LETTERS

Backtracking determines the force sensitivity of RNAP II in a factor-dependent manner

Eric A. Galburt[1]*†, Stephan W. Grill[1]*†, Anna Wiedmann[2], Lucyna Lubkowska[5], Jason Choy‡, Eva Nogales[1,2,4], Mikhail Kashlev[5] & Carlos Bustamante[1,2,3,4]

RNA polymerase II (RNAP II) is responsible for transcribing all messenger RNAs in eukaryotic cells during a highly regulated process that is conserved from yeast to human[1], and that serves as a central control point for cellular function. Here we investigate the transcription dynamics of single RNAP II molecules from *Saccharomyces cerevisiae* against force and in the presence and absence of TFIIS, a transcription elongation factor known to increase transcription through nucleosomal barriers[2]. Using a single-molecule dual-trap optical-tweezers assay combined with a novel method to enrich for active complexes, we found that the response of RNAP II to a hindering force is entirely determined by enzyme backtracking[3–6]. Surprisingly, RNAP II molecules ceased to transcribe and were unable to recover from backtracks at a force of 7.5 ± 2 pN, only one-third of the force determined for *Escherichia coli* RNAP[7,8]. We show that backtrack pause durations follow a $t^{-3/2}$ power law, implying that during backtracking RNAP II diffuses in discrete base-pair steps, and indicating that backtracks may account for most of RNAP II pauses. Significantly, addition of TFIIS rescued backtracked enzymes and allowed transcription to proceed up to a force of 16.9 ± 3.4 pN. Taken together, these results describe a regulatory mechanism of transcription elongation in eukaryotes by which transcription factors modify the mechanical performance of RNAP II, allowing it to operate against higher loads.

Promoter-based initiation of the 12-subunit RNAP II requires the complex assembly of a host of multi-component general transcription factors[1,9] and has therefore eluded researchers attempting to follow eukaryotic transcription at the single-molecule level. To overcome this difficulty, we adapted a previously described method that bypasses promoter-based initiation and assembles elongation complexes piecewise in the absence of factors[10] (Supplementary Information). Sporadic single-molecule activity was observed in this way (data not shown). To establish a robust assay, we increased the proportion of active elongation complexes by selecting for enzymes that responded to an initial nucleotide triphosphate (NTP) pulse (Fig. 1a, and Supplementary Information). Complexes that transcribed to a nucleotide starvation stall site protected an overlapping restriction site from digestion by the corresponding endonuclease. Inactive complexes failed to protect the restriction site and were digested away (Fig. 1a, and Supplementary Information). This pulse–digest method increased the yield of active elongation complexes approximately fivefold (data not shown).

After pulse–digest, a single ternary complex with 9.8 kb of template DNA was tethered between two polystyrene beads held in place by two single-beam optical traps[6,11,12] (Supplementary Information). On addition of 1 mM NTPs, RNAP II began to translocate along the DNA, shortening the tether between the two beads (Fig. 1b, and Supplementary Fig. 1). Force was monitored in both traps using laser beam deflections and the average value was converted to enzyme position along the template with the worm-like-chain model for DNA elasticity[13] (Supplementary Information). We observed continuous runs of transcription interrupted by pauses (Fig. 1c), which were scored and removed with a velocity-threshold algorithm to obtain a pause-free elongation velocity[14]. The average pause-free velocity was 12.2 ± 4.5 nucleotides per second ($N = 33$, mean ± s.d. unless otherwise noted; Fig. 2b, Supplementary Fig. 7 and Supplementary Table 1), which is comparable to bulk data from RNAP II[15] and to single-molecule data from the bacterial enzyme[7,11,16]. An experiment ended when, having ceased to transcribe, no resumption of activity was observed for 10 min. These experiments revealed that RNAP II was able to transcribe up to forces of 7.5 ± 2.0 pN (Fig. 2a, and Supplementary Table 1). Interestingly, although the eukaryotic enzyme translocates at rates comparable to those of its prokaryotic counterpart, it can only transcribe against a force about one-third that of the bacterial enzyme[8,16,17].

Next, we looked at the relationship between pause-free velocity and force[8,16]. Because velocity differed significantly between enzymes[8,16,17] (Fig. 2b), we preserved the force–velocity (F–V) relationship of individual enzymes by normalizing both variables[8]. Pause-free velocity shows no force dependence up to the average force of 7.5 pN (Fig. 2c), indicating that under physiological and saturating NTP concentrations, translocation is not rate limiting at these forces (also true at 120 μM NTP, data not shown). At 7.5 pN, transcription rates drop sharply to zero in contrast to the gradual decrease observed for the bacterial enzyme[8,11]. We then asked whether this sharp decrease is due to enzyme translocation becoming rate limiting at 7.5 pN or if it reflects the force sensitivity of another process. A single-parameter fit of the F–V relationship to a generalized Boltzmann scheme[8] yielded an unphysical distance to the translocation transition state of 152 ± 8 nucleotides. This suggests that the force dependence of a process other than NTP-dependent forward translocation determines the behaviour of the eukaryotic enzyme in this force regime. Thus, 7.5 pN is not the maximum force that RNAP II can generate (that is, its thermodynamic stall force), but instead represents an 'operational force limit'.

What is the physical origin of the strong force sensitivity observed around 7.5 pN? Data obtained at the maximum spatial resolution allowed by our experiments (~3 nucleotides) revealed that arrest strongly correlates with a lengthening of the tether, an observation consistent with backtracking (Fig. 1c, inset). Backtracking is independent of NTP hydrolysis and involves a process wherein the enzyme moves upstream on the DNA template while the RNA–DNA register is maintained. This process results in an inactive state

[1]Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, California 94720, USA. [2]Department of Molecular and Cell Biology, [3]Department of Chemistry and [4]Howard Hughes Medical Institute, University of California, Berkeley 94720, USA. [5]NCI Center for Cancer Research, Frederick, Maryland 21702, USA. †Present address: Max Planck Institute for the Physics of Complex Systems, 01187 Dresden, Germany. ‡Deceased.
*These authors contributed equally to this work.

because the 3′-end of the transcript is no longer at the active site[3,4,18] (Supplementary Fig. 2). Fifty per cent of all the experiments ended with a terminal backtrack, with an average length of 7.2 ± 3.0 nucleotides. We note that the real average terminal backtrack distance must be considerably smaller (we estimate ~3 nucleotides) because we expect to miss a significant fraction of backtrack events less than 3 nucleotides in size (Supplementary Fig. 3). These observations strongly implicate backtracking as the origin of the force sensitivity of the enzyme at 7.5 pN.

Because the backtracked state is physically displaced along the template, an applied force should affect either the rate of entry to or the rate of exit from this backtracked state, or both. Thus, we sought to characterize the force dependence of these rates. By looking at the effects of assisting force on arrest, studies on the bacterial polymerase have inferred that force primarily affects the ability of the enzyme to recover from backtracks[16]. To determine the force dependence of backtrack return for the eukaryotic enzyme, we measured the fraction of observed backtracks that did not recover within 10 min in different force ranges (Fig. 3a, filled red circles). The return probability is highly sensitive to force: above 8 pN, less than 50% of the enzymes are able to recover from the backtracked state. To determine the force dependence of backtrack entry, we measured the probability of observing a backtrack event within one second of active transcription. Below the operational force limit of the enzyme, this probability is 0.02 ± 0.012 and exhibits no force dependence (Fig. 3a, filled blue circles). To conclude, force does not significantly modulate the probability of entering a backtracked state in the operational force range of the enzyme. The probability of returning from the backtracked state is, however, highly force sensitive and the response of RNAP II to force in the absence of factors is entirely dominated by this effect.

We have shown that RNAP II can only transcribe against a force about one-third that of its bacterial counterpart as it can no longer return from the backtracked state. The maximum force that RNAP II can generate during translocation, however, should be higher than this operational force limit. To measure this thermodynamic stall force, we devised an experiment that would allow us to observe transcription at forces beyond the operational force limit of RNAP II. To this end, we performed 'force jump' experiments during active enzyme translocation[19]. In these experiments, the force on the enzyme was rapidly increased by suddenly displacing one of the two traps (Supplementary Fig. 4). After one second, the original position of the trap was restored, and the velocity of the enzyme during the jump was measured. In these experiments, enzymes were seen to transcribe beyond the operational force limit determined above. Transcription was observed for 50% (6/12) of jumps between 14 to 20 pN, for 17% (1/6) of jumps between 20 to 25 pN, and 0% (0/12) of

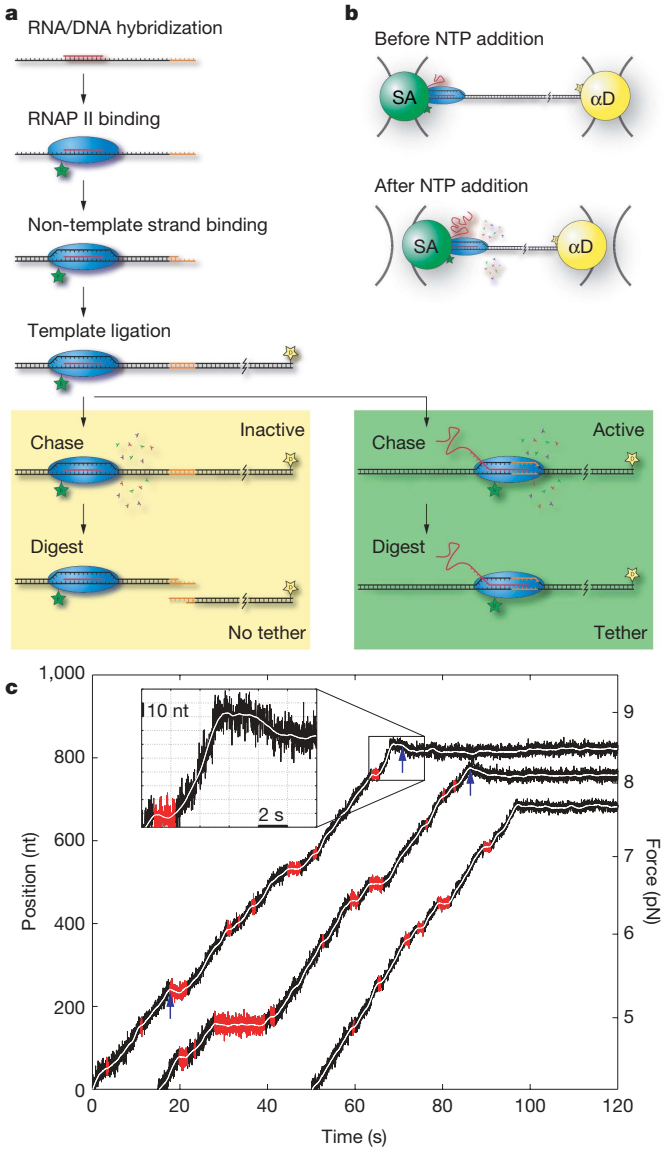

Figure 1 | Single-molecule transcription. **a**, Elongation complexes are formed as previously described[10] (Supplementary Methods; biotinylated RNAP II, blue with green star; digoxigenin, yellow star) and ligated to the transcription template. After pulse–digest, inactive complexes (yellow shading) are digested away and only active complexes (green shading) can form tethers. **b**, Passive mode dual-trap optical tweezer. After adding NTPs, transcription begins, the tether shortens and the load increases (Supplementary Fig. 1). **c**, Template position and force versus time (100 Hz bandwith, 3rd order Savitzky–Golay filter with time constant of 2.5 s). Enzymes exhibit elongation (black), pausing (red), backtracking (blue arrows and inset) and arrest. Nucleotides, nt.

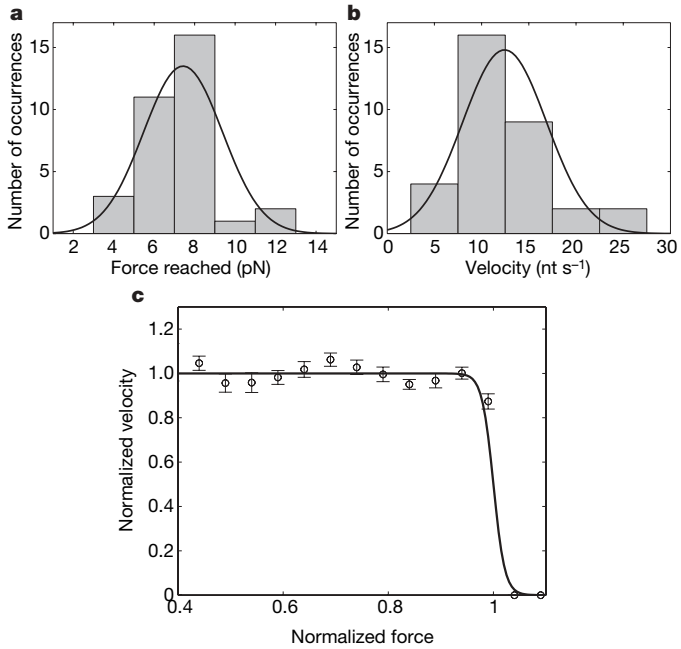

Figure 2 | Force–velocity analysis. **a**, A gaussian fit of the histogram of forces reached shows that RNAP II ceases transcription at a force of 7.5 ± 2.0 pN (mean ± s.d., $R^2 = 0.82$, $N = 33$). **b**, A gaussian fit of the pause-free transcription velocity histogram yields an average of 12.2 ± 4.5 nt s^{-1} (mean ± s.d., $R^2 = 0.85$, $N = 33$). **c**, Normalized force–velocity curve (Supplementary Information). Velocity is uncorrelated with force (error bars, s.e.m.) for normalized forces smaller than 1 ($r^2 = 7 \times 10^{-3}$). The solid line represents a fit to a generalized Boltzmann scheme[8] (single parameter fit, $R^2 = 0.97$).

jumps over 25 pN. Enzymes that continued to transcribe in these experiments displayed pause-free velocities not significantly different from velocities measured at lower forces (data not shown), indicating that translocation is still not rate limiting at these increased forces. These results indicate that the thermodynamic stall force of RNAP II is higher than 20 pN at physiological NTP concentrations. Significantly, measurements of transcriptional velocity beyond ~25 pN were foiled because the probability of backtracking within one second increased dramatically (Fig. 3a, filled blue squares): 17/21 enzymes backtracked during the jump at jump forces beyond 20 pN, and 100% (7/7) of enzymes backtracked above 30 pN. In conclusion, both backtrack entry and exits are sensitive to force, however, the former only at very high forces, whereas the latter determines the operational force limit of the enzyme.

We have shown that the main response of RNAP II to force is backtracking, which causes the enzyme to pause. Next we ask if it is possible that all transcriptional pauses in RNAP II are due to backtracking. However, with a resolution of 3 to 4 nucleotides at physiological NTP concentrations[11], it is not possible to determine whether or not brief pauses are associated with backtracking events. Specifically, backtrack displacements of a couple of nucleotides will not be recognized (Supplementary Fig. 3 and Supplementary Information). Because it is easier to determine the duration of a pause than its backtracking distance, we identified enzyme pauses with a computer algorithm and analysed the distribution of pause lifetimes[14]. Analysis of the pause time durations (t) revealed that the distribution of pause lifetimes follows a $t^{-3/2}$ power law (Fig. 3c, and Supplementary Information). This observation implies a mechanism in which, during a pause, a polymerase diffuses among many intermediate states and ends the pause when it diffusively realigns the dislocated 3′-end of the transcript with the active site to resume elongation. Significantly, all pause durations, short and long, follow this power law distribution, implying that the molecular mechanism underlying most transcriptional pauses in RNAP II is the same, that is, diffusive backtracking. Note that the distribution deviates from the $t^{-3/2}$ power law only for pauses longer than 10 s, as is to be expected from the effect of the opposing force on the polymerase (manuscript in preparation).

Because the operational force limit of the enzyme is determined by the force dependence of exit from backtracked states, it follows that the enzyme should be able to transcribe to higher forces in conditions that increase its probability to return from these states. Specifically, the polymerase would be expected to transcribe up to forces in the 20 pN range, where the probability of backtracking would limit transcriptional progress (Fig. 3a). To test this prediction, we performed experiments in the presence of the transcription factor TFIIS. This factor is involved in both the initiation and elongation of RNAP II[20], is functionally homologous to the Gre factors in bacteria[21], and may have an important role in RNA proofreading[22]. TFIIS binds RNAP II, catalyses the enzyme's intrinsic RNA cleavage activity, and rescues backtracked states by producing a new 3′-end that is aligned with the enzyme's active site[23,24] (Supplementary Fig. 2).

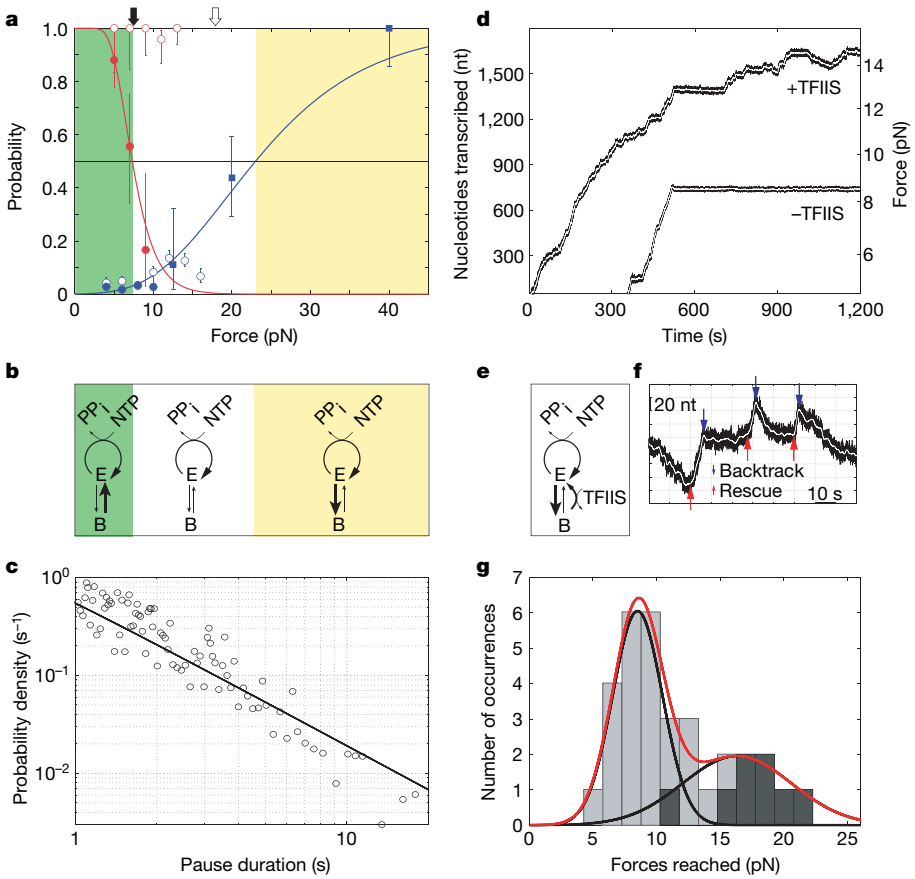

Figure 3 | Backtrack entry and exit. **a**, The probability of entering a backtrack within one second of transcription (blue markers) and the probability of returning from a backtrack within 10 min (red markers) versus force. Data are shown in the absence (filled markers) and presence (open markers) of TFIIS, and arrows indicate the respective forces reached. Square markers represent data from force jump experiments. Error bars represent s.e.m. and solid lines indicate trends. **b**, Illustrated kinetic schemes for three regions of force where return to elongation (E) from a backtrack (B) is likely (green), unlikely (white and yellow) and where backtrack entry is likely (yellow). **c**, Distribution of measured pause durations with a single-parameter fit of a $t^{-3/2}$ power-law ($R^2 = 0.84$). **d**, Template position and force versus time in the presence and absence of TFIIS (Supplementary Fig. 5). **e**, Illustrated scheme in the presence of TFIIS. **f**, An example of cycles of backtracking and TFIIS rescue at 18 pN. **g**, Histogram of forces reached in the presence of TFIIS. Instances of cycling between backtracking and rescue are indicated in dark grey. Solid lines indicate a double gaussian fit with means of 8.5 ± 2.0 and 16.5 ± 3.5 pN (mean ± s.d., $R^2 = 0.96$, $N = 32$).

TFIIS was expressed and purified as described[23] and introduced at a saturating concentration (600 nM), concomitant with NTPs. Seventy five per cent of the runs obtained in the presence of TFIIS (24/32) were not significantly different from runs obtained in the absence of TFIIS (Fig. 3g, and Supplementary Information). However, 25% of the transcription runs (8/32) exhibited a dramatic change in behaviour (Fig. 3d): instead of arresting after a terminal backtrack, these enzymes never ceased to transcribe but repeatedly switched between backtracking and active transcription (Fig. 3d, f, and Supplementary Fig. 5) and translocated to significantly higher forces (16.9 ± 3.4 pN, Fig. 3d, g). This behaviour was never observed in the absence of TFIIS (0/33). At this new operational force limit, the average distance backtracked before rescue equals the average distance transcribed before entering a backtracked state (Fig. 3d, f, g). Again, pause-free velocity was unchanged even at the increased opposing forces accessible in the presence of TFIIS (Supplementary Fig. 6). To conclude, TFIIS increases the operational force limit of RNAP II more than twofold by accelerating backtrack exit and allowing the polymerase to proceed to forces where the rate of backtrack entry is significantly increased (Fig. 3a). These results rationalize observations that TFIIS promotes transcription through transcriptional blocks such as the *lac* operon repressor[25] and the nucleosome *in vitro*[2]. Additionally, they predict that bacterial enzymes might transcribe to higher forces in GreB's presence.

The low intrinsic operational force limit of RNAP II, coupled with the dramatic effect of TFIIS, indicates the possibility of transcriptional regulation through a TFIIS-dependent switch of the polymerase's operational force limit. Although it does not seem that TFIIS levels are regulated directly in yeast (C. M. Kane, personal communication), the ability of TFIIS to bind the polymerase is regulated by additional factors in multi-cellular eukaryotes[26]. The results described here set the stage for the investigation of eukaryotic transcription elongation through nucleosomal arrays in the presence and absence of transcription elongation factors, using single-molecule manipulation methods.

METHODS

A detailed description of materials and methods is given in Supplementary Information.

Transcription initiation. Elongation complexes were formed through the ordered addition of a 54 nucleotide template DNA, 9 nucleotide RNA primer, biotinylated RNAP II, and complementary 50 nucleotide non-template DNA strand[10,27]. Elongation complexes were then ligated to a 9.8 kilobase double-stranded DNA template with a downstream digoxigenin label. Oligonucleotide sequences and other details are provided in Supplementary Information.

Optical trapping. The optical trap used for these experiments is based on a setup previously described[28]. To reduce drift, the dual-beam, single-trap setup was converted to a single-beam, dual-trap machine by slightly overfilling each objective. The experimental setup is described in more detail in the Supplementary Information.

Backtrack detection. Backtracks were detected by looking at the first 4 s of a pause and asking whether the enzyme moved backwards by more than 3 nucleotides. These strict limits ensured that backtrack signals were not generated by noise.

Received 11 November 2006; accepted 23 February 2007.
Published online 14 March 2007.

1. Roeder, R. G. The role of general initiation factors in transcription by RNA polymerase II. *Trends Biochem. Sci.* **21,** 327–335 (1996).
2. Kireeva, M. L. *et al.* Nature of the nucleosomal barrier to RNA polymerase II. *Mol. Cell* **18,** 97–108 (2005).
3. Komissarova, N. & Kashlev, M. Transcriptional arrest: *Escherichia coli* RNA polymerase translocates backward, leaving the 3′ end of the RNA intact and extruded. *Proc. Natl Acad. Sci. USA* **94,** 1755–1760 (1997).
4. Komissarova, N. & Kashlev, M. 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 (1997).
5. Nudler, E., Mustaev, A., Lukhtanov, E. & Goldfarb, A. The RNA–DNA hybrid maintains the register of transcription by preventing backtracking of RNA polymerase. *Cell* **89,** 33–41 (1997).
6. Shaevitz, J. W., Abbondanzieri, E. A., Landick, R. & Block, S. M. Backtracking by single RNA polymerase molecules observed at near-base-pair resolution. *Nature* **426,** 684–687 (2003).
7. Davenport, R. J., Wuite, G. J., Landick, R. & Bustamante, C. Single-molecule study of transcriptional pausing and arrest by *E. coli* RNA polymerase. *Science* **287,** 2497–2500 (2000).
8. Wang, M. D. *et al.* Force and velocity measured for single molecules of RNA polymerase. *Science* **282,** 902–907 (1998).
9. Hahn, S. Structure and mechanism of the RNA polymerase II transcription machinery. *Nature Struct. Mol. Biol.* **11,** 394–403 (2004).
10. Komissarova, N., Kireeva, M. L., Becker, J., Sidorenkov, I. & Kashlev, M. Engineering of elongation complexes of bacterial and yeast RNA polymerases. *Methods Enzymol.* **371,** 233–251 (2003).
11. Abbondanzieri, E. A., Greenleaf, W. J., Shaevitz, J. W., Landick, R. & Block, S. M. Direct observation of base-pair stepping by RNA polymerase. *Nature* **438,** 460–465 (2005).
12. Moffitt, J., Chemla, Y., Izhaky, D. & Bustamante, C. Differential detection of dual traps improves the spatial resolution of optical tweezers. *Proc. Natl Acad. Sci. USA* **103,** 9006–9011 (2006).
13. Bustamante, C., Marko, J. F., Siggia, E. D. & Smith, S. Entropic elasticity of lambda-phage DNA. *Science* **265,** 1599–1600 (1994).
14. Neuman, K. C., Abbondanzieri, E. A., Landick, R., Gelles, J. & Block, S. M. Ubiquitous transcriptional pausing is independent of RNA polymerase backtracking. *Cell* **115,** 437–447 (2003).
15. Izban, M. G. & Luse, D. S. Factor-stimulated RNA polymerase II transcribes at physiological elongation rates on naked DNA but very poorly on chromatin templates. *J. Biol. Chem.* **267,** 13647–13655 (1992).
16. Forde, N. R., Izhaky, D., Woodcock, G. R., Wuite, G. J. L. & Bustamante, C. Using mechanical force to probe the mechanism of pausing and arrest during continuous elongation by *Escherichia coli* RNA polymerase. *Proc. Natl Acad. Sci. USA* **99,** 11682–11687 (2002).
17. Yin, H. *et al.* Transcription against an applied force. *Science* **270,** 1653–1657 (1995).
18. Kulish, D. & Struhl, K. TFIIS enhances transcriptional elongation through an artificial arrest site *in vivo*. *Mol. Cell. Biol.* **21,** 4162–4168 (2001).
19. Li, P. T. X., Collin, D., Smith, S. B., Bustamante, C. & Tinoco, I. Probing the mechanical folding kinetics of TAR RNA by hopping, force-jump, and force-ramp methods. *Biophys. J.* **90,** 250–260 (2006).
20. Fish, R. N. & Kane, C. M. Promoting elongation with transcript cleavage stimulatory factors. *Biochim. Biophys. Acta* **1577,** 287–307 (2002).
21. Borukhov, S., Lee, J. & Laptenko, O. Bacterial transcription elongation factors: new insights into molecular mechanism of action. *Mol. Microbiol.* **55,** 1315–1324 (2005).
22. Jeon, C. & Agarwal, K. Fidelity of RNA polymerase II transcription controlled by elongation factor TFIIS. *Proc. Natl Acad. Sci. USA* **93,** 13677–13682 (1996).
23. Awrey, D. E. *et al.* Yeast transcript elongation factor (TFIIS), structure and function. II: RNA polymerase binding, transcript cleavage, and read-through. *J. Biol. Chem.* **273,** 22595–22605 (1998).
24. Weilbaecher, R. G., Awrey, D. E., Edwards, A. M. & Kane, C. M. Intrinsic transcript cleavage in yeast RNA polymerase II elongation complexes. *J. Biol. Chem.* **278,** 24189–24199 (2003).
25. Reines, D. & Mote, J. Jr. Elongation factor SII-dependent transcription by RNA polymerase II through a sequence-specific DNA-binding protein. *Proc. Natl Acad. Sci. USA* **90,** 1917–1921 (1993).
26. Palangat, M., Renner, D. B., Price, D. H. & Landick, R. A negative elongation factor for human RNA polymerase II inhibits the anti-arrest transcript-cleavage factor TFIIS. *Proc. Natl Acad. Sci. USA* **102,** 15036–15041 (2005).
27. Kireeva, M. L., Lubkowska, L., Komissarova, N. & Kashlev, M. Assays and affinity purification of biotinylated and nonbiotinylated forms of double-tagged core RNA polymerase II from *Saccharomyces cerevisiae*. *Methods Enzymol.* **370,** 138–155 (2003).
28. Smith, S. B., Cui, Y. & Bustamante, C. Optical-trap force transducer that operates by direct measurement of light momentum. *Methods Enzymol.* **361,** 134–162 (2003).

Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

Acknowledgements We thank Y. R. Chemla, W. Cheng, M. Cruse, S. Dumont, N. R. Forde, B. Ibarra, D. Izhaky, C. Kane, S. Kostek, J. Moffit, J. M. R. Parrondo, M. Peris, S. Plyasunov, A. Ruiz and S. B. Smith for experimental assistance and helpful discussions. This research was supported by a DOE grant. E.A.G. was supported by a Jane Coffin Childs Postdoctoral Fellowship. S.W.G. was supported first by an EMBO Long Term Fellowship and subsequently by a Helen Hay Whitney Postdoctoral Fellowship. We dedicate this manuscript to Jason Choy.

Author Information Reprints and permissions information is available at www.nature.com/reprints. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to C.B. (carlos@alice.berkeley.edu).