation, respectively. As before, the activity declined rapidly (to ~7% of the initial activity in 10 min). As in the case of RNase A, RNase H rescued transcription in a dose-dependent manner, albeit to a much lesser extent. Approximately 25% of the initial activity was retained at the highest



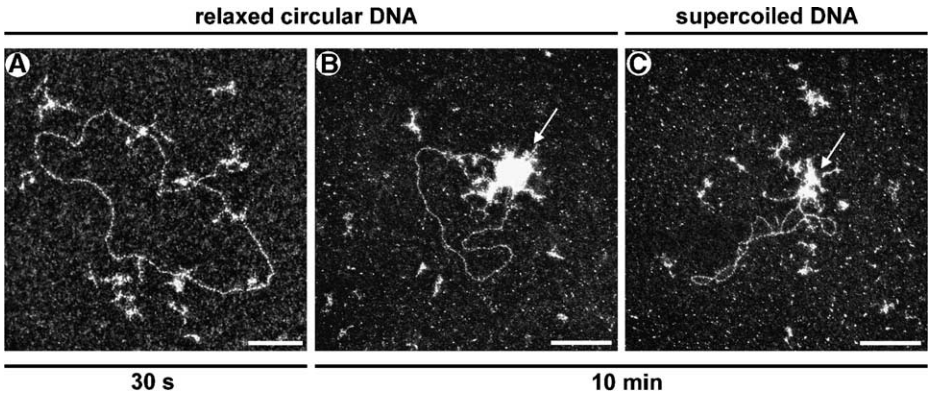



Fig. 4. EC–EC collisions as a possible cause of transcription inhibition. Electron microscopy imaging of transcription complexes. Transcription reactions containing relaxed circular DNA (A and B) or supercoiled DNA (C) as a template were incubated using a molar ratio of RNAP to DNA ~20 for 30 s (A) or 10 min (B and C) and otherwise as described in Experimental procedures. Aliquots of 1 μl were withdrawn at the indicated time and transcription was terminated by 200-fold dilution into 10 mM Tris pH 7.6, 50 mM NaCl, and 0.1 mM EDTA. 20 μl of this solution was subject to electron microscopy. Images were taken in a dark-field mode. Bars indicate 100 nm. Arrows indicate RNA aggregates possibly containing arrested ECs.

RNase H concentration used. These results suggest that R-loop structures could play a role in the observed transcription inhibition, as the RNase H would degrade the RNA moiety of an RNA–DNA hybrid thus allowing passage of trailing RNAP(s).

If the formation of nascent RNA dependent roadblocks were in fact a cause of transcription inhibition, this effect should depend on the number of ECs per template. We therefore repeated the experiment described in Fig. 1 at reduced RNAP concentration to establish equal molar amounts of RNAP and DNA template (Fig. 6). Under these conditions RNA accumulation was roughly constant up to ~10 min of transcription. This implies that the number of nascent RNA chains per template was indeed crucial to the inhibitory process. We also note that transcription appeared particularly sustained when using a relaxed circular template as compared with the supercoiled DNA.

Table 1
The number of ECs/DNA

| Incubation time (min) | ECs/DNA | |
|---|---|---|
| | Initiation by RNAP | Initiation by CTP |
| 0 | 50–60% | 100% |
| 0.5 | 1.3∓0.4 (40) | 2.8∓1.3 (69) |
| 1 | 1.8∓0.6 (76) | 2.6∓1.3 (85) |
| 3 | 1.8∓1 (62) | 1.9∓1.1 (63) |
| 10 | 1.3∓1 (80) | 1.4∓0.7 (66) |

Estimate of the number of ECs per template as based on the inspection of electron microscopy images. The samples were incubated with supercoiled pT3T7 as a template and transcription was initiated by the addition of RNAP (middle column) or CTP (right column) to a master mix containing the remaining transcription components. Aliquots were withdrawn for EM analysis at the indicated times and treated as described in Materials and methods. The mean number of ECs/template±standard deviation is reported. The numbers in brackets show the number of complexes analysed. The numbers given in percentages indicate the template RNAP occupancy before adding the full complement of ribonucleotides.

## 4. Discussion

The major conclusion from the present results is that long nascent RNA molecules can be transcription limiting in vitro and that this effect can be relieved by the action of RNases. Several molecular mechanisms can, solely or in combination, account for the inhibitory effect on transcription.

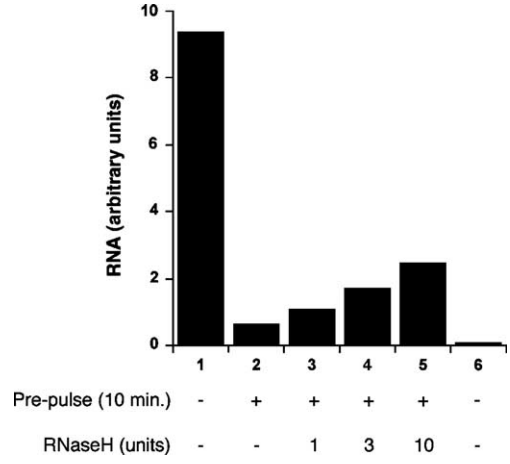



Fig. 5. RNase H mediated transcription rescue. (A) RNA produced during RNase H mediated transcription rescue as analysed by dot blot analysis. A two-step reaction was conducted. In the first step 0.5 pmol of T3 RNAP and 5 fmol of supercoiled DNA (molar RNAP/DNA ratio ~100) were incubated in TB containing RNasin and supplemented with RNase H as indicated. The reactions were initiated by the addition of NTP to a final concentration of 0.5 mM each of ATP, CTP, GTP, and UTP in a final volume of 50 μl and incubated as indicated. In the second step, the pre-pulse reaction was combined with equal volumes of pulse-mix containing 0.5 mM each of ATP, CTP, and GTP and 0.4 MBq [α-$^{32}$P]UTP in TB. The samples were incubated for 1 min, EDTA quenched and further processed by dot blot analysis as described (Experimental procedures). Column 1: transcription for 30 s prior to $^{32}$P-UTP pulse; column 2: transcription for 10 min prior to $^{32}$P-UTP pulse; column 3–5: transcription for 10 min with the indicated amount of RNase H prior to $^{32}$P-UTP pulse; column 6: background.

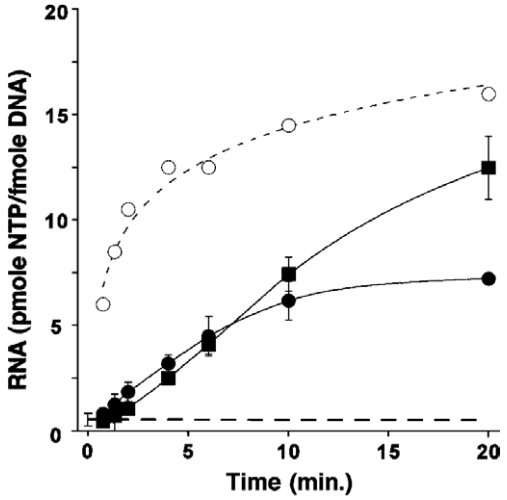



Fig. 6. Transcription sustained at reduced RNAP:DNA ratio. Overall transcription activity as a function of time using a 1:1 molar RNAP/DNA ratio as analysed by dot blot analysis. One-step reactions were incubated as described in the legend to Fig. 1 using 5 fmol of T3 RNAP and 5 fmol of supercoiled DNA. Symbols: (●) supercoiled DNA, (■) relaxed circular DNA. As a reference, the data from Fig. 1 using supercoiled DNA as template has been included (○). [Note that transcription with relaxed circular and supercoiled DNA at the RNAP:DNA ratio of 100:1 is very similar (Fig. 1)].

### 4.1. R-loops

It has been shown that stable R-loops can form during transcription in vitro using a supercoiled template, and it was speculated that R-loops could form roadblocks to transcription [25,26]. Furthermore, it was recently shown that the transcription of rDNA in *E. coli topA* (topoisomerase I null mutant) cells is impaired at the level of transcription elongation and that RNase H overexpression corrects this defect. Thus it was proposed that R-loops could form roadblocks to ECs [24]. Our RNase H data support this conclusion because the enzyme partially relieved the observed transcription inhibition as would be expected if R-loops blocked the elongation of trailing polymerases.

### 4.2. Topology dependent inactivation

We suspect that a component of the striking inhibition of transcription seen with cccDNA as a template could be due to topological restrictions in the framework of the twin-supercoiled-domain model [27]. The entanglement of multiple nascent RNA chains [28] might cause transcription inactivation by restricting free rotary movement of ternary elongation complexes. Indeed the RNA aggregates observed by electron microscopy appear like entangled yarn inside which it is difficult to envision efficient transcription elongation. Alternatively, RNAP could drive structural alterations of the cccDNA template, forming transient structures incompatible with efficient elongation. To this end it has been shown that a knotted template is a very poor substrate for T7 polymerase [29].

### 4.3. Enzyme collision mediated inactivation

At an elongation rate of 170 nt/s, even small variations in this rate and uneven pausing by individual ECs could rapidly create a "phasing" problem. Such variations in elongation rate (that go unnoticed in the present analysis) might be due to topological restrictions (see above) or result from other slowed species such as RNAPs in the process of transcription initiation. Long nascent RNA chains might exacerbate such problems by forming an entangled web. Because it takes less than 20 s to transcribe the entire plasmid circle, the association of multiple RNAPs with the DNA would place the enzymes only a few seconds apart as is also the case in the *E. coli* rDNA operons where the distance between consecutive RNAP molecules is only 85 bp [30,31]. To this end, our transcription conditions with an RNAP:template ratio of 30:1 (Fig. 3) or 100:1 (Fig. 1) corresponds to a maximal theoretical template load of 1 RNAP per 30–90 bp provided that all RNAPs were active and associated with the DNA. Moreover, electron microscopy analysis revealed only a few ECs/template (Table 1) strongly arguing that transcription inhibition occurs with a physiologically relevant template load.

We note that previous investigations lead to the realization that collision events between polymerases can have pronounced effects on enzyme function. For instance, when a replication apparatus of bacteriophage T4 meets an EC containing *E. coli* RNAP, the replication fork may pause from a few seconds to several minutes in vitro (depending on the presence or absence of a helicase) before passing the RNAP [32]. Furthermore, the replication machinery encompassing bacteriophage ϕ29 DNA polymerase has been observed to slow down in vitro when encountering a RNAP from *Baccillus subtillis* [33,34]. However, recent data have shown that transcription in *E. coli* by RNAPs transcribing in tandem actually enhance transcription elongation [35] and this cooperative effect of trailing RNAPs could argue against collisions as a cause of transcription inhibition in our system.

As discussed above, the irregular phasing of ECs due to changes in transcription elongation rate may cause EC collision and transcription arrest. The observation that transcription inhibition is strongly influenced by the molar RNAP to DNA template ratio, and therefore also by the EC/template occupancy, may be relevant to the discussion of ribosomal DNA (rDNA) transcription in vivo. While yeast episomal rDNA transcription initiation is stimulated in top1–top2 double mutants, the generation of full-length transcripts is dramatically inhibited suggesting that the transcription elongation on rDNA templates require topoisomerase activity [36]. In contrast, transcription of the less frequently transcribed yeast *GAL1*, *MATα1*, *SIR3* and tRNA genes is much less affected [37]. Topoisomerase I inhibits R-loop formation in *E. coli* by the removal of negative supercoils [38] and perhaps this activity is necessary to maintain a constant EC phasing on ribosomal DNA

templates and hence for sustained rDNA transcription. This could also be a reason why topoisomerase I is part of the RNAP I transcription machinery [39,40].

Thus, the degree by which nascent RNA might inhibit further transcription of a gene most certainly could be heavily influenced by parameters such as promoter strength (transcription initiation rate), transcript length, and template topology. Also, it is likely that mechanisms in Nature such as RNase H and also topoisomerase activity contribute to avoid this potential problem in vivo.

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