

Synergistic action of RNA polymerases in overcoming the nucleosomal barrier

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During gene expression, RNA polymerase (RNAP) encounters a major barrier at a nucleosome and yet must access the nucleosomal DNA. Previous *in vivo* evidence has suggested that multiple RNAPs might increase transcription efficiency through nucleosomes. Here we have quantitatively investigated this hypothesis using *Escherichia coli* RNAP as a model system by directly monitoring its location on the DNA via a single-molecule DNA-unzipping technique. When an RNAP encountered a nucleosome, it paused with a distinctive 10–base pair periodicity and backtracked by ~10–15 base pairs. When two RNAPs elongate in close proximity, the trailing RNAP apparently assists in the leading RNAP's elongation, reducing its backtracking and enhancing its transcription through a nucleosome by a factor of 5. Taken together, our data indicate that histone-DNA interactions dictate RNAP pausing behavior, and alleviation of nucleosome-induced backtracking by multiple polymerases may prove to be a mechanism for overcoming the nucleosomal barrier *in vivo*.

Nucleosomes are known to play an important role in the regulation of gene expression. During transcription, RNA polymerase (RNAP) must access DNA associated with nucleosomes, the fundamental packing units of chromatin. *In vitro* studies have shown that even a mononucleosome imposes a substantial barrier to transcription elongation by a single RNAP^{1–10}. The presence of a nucleosome induces RNAP to pause or arrest due to backtracking, during which RNAP disengages its active site from the 3' end of RNA and slides backwards noncatalytically along the DNA, resulting in an extrusion of 3' RNA through its secondary channel⁶.

In contrast, *in vivo* data have shown that RNAP is able to elongate rapidly in the presence of nucleosomes^{11–13}. If so, how does RNAP overcome the nucleosome barrier during elongation? To date, several mechanisms have been recognized, including direct elongation-rate enhancement by transcription factors^{6,14–16} and increasing DNA accessibility via histone modifications^{17–19} and/or nucleosome remodeling^{20,21}.

Additionally, *in vivo* evidence shows that multiple RNAPs often occur on active genes. A large number of human genes are found to have two or more active promoters, which greatly increases the chance of recruitment of multiple RNAPs²². On a fully induced *Drosophila melanogaster hsp70* gene, ~30 transcribing polymerase molecules have been detected¹¹. Live-cell imaging of transcription indicates that mammalian RNAP often enters a paused state for unexpectedly long times, which may allow trailing RNAPs to catch up to it¹³. More importantly, it has been shown that the density of RNAP is a major factor for defining the regions of nucleosome removal in transcribed genes^{23,24}. Therefore, it is appealing to

hypothesize that cooperation by multiple RNAPs may also contribute to efficient RNAP progression through a nucleosomal barrier.

Several observations suggest that such a cooperative effect may be plausible. Biochemical studies of *E. coli* RNAP show that, when multiple initiation events happen from the same promoter, the leading RNAP is able to more efficiently forward-translocate through a bound protein such as EcoRQ111 or *lac* repressor, with a concomitant reduction in the probability of RNAP arrest^{25,26}. In addition, single-molecule studies show that both *E. coli* RNAP and RNA polymerase II (Pol II) are powerful molecular motors capable of exerting forces and displacing proteins^{16,27}. Thus, an assisting force may be exerted by a trailing RNAP on a leading RNAP as the leading RNAP encounters a nucleosome barrier. Indeed, an assisting external force has been shown to reduce RNAP backtracking and to facilitate its forward translocation^{16,28}.

Here we have tested this hypothesis using *E. coli* RNAP as a model system because *E. coli* RNAP and Pol II are evolutionarily conserved in sequence, structure and function^{29,30}, yet *E. coli* RNAP is structurally simpler and requires only the holoenzyme for initiation. Furthermore, *E. coli* RNAP has been shown to resemble yeast Pol II in all tested properties of transcription through a nucleosome *in vitro*⁵. In this work, we have ascertained how two RNAPs may work together to transcribe through a nucleosome.

RESULTS

Locating RNAP by unzipping DNA

To monitor how RNAP progresses through a nucleosome, we needed to be able to detect its physical location along DNA. This cannot

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be readily achieved by conventional bulk transcription gel assays, which measure the length of the RNA transcript (that is, the 3'-RNA location along the DNA). Instead, we used a single-molecule assay to locate RNAP by mechanically unzipping double-stranded DNA (dsDNA) through a bound RNAP. Previously, we had developed the DNA-unzipping technique and had shown that it is a versatile and powerful tool for measurements of protein-DNA interactions with near-base pair precision and accuracy^{31–33}.

We first constructed a DNA template containing a single T7A1 promoter and then allowed a paused transcription complex (PTC) to form at the +20-nt position via depletion of UTP at room temperature (Fig. 1a and Online Methods). The PTC formation reaction was quenched by EDTA after 2 min. To unzip DNA through a PTC, we used an optical trap to sequentially convert dsDNA into single-stranded DNA by mechanical separation of base pairs (Fig. 1a, Supplementary Fig. 1b and Online Methods). We detected an RNAP-DNA interaction whenever the unzipping force substantially deviated from the corresponding naked DNA unzipping force, a sequence-dependent baseline around 15 pN.

When a single DNA molecule was unzipped starting from upstream of the RNAP (Fig. 1b), the unzipping force initially followed that of the corresponding naked DNA. However, as the unzipping fork encountered the transcription bubble formed by the RNAP, the force dropped below the naked DNA baseline. Subsequently, the force rose sharply above the baseline as the unzipping fork encountered the beginning of the dsDNA that was clamped downstream by the RNAP. The force then continued to follow that of the corresponding naked DNA. As expected for a thermally activated off-equilibrium process, the magnitude of the force drop and the force rise varied from trace to trace.

For a PTC at +20 nt, the active site of the RNAP should be at +20 base pairs (bp) from the transcription start site, and the downstream

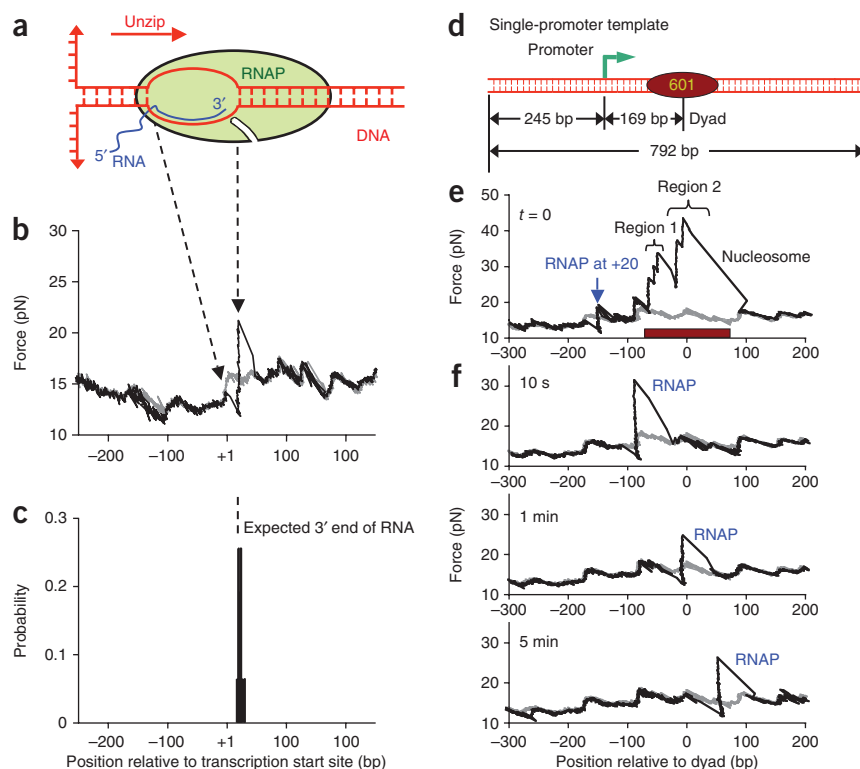
dsDNA should begin at around $+23 \pm 1$ bp^{34–36}. We detected the location of the force rise, indicative of the beginning of the downstream dsDNA, at +22 bp, in agreement with the expected location (Fig. 1c). Additional experiments also showed that depletion of Mg^{2+} by EDTA quenching minimized RNAP diffusive motion along the DNA in an elongation complex (Supplementary Fig. 2). Thus, the unzipping force signature of an RNAP serves as a convenient and distinctive indicator of the RNAP location. We then took the active-site location to be 2 bp upstream from the measured force-rise location for all subsequent experiments.

Locating RNAP during elongation on nucleosomal DNA

We next showed that the DNA-unzipping assay could also be used to locate an RNAP during elongation on nucleosomal DNA. For these experiments, we constructed a single-promoter DNA template containing a single T7A1 promoter followed by a 601 nucleosome positioning element (NPE) that is known to uniquely position a nucleosome³⁷ (Fig. 1d). In this design, the 601 NPE was flanked by long stretches of DNA, in contrast to the short DNA templates typically used in conventional biochemistry experiments. We then assembled a single nucleosome onto the 601 NPE using a salt-dialysis method and subsequently formed a PTC at the +20-nt position (Online Methods). When this DNA template was unzipped, we observed the characteristic force signatures for both the RNAP and the nucleosome at their expected locations (Fig. 1e).

We found that the nucleosome was uniquely positioned within the 601 NPE, and its unzipping force signatures were consistent with those of our previous work³². For a given nucleosome, there were three broad regions of strong interactions, with one around the dyad and the other two approximately ± 40 bp from the dyad. Unzipping from one direction typically revealed the first two regions encountered

Figure 1 Locating an RNAP during elongation on nucleosomal DNA. (a) A cartoon of the transcription elongation complex. Unzipping direction is indicated by a red arrow. (b) An example trace of unzipping DNA through a PTC. RNAP was stalled at the +20-nt position relative to transcription start site. The RNAP unzipping force signature (black) shows a distinctive force drop immediately followed by a sharp force rise. The unzipping force of the corresponding naked DNA is shown for comparison (gray). (c) Location distribution of the unzipping force rise obtained by pooling a number of measurements such as that shown in b. The dashed line indicates the expected location of the 3' end of the transcribed RNA. (d) The single-promoter transcription template construct containing both a single T7A1 promoter and a 601 NPE. (e) An example unzipping trace of a template containing both a PTC stalled at +20 nt and a positioned nucleosome. Unzipping confirmed that the RNAP and the nucleosome were at their expected locations. Two regions of strong histone-DNA interactions in a nucleosome, region 1 (off-dyad interactions) and region 2 (dyad interactions), are indicated. The brown bar indicates the 147-bp 601 NPE. (f) Representative traces of unzipping through an elongation complex. After transcription was resumed for an indicated duration, it was quenched and histones were dissociated. Unzipping revealed the location of the remaining RNAP. Each trace is from a different DNA molecule. The unzipping force of the corresponding naked DNA is shown for comparison (gray).



but not the last one, due to histone dissociation from the 601 NPE upon disruption of the dyad region of interactions.

To resume elongation, we supplemented 1 mM of NTPs together with competitor DNA containing a T7A1 promoter to prevent reinitiation (Online Methods and **Supplementary Fig. 3a**). We then quenched the reaction by excess EDTA at specified time points. When the RNAP was not in the immediate vicinity of the nucleosome, the unzipping force signatures for both the RNAP and the nucleosome were readily discernable (as shown in **Fig. 1e**). However, when the RNAP had encountered a nucleosome, we observed a much more complex and variable force signature that did not readily distinguish between the RNAP and the nucleosome. To examine only the RNAP location, we used heparin to dissociate the histones from the DNA immediately after we quenched the chase reaction (Online Methods and **Supplementary Fig. 3b**). Control experiments showed that neither the competitor DNA nor heparin dissociated RNAP or altered RNAP locations (**Supplementary Fig. 3a,d**). As shown from the representative traces at three transcription times (**Fig. 1f**), RNAP was clearly distinguishable on DNA molecules after histone dissociation by heparin. When RNAP moved through a nucleosome, it encountered strong interactions preceding the dyad region (10-s trace) followed by strong interactions at the dyad region (1-min trace); the RNAP then moved out of the nucleosome (5-min trace).

Transcription-pausing pattern at a nucleosome

We carefully examined nucleosome-induced pause sites using bulk transcription assays on a single-promoter DNA template (**Fig. 2a**, top, and Online Methods). We determined the lengths of the RNA, indicative of the 3'-end location of the RNA transcript on DNA, using denaturing PAGE. Consistent with previous observations^{1,4-8}, the presence of a nucleosome markedly reduced the transcription rate. Although essentially all RNAPs reached the runoff end of a naked DNA template within 1 min, only ~50% of RNAPs were able to reach the runoff end in the presence of a nucleosome, even after 30 min. In addition, as RNAP proceeded into the nucleosome, a distinct periodicity of ~10 bp highlighted the nucleosome-induced pause sites: -60 bp (strong), -50 bp (weak), -40 bp (strong) and -30 bp (strongest) from the dyad. Because the RNAP leading edge is located ~20 bp downstream of the active site³⁸, these pause sites coincided with the two strong histone-DNA

interaction regions that the RNAP encountered. As the leading edge of the RNAP passed the dyad region, pausing immediately disappeared, indicating the absence of major obstacles. It is noteworthy that the pausing pattern, including the 10-bp periodicity, remained unchanged when the DNA downstream of the nucleosome was truncated, indicating that this segment of the DNA was not essential for the pausing pattern (**Supplementary Fig. 4**).

To examine whether these observations were specific to the DNA sequence transcribed, we placed the promoter on the distal site of the 601 NPE and allowed the RNAP to elongate into the nucleosome from the reverse direction (**Fig. 2b**). Because the 601 NPE sequence is not palindromic, RNAP effectively transcribed a new sequence. We found that all nucleosome-induced pauses were still highlighted by a distinctive 10-bp periodicity. The pause patterns from the two sequences share substantial similarities, indicating that the nucleosome-induced pausing pattern described here is not specific to the sequences used here, although DNA sequence may influence the strengths of the pause sites.

To substantiate this conclusion, we compared the intrinsic pause sites obtained at low NTP concentration on naked DNA with nucleosome-induced pause sites (**Supplementary Fig. 5**). As shown, the intrinsic pausing sites do not show a 10-bp periodicity and in general do not completely coincide with the pausing sites at a nucleosome. Therefore, a nucleosome does not simply enhance intrinsic pausing.

Notably, these pausing features bear resemblance to the resistance encountered during mechanical unzipping through a nucleosome³²: the unzipping fork paused at the first off-dyad and dyad regions of interactions. In addition, the unzipping fork paused with a 5-bp periodicity, likely resulting from alternating interactions of the histone core with the two strands of dsDNA at each minor groove^{32,39}. Because RNAP paused every 10 bp, it may cooperatively disrupt a pair of interactions at each DNA minor groove. Therefore, we conclude that the transcription-pausing pattern at a nucleosome is predominantly determined by the nucleosome structure. Other factors, such as the type of RNAP, DNA sequence and the uniqueness of nucleosome positioning, may also contribute to the pausing pattern.

RNAP backtracking at a nucleosome

We investigated the extent of backtracking during nucleosome-induced transcription pausing by comparing the location of the RNAP

Figure 2 Transcription through a nucleosome shows a distinctive 10-bp periodicity pausing pattern. **(a)** RNAP transcribed through a nucleosome in the forward direction of the 601 NPE as indicated by the template cartoon (identical to **Fig. 1d**). PAGE analysis of transcription through naked DNA and nucleosomal DNA shows that, as RNAP proceeded into the nucleosome, a distinctive periodicity of ~10 bp highlighted all nucleosome-induced pause sites within regions 1 and 2. Transcription pause sites are marked as distances from the dyad. **(b)** RNAP transcribed through a nucleosome from the reverse direction of 601 NPE as indicated by the template cartoon. Although RNAP effectively transcribed a different sequence, all nucleosome-induced pauses were again highlighted by a distinctive ~10-bp periodicity within regions 1 and 2. The pause site at the end of the 601 NPE might be intrinsic pausing (compare transcription through naked DNA and nucleosomal DNA). Also note that, at this pause site, the leading edge of the RNAP was ~20 bp downstream of the 601 NPE.

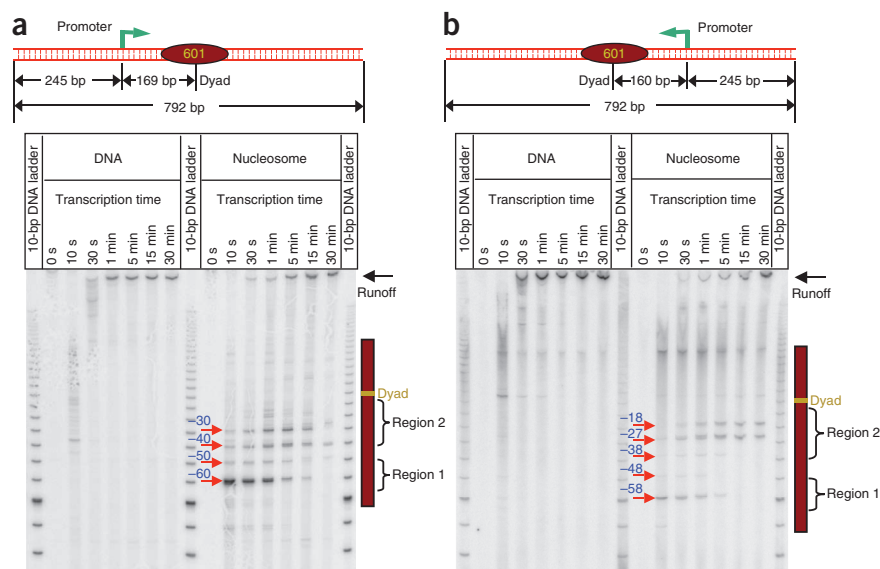
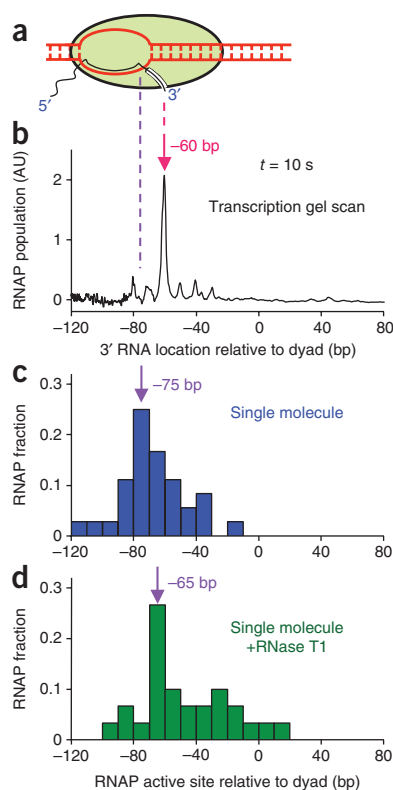


Figure 3 Histone-DNA interactions induce RNAP backtracking, and prevention of backtracking facilitates transcription. All experiments were conducted using the single-promoter DNA template and for 10 s of transcription time. The predominant peak position in each distribution is indicated by an arrow. (a) A cartoon of a backtracked transcription elongation complex. Pink dashed line indicates the location of the 3' end of RNA, and the purple dashed line indicates the location of RNAP active site. (b) An intensity scan of the gel shown in **Figure 2a**. The 3' RNA location is specified relative to the dyad. (c) Distribution of RNAP active-site location as determined by the unzipping method. The active-site location is specified relative to the dyad. The displacement between the peak location of the active site and that of the 3' end of the RNA indicates the backtracking distance. (d) Distribution of RNAP active-site location in the presence of RNase T1.



active site on DNA with the corresponding transcript length (**Fig. 3**). This allowed a direct measurement of the backtracking distance, as compared with conventional methods, which typically can only detect transcript length and therefore rely on sensitivity to cleavage factors (TFIIS or GreA/B) for evidence of backtracking.

A line scan (**Fig. 3b**) of the transcription gel of the single-promoter nucleosome template (**Fig. 2a**) shows that the distribution of the 3' end of RNA peaked at the -60 -bp position from the dyad (upon encountering the off-dyad region of interactions) after 10 s of transcription (**Fig. 3b**). The corresponding distribution of the location of the RNAP active site, as determined by DNA unzipping, resembles that of the 3' end of RNA but peaked at -75 bp from the dyad (**Fig. 3c**), with a broader distribution that lacked the 10-bp periodicity. This clearly shows that a substantial fraction of RNAP was backtracked to various distances at a given pause, and on average, the nucleosome-induced backtracking was ~ 15 bp (**Fig. 3a**). After 5 min of transcription, the RNAP progressed further into the nucleosome and encountered the dyad region of strong interactions, as indicated by the strong pause sites at -40 bp and -30 bp before the dyad (**Fig. 2a** and **Supplementary Fig. 6a**). RNAP again backtracked, with a mean backtracking distance of ~ 10 bp, and a small fraction elongated through the nucleosome (**Supplementary Fig. 6b**). Compared with the 10-s data, a fraction of RNAP that initially paused continued to elongate, indicating that this fraction either was not backtracked or was not backtracked extensively, as has been previously reported^{6,8}. However, a substantial fraction was not able to elongate through the

nucleosome even after 30 min of transcription (**Fig. 2**), indicating that extensive backtracking occurred in this fraction.

To substantiate this conclusion, we conducted an experiment in which we added RNase T1 during the transcription chase reaction (Online Methods) to remove most of the 5' end of the exposed nascent RNA. This truncation is expected to reduce the extent of backtracking to facilitate transcription through a nucleosomal template⁶. Such an effect is difficult to observe using traditional methods that typically measure the length of intact RNA, but the unzipping assay allows direct detection of the RNAP position and thus circumvents this problem. As a control experiment, we verified that the presence of RNase T1 did not alter the unzipping force signature of the RNAP or the nucleosome (**Supplementary Fig. 3c**).

In the presence of RNase T1, after 10 s of transcription, the active-site location distribution peaked at -65 bp from the dyad, and the peak was better defined (**Fig. 3d**). This indicates that, when the leading edge of the RNAP encountered the first off-dyad region of interactions in the nucleosome, it still paused, but the backtracking distance was largely reduced (compare **Fig. 3b-d**). The reduced backtracking is expected to be less inhibitory to elongation. Consistent with this, a greater fraction of RNAP elongated further along the template (compare **Fig. 3c,d**; also compare **Supplementary Fig. 6b,c** for the 5-min transcription).

Taken together, these results suggest that backtracking is the major cause of nucleosome-induced RNAP pausing, and any mechanism that reduces backtracking should facilitate transcription through nucleosomes.

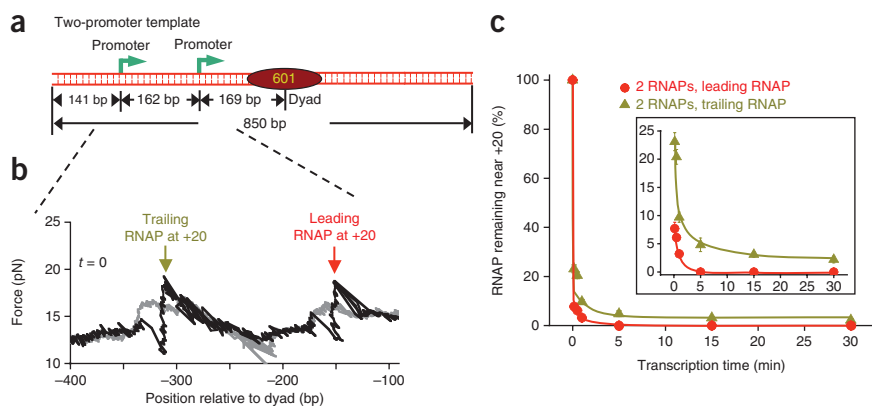


Figure 4 The trailing RNAP assists the leading RNAP to exit an arrested state. (a) The two-promoter transcription template construct contains two T7A1 promoters followed by a single 601 NPE. (b) Example of an unzipping trace from the template shown in **a** containing two PTCs at their respective $+20$ nt positions. The two RNAPs were detected at their expected locations. (c) Percentage of RNAP that remained near the $+20$ nt position versus transcription time for leading and trailing RNAPs. The inset more clearly shows the percentage of the RNAP remaining near the $+20$ nt position.

Elongation by two RNAPs through a nucleosome

In vivo, the concerted action of multiple RNAPs that elongate in the same direction may facilitate transcription through nucleosomal DNA. In order to test this hypothesis, we constructed a two-promoter DNA template containing two T7A1 promoters, each followed by identical sequences of 36 bp, and both oriented toward a downstream 601 NPE (Fig. 4a). We then monitored the locations of the two RNAPs by the unzipping method, which, unlike a bulk transcription assay, does not suffer from complications caused by overlapping in pause sites from the two RNAPs.

The experimental procedures were similar to those described for single-promoter DNA template experiments (Online Methods). First, we examined PTCs that remained near the +20-nt position. Before the NTP chase, we allowed PTCs to equilibrate among their translocation states. Unzipping experiments showed clear force signatures for the two RNAPs stalled at their respective +20-nt loci (Fig. 4b). Upon NTP addition, a majority of the PTCs at each promoter escaped almost instantly. However, a small fraction escaped more slowly and then leveled off with time. For the trailing RNAP, the fraction remaining was clearly backtracked, as indicated by the average location of remaining RNAPs relative to the expected RNAP location (Supplementary Fig. 7b, dark yellow). Furthermore, the more extensive the backtracking, the

longer it took for the RNAP to escape (Fig. 4c and Supplementary Fig. 7a, dark yellow). After 30 min of NTP chase, ~5% of the trailing RNAPs remained, and they were backtracked by ~12 bp. These backtracked complexes were extremely stable and were considered to be arrested on the experimental time scale. These properties were essentially identical to those shown by PTCs on the single-promoter template (Supplementary Fig. 7a,b). This result provides direct evidence for nucleosome-independent backtracking. In contrast, the leading RNAP escaped to completion in <5 min (Fig. 4c, red). Given that both PTCs were identical, the different escape behaviors were a result of the interaction between the two RNAPs. This indicates that the trailing RNAP is capable of assisting the leading RNAP in escaping from a backtracked state, rescuing it from an arrested state.

Second, we examined the RNAPs that escaped after NTP addition (Fig. 5). Before the NTP chase, unzipping experiments showed clear force signatures for the two RNAPs stalled at their respective +20-nt loci followed by a nucleosome (Fig. 5b). As shown in the representative traces (Fig. 5c), upon the resumption of transcription, the locations of both RNAPs were clearly discernible for each trace after nucleosomes were dissociated by heparin. Notice that we did not always find the two RNAPs to be in the immediate vicinity of each other. Although the interaction between two RNAPs would assist the leading RNAP's elongation, this interaction would also possibly induce backtracking of the trailing RNAP. Thus, a separation could be created between the two RNAPs.

The distribution of leading RNAP location (Fig. 5d) shows that the peak location of the RNAP positions was shifted toward the nucleosome to -60 bp from the dyad, with a substantial fraction transcribing beyond the -60 bp pause site. As compared with the single RNAP experiments (Fig. 3c), the fraction elongating through the nucleosome was also increased.

Rate enhancement by a trailing RNAP at a nucleosome

To provide a quantitative measure of elongation-rate enhancement of a leading RNAP due to a trailing RNAP, we examined the transcription runoff efficiency of each RNAP as a function of transcription time (Fig. 6a). We computed runoff efficiency based on the percentage of DNA templates that showed an absence of RNAP during the DNA-unzipping experiments, as an RNAP did not dissociate until it reached the runoff end (Supplementary Fig. 8 and Supplementary Discussion). The runoff efficiencies are more concisely summarized using the initial transcription rate at near-zero transcription times and the rate to achieve 50% runoff to quantify the comparison (Fig. 6b). When a single RNAP transcribed through a mononucleosomal template of ~550 bp in total transcript size, the transcription rate was reduced by a factor of ~20–35 relative to that of naked DNA. However, this rate was increased by a factor of 5 with the assistance of a trailing RNAP, a rate enhancement comparable to that achieved by using RNase T1. Even the trailing RNAP showed a rate enhancement by

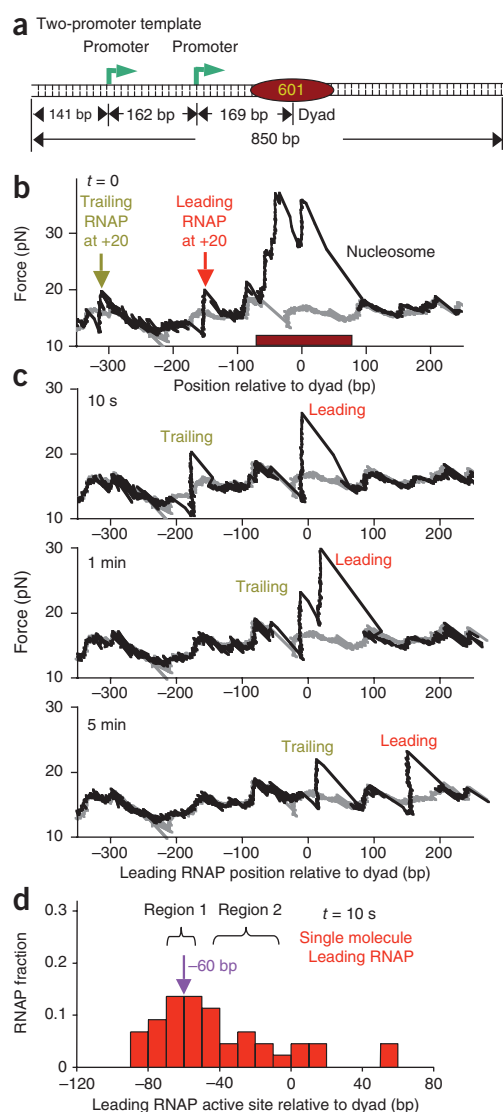


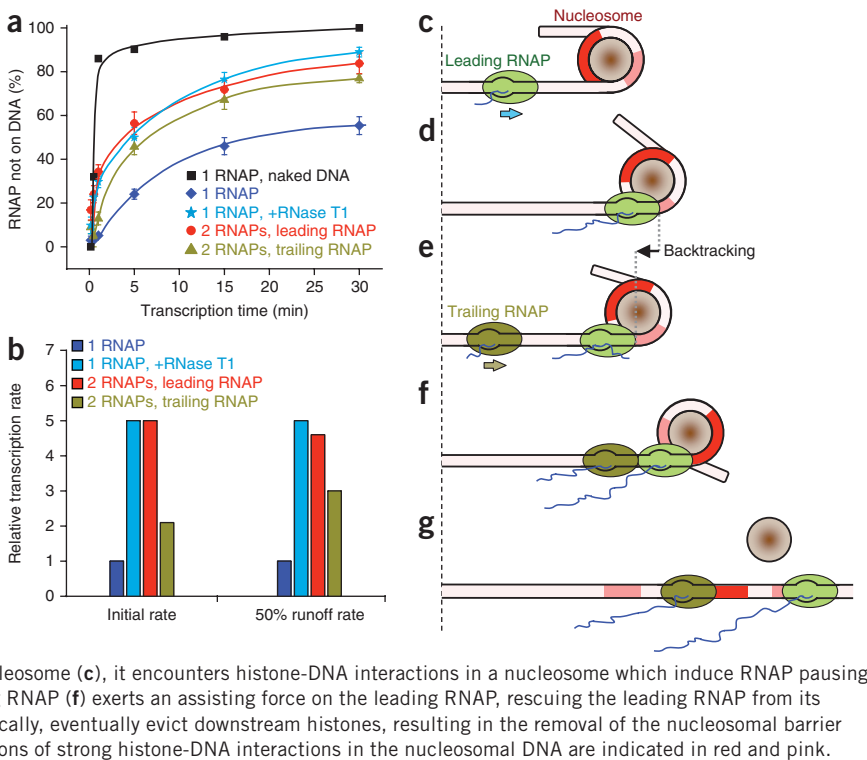
Figure 5 Two RNAPs work synergistically to overcome a nucleosomal barrier. (a) The two-promoter transcription template construct contains two T7A1 promoters followed by a single 601 NPE (same as Fig. 4a). (b) Example of an unzipping trace from the template shown in a containing two PTCs at their respective +20 bp positions and a positioned nucleosome before transcription resumption. The two RNAPs and the nucleosome were detected at their expected locations. The brown bar indicates the 147-bp 601 NPE. (c) Representative unzipping traces through two elongation complexes on a single DNA molecule after transcription for the indicated durations and after removal of histones. Each trace was from a different DNA molecule. Both the leading and trailing RNAPs were detected by their unzipping signatures. (d) Distribution of the leading RNAP active-site location after 10 s of transcription reaction.

Figure 6 Transcription efficiency comparison and cartoon illustrating the mechanism of transcription through nucleosomal DNA.

(a) Transcription runoff efficiencies versus transcription time. A runoff efficiency was represented by the percentage of DNA template that showed an absence of RNAP during DNA-unzipping experiments; error bars, s.e.m. Smooth curves passing through the data points for each transcription condition were drawn for ease of comparison (not fits). Naked DNA runoff efficiency (black) was obtained from PAGE gel analysis and is shown for comparison.

(b) Bar plot of relative transcription rates through nucleosomal DNA. The rate of a single RNAP transcribing through a nucleosomal template is used as a reference. The initial rates were estimated from the slopes of linear fits to the near-zero transcription times (≤ 1 min). Note that because the trailing promoter is about 162 bp upstream of the leading promoter, a 10-s time delay was taken into account for the trailing RNAP transcription rate calculation. The 50% runoff rates were the reciprocal of the time to achieve 50% runoff.

(c–g) Cartoon illustrations of the mechanism of transcription through a nucleosome. As an RNAP approaches a nucleosome (c), it encounters histone-DNA interactions in a nucleosome which induce RNAP pausing (d) and likely backtracking (e). The arrival of a trailing RNAP (f) exerts an assisting force on the leading RNAP, rescuing the leading RNAP from its backtracked state. The two RNAPs, working synergistically, eventually evict downstream histones, resulting in the removal of the nucleosomal barrier and the resumption of efficient transcription (g). Regions of strong histone-DNA interactions in the nucleosomal DNA are indicated in red and pink.



a factor of 2–3 compared with that from a single RNAP alone. This is consistent with at least partial eviction of histones by the leading RNAP as evidenced by the lack of pausing sites after RNAP moves beyond the dyad region of interactions (Fig. 2).

DISCUSSION

This work provides a coherent picture of transcription through a nucleosome (Fig. 6c–g). As an RNAP encounters a nucleosome barrier, it must sequentially overcome the histone-DNA interactions within the nucleosome. The locations and strengths of these interactions dictate the pausing pattern of the RNAP, yielding pausing behaviors that are characteristic of these interactions. Pauses occur approximately every 10 bp (when RNAP encounters DNA minor-groove interactions with the core histone surface), with the strongest pausing at around –60 bp before the dyad (upon encountering the first off-dyad region of strong interactions) and at around –30 bp before the dyad (upon encountering the dyad region of strong interactions), but no pausing occurs once the leading edge of the RNAP passes the dyad region (possibly due to histone dissociation). At each pause site, before reaching the dyad region, RNAP may backtrack to a variable distance, and the mean backtracking distance is ~10–15 bp. Such a large backtracking distance makes it difficult for RNAP to resume active elongation. Thus, any mechanism that would reduce backtracking should facilitate the escape of RNAP from a nucleosome-induced backtracking pause. A trailing RNAP, which initiates from the same or a different promoter, may then catch up with a leading RNAP and interact with it to facilitate its exit from the backtracked state and its entry into productive elongation. Once the leading RNAP overcomes the dyad region of interactions, it may then proceed forward with little resistance. The current work used *E. coli* RNAP, but many findings here may also be more generally applicable to Pol II.

First, we showed that *E. coli* RNAP has a characteristic 10-bp periodic pausing pattern when encountering the promoter-proximal half of the nucleosome. Such periodicity has not been explicitly reported for

Pol II or *E. coli* RNAP, and the apparent lack of reported periodicity may be due to nucleosome positioning heterogeneity. The 5S rRNA NPE generates several major and minor nucleosome positions⁴⁰, and in previous studies where it was used^{4–6}, nucleosome-specific pauses might have been masked by multiple sequence-specific pause sites enhanced by the presence of the nucleosome. The 601 and 603 NPEs can position a nucleosome more uniquely, but the positioning accuracy may still be influenced by the length of the DNA template used and the position of a nucleosome relative to the DNA ends^{7,8}. Nonetheless, there have been hints of the presence of a 10-bp pausing periodicity of Pol II from previous studies that used 601 and 603 NPEs^{7,8}. Also, the 10-bp pausing periodicity was observed for Pol III³ but was interpreted as a restricted rotation of Pol III due to DNA loop formation. Our work offers an alternative and much simpler explanation. Despite the evidence discussed above, we cannot fully exclude the possibility that the lack of strong 10-bp pausing periodicity during Pol II transversal of a nucleosome could be due to a difference between bacterial and eukaryotic RNA polymerases.

Second, we found that the strongest pause sites occurred at around –60 bp and –30 bp before the dyad. Essentially identical pausing regions were identified for Pol II, albeit with a lack of distinct (or with less pronounced) periodicity^{6–8}. This again suggests a high degree of similarity in the nature of the nucleosome barrier encountered by *E. coli* RNAP and Pol II, as has been previously reported⁵.

Third, we have provided direct evidence for *E. coli* RNAP backtracking upon encountering a nucleosome barrier. We have further shown that the mean backtracking distance is ~10–15 bp and that RNase T1 can facilitate transcription through a nucleosome. These findings are consistent with previous work that showed cleavage sensitivity of transcripts to transcription factor IIS for Pol II⁶. However, the current work has provided a more direct method to quantitatively determine the extent of backtracking.

The nucleosome barrier encourages the RNAP to backtrack extensively, and the backtracked state may be further stabilized or ‘trapped’

by the histones due to the exposed 3'-RNA interaction with histones⁴¹. Although nucleosome-induced backtracking has been identified as an important contributor to the nucleosome barrier, transcript cleavage factors such as GreB and TFIIS that rapidly rescue backtracked complexes reduce, but do not eliminate, the nucleosome barrier^{4,5,7}. These results argue for the existence of pausing mechanisms other than backtracking at the nucleosome. During elongation, RNA polymerase rapidly shifts between the pre- and post-translocation states at each template position. A physical blockage imposed by the nucleosome should increase the dwell time at the pretranslocated state, leading to pausing⁴². At the nucleosome barrier, pretranslocation pausing is poised to occur during each elongation cycle and thus may be an important mechanism of polymerase pausing at a nucleosome. Cleavage factors would have no effect on this type of pausing.

In this work, we have provided direct evidence for the synergistic actions of multiple RNAPs working in concert to overcome the nucleosome barrier. In the presence of a trailing RNAP, we found that a leading RNAP could transcribe through a nucleosome with a rate enhancement by a factor of 5. The trailing RNAP is capable of assisting the leading RNAP, likely by exerting an assisting force on it⁴³, and facilitating the leading RNAP to exit the backtracked state and resume elongation. Indeed, RNAPs are known to be powerful molecular motors that can exert forces and work against resistance. *E. coli* RNAP is able to generate ~27 pN of force²⁷ and Pol II at least ~8 pN of force¹⁶. Forces of such magnitude have been shown to greatly speed active elongation rates on naked DNA^{44,45}. Alternatively, the trailing RNAP can form a steric hindrance to prevent the leading one from entering the backtracked state.

In vivo, multiple initiation is common among highly expressed genes. It has been shown that the rates and efficiencies of transcription elongation in various eukaryotic and prokaryotic cells are directly proportional to the rates of transcriptional initiation^{26,46}. Although transcription elongation factors have been found to associate with coding regions *in vivo*, there is also evidence that many transcription factors that travel along with Pol II do not affect the Pol II elongation rate^{21,47}. Notably, cleavage factors that have been suggested to reactivate backtracked RNAP and contribute to the rapid progression of RNAP elongation are dispensable *in vivo* under physiological conditions^{48,49}. Therefore, it is likely that multiple initiation may serve as an alternative mechanism to remove roadblocks such as nucleosomes and other DNA binding proteins during transcription. In addition, it has been increasingly evident that promoter-proximal pausing is a common feature in the expression of many genes⁵⁰⁻⁵⁵. It is possible that, if a second Pol II initiates, it may collide with the leading one, and thus this collision may function as a control of Pol II escape at these pause sites.

It has recently been suggested that, during multiple initiation, the leading RNAP that first encounters nucleosomes might be a specialized 'pioneer' polymerase equipped with additional factors to open unmodified, fully repressed chromatin⁵⁶. However, there is little evidence that such a pioneer RNAP differs from its trailing RNAPs. So how does a pioneer RNAP work so effectively? Our study suggests a much simpler explanation without invoking a pioneer RNAP with unique properties. The initial few RNAPs may function together as a group, effectively acting as pioneer RNAPs so that their additive force is sufficient to evict histones and thereby establish a more accessible chromatin for trailing RNAPs.

METHODS

Methods and any associated references are available in the online version of the paper at <http://www.nature.com/nsmb/>.

Note: Supplementary information is available on the Nature Structural & Molecular Biology website.

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AUTHOR CONTRIBUTIONS

J.J. designed and constructed transcription templates, designed and performed bulk and single-molecule transcription experiments, analyzed the data, participated in all discussions and proposal of the model and also wrote the manuscript; L.B., D.S.J. and M.L.K. offered suggestions and technical advice and helped troubleshoot the experiments; R.M.F. purified *E. coli* RNAP and histones and revised the manuscript; M.K. helped with the design of the project, offered advice on biochemical studies and contributed significantly to the manuscript revision; M.D.W. supervised the study throughout all stages of the project and wrote the manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

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ONLINE METHODS

Nucleosomal DNA templates for transcription. We prepared nucleosomal DNA templates using methods similar to those previously described^{31,32,57}, except that these templates contained either one or two promoters. Briefly, each DNA construct consisted of an anchoring and an unzipping segment (**Supplementary Fig. 1a**). We labeled a ~1.3-kbp anchoring segment with digoxigenin at one end and a ligatable *Dra*III overhang at the other end. We constructed two unzipping segments. The single-promoter segment was 792 bp long and composed of one T7A1 promoter followed by one 601 NPE (**Fig. 1d**). The two-promoter segment was 850 bp long and contained two T7A1 promoters 162 bp apart followed by one 601 NPE (**Fig. 5a**). We achieved this by inserting a second T7A1 promoter upstream of the original one shown in **Figure 1d**. We synthesized both segments by PCR using a biotin-labeled primer. The PCR products were then digested by the restriction enzyme *Dra*III to generate a ligatable end and dephosphorylated using CIP (NEB) to introduce a nick into the final DNA templates. We assembled nucleosomes onto the unzipping segments using purified HeLa histones by a well-established salt dialysis method. We joined the anchoring and unzipping segments by ligation immediately before use. This produced a complete template that was labeled with a single dig tag on one end and a biotin tag located 5 bp away from the nick in one DNA strand.

Bulk transcription assays. We first initiated transcription by incubation of 20 nM *E. coli* RNAP, 4 nM transcription DNA template, 250 μ M ApU initiating dinucleotide, 50 μ M ATP and GTP and anti-[³²P]-CTP antibody [5 μ Ci (1 μ Ci = 37 GBq) at 3,000 Ci mmol⁻¹] in transcription buffer (25 mM Tris-Cl, pH 8.0, 100 mM KCl, 4 mM MgCl₂, 1 mM DTT, 3% (v/v) glycerol, 0.15 mg ml⁻¹ acetylated BSA) for 20 min at 37 °C to form PTCs, which contained DNA, RNAP and 20 nt RNA transcript. We then diluted PTCs by a factor of 10 in transcription buffer, and transcription was resumed at room temperature (23 °C \pm 1 °C) by addition of 1 mM of all four unlabeled NTPs. To prevent re-initiation, competitor DNA was added to 15 nM to serve as an RNAP sink immediately before the resumption of transcription (**Supplementary Fig. 3a**). Transcription reactions were quenched at predetermined time points by

addition of EDTA to 10 mM. Transcripts were analyzed on polyacrylamide sequencing gels and imaged with PhosphorImager (Molecular Dynamics)²⁸.

Single-molecule transcription assays. Transcription reactions were typically performed using identical protocols as in bulk transcription assays, except that 50 μ M unlabeled CTP was used instead of anti-[³²P]-CTP antibody during PTC formation. After the transcription reactions were quenched, 4 mg ml⁻¹ heparin was used to chemically dissociate histone proteins. Single-molecule sample preparation was then immediately performed using protocols similar to those previously described⁵⁷. In the experiments where RNase T1 was needed, 5 units per μ l was added right before the addition of NTPs. For experiments described in **Figure 1c**, PTCs at +20 nt were formed by incubating 2 nM RNAP, 0.4 nM DNA template and 1 mM ApUTP and ATP, GTP and CTP in transcription buffer for 2 min at room temperature before the reaction was quenched by EDTA.

Single-molecule DNA-unzipping experiments. The experimental configuration for optical trapping was similar to that previously described⁵⁷ (**Supplementary Fig. 1b**). Briefly, one end of an anchoring segment was attached to a microscope-coverslip via a digoxigenin-antidigoxigenin connection. The 5'-nicked unzipping segment was attached to a 0.48 μ m-diameter microsphere via a biotin-streptavidin connection. A single-molecule optical trapping setup was used to unzip the DNA template by moving the coverslip horizontally away from the optical trap. When a bound protein was encountered, a computer-controlled feedback loop increased the applied load linearly with time (8 pN s⁻¹) as necessary to unzip through the protein-DNA interactions. Data were digitized at 12 kHz and boxcar-averaged to 60 Hz. The acquired data signals were converted into force and number of base pairs unzipped as described. Additionally, the force-versus-base-pairs-unzipped curves were aligned as previously described to achieve high-precision position detection³².

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