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Mechanism of transcriptional repression at a bacterial promoter by analysis of single molecules

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The molecular basis for regulation of lactose metabolism in *Escherichia coli* is well studied. Nonetheless, the physical mechanism by which the Lac repressor protein prevents transcription of the lactose promoter remains unresolved. Using multi-wavelength single-molecule fluorescence microscopy, we visualized individual complexes of fluorescently tagged RNA polymerase holoenzyme bound to promoter DNA. Quantitative analysis of the single-molecule observations, including use of a novel statistical partitioning approach, reveals highly kinetically stable binding of polymerase to two different sites on the DNA, only one of which leads to transcription. Addition of Lac repressor directly demonstrates that bound repressor prevents the formation of transcriptionally productive open promoter complexes; discrepancies in earlier studies may be attributable to transcriptionally inactive polymerase binding. The single-molecule statistical partitioning approach is broadly applicable to elucidating mechanisms of regulatory systems including those that are kinetically rather than thermodynamically controlled.

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Introduction

In all organisms, messenger RNAs are synthesized by a multisubunit RNA polymerase (RNAP) that binds to promoter regions of DNA, separates the DNA strands to form an 'open' promoter complex and then escapes from the promoter, moving along the DNA with the concomitant synthesis of a transcript RNA. Both open complex formation and promoter escape steps are key stages at which DNA transcription, and thus gene expression, is regulated.

The lactose promoter (P*lac*) of *Escherichia coli* drives transcription of genes needed for the uptake and utilization of lactose as a source of carbon when glucose is absent in the extracellular media (Muller-Hill, 2004). P*lac* and its repressor protein have served as a model system for studying gene regulation (Muller-Hill, 2004; Wilson *et al*, 2007), and have become an important module for building synthetic gene circuits (Gardner *et al*, 2000; Kobayashi *et al*, 2004; Ramalingam *et al*, 2009; Chang *et al*, 2010). Particularly in recent years, the P*lac* promoter has also served as a model system for investigating the mechanisms of molecular evolution (Dekel and Alon, 2005; Poelwijk *et al*, 2007; Stoebel *et al*, 2008), the behaviour of gene regulatory networks at the systems level (Ozbudak *et al*, 2004; Kuhlman *et al*, 2007), and for understanding the dynamic aspects of gene regulation in single cells (Golding *et al*, 2005; Cai *et al*, 2006; Yu *et al*, 2006; Elf *et al*, 2007; Choi *et al*, 2008). In all of these applications it is highly desirable to have an understanding, based on molecular mechanisms, that enables quantitative prediction of transcription output from regulatory inputs. Unfortunately, this understanding is lacking for essentially all promoters, even one as well documented as P*lac*. For example, recent experiments that examined transcription in individual cells yielded surprising results, including the fact that a P*lac* variant fires in bursts and remains turned off most of the time even under full induction conditions (Golding *et al*, 2005). Understanding the mechanism of gene regulation for this promoter is essential in order to rationalize these results, as well as to engineer synthetic gene circuits that use the lac system as a building block.

A key feature of P*lac* regulation, the mechanism of repression by Lac repressor, has remained unresolved in spite of decades of research (Gralla, 1996; Muller-Hill, 1998, 2004; Rojo, 1999; Hsu, 2002; Wilson *et al*, 2007). A variety of experimental results (Majors, 1975; Nick and Gilbert, 1985; Schlax *et al*, 1995) are consistent with a mechanism in which Lac repressor shuts off transcription by competing with RNAP for binding at the promoter. This possibility was referred to as the *steric occlusion* mechanism. Here, we use the more generic term 'inhibition of open complex formation' (Figure 1, top) to include both the original steric occlusion proposal as well as mechanisms in which the polymerase can bind to DNA when repressor is bound, but cannot isomerize into a stable open complex. Other results (Straney and Crothers, 1987a; Lee and Goldfarb, 1991) appear inconsistent with inhibition of open complex formation. These experiments have instead been interpreted (Krummel and Chamberlin, 1989; Lee and Goldfarb, 1991) as implicating an 'inhibition of promoter escape' mechanism (Figure 1, bottom), in which a repressor-bound open complex is formed and from which promoter escape and transcript elongation are blocked.

The discrepancies between earlier studies of the Plac repression mechanism may arise in part from the difficulty

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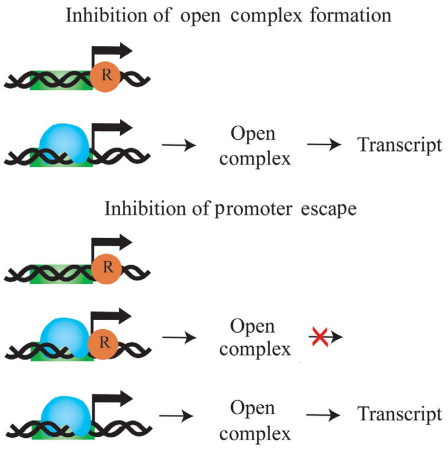

Figure 1 Proposed mechanisms of repression of P*lac*. (Top) Inhibition of open complex formation. Lac repressor (orange) binding to the primary operator sequence located on the DNA immediately downstream of the transcription start site (arrow) prevents binding of RNAP (blue) to the promoter, or allows RNAP binding but prevents isomerization to an open complex. Thus RNAP cannot produce transcript when repressor is bound. (Bottom) Inhibition of promoter escape. RNAP can bind to the promoter and form an open complex even while repressor is also bound. However, repressor prevents RNAP escape from the promoter and thereby prevents transcript production.

of interpreting bulk biochemical measurements on samples, in which RNAP can bind to DNA in multiple states and at multiple sites. Here we introduce a methodology based on colocalization of single-molecule spectroscopy (CoSMoS; Friedman *et al*, 2006; Hoskins *et al*, 2011) and a statistical partitioning analysis that allows us to directly observe, count and determine the stoichiometry of individual RNAP–DNA open complexes in the presence and the absence of repressor. These single-molecule analyses are correlated with bulk experiments that measure transcriptional output under the same conditions. The results are fully consistent with an inhibition of open complex formation mechanism, in which Lac repressor binding is in kinetic competition with open complex formation. Our results further suggest that discrepancies between the results of earlier studies may originate in the presence of one or more transcriptionally inactive tight-binding sites (TBSs) for RNAP.

Results

Formation and visualization of individual competitor-resistant DNA–RNAP complexes

A P*lac* variant, P*lac*UV5, is strongly expressed in the absence of activation by the cyclic AMP receptor protein. P*lac*UV5 is used here, as it has been elsewhere (Stefano and Gralla, 1979; Buc and McClure, 1985; Straney and Crothers, 1987a, b; Schlax *et al*, 1995), as a simplified system in which to investigate the repression mechanism.

The principal binding site for the lac repressor, O1, is a 21 bp sequence beginning at the first transcribed nucleotide of the lac operon. The operon also contains two weakly binding operators O2 and O3, but these are not used here because they act only through O1 (by increasing its occupancy) and thus are not needed to achieve maximal repression at the saturating repressor concentrations that we used (Oehler *et al*, 1994).

A key difference between the alternative repression mechanisms shown in Figure 1 is whether or not RNAP can form open complexes in the presence of repressor. We therefore used the CoSMoS technique to examine open complex formation on individual P*lac*UV5 DNA molecules in the presence and absence of repressor.

To form open complexes that could be directly observed by single-molecule fluorescence microscopy, we used an *E. coli* RNAP holoenzyme construct labelled with a red-absorbing fluorescent dye (BG-647), and a P*lac*UV5-containing duplex DNA linked to biotin and labelled with a blue-absorbing dye (Alexa488). RNAP and DNA were incubated together at concentrations and temperature known to favour the formation of open complexes (Buc and McClure, 1985; Straney and Crothers, 1987b). Next, we sequestered RNAP that had not formed an open complex with DNA by diluting the sample into a solution containing a high concentration of competitor DNA or heparin, challenges to which open complexes are resistant. Finally, the sample was introduced into a flow chamber, the glass surface of which had been coated with streptavidin, to capture the biotinated complexes (Figure 2A). Individual surface-tethered RNAP and DNA molecules were imaged as discrete spots of fluorescence by total internal reflection fluorescence (TIRF) microscopy with excitation by blue and red lasers, respectively (Figure 2B). In a representative field of view, 17 of 20 (85%) RNAP spots colocalized with DNA spots. In an otherwise identical control sample with no DNA attached to the surface, typically <1 RNAP spot on average was seen in a field. These results show that nearly all DNA molecules are labelled with dye and that there is little, if any, DNA-independent adsorption of RNAP to the glass surface. More importantly, they show that essentially all of the RNAP spots observed on the surface correspond to RNAP bound to DNA in competitor-resistant complexes, some or all of which may be open complexes.

Distinguishing open-promoter complexes and a separate class of promoter-independent RNAP–DNA complexes

In order to determine whether the observed RNAP spots correspond to open complexes, we repeated the same experiment with a DNA that is identical to that utilized before, except that the promoter region from -36 to -6 was deleted. In measurements of RNA synthesis from radiolabelled nucleotides, this construct directed little or no synthesis ($\leqslant 7\%$ that of the promoter-containing DNA) and showed no detectable product that initiated from the promoter region (Supplementary Figure S7). By counting RNAP and DNA spots, we determined the fractions of promoter or non-promoter DNA molecules that were bound by one or more RNAP molecules (Figure 2C). As expected, promoter DNA had more bound RNAP than non-promoter, but stable RNAP binding was also observed on a substantial fraction of the non-promoter DNA molecules under these conditions. The existence of competitor-resistant complexes between non-promoter DNA and RNAP has been thoroughly documented (Melancon *et al*, 1982, 1983). In particular, RNAP forms stably bound complexes with DNA sequences known as TBS (Melancon *et al*, 1982). Our observations suggest that the RNAP binding to the promoter DNA consists of roughly equal parts of promoter-independent and promoter-dependent binding, both competitor-resistant.

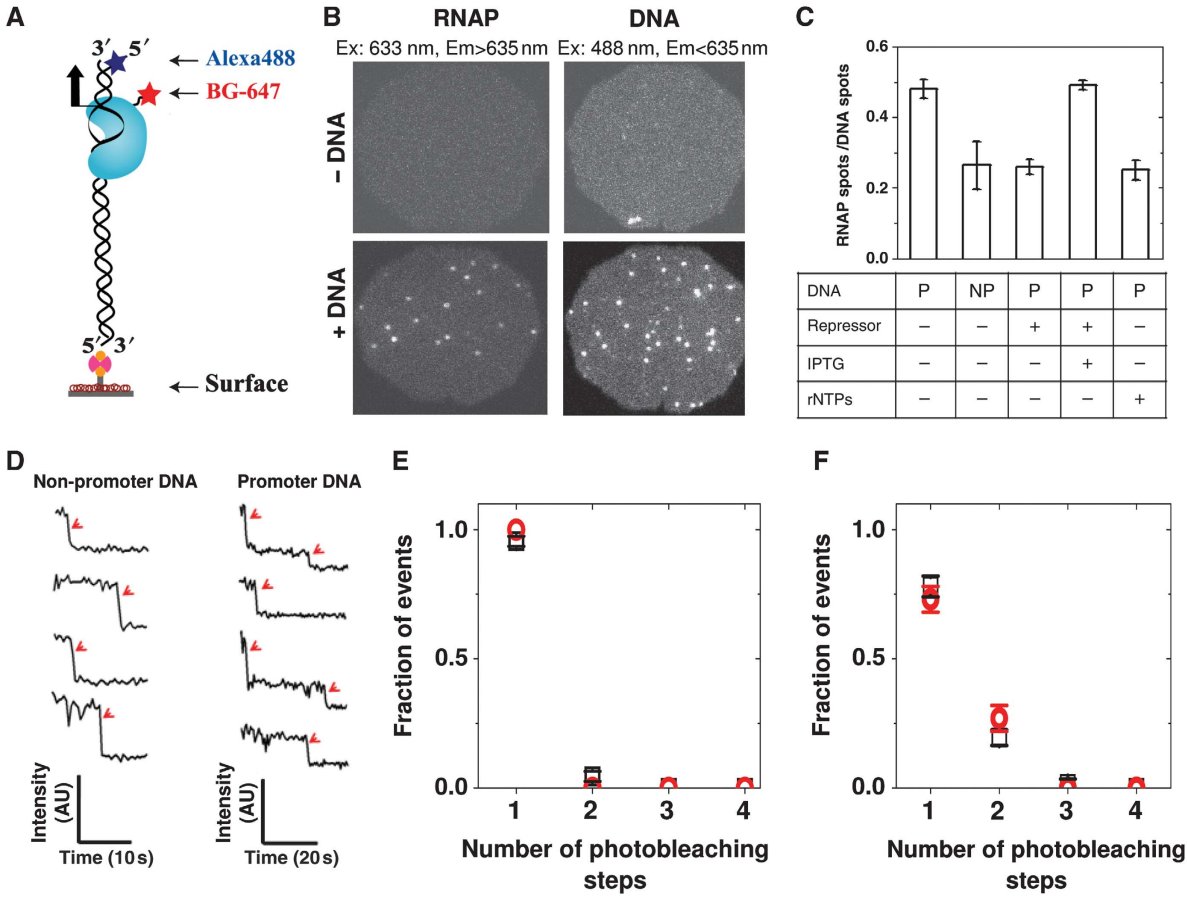

Figure 2 Visualization of individual RNA polymerase molecules bound to tethered DNAs. (**A**) Experimental design. RNAP labelled with a red dye (BG-647) is allowed to bind in solution to duplex DNA labelled with biotin at one end and with a blue dye (Alexa488) at the other. After incubation, the protein–DNA complex is tethered to the surface of a streptavidin (orange)-coated flow chamber and observed by multi-wavelength TIRF microscopy. (**B**) TIRF microscope images of a sample containing RNAP-promoter DNA complexes (bottom) and a control sample that lacked surface-tethered DNA (top). For both samples, the same field of view was separately imaged at the excitation (Ex) and emission (Em) wavelengths that detect BG-647-RNAP (left) and Alexa488 DNA (right). (**C**) The ratio of the number of RNAP spots to the number of DNA spots (mean ± s.e. of three independent measurements) for complexes formed on promoter (P) and non-promoter (NP) DNA in the presence or the absence of Lac repressor, IPTG and rNTPs. (**D**) Example time records of fluorescence emission from individual BG-647–RNAP spots (1.3 mW 635 nm excitation). Photobleaching of a dye moiety was observed as an abrupt change (arrow) in intensity. (**E**, **F**) Observed distribution of number (± s.e.) of bleaching steps per RNAP spot (black squares) and number distribution of fluorescently labelled RNAP molecules per DNA predicted by the statistical partitioning model (red circles) for the non-promoter (**E**) and promoter (**F**) DNAs.

To test the hypothesis that the observed promoter-dependent binding corresponds to the formation of functional open complexes, we examined the effect of incubating the complexes for 15 min with the four ribonucleoside triphosphates (rNTPs) required for promoter escape and RNA synthesis. After rNTP addition, the fraction of RNAP-occupied DNA dropped to levels comparable to those previously observed in the non-promoter control (Figure 2C). This supports the conclusion that those complexes that vanished upon addition of rNTPs correspond to open complexes that escaped the promoter and ran off the end of the DNA. Conversely, the competitor-resistant complexes that survived the rNTP challenge are polymerases stably bound elsewhere on the DNA (i.e., to TBS) and therefore cannot initiate transcription.

The conclusion that the same P*lac*-containing DNA can support both promoter-dependent and TBS binding of RNAP raises the possibility that some of the promoter DNA molecules in our experiments are bound by two RNAP molecules simultaneously, whereas each non-promoter DNA may have at most one RNAP. To determine the stoichiometry of individual DNA–RNAP complexes, we performed additional experiments, in which the red excitation laser power was increased 10-fold so that photobleaching of the RNAP spots was readily observable (Leake *et al*, 2006; Ulbrich and Isacoff, 2007). For complexes with non-promoter DNA, almost all (96 ± 4%) of RNAP spots bleached in a single step. In contrast, with promoter DNA containing the P*lacUV5* promoter, RNAP spots bleached in either one (78 ± 5%) or two (19.5 ± 3%) steps (Figure 2D–F). The number that bleached in three or more steps was negligible (1.7 ± 2%). The numbers of steps and their frequencies observed on the two DNAs agreed precisely with a simple statistical partitioning model (see Supplementary data), which has no adjustable parameters and assumes that TBS and promoter binding are independent (Figure 2E and F). Thus, it is likely that the promoter-containing DNA can bind to RNAP both at the promoter and at a separate second site. This may be important for the

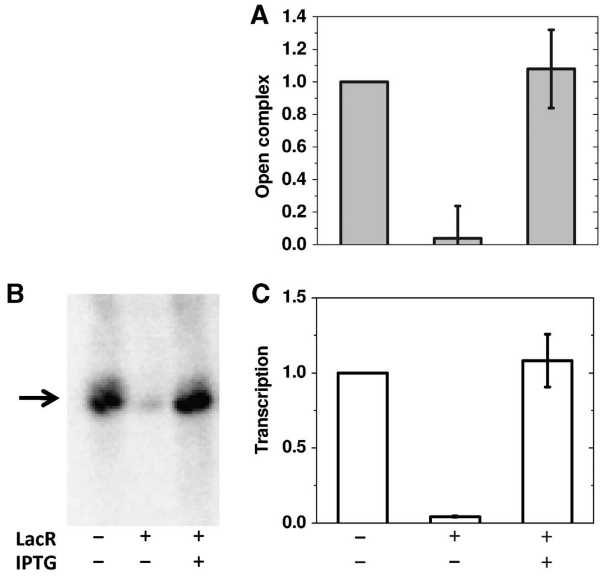

Figure 3 Parallel inhibition of open complex formation and transcript synthesis by Lac repressor (LacR) and its reversal by IPTG. (**A**) Relative amounts of open complex formation determined by applying the statistical partitioning model to the data of Figure 2C (see text). (**B**) Transcript synthesis observed by denaturing gel electrophoresis of bulk samples of radiolabelled transcript RNA (arrow) produced in a single round of transcription. (**C**) Relative amounts of transcript in **B**.

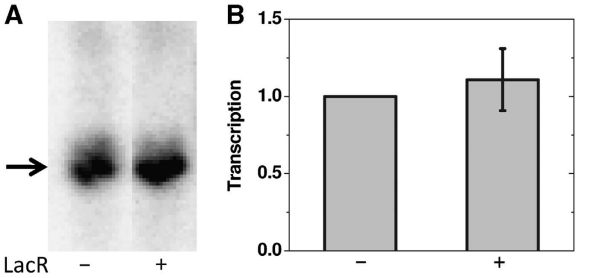

Figure 4 Lac repressor (LacR) does not inhibit transcription from pre-formed open complexes. (**A**) Denaturing gel electrophoresis showing radiolabelled run-off transcripts (arrow) produced from pre-formed open complexes with or without challenge by 200 nM Lac repressor for 15 min at 37°C. Images are of non-adjacent lanes of the same gel shown in Figure 3B. (**B**) Relative amounts of transcript in **A**.

interpretation of previous work on the repression mechanism (see Discussion).

Lac repressor represses transcription by inhibiting open complex formation

Having examined the nature of RNAP interactions with the promoter DNA, we proceeded to examine the effects of Lac repressor on these interactions. When RNAP and promoter DNA are incubated together in the presence of Lac repressor, the number of competitor-resistant complexes dropped to the level observed in the non-promoter control (Figure 2C), and a separate photobleaching experiment shows that 100% of complexes (25 of 25 observed) now contain only a single RNAP molecule. Addition of IPTG, a small-molecule inducer that largely eliminates repressor binding to DNA, restores the number of complexes to the level seen in the absence of repressor (Figure 2C). In contrast, non-promoter DNA binding is unaffected by Lac repressor. Thus, the binding attributed to open complexes is inhibited by Lac repressor (and not by Lac repressor plus inducer), consistent with an inhibition of open complex formation mechanism and inconsistent with inhibition of promoter escape.

We can use the statistical partitioning model, together with the data in Figure 2C, to measure whether an experimentally detectable quantity of open complexes form on promoter DNA molecules in the presence of Lac repressor (see Supplementary Methods). The amount of open complex formation in the presence of repressor is reduced by $96 \pm 20\%$ (Figure 3A). Thus, to the limit of detection in this experiment Lac repressor completely blocks open complex formation.

To confirm that the elimination of open complex formation by Lac repressor results in a quantitatively equivalent reduction of RNA transcript synthesis, we performed conventional bulk experiments that measured the amount of run-off transcripts produced from open complexes in a single round of transcription (Figure 3B). We measured the amount of mRNA produced in bulk samples of competitor-resistant promoter DNA–RNAP complexes in the presence and the absence of Lac repressor (Figure 3C). Under the conditions of this experiment, repressor caused a $95.8 \pm 0.3\%$ decrease in the amount of run-off transcript. This agrees within experimental uncertainty with the reduction in open complex formation presented above. Thus, repression of transcription at the P*lacUV5* promoter by the Lac repressor can be entirely attributed to inhibition of open complex formation.

Kinetic control of transcription initiation at Plac

Early studies of P*lac* demonstrated that open complexes are stable for >140 min under the conditions studied here (Buc and McClure, 1985; Straney and Crothers, 1987b). Therefore, if Lac repressor works by inhibiting open complex formation rather than suppressing promoter escape, it should not significantly repress transcription when it is added to *pre-formed* open complexes and subjected to the 15 min incubations used here. This prediction was confirmed in our experiments (Figure 4). These results further confirm that Lac repressor works by inhibiting open complex formation. The rate of transcription initiation *in vitro* from the open complex at the P*lacUV5* promoter is $\sim$10-fold larger than the rate of decay of the open complex into a closed, competitor-sensitive complex (Straney and Crothers, 1987b). Therefore, open complex formation is essentially irreversible under the conditions of these experiments. Once the open complex is formed, the repressor does not exert any transcriptional control.

Discussion

We used multi-wavelength single-molecule fluorescence microscopy to directly visualize individual open complexes of *E. coli* RNAP on the LacUV5 promoter. The CoSMoS technique allowed us to directly count the number of RNAP molecules bound to each promoter or non-promoter DNA tethered to a surface. Quantitative analysis of these data using a statistical partitioning model with no free parameters demonstrated that RNAP can form a competitor-resistant, kinetically stable interaction with the DNA that is independent of the canonical

promoter sequences and is transcriptionally inactive under the conditions of the experiments. Eliminating the contribution of this promoter-independent binding allowed accurate counting of the number of open complexes formed at the promoter, which in turn allowed us to demonstrate that Lac repressor completely inhibits open complex formation. Furthermore, we showed that once an open complex is formed, it is resistant to repression under our experimental conditions. Taken together, the results show that Lac repressor does not promote dissociation of competitor-resistant open complexes on a timescale that is relevant for repression, and that it does not suppress promoter escape. Each of these findings is consistent with an inhibition of open complex formation mechanism of repression (Figure 1, top). Conversely, each is inconsistent with an inhibition of promoter escape mechanism (Figure 1, bottom), in which repressor can bind to competitor-resistant open complexes and prevent promoter escape.

Repressor inhibition of open complex formation could in principle occur either by repressor preventing the initial formation of a closed complex or by repressor allowing closed complex formation, but blocking isomerization to an open complex. Both of these possibilities have been previously proposed to explain the action of other bacterial repressors. For example, the former is proposed for repression of pR by λ cI (Hawley *et al*, 1985), the latter for repression of *merTPCAD* by MerR (Heltzel *et al*, 1990). Our experiments do not distinguish between these two subcategories of the inhibition of open complex formation mechanism.

Although both classes of mechanism depicted in Figure 1 predict reduction of transcription by repressor, determining which is used to repress a given promoter is important because the two mechanisms can lead to qualitatively distinct regulatory behaviours (Supplementary Figure S6). For the inhibition of open complex formation mechanism, a minimal kinetic model predicts that transcription can be reduced to an arbitrarily low level by increasing the concentration of repressor. In contrast, the minimal model based on inhibition of promoter escape, predicts a minimum level of transcription no matter how large the repressor concentration. This is because in this model the rate of transcription initiation in the limit of large repressor concentrations is controlled by the dissociation of the repressor from the promoter, which is independent of repressor concentration. In contrast, in the inhibition of open complex formation minimal kinetic model the initiation rate is proportional to RNAP occupancy of the promoter, which can be made arbitrarily small by increasing repressor concentration.

In our experiments, whether a round of transcription occurs on a given DNA molecule is completely dependent on whether Lac repressor binding or open complex formation by RNAP happens to occur first, demonstrating that the two processes are not in equilibrium on the timescale relevant to transcription initiation at P*lac*. This extends earlier studies demonstrating that the P*lac* initial transcribing complex, which forms after the open complex, is immune to the effects of Lac repressor (Majors, 1975). It is commonplace in modelling of both individual promoters (Buchler *et al*, 2003; Bintu *et al*, 2005) and of transcriptional regulatory networks (Aurell *et al*, 2002; Dodd, 2004) to assume that the rate of transcript production depends only on the equilibrium occupancy of the promoter by RNAP, which is in turn modulated by transcription factors. Our results support the idea that this assumption is not always valid, a notion consistent with studies of the regulation of other promoters (e.g., Barker *et al*, 2001). Thus, when constructing models of gene regulatory systems it may be necessary to consider that transcription output is a non-equilibrium phenomenon controlled by the kinetic properties of the system, not simply its thermodynamics.

Our results give insight into the apparently contradictory conclusions of earlier studies on the repression mechanism at P*lac*UV5. Electromobility shift assays showed evidence for the formation of both competitor-sensitive and -resistant ternary complexes between P*lac*UV5-containing DNA, RNAP, and Lac repressor (Straney and Crothers, 1987a). The competitor-sensitive complexes were interpreted as evidence for repression by inhibition of open complex formation, but it was later noted (Krummel and Chamberlin, 1989; Lee and Goldfarb, 1991) that the formation and properties of the resistant ternary complexes support an inhibition of promoter escape mechanism. Consistent with this earlier work, our experiments conducted under similar ionic conditions demonstrate the formation of competitor-resistant RNAP–DNA complexes in the presence of repressor (Figure 2D). However, we demonstrate that these complexes are not formed at the promoter, but at a promoter-independent binding site on the DNA. We favour the hypothesis that the site is a unique DNA sequence, but we cannot rule out the possibility of competitor-resistant binding to a structural feature, such as one of DNA ends. Support for an inhibition of promoter escape mechanism also came from abortive initiation and single round transcription experiments (Lee and Goldfarb, 1991). We repeated those experiments attempting to closely duplicate the conditions of that earlier work (Supplementary Figure S4), but were unable to reproduce the results.

Conversely, other studies confirmed various predictions of the inhibition of open complex formation mechanism using a variety of experimental methods (Schlax *et al*, 1995, and references cited therein). Steric blockage of open complex formation by repressor is consistent with the structure of the open complex inferred by modelling the DNA segment downstream of the transcription start site (the segment in which the primary Lac operator is located) into an upstream DNA fork junction–RNAP crystal structure (Murakami *et al*, 2002). A landmark study (Schlax *et al*, 1995) demonstrated that formation and breakdown kinetics of RNAP-promoter complexes catalysing abortive transcript synthesis are consistent with a simple steric occlusion mechanism. However, this work did not directly detect the initial open complex and was not able to measure repressor effects on rate constants for the individual steps between the first closed complex and the catalytic initial transcription complex. Consequently, the data do not unambiguously exclude some inhibition of promoter escape mechanisms, such as one in which binding of repressor prevents transition from an initial open complex formed before the rate-limiting step for dissociation to a complex that is active in RNA synthesis. It was also noted that possible association of repressor with an open complex might be unfavourable at the monovalent ion concentrations used by Schlax *et al* (1995), which were significantly higher than those used in the studies (Straney and Crothers, 1987a; Lee and Goldfarb, 1991) that provided the strongest evidence for an inhibition of promoter escape mechanism.

In contrast to earlier studies, our work directly demonstrates that Lac repressor blocks open complex formation,

and does so at ionic conditions identical to those used in studies that were viewed as supporting an inhibition of promoter escape mechanism. Our results are fully consistent with the data in the cited previous studies (with the exception noted above of Lee and Goldfarb, 1991) and provide an explanation for the apparent discrepancies concerning the mechanism of repression.

Hundreds of promoters in *E. coli* are regulated by the simple repression motif (Garcia *et al*, 2010). In order to achieve a satisfactory understanding of how evolution has shaped promoter architecture and gene regulation, it is necessary to understand the physical mechanisms by which gene expression is controlled (Dekel and Alon, 2005; Poelwijk *et al*, 2007). Quantitative understanding of the molecular mechanisms of gene regulation at bacterial promoters is also important to diverse applications in biotechnology, medicine and synthetic biology (for example, engineering synthetic gene circuits that use these promoters and their regulatory modules as building blocks (Gardner *et al*, 2000; Ramalingam *et al*, 2009; Chang *et al*, 2010)). The single-molecule statistical partitioning approach used here is a powerful tool that should be generally applicable to elucidating transcription regulatory mechanisms.

Materials and methods

DNA constructs

The 516-bp promoter DNA, which contains P*lac*UV5 from −60 to +38 plus flanking vector and *lac*Z sequences (see Supplementary Figure S3), was synthesized by PCR from plasmid BN1824 (a generous gift from Anne Hochschild and Bryce Nickels; Nickels *et al*, 2004) and the labelled primers 5′-/biotin/ACTGGGCCTTTC GTTTTATCTGTTGTTTG-3′ and 5′-/Alexa488N/CGGGCCTCTTCGCT ATTAC-3′ (IDT; Coralville, IA). The non-promoter DNA was prepared the same way using plasmid AS1, a derivative of BN1824 in which P*lacUV5* from −36 to −6 was deleted using a QuikChange mutagenesis kit (Stratagene/Agilent, Santa Clara, CA). The deletion removes the −35 and −10 boxes of LacP1 and the −10 box of LacP2 and consequently ablates promoter activity (Supplementary Figure S7). Amplified segments of the plasmids were verified by sequencing.

Proteins and other reagents

Lac repressor was purified and stored at −80°C as described (Velkov *et al*, 2008). RNAP holoenzyme was purchased from Epicentre (Madison, WI). σ^{70} was a kind gift from Robert Landick. IPTG was purchased from Novagen (Merck, Darmstadt, Germany). Streptavidin was purchased from Prozyme (Hayward, CA), diluted in diethylpyrocarbonate-treated water to a final concentration of 10 mg/ml, and stored at −80°C. Radiolabelled rNTPs were obtained from Perkin Elmer (Waltham, MA). Unlabelled rNTPs were from Roche Applied Science (Indianapolis, IN). Polyethylene glycol (PEG)-5000 succinimidyl carbonate and biotin-PEG5000-succinimidyl carbonate were purchased from Laysan Inc. (Arab, AL).

Labelled RNAP holoenzyme

SNAP-tagged *E. coli* core RNAP ($\alpha_2\beta\beta'\omega$) was a generous gift from Robert Landick, Rachel Mooney and Abbey Vangeloff. In this preparation, the C terminus of β′ is fused to the linker Leu-Asp followed by the initiating Met of the SNAP26b tag sequence encoded by the plasmid pSNAP-tag(T7) (New England Biolabs), the SNAP26b sequence and a His10 tag inserted at the *Bam*HI site at the end of the SNAP26b sequence. The enzyme (1.6 μM) in 20 mM Tris–Cl^-, pH 8.0, 130 mM KCl, 8 mM $MgCl_2$, 10 μM $ZnCl_2$, 1 mM DTT was incubated with an equimolar amount of SNAP-Surface 647 (New England Biolabs) at room temperature for 30 min, and then supplemented with 30% glycerol, frozen in liquid nitrogen, and stored at −80°C. Core RNAP labelling stoichiometry was measured (Supplementary Methods) to be 68 ± 5%. Holoenzyme was reconstituted by mixing 800 nM σ^{70} with 800 nM labelled core RNAP, at 25°C for 20 min (19 mM Tris–Cl^-, pH 8.0, 115 mM KCl, 10 mM NaCl, 7 mM $MgCl_2$, 9 μM $ZnCl_2$, 0.9 mM DTT, 0.01 mM EDTA, 29% glycerol). The rate of initiation on P*lac*UV5 by the labelled holoenzyme was similar to the one determined for the untagged holoenzyme (Supplementary Figure S2).

RNAP–DNA complexes

RNAP–DNA complexes were formed by incubating 8 nM DNA and 80 nM RNAP holoenzyme in 10 μl of binding buffer (110 mM KCl, 50 mM Tris–Cl, pH 7.9, 8 mM $MgCl_2$, 0.11 mg/ml bovine serum albumin, 1 mM dithiothreitol) for 25 min at 37°C. When Lac repressor was added, 200 nM repressor was either preincubated with the DNA for 15 min prior to the addition of RNAP, or else added to preformed open complexes. IPTG, when present, was added to the binding buffer to a final concentration of 1 mM.

Single-molecule fluorescence microscopy

Flow chambers were prepared and the glass surfaces were derivatized with a mixture of PEG and PEG-biotin as described (Friedman *et al*, 2006). Excess PEG was flushed from the flow chamber with 400 μl binding buffer. The chambers were then flushed with 100 μl streptavidin (200 nM in binding buffer), incubated 30 s, and then flushed with 200 μl binding buffer. Next, RNAP–DNA complexes (1 μl) were diluted into 99 μl of 37°C buffer containing an O_2 scavenging system (Friedman *et al*, 2006), 20 μg/ml of competitor DNA (salmon sperm DNA, Sigma-Aldrich, cat. no. D1626) or 16 μg/ml heparin (Sigma-Aldrich, cat. no. H4784) and incubated for 5 min at 37°C. The mixture was then introduced into the flow chamber and imaged using the multi-wavelength TIRF microscope, as previously described (Friedman *et al*, 2006). Laser powers measured at an intermediate point in the excitation optical path were 130 μW (632 nm) and 500 μW (488 nm), except where otherwise noted. Bulk assays confirmed that the O_2 scavenging system had no significant effect on transcription (Supplementary Figure S5). IPTG was found to have no inducer effect in the presence of O_2 scavenger, presumably because the compound was degraded by glucose oxidase.

TIRF images were analysed using custom software implemented in Matlab (Mathworks, Natick, MA). In spot-counting experiments, accidental colocalization of spots due to overlap of their point-spread function had only a negligible effect on the measured colocalization fraction when the number of spots per field of view was <150 (Supplementary data), which was the case in all experiments. In a typical photobleaching experiment, <10% of complexes did not show distinct bleaching steps; these were excluded from analysis. The mean photobleaching time was 7.9 ± 1.8 s, whereas the time resolution of our experiment was 0.5 s. Therefore, the fraction of events missed due to photobleaching prior to the first time point was small (6%), and was therefore neglected in our analysis.

Bulk transcription assays

To 10 μl RNAP–DNA complexes, we added 1 μl of 10 × transcription start buffer (10 mM ATP, 500 μM GTP, 500 μM UTP, 500 μM CTP, 1 μCi/μl [α-^{32}P] UTP and 0.1 mg/ml heparin). The resulting single round transcription reaction was incubated at 37°C for 15 min, and then stopped by addition of an equal volume of 2 × stop buffer (10 mM EDTA, 7 M urea, 1 mg/ml bromophenol blue, 1 mg/ml xylene cyanol). The samples were heated (95°C, 7 min), and a 4 μl aliquot was then loaded onto a 12% polyacrylamide gel (19:1 acrylamide/bis-acrylamide in 0.5 × TBE) and run at 1000 V for ~2 h. Radioactive RNA bands were detected via a phosphorimager (Typhoon 9410, GE Life Sciences) and quantified using ImageQuant software (Amersham, Piscataway, NJ).

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (http://www.embojournal.org).

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Conflict of interest

The authors declare that they have no conflict of interest.

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