Transcription through the roadblocks: the role of RNA polymerase cooperation

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During transcription, cellular RNA polymerases
(RNAP) have to deal with numerous potential road-
blocks imposed by various DNA binding proteins.
Many such proteins partially or completely interrupt
a single round of RNA chain elongation *in vitro*. Here
we demonstrate that *Escherichia coli* RNAP can
effectively read through the site-specific DNA-binding
proteins *in vitro* and *in vivo* if more than one RNAP
molecule is allowed to initiate from the same pro-
moter. The anti-roadblock activity of the trailing
RNAP does not require transcript cleavage activity
but relies on forward translocation of roadblocked
complexes. These results support a cooperation model
of transcription whereby RNAP molecules behave as
'partners' helping one another to traverse intrinsic
and extrinsic obstacles.
Keywords: protein roadblocks/RNA polymerase/
transcription elongation

Introduction

In bacteria and eukaryotes DNA is organized into a
condensed structure composed, in part, of histones and
histone-like proteins. Despite the constant presence of
such nucleoprotein complexes as well as various site-
specific DNA binding proteins within intragenic regions,
rapid progression of elongating RNAP is not compromised
in vivo, although *in vitro*, protein roadblocks often impede
transcription (e.g. Pavco and Steege, 1990; Izban and
Luse, 1991; Reines and Mote, 1993). Eukaryotic and
bacterial general elongation factors SII and GreA/GreB,
respectively (Reinberg and Roeder, 1987; Borukhov *et al*.,
1993) have been shown to stimulate transcription through
site-specific DNA binding proteins (Reines and Mote,
1993; Toulmé *et al*., 2000). These factors function by
inducing transcript cleavage in the RNAP catalytic site
(Izban and Luse, 1992; Borukhov *et al*., 1993; Rudd *et al*.,
1994; Orlova *et al*., 1995). Cleavage of the 3′ portion of
the nascent transcript reactivates the elongation complex
(EC) that has been backtracked for one or more
nucleotides along DNA and RNA (Komissarova and
Kashlev, 1997; Nudler *et al*., 1997). It is assumed that the
repeated rounds of cleavage and re-extension of RNA in

blocked EC can eventually displace the roadblock, allow-
ing transcription to proceed through the physical barrier.
More specific elongation factors have been also described
that can either suppress EC backtracking (e.g. Elongin;
Aso *et al*., 1995) or reactivate the backtracked EC by
actively translocating it forward (Mfd; Park *et al*., 2002).

Remarkably, transcript cleavage factors, as well as
Elongin and Mfd, are dispensable for cell viability under
physiological conditions (Archambault *et al*, 1992; Selby
and Sancar, 1993; Orlova *et al*., 1995; Yamazaki *et al*.,
2003), suggesting that a more general transcriptional anti-
arrest and anti-roadblock mechanism exists. Although
specific protein complexes, such as FACT and Elongator,
have been shown to facilitate RNAP II transcription to
some extent on chromatin templates *in vitro* (Orphanides
et al., 1998; Kim *et al*., 2002), it is unclear whether these
factors are sufficient for rapid chromatin transcription
in vivo by RNAP II, and also RNAP I and III. Even less
clear is how various RNAPs deal with DNA-binding
proteins other than histones during elongation. Con-
sidering the common structural organization of bacterial
and eukaryotic RNAP (Ebright, 2000; Darst, 2001; Gnatt
et al., 2001), it is likely that the basic anti-roadblock
mechanism is also conserved in evolution.

The elongation phase of the transcription cycle often
involves multiple RNAP molecules moving, one after
another, along the same DNA molecule. Recently, we
showed that trailing ECs directly stimulate the activity of
leading ECs. Using a reconstituted transcription system
from *Esherichia coli*, we demonstrated that trailing ECs
reactivate leading backtracked ECs at pause or arrest sites
by translocating them forward (Epshtein and Nudler,
2003). In the present work, we tested the hypothesis that
such a cooperation mechanism between RNAP molecules
also functions in overcoming elongational roadblocks.
Using two different site-specific DNA-binding proteins
with high affinity for their cognate sites, we demonstrate
that, in both cases, trailing ECs rescue roadblocked ECs by
'pushing' them forward. Transcript cleavage activity is not
required during this process. Taken together our *in vitro*
and *in vivo* results suggest that cooperation between
RNAP molecules plays an important role in rendering
transcription fast and processive on protein covered
(physiological) DNA.

Results and discussion

Efficient transcription through the roadblocks depends on multiple rounds of initiation

DNA-bound *Eco*RI restriction endonuclease (*Eco*RQ111),
defective in DNA cleavage (Wright *et al*., 1989), is a
formidable barrier to transcription. It halts the majority of
ECs 14 ± 1 bp upstream of the *Eco*RI site (Pavco and
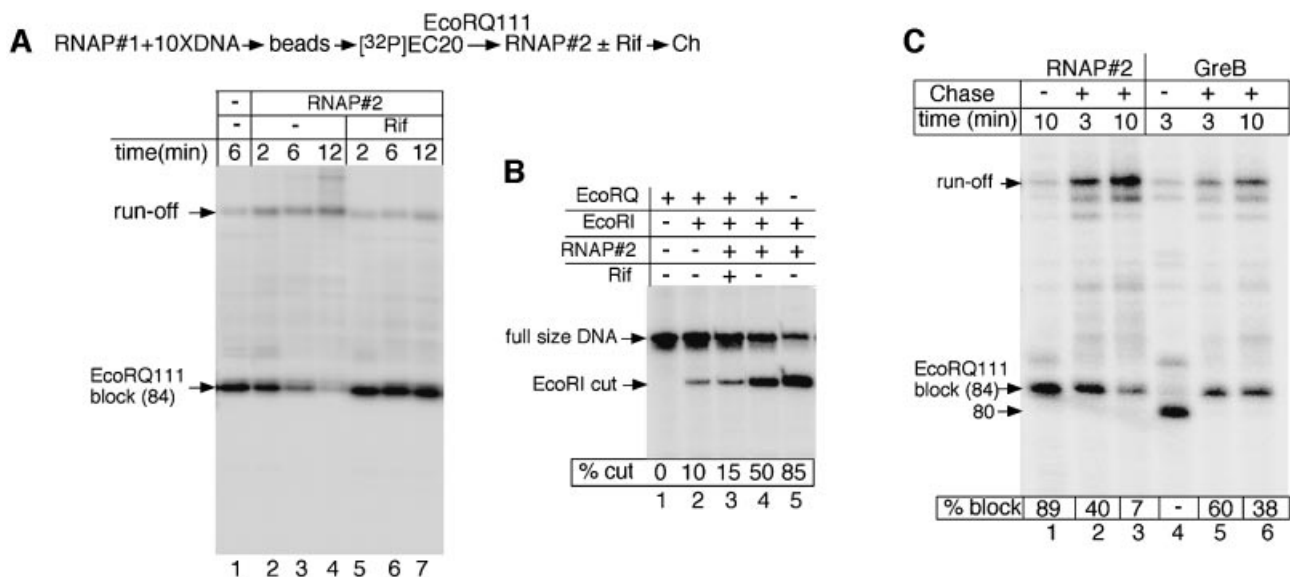Steege, 1990; Nudler *et al*., 1995). To monitor the effect of

Fig. 1. Cooperation between RNAP molecules in overcoming *Eco*RQ111 roadblock. (**A**) Stimulating effect of trailing RNAP molecules on transcription through DNA-bound *Eco*RQ111. An *Eco*RI restriction site is located at position +98 from the +1 start of transcription of template 1. A step-by-step diagram of the experiment is shown at the top (see details in the text and Materials and methods). The autoradiogram displays RNA products synthesized during a single round of transcription by RNAP1. The washed roadblocked complexes were chased in the presence of RNAP2 with or without rifampicin (Rif) for the indicated time intervals (lanes 2–7). (**B**) Dissociation of *Eco*RQ111 from DNA during transcription. The autoradiogram shows end-labeled DNA template fragments (template 1) recovered from transcription reactions with RNAP1 alone (lanes 1 and 2) or together with RNAP2 (lanes 3–5). The experiment was performed as shown in (A) except that native *Eco*RI was also added prior to the chase reaction (lanes 2–5). The increase in *Eco*RI-mediated DNA cleavage (% cut) during multi-round transcription (lane 4) reflects the increased accessibility of the restriction site, i.e. dissociation of *Eco*RQ111. (**C**) Comparison of the anti-roadblock effect of RNAP2 with that of GreB. The experiment is performed as in (A) except that equal molar amounts of RNAP2 and GreB (3 pmol) were added prior to the chase reaction. The amount of the roadblocked complexes (% block) was calculated by dividing the amount of radioactivity in the '*Eco*RQ111' band by the total radioactivity present in all readthrough bands.

trailing ECs on roadblocked ECs, we utilized T7A1 promoter DNA and two kinds of *E.coli* RNAPs, one that carries a His$_6$ tag at the C-terminus of the β′ subunit and another without the His tag. The non-tagged holoenzyme was purified from *greA⁻/greB⁻* cells and is free of transcript cleavage factors (Orlova *et al.*, 1995). For convenience, the His-tagged enzyme is designated as 1 and non-His-tagged as 2. The experiment began with preparation of EC stalled at position +20 (EC20) using RNAP1 (Figure 1A). EC20 was labeled with [^{32}P]CTP at three positions and immobilized on Ni^{2+}-chelating agarose beads. Ten molar excess of the DNA template (template 1) was used to ensure that no more than one RNAP molecule initiates on a single DNA molecule. EC20 was then washed several times with high-salt (1 M KCl) and standard (100 mM KCl) transcription buffer (TB) to remove free DNA and unstable complexes, and chased to the *Eco*RQ111 roadblock. Almost 90% of ECs were blocked by *Eco*RQ111 (Figure 1A, lane 1). Next, RNAP2 was added with or without rifampicin (Rif). RNAP2 transcription allowed most of the roadblocked ECs to move through the block in 12 min of the chase reaction (Figure 1A, lanes 2–4). Rif, which inhibits promoter clearance (Campbell *et al.*, 2001), completely abolished this effect (lanes 5–7). Transcription through *Eco*RQ111 was accompanied by dislocation of the block from the DNA, as evident from the loss of the restriction site protection against native *Eco*RI (Figure 1B).

To address whether transcript cleavage activity contributed to the anti-roadblock effect of trailing RNAP, we compared the ability of RNAP2 to potentiate the roadblock readthrough with that of GreB (Figure 1C). The same molar amounts (3 pmol) of RNAP2 holoenzyme and GreB were used in the experiment. Without NTPs, the transcript in the roadblocked complex remained intact after 10 min of incubation with RNAP2, thus excluding any transcript cleavage activity (Figure 1C, lane 1). Under the same conditions, GreB induced cleavage of 4 nt from the RNA 3′ terminus in ~95% of blocked complexes (Figure 1C, lane 4). Upon addition of NTPs, RNAP1 proceeded through the block at a much faster rate in the presence of RNAP2 than GreB (Figure 1C, compare lanes 2 and 3 with 5 and 6), indicating that at least under the specified conditions, GreB has a lower anti-roadblock potential than trailing ECs.

Taken together these results indicate that transcription through the roadblock by leading RNAP relies on actively transcribed trailing RNAP molecules and does not involve transcript cleavage activity.

A similar study was performed using template 2 and the DNA-bound *lac* repressor as a different roadblock (Figure 2). Like the situation with *Eco*RQ111, RNAP2 markedly potentiated roadblock readthrough by RNAP1 (Figure 2, lanes 4 and 5). The stimulating effect was observed only with RNAP2 holoenzyme, not the core enzyme (Figure 2, lane 7), directly implicating transcription initiation by RNAP2 in this process. Addition of RNAP2 holoenzyme to the washed roadblocked complex lacking NTPs did not change the size of the nascent transcript (Figure 2, lanes 9 and 10), confirming the
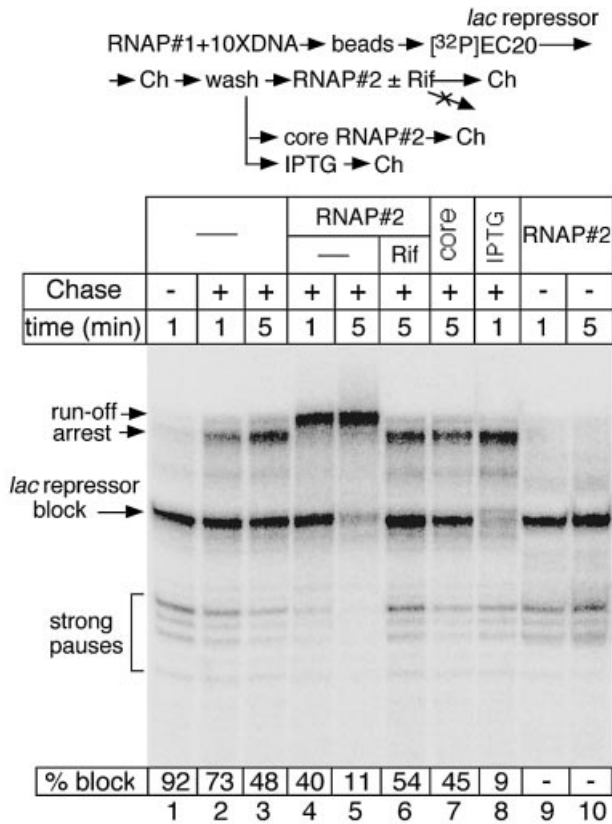
Fig. 2. Cooperation between RNAP molecules in overcoming the *lac* repressor roadblock. A step-by-step diagram of the experiment is shown at the top. The autoradiogram displays RNA products synthesized during a single round of transcription by RNAP1. The washed roadblocked complexes were chased in the presence of RNAP2 holoenzyme [with or without rifampicin (Rif)], core RNAP2 (core) or IPTG (200 μM) for the indicated time intervals. The amount of the road-blocked complexes (% block) was calculated by dividing the amount of radioactivity in the '*lac* repressor' band by the total radioactivity present in all readthrough bands. Note that not only the roadblock band, but also specific arrest and pausing bands disappear much faster if transcription performed in the presence of RNAP2 (lanes 4 and 5).

absence of any transcript cleavage activity. The release of *lac* repressor by IPTG immediately reactivated road-blocked ECs without RNAP2 (Figure 2, lane 8).

Notably, RNAP2 transcription also stimulated RNAP1 elongation through the strong arrest site near the end of template 2, as well as several other minor arrest/pause sites. This observation exemplifies the role of cooperation between RNAP molecules in overcoming intrinsic blocks, i.e. pauses and arrests (Epshtein and Nudler, 2003), in addition to roadblocks.

The anti-roadblock mechanism

To investigate the mechanism of the anti-roadblock activity of trailing RNAPs, we analyzed the conformation of roadblocked ECs. Judging by its high sensitivity to GreB (Figure 3A, lanes 2 and 3), EC blocked by *Eco*RQ111 should be in the backtracking state (Nudler *et al.*, 1995). Removing *Eco*RQ111from the DNA by washing with high-salt TB renders EC resistant to GreB (Figure 3A, lanes 4 and 5). The drastic change in GreB sensitivity in response to the roadblock and also the size of GreB cleavage products (4–5 nt) indicate that *Eco*RQ111

forces EC to backtrack for 4–5 nt from the position that would otherwise keep EC in the active non-backtracking state (Figure 3B). A similar EC backtracking was induced by the *lac* repressor roadblock *in vitro* (Pavco and Steege, 1990; Reines and Mote, 1993; Syroid and Capone, 1994) and *in vivo* (see below). These results also indicate that roadblock-induced backtracking is reversible. Addition of high-salt or IPTG not only removes *Eco*RQ111 or lac repressor, respectively, but also reactivates EC, i.e. the blocked complex rapidly assumes its default (active) configuration. Since trailing ECs have been shown to reactivate various spontaneously backtracked complexes by translocating them forward (Epshtein and Nudler, 2003), the present observations strongly suggest that the anti-roadblock mechanism also relies on forward trans-location of the blocked complex. In other words, trailing RNAPs help the blocked complex to pass through the block by keeping it in the active state even in the presence of the roadblock. Once the blocked EC assumes its active configuration, it has a chance to move through the roadblock as soon as the latter dissociates (Figure 3B).

This model also explains why GreB is less effective in stimulating readthrough of the roadblocked EC than a trailing EC. Unlike simple forward translocation induced by a trailing EC, the GreB-mediated assistance requires two steps: RNA cleavage and re-synthesis. While cleavage is fast, the re-synthesis step is slow, since the complex starts to pause 4–5 nt before reaching the final stoppage point in front of the roadblock. Pausing, in this case, is a result of progressive backtracking.

Trailing RNAP molecules rescue roadblocked complexes in vivo by 'pushing' them forward

To confirm that the cooperation mechanism described above also operates in living cells, we designed an *in vivo* system to monitor the effect of a trailing RNAP on a roadblocked EC. In this system, *E.coli* RNAP that initiates transcription from a constitutive promoter within a plasmid is temporarily halted at a downstream position (at +64 relative to the transcription start site) by the *lac* repressor bound to its operator motif (Figure 4A). The dynamic feature of the roadblocked EC was investigated by *in situ* footprinting with the single-strand-specific probe, chloroacetaldehyde (CAA) and RNA 3′ end mapping with S1 nuclease. The analysis of the CAA modifications on the non-template strand of the plasmid pATC10 revealed two RNAPs transcribing in tandem along the DNA region between the promoter and the *lac* repressor binding site (Figure 4B, lane 1). The roadblocked EC hereafter called 1 is in close apposition to the *lac* repressor whereas the trailing EC2 is halted further upstream (at around +34). As previously reported (Toulmé *et al.*, 1999, 2000), the roadblocked EC1 slides back and forth between positions –6 and –9 with accompanying Gre-mediated transcript cleavage and re-synthesis (the positions refer to the 3′ terminal nucleotide in the RNA transcript with respect to the upstream edge of the operator motif). This is revealed by the size of the apparent CAA footprint and the detection of a cluster of RNA 3′ ends between –6 and –9 (Figure 4C, lane 3).

To investigate the potential effect of the trailing EC2 on the roadblocked EC1, we constructed a plasmid derivative (pATC10Ter) in which the *trp* transcription terminator
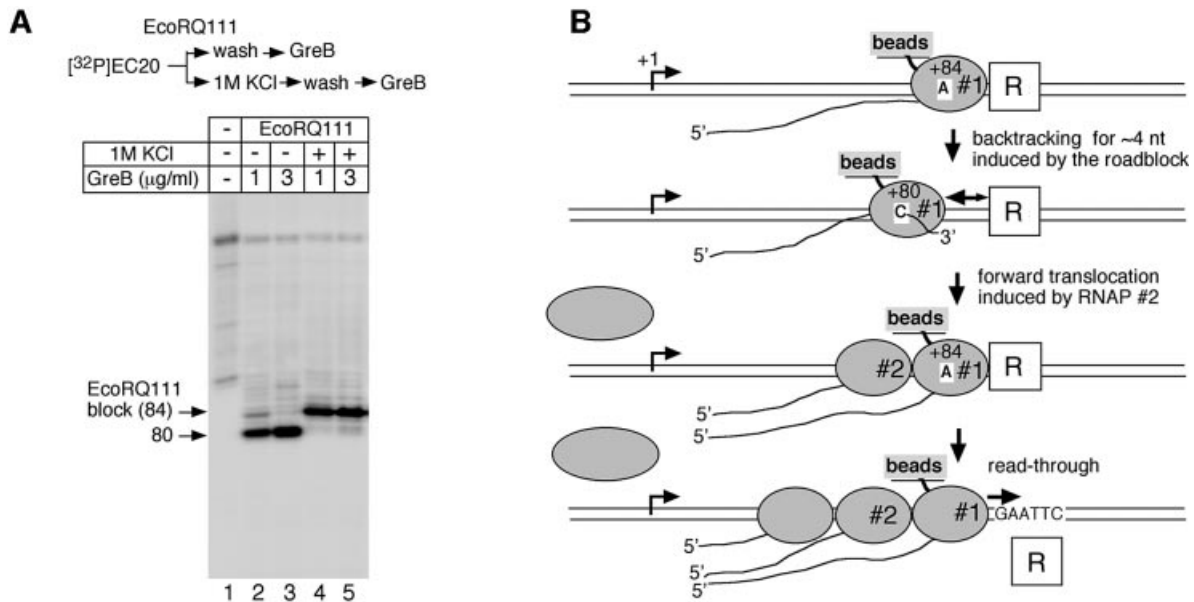
Fig. 3. The anti-roadblock mechanism. (**A**) Analysis of the conformation of the roadblocked EC. A step-by-step diagram of the experiment is shown at the top. The autoradiogram displays RNA products synthesized during a single round of transcription by RNAP1 with (lanes 2–5) or without *Eco*RQ111 (lane 1). All conditions are as in Figure 1. The washed roadblocked complex was treated with GreB before (lanes 2 and 3) or after washing with 1 mM KCl followed by equilibration with standard TB (lanes 4 and 5). Numbers on the left indicate the size of RNA transcripts. (**B**) The model for the anti-roadblock mechanism. The scheme summarizes results of Figure 1 and this figure. Trailing EC first reactivates the roadblocked EC by translocating it forward. Next, the active blocked EC has an opportunity to move through the block as soon as the latter dissociates. In addition, the local distortion of DNA and/or steric clash between EC and the block may facilitate dissociation of the block. R, *Eco*RQ111 roadblock. The model can be generalized for other types of blocking molecules.

was inserted between the promoter and the *lac* operator upstream from the position occupied by EC2 in pATC10. The rational of this design is that the relatively high efficiency of the terminator *in vivo* (80–85%) should dramatically decrease the flow of RNAPs transcribing downstream from the termination point. Under the steady state conditions of transcription, the operator-bound *lac* repressor is expected to impede RNAP progression only for a certain period of time, which is determined by the repressor concentration and operator affinity. Thus, in the context of a low level of RNAPs bypassing the terminator, the roadblocked EC1 may have enough time to escape the repressor impediment before a trailing EC2 arrives behind. As shown in Figure 4B (lanes 3 and 4), the CAA footprinting of pATC10Ter revealed strong reactivity of the DNA region between the promoter and the termination site, in both the absence and in the presence of IPTG. This reflects the overall transcriptional activity in the region, with a possible queuing effect due to RNAP pausing at the terminator before RNA release (Gusarov and Nudler, 1999). In this case, the CAA footprint in the absence of IPTG revealed a single roadblocked EC, which is isolated between the terminator and the operator (Figure 4B, lane 3). The footprint is significantly different from the one observed for EC1 in the construct lacking the terminator (compare lanes 1 and 3 and the corresponding scans in Figure 4B). Obviously, the isolated EC1 in pATC10Ter backtracks over a longer distance, since new CAA reactive sites are detected upstream and the reactivity of the downstream margin of the footprint (around position −6) is strongly decreased. The retreat of isolated EC1 is also clearly seen with the S1 mapping experiments where the

cluster of RNA 3′ ends is now shifted toward more upstream positions (compare the scans of lanes 3 and 4 in Figure 4C).

In the context of the back and forth movements of the roadblocked EC, the efficiency with which RNAP will read through the operator motif, following repressor dissociation, depends upon whether the enzyme is able to shift forward rapidly before the repressor rebinds DNA. Therefore, since the trailing RNAP restricts backtracking of the leading EC, this physical constrain should have an effect on the transcriptional readthrough. This hypothesis was tested by measuring the amounts of downstream *cat* mRNA produced with the two plasmids *in vivo*. A ^{32}P end-labeled oligonucleotide complementary to the early region of the *cat* message was used to quantitate mRNA by primer extension with reverse transcriptase. A second primer was also included to detect the plasmid-encoded β-lactamase transcript (*bla*), for normalization. As shown in Figure 5A and B, a fraction of the RNAPs transcribed beyond the operator motif under repressing conditions on both constructs, yet the level of readthrough was particularly low in the case of the terminator-containing plasmid. To deduce the roadblock effect, we normalized the level of *cat* mRNA produced under repressing conditions to that obtained under inducing conditions. This analysis (Figure 5C) revealed a significant *in vivo* cooperation between two RNAPs in overcoming the roadblock, with a 30% readthrough reduction for the terminator-containing plasmid as compared with pATC10. This effect is independent of Gre factors, since the same stimulation by the trailing RNAP was observed when the two plasmids were analyzed in *greA⁻/greB⁻* cells (A.R.Rahmouni and
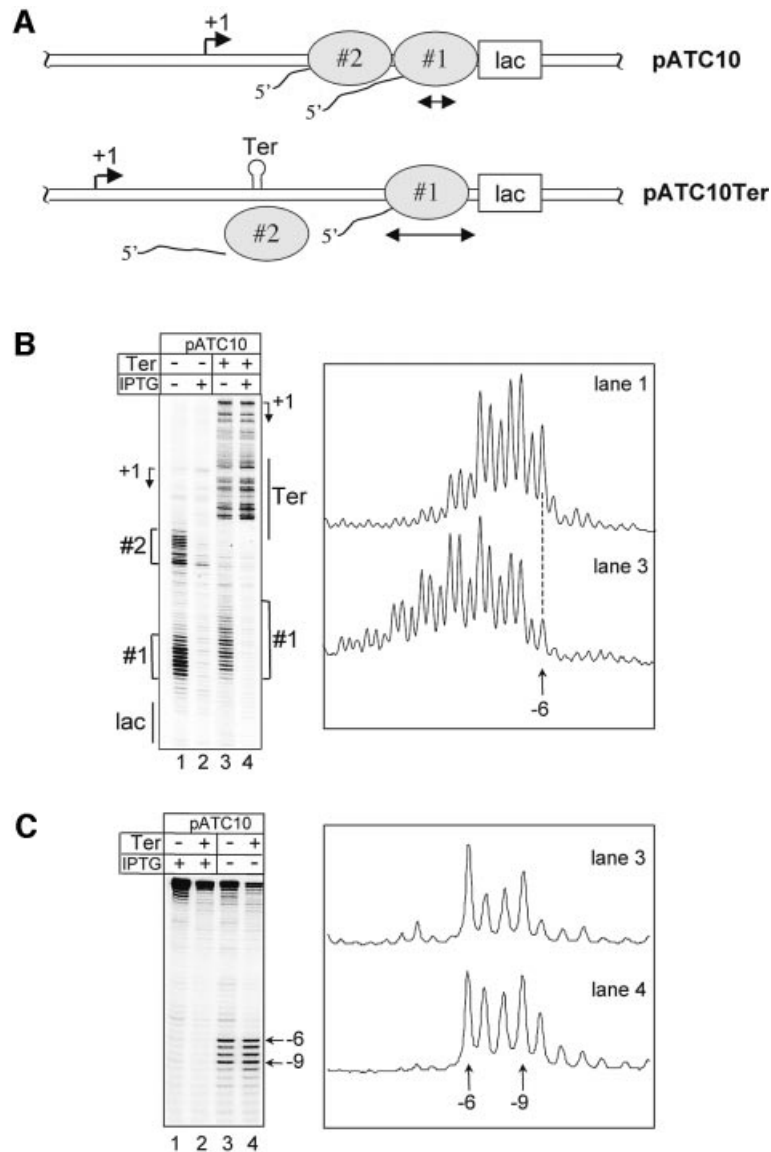
Fig. 4. Physical interaction between RNAP molecules *in vivo*. (**A**) Schematic representation of RNAP molecules roadblocked by the *lac* repressor within the two plasmids, pATC10 and its terminator-containing variant pATC10Ter. (**B**) Primer extension analyses of the *in situ* CAA modifications on the non-template strand in pATC10 and pATC10Ter. The positions of the operator (lac) and the terminator (Ter) are depicted, as well as the locations of EC1 and EC2 within the two plasmids. The arrows mark the positions of the transcription start sites in each construct. The + and – IPTG indicate the presence or absence of the inducer in the growth media during the footprinting experiment. Densitometer scans of lanes 1 and 3 are shown on the right. (**C**) S1 mapping of the 3′ ends of the RNA transcripts produced *in vivo* by the roadblocked ECs from the two plasmid derivatives. Bands corresponding to positions –6 and –9 are indicated by arrows. Densitometer scans of lanes 3 and 4, obtained as in (B), are shown on the right.

E.Nudler, unpublished observations). Altogether, these results provide a clear view of the dynamic interactions between two RNAPs moving one after another along a transcription unit *in vivo*. They also highlight a transcription regulation mechanism by which transcribing RNAPs may help each other to traverse protein roadblocks inside the cell.

The cumulative anti-roadblock effect by trailing RNAP molecules

Since each active EC exerts ~20 pN of force (Wang *et al*., 1998; Davenport *et al*., 2000), the cooperation mechanism described above is expected to be cumulative, i.e. the efficiency of the anti-roadblock process should depend on

a number of RNAPs trailing behind the blocked EC. This hypothesis was tested *in vivo* by comparing the efficiency of the *lac* repressor readthrough between two plasmids, pATC10 and its derivative pATC21. The latter construct has an additional 33 bp between the promoter and *lac* operator, so that it could accommodate at least one extra EC transcribing behind the roadblocked EC (Figure 6A). As depicted in Figure 6B, the ability to load more RNAPs on the same DNA molecule by increasing the distance between the promoter and the roadblock has a marked effect on transcriptional readthrough, with the efficiency rising from 20 to 45%. A similar distance effect was reported previously for T7 RNAP halted by the *lac* repressor *in vitro* (Lopez *et al*., 1998). These results argue
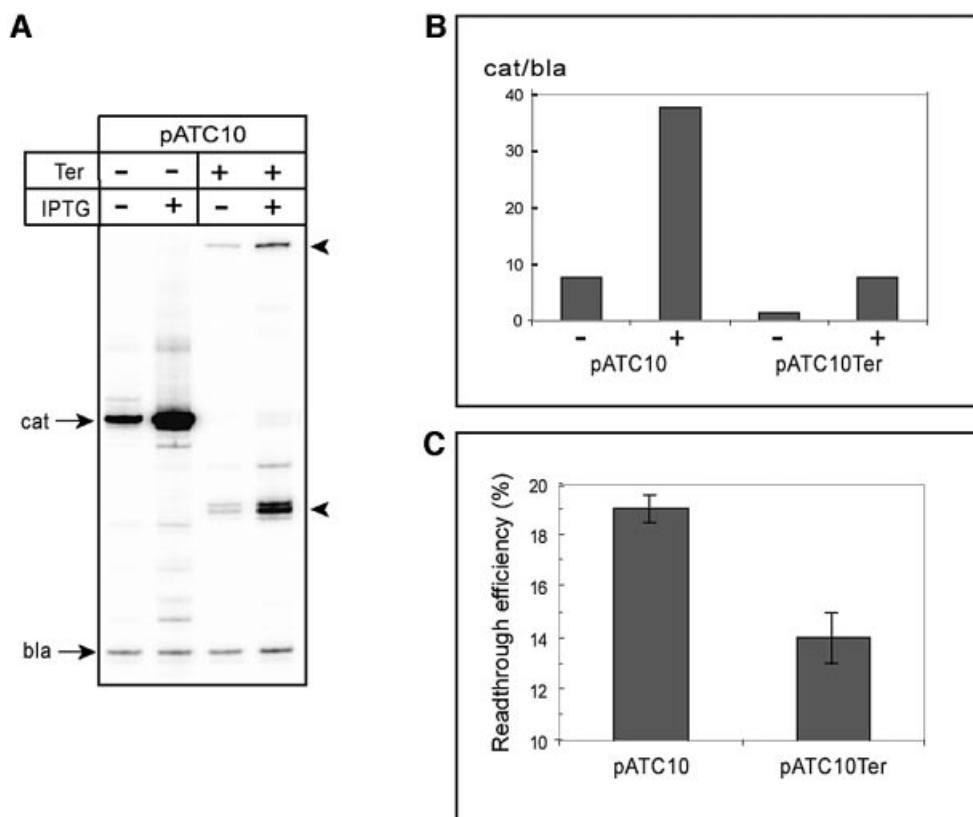
Fig. 5. Anti-roadblock activity by the trailing EC *in vivo*. (**A**) Comparison of the transcriptional readthrough within plasmids pATC10 and pATC10Ter. Autoradiogram of the extension products obtained after *in vitro* reverse transcription of the RNAs with ^{32}P primers hybridizing to either *cat* or *bla* transcripts. Note, that the reverse transcriptase does not read efficiently across the terminator hairpin. As a result, the extension reaction with the terminator-containing RNAs generates two products (indicated by arrowheads), the runoff at +1 of the transcript, and additional products resulting from the extension up to the base of the terminator hairpin stem. (**B**) Quantification of the primer extension products from (A) using STORM PhosphorImager. The value plotted in each lane indicates the level of *cat* mRNA relative to the internal control *bla* transcript. (**C**) Determination of the readthrough efficiency within the two plasmids. The readthrough was calculated using the following formula: [(*cat/bla* transcripts)$_{-\text{IPTG}}$/(*cat/bla* transcripts)$_{+\text{IPTG}}$] $\times$ 100. The error bars reflect the standard deviation from the mean of three independent experiments.

that in the anti-roadblock mechanism, every additional RNAP contributes to the forward 'push'.

Physiological implications of the cooperation mechanism of transcription

Considering the presence of numerous specific and non-specific DNA binding proteins that could potentially impede transcription *in vivo*, the ability of cellular RNAPs to transcribe DNA at the rate of 20–80 nt/s (Vogel and Jensen, 1994; Reines *et al.*, 1996; Uptain *et al.*, 1997) is remarkable. Although, this general property of RNAP must be essential for efficient transcription of chromosomal DNA, its mechanism is poorly understood. There are many examples of bacterial and eukaryotic RNAP being able to transcribe beyond a potential roadblock in natural systems. For example, in *E.coli*, DnaA protein bound to its box within the *dnaA* coding region does not repress transcription of the gene (Perez-Roger *et al.*, 1995). RNAP I initiated from the upstream promoter freely reads through downstream promoter-bound transcription initiation factor (TIF) by disrupting the TIF–DNA complex (Bateman and Paule, 1988). RNAP III proceeds without impediment through TFIIIC, which is bound downstream of the promoter (Bardeleben *et al.*, 1994; Matsuzaki *et al.*, 1994). Most significantly, tran-

scription through multiple nucleosomes by RNAP I and II, or histone-like proteins by bacterial RNAP, seems to occur without any delay *in vivo*. However, the ability of RNAP II to transcribe through nucleosomes is severely compromised in a reconstituted system and inversely proportional to the number of nucleosomes bound to template DNA (Izban and Luse, 1991; Chang and Luse, 1997). Although factors such as IIS, IIF, FACT and Elongator have been shown to relieve the nucleosome block *in vitro* (Chang and Luse, 1997; Orphanides *et al.*, 1998; Kim *et al.*, 2002), the effect was only partial if compared with transcription *in vivo*.

Intuitively, the anti-roadblock mechanism of transcription should rely on some common strategy, since various RNAPs have to deal with many obstructive proteins that bind DNA in different ways and with different affinity. In the present study, we compared the ability of an *E.coli* RNAP molecule alone or in the presence of other RNAP molecules trailing behind to read through two different roadblocks *in vitro* and *in vivo*. The results clearly demonstrate that in both cases cooperation between RNAP molecules facilitates transcription through the blocks. In the case of *Eco*RQ111, the cooperation was virtually absolute, i.e. without trailing molecules, the first round of transcription was almost completely and per-
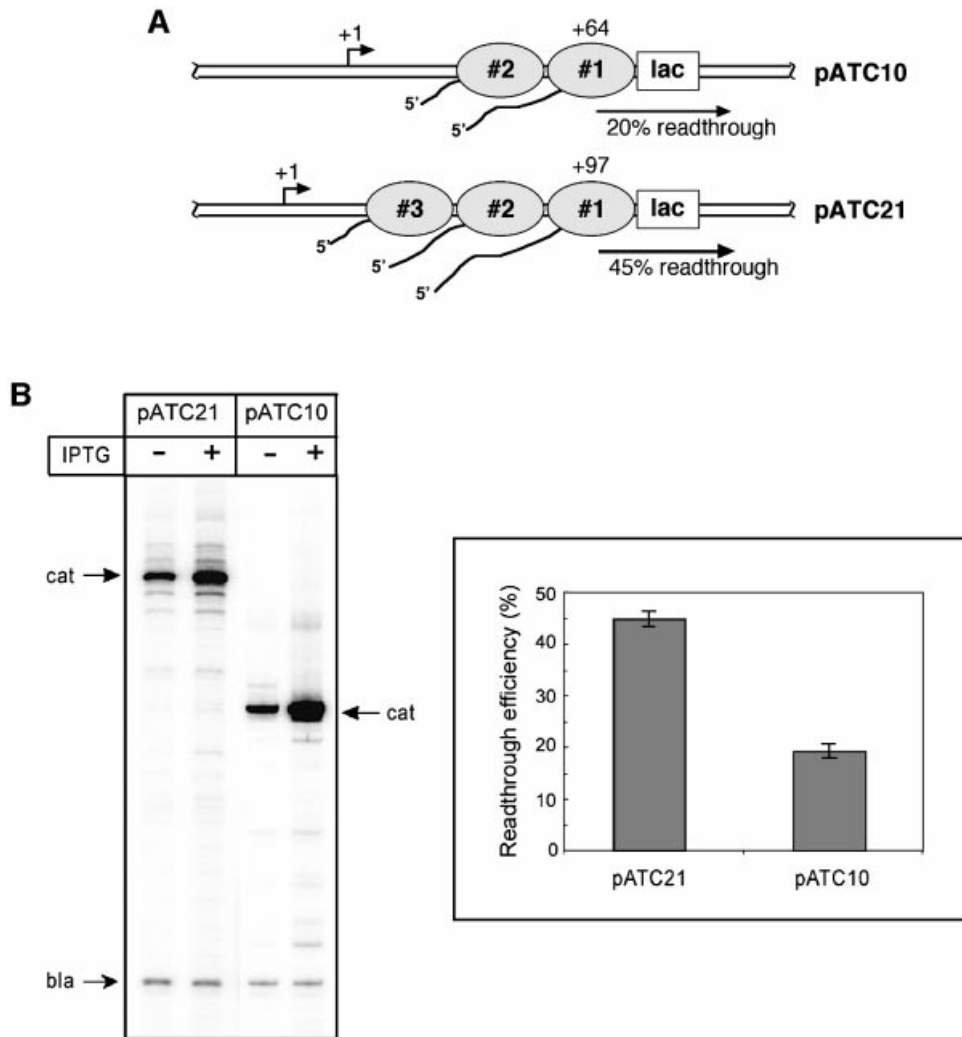
Fig. 6. Transcriptional readthrough *in vivo* as a function of a distance between the promoter and roadblock. (**A**) Schematic comparison of the pATC10 and pATC21 constructs and their capacity for ECs. (**B**) The amount of *cat* mRNA produced by the plasmids was determined by the reverse transcriptase assay, as in Figure 5B. The efficiencies of transcriptional readthrough were calculated and compared as in Figure 5C. The error bars reflect the standard deviation from the mean of three independent experiments.

manently blocked (Figure 1; data not shown). *Eco*RQ111 has an exceptionally high affinity for its site ($K_d = 10^{-15}$ M; Wright *et al.*, 1989), suggesting that this mechanism would work even better with other less avid DNA binding molecules. Our results with the *lac* repressor ($K_d = 10^{-13}$ M; Barkley, 1981) are consistent with this notion (Figure 2). The analysis of the conformation of EC blocked by either *Eco*RQ111 or *lac* repressor indicates that in both cases EC was in the backtracking (inactive) state, suggesting that backtracking may be a common outcome of the collision between EC and a roadblock. The observation that the distance between the RNA 3′ terminus and the rear edge of the roadblock (*lac* repressor and *Eco*RQ111) is shorter than expected from the footprint of halted EC (Deuschle *et al.*, 1990; Pavco and Steege, 1990; Reines and Mote, 1993; Syroid and Capone, 1994) supports this model. Thus, there must be at least two consecutive steps in the process of passing a transcriptional roadblock: (i) EC reactivation and (ii) relocation and/or dissociation of the roadblock. Cooperation between RNAP molecules serves to increase the rate of the first

step, i.e. reactivation of EC via its forward translocation. The second step, which is primarily determined by the nature of the roadblock–DNA contacts, may be also affected by the first step due to local distortion of the DNA and/or steric clash between two proteins. The same mechanism may work to facilitating transcription through nucleosomes. Indeed, the increased TF$_{II}$S sensitivity of the EC blocked by a nucleosome (Izban and Luse, 1992) suggests that pausing due to nucleosome-induced backtracking of RNAP II is the rate-limiting step in chromatin transcription. Thus, preventing backtracking by trailing ECs may contribute to relief of the nucleosomal block.

The anti-roadblock mechanism that relies on RNAP–RNAP cooperation fits the criteria of a general mechanism of transcription. It does not require transcript cleavage activity, which is consistent with the dispensability of the transcript cleavage factors (TF$_{II}$S or GreA/GreB) *in vivo* under physiological conditions (Archambault *et al*, 1992; Orlova *et al*., 1995), and should only depend on the density of elongating RNAP molecules (Epshtein and Nudler, 2003). In other words, the efficiency of this mechanism

should be directly proportional to the transcriptional activity of a gene and a number of ECs trailing behind. Experimental evidence supports this model. We detected strong blockage of elongation by the *lac* repressor *in vivo* when it was initiated from the relatively weak *his* promoter, which is located at a short distance from the *lac* operator site (Figure 4). The readthrough increased with increasing distance between the *his* promoter and *lac* operator since additional ECs could be accommodated on the same DNA molecule and exert their cumulative 'pushing' effect (Figure 6). Furthermore, much higher readthrough levels were observed even at two or three consecutive *lac* operator sites, if transcription was driven from the very strong T7 A1 promoter located at a relatively long distance from the operator (S.Borukhov, unpublished results). The difference in the promoter activity and its location relative to the roadblock may also explain the previously observed broad variations in the abilities of RNAP to read through the *lac* repressor *in vivo* (Horowitz and Platt, 1982; Sellitti *et al.*, 1987) and also *Eco*RQ111 *in vitro* (Pavco and Steege, 1991). Moreover, we have demonstrated recently that the rate and efficiency of transcription elongation in living *E.coli* cells is directly proportional to the transcriptional initiation rate (Epshtein and Nudler, 2003). A similar correlation was observed with RNAP II transcription in various eukaryotic cells (Yankulov *et al.*, 1994). In this regard, one can argue that the highly active genes (e.g. rRNA or heat shock genes) should have the least problems with protein roadblocks or numerous intrinsic pauses and arrests associated with EC backtracking. This explains, at least in part, the highest elongational rate observed with these genes *in vivo* (Giardina and Lis, 1993; Condon *et al.*, 1995). Alternatively, many genes (particularly in eukaryotes) are constantly weak, having only a few RNAP molecules engaged in transcription. We believe that various stimulating elongation factors are needed in such cases to compensate for lack of efficient cooperation between RNAP molecules.

Materials and methods

DNA templates and proteins
Templates 1 and 2 were generated by PCR-directed mutagenesis from 'template 1' (Nudler *et al.*, 1995) using Deep Vent DNA polymerase (New England Biolabs) and synthetic DNA oligos (IDT). They have the identical T7 A1 promoter and initial transcribed sequence up to position +36 (from the +1 start of the transcription). The transcribed sequence of template 1: atcgagagggccacggcgaatagccaaccccaatcgaacagccatcatcctcag-tattcaggtagctgttgagcctgggcggtagcgtgctttttttcgaattcacttaatggtaatctcgc.
The *Eco*RI site is underlined. The transcribed sequence of template 2: atcgagagggccacggcgaatagccaaccccaatcgacaccggatccccgggttgtgtgaaattgt-tatccgctcacaattccacacatgatacg. The *lac* operator site is underlined. Both templates were purified from a low-melting agarose gel and diluted in TE buffer to a concentration ~1 pmol/μl. His$_6$-tagged RNAP (1) and GreA/GreB-free RNAP (2) and its core were purified as described previously (Orlova *et al.*, 1995; Nudler *et al.*, 1996). GreB was isolated from GreA$^-$/GreB$^-$ *E.coli* AD8571 strain carrying pMO1.4 plasmid as described by Koulich *et al.* (1997). *Eco*RQ111 was a gift from Dr P.Modrich (Duke University). The *lac* repressor was purified from MG1665 *E.coli* cells harboring a placI^q plasmid (Gusarov and Nudler, 2001). T4 polynucleotide kinase and *Eco*RI were from New England Biolabs.

In vitro transcription
Two to 5 pmol RNAP were mixed with 10× molar excess of DNA in 20 μl of TB (100 mM KCl, 10 mM MgCl$_2$, 10 mM Tris–HCl pH 7.2) for 5 min at 37°C followed by addition of ApUpC (10 μM), GTP and ATP (25 μM)

for 7 min. Next, 5 μl TB-equilibrated Ni^{2+}-chelating agarose beads (Qiagen) were added for 5 min at room temperature followed by washing with 2× 1.5 ml of TB1000 (1 M KCl) and 2× 1.5 ml of TB. To produce EC20, ATP, GTP (5 μM) and 1 μl of [α-^{32}P]CTP were added for 5 min at room temperature followed by unlabeled CTP (5 μM) for a further 2 min. Beads were washed five times with TB. Probes were treated with roadblocking proteins as described below. RNAP2 holoenzyme (3 pmol), RNAP2 core (3 pmol), GreB (3 pmol), RNAP2 holoenzyme preincubated with rifampicin (100 μM, 5 min at 37°C) were added at 37°C as indicated in each figure. All chase reactions were performed with 100 μM NTPs and quenched by an equal volume of stop solution (8 M urea, 20 mM EDTA, 1× TBE, 0.25% bromphenol blue, 0.25% xylene cianol). The products were separated by electrophoresis in 12% sequencing PAGE (8 M urea). Relative amounts of [^{32}P]RNA and DNA species were determined using a PhosphorImager and software from Molecular Dynamics.

*Eco*RQ111 mutant protein was diluted immediately prior to use with ice-cold 20 mM KPO$_4$ pH 7.4, 0.2 M KCl, 0.2 mM DTT, 1 mM EDTA. *Eco*RQ111 (2 pmol) was added to the TB-washed EC20 for 1 min at 37°C. RNAP2 (2 pmol) was added together with 100 μM NTPs with or without rifampicin for the indicated intervals at 37°C. To remove *Eco*RQ111, EC was washed once with TB1000 (1 min) followed by several washes with TB. The completeness of *Eco*RQ111 removal was verified by a control demonstrating 100% readthrough from the upstream position. For the experiment of Figure 1B, Template 1 was obtained by PCR using non-phosphorylated (left) and 5′-phosphorylated (right) primers to produce a 5′-OH group in the non-template strand for subsequent enzymatic phosphorylation. DNA was labeled by T4 polynucleotide kinase (25 U) and [γ-^{32}P]ATP for 10 min at room temperature in TB. Ten units of *Eco*RI per reaction were added for 3 min at 37°C. The *lac* repressor was added to the final concentration 0.5 μM for 5 min at 25°C prior to the chase reaction.

In vivo studies
The pATC10 plasmid was constructed as previously described using pKK232-8 as the starting vector (Toulmé *et al.*, 1999). It contains a constitutive promoter that drives transcription through an (ATC/TAG)$_{10}$ repeat upstream of the operator motif and followed by the *cat* gene. The (ATC/TAG)$_{10}$ repeat with the downstream operator are inserted at position +40 relative to the transcription start site with the ATC sequence on the non-template strand. pATC21 is the same as pATC10 except that it has 21 (ATC/TAG) repeats, which increase the distance between the promoter and the *lac* operator site by 33 bp. Its construction was reported previously (Toulmé *et al.*, 1999). The plasmid pATC10Ter was obtained by inserting the trp transcription terminator as a 36mer duplex oligonucleotide into the filled-in *Bam*HI site in pATC10. The plasmids were maintained in *E.coli* strain SU1675 (Toulmé *et al.*, 1999). To have a high yield of intracellular *lac* repressor, the cells were co-transformed with a pACYC177 derivative (pAC177IQ) that harbors the *lacI^Q* gene. The two plasmids were maintained within the cell by double selection in ampicillin (100 μg/ml) and kanamycin (30 μg/ml). The *in situ* DNA footprinting with CAA and the subsequent analyses of the modifications by primer extension with Klenow fragment of DNA polymerase I as well as total RNA extraction and 3′ end mapping with S1 nuclease protection experiments were carried out as previously reported (Toulmé *et al.*, 1999, 2000). Densitometer scans were obtained on a Molecular Dynamics PhosphorImager using ImageQuant software version 5.1 for data processing. Quantitative analyses of the *cat* transcripts by primer extension with reverse transcriptase were also performed exactly as described previously (Toulmé *et al.*, 2000). In these experiments, a primer that hybridizes to the plasmid encoded β-lactamase (*bla*) transcript was also included for normalization.

References

Archambault,J., Lacroute,F., Ruet,A. and Friesen,J.D. (1992) Genetic interaction between transcription elongation factor TFIIS and RNA polymerase II. *Mol. Cell. Biol.*, **12**, 4142–4152.
Aso,T., Lane,W.S., Conaway,J.W. and Conaway,R.C. (1995) Elongin

(SIII): a multisubunit regulator of elongation by RNA polymerase II. *Science*, **269**, 1439–1443.

Bardeleben,C., Kassavetis,G.A. and Geiduschek,E.P. (1994) Encounters of *Saccharomyces cerevisiae* RNA polymerase III with its transcription factors during RNA chain elongation. *J. Mol. Biol.*, **235**, 1193–1205.

Barkley,M.D. (1981) Salt dependence of the kinetics of the lac repressor–operator interaction: role of nonoperator deoxyribonucleic acid in the association reaction. *Biochemistry*, **20**, 3833–3842.

Bateman,E. and Paule,M.R. (1988) Promoter occlusion during ribosomal RNA transcription. *Cell*, **54**, 985–992.

Borukhov,S., Sagitov,V. and Goldfarb,A. (1993) Transcript cleavage factors from *E. coli*. *Cell*, **72**, 459–466.

Campbell,E.A., Korzheva,N., Mustaev,A., Murakami,K., Nair,S., Goldfarb,A. and Darst,S.A. (2001) Structural mechanism for rifampicin inhibition of bacterial RNA polymerase. *Cell*, **104**, 901–912.

Chang,C.H. and Luse,D.S. (1997) The H3/H4 tetramer blocks transcript elongation by RNA polymerase II *in vitro*. *J. Biol. Chem.*, **272**, 23427–23434.

Condon,C., Squires,C. and Squires,C.L. (1995) Control of rRNA transcription in *Escherichia coli*. *Microbiol. Rev.*, **59**, 623–645.

Darst,S.A. (2001) Bacterial RNA polymerase. *Curr. Opin. Struct. Biol.*, **11**, 155–162.

Davenport,R.J., Wuite,G.J, Landick R and Bustamante,C. (2000) Single-molecule study of transcriptional pausing and arrest by *E. coli* RNA polymerase. *Science*, **287**, 2497–2500.

Deuschle,U., Hipskind R.A. and Bujard,H. (1990) RNA polymerase II transcription blocked by *Escherichia coli lac* repressor. *Science*, **248**, 480–483.

Ebright,R.H. (2000) RNA polymerase: structural similarities between bacterial RNA polymerase and eukaryotic RNA polymerase II. *J. Mol. Biol.*, **304**, 687–698.

Epshtein,V. and Nudler,E. (2003) Cooperation between RNA polymerase molecules in transcription elongation. *Science*, **300**, 801–805.

Giardina,C. and Lis,J.T. (1993) Polymerase processivity and termination on *Drosophila* heat shock genes. *J. Biol. Chem.*, **268**, 23806–23811.

Gnatt,A.L., Cramer,P., Fu,J., Bushnell,D.A and Kornberg,R.D. (2001) Structural basis of transcription: an RNA polymerase II elongation complex at 3.3 Å resolution. *Science*, **292**, 1876–1882.

Gusarov,I. and Nudler,E. (1999) The mechanism of intrinsic transcription termination. *Mol. Cell*, **3**, 495–504.

Gusarov,I. and Nudler,E. (2001) Control of intrinsic transcription termination by N and NusA: the basic mechanisms. *Cell*, **107**, 437–449.

Horowitz,H. and Platt,T. (1982) Regulation of transcription from tandem and convergent promoters. *Nucleic Acids Res.*, **10**, 5447–5465.

Izban,M.G. and Luse,D.S. (1991) Transcription on nucleosomal templates by RNA polymerase II *in vitro*: inhibition of elongation with enhancement of sequence-specific pausing. *Genes Dev.*, **5**, 683–696.

Izban,M.G. and Luse,D.S. (1992) The RNA polymerase II ternary complex cleaves the nascent transcript in a 3′–5′ direction in the presence of elongation factor SII. *Genes Dev.*, **6**, 1342–1356.

Kim,J.H., Lane W.S. and Reinberg,D. (2002) Human Elongator facilitates RNA polymerase II transcription through chromatin. *Proc. Natl Acad. Sci. USA*, **99**, 1241–1246.

Komissarova,N. and Kashlev,M. (1997) RNA polymerase switches between inactivated and activated states by translocating back and forth along the DNA and the RNA. *J. Biol. Chem.*, **272**, 15329–15338.

Koulich,D., Orlova,M., Malhotra,A., Sali,A., Darst,S.A. and Borukhov,S. (1997) Domain organization of *Escherichia coli* transcript cleavage factors GreA and GreB. *J. Biol. Chem.*, **272**, 7201–7210.

Lopez,P.J, Guillerez,J., Sousa,R. and Dreyfus,M. (1998) On the mechanism of inhibition of phage T7 RNA polymerase by lac repressor. *J. Mol. Biol.*, **276**, 861–875.

Matsuzaki,H., Kassavetis,G.A. and Geiduschek,E.P. (1994) Analysis of RNA chain elongation and termination by *Saccharomyces cerevisiae* RNA polymerase III. *J. Mol. Biol.*, **235**, 1173–1192.

Nudler,E., Kashlev,M., Nikiforov,V. and Goldfarb,A. (1995) Coupling between transcription termination and RNA polymerase inchworming. *Cell*, **81**, 351–357.

Nudler,E., Avetissova,E., Markovtsov,V. and Goldfarb,A. (1996) Transcription processivity: protein-DNA interactions holding together the elongation complex. *Science*, **273**, 211–217.

Nudler,E., Mustaev,A., Lukhtanov,E. and Goldfarb,A. (1997) The RNA–DNA hybrid maintains the register of transcription by preventing backtracking of RNA polymerase. *Cell*, **89**, 33–41.

Orlova,M., Newlands,J., Das,A., Goldfarb,A. and Borukhov,S. (1995) Intrinsic transcript cleavage activity of RNA polymerase. *Proc. Natl Acad. Sci. USA*, **92**, 4596–4600.

Orphanides,G., LeRoy,G., Chang,C.H., Luse,D.S. and Reinberg,D. (1998) FACT, a factor that facilitates transcript elongation through nucleosomes. *Cell*, **92**, 105–116.

Park,J.S., Marr,M.T. and Roberts,J.W. (2002) *E. coli* transcription repair coupling factor (Mfd protein) rescues arrested complexes by promoting forward translocation. *Cell*, **109**, 757–767.

Pavco,P.A. and Steege,D.A. (1990) Elongation by *Escherichia coli* RNA polymerase is blocked *in vitro* by a site-specific DNA binding protein. *J. Biol. Chem.*, **265**, 9960–9969.

Pavco,P.A. and Steege,D.A. (1991) Characterization of elongating T7 and SP6 RNA polymerases and their response to a roadblock generated by a site-specific DNA binding protein. *Nucleic Acids Res.*, **19**, 4639–4646.

Perez-Roger,I., Macian,F. and Armengod,M.E. (1995) Transcription termination in the *Escherichia coli dnaA* gene is not mediated by the internal DnaA box. *J. Bacteriol.*, **177**, 1896–1899.

Reinberg,D. and Roeder,R.G. (1987) Factors involved in specific transcription by mammalian RNA polymerase II. Transcription factor IIS stimulates elongation of RNA chains. *J. Biol. Chem.*, **262**, 3331–3337.

Reines,D. and Mote,J.,Jr (1993) Elongation factor SII-dependent transcription by RNA polymerase II through a sequence-specific DNA-binding protein. *Proc. Natl Acad. Sci. USA*, **90**, 1917–1921.

Reines,D., Conaway,J.W. and Conaway,R.C. (1996) The RNA polymerase II general elongation factors. *Trends Biochem. Sci.*, **21**, 351–355.

Rudd,M.D., Izban,M.G. and Luse,D.S. (1994) The active site of RNA polymerase II participates in transcript cleavage within arrested ternary complexes. *Proc. Natl Acad. Sci. USA*, **91**, 8057–8061.

Selby,C.P. and Sancar,A. (1993) Molecular mechanism of transcription–repair coupling. *Science*, **260**, 53–58.

Sellitti,M.A., Pavco,P.A. and Steege,D.A. (1987) *lac* repressor blocks *in vivo* transcription of lac control region DNA. *Proc. Natl Acad. Sci. USA*, **84**, 3199–3203.

Syroid,D.E. and Capone,J.P. (1994) RNA chain elongation and termination by mammalian RNA polymerase III. Analysis of tRNA gene transcription by imposing a reversible factor-mediated block to elongation using a sequence-specific DNA binding protein. *J. Mol. Biol.*, **244**, 482–493.

Toulmé,F., Guerin,M., Robichon,N., Leng,M. and Rahmouni,A.R. (1999) *In vivo* evidence for back and forth oscillations of the transcription elongation complex. *EMBO J.*, **18**, 5052–5060.

Toulmé,F., Mosrin-Huaman,C., Sparkowski,J., Das,A., Leng,M. and Rahmouni,A.R. (2000) GreA and GreB proteins revive backtracked RNA polymerase *in vivo* by promoting transcript trimming. *EMBO J.*, **19**, 6853–6859.

Uptain,S.M., Kane,C.M. and Chamberlin,M.J. (1997) Basic mechanisms of transcript elongation and its regulation. *Annu. Rev. Biochem.*, **66**, 117–172.

Vogel,U. and Jensen,K.F. (1994) The RNA chain elongation rate in *Escherichia coli* depends on the growth rate. *J. Bacteriol.*, **176**, 2807–2813.

Wang,M.D., Schnitzer,M.J., Yin,H., Landick,R., Gelles,J. and Block,S.M. (1998) Force and velocity measured for single molecules of RNA polymerase. *Science*, **282**, 902–907.

Wright,D.J., King,K. and Modrich,P. (1989) The negative charge of Glu-111 is required to activate the cleavage center of *Eco*RI endonuclease. *J. Biol. Chem.*, **264**, 11816–11821.

Yamazaki,K., Aso,T., Ohnishi,Y., Ohno,M., Tamura,K., Shuin,T., Kitajima.S. and Nakabeppu,Y. (2003) Mammalian Elongin A is not essential for cell viability but is required for proper cell cycle progression with limited alteration of gene expression. *J. Biol. Chem.*, **278**, 13585–13589.

Yankulov,K., Blau,J., Purton,T., Roberts,S. and Bentley,D.L. (1994) Transcriptional elongation by RNA polymerase II is stimulated by transactivators. *Cell*, **77**, 749–759.