7.3.4 Clamp conformation during initial RNA synthesis

A previous single-molecule confocal spectroscopy study produced results suggesting that the clamp does not open during de novo RNA synthesis (27). However, as this study was not able to monitor molecules in real-time, it remained a possibility that transient or partial clamp opening events occurred during RNA synthesis but were not captured. Using TIRF microscopy and building upon the success we have had in observing real-time RNA synthesis (see chapter 6), we are able to confirm that clamp opening does not occur during RNA synthesis.

We immobilized pre-formed RP₀ (using fully complementary DNA) and added NTP reaction mixtures to the observation chamber during data acquisition. In one experiment our NTP reaction mixture permitted the synthesis of short RNAs up to 7-nt in length (RP₁RTC57). In a second experiment, our NTP mixture permitted the synthesis of RNAs up to 11-nt in length (RD₁₁). In the RD₁₁ experiment, it was expected that some molecules would escape the promoter and form stable elongation complexes. We performed experiments at both 20 and 200 ms temporal resolutions but failed to see any indications of clamp opening – fully or partially – upon the addition of the NTP reaction mixtures (Fig. 7.8). In addition, no FRET transitions were observed in these experiments.

Our observations suggest that each nucleotide addition cycle is not accompanied by a clamp opening and closing cycle, and that RNAP translocates along the promoter DNA during initial RNA synthesis with the clamp in a closed conformation. We propose that it is this closed clamp conformation that confers processivity to the transcribing RNAP. Our results also suggest that the clamp remains closed during the release of abortive RNAs. This leaves the NTP-entry channel as the only possible route through which abortive RNAs might be released. We note that our 20 ms temporal resolution data is not able to eliminate the possibility that each nucleotide addition cycle is accompanied by clamp opening and closing cycles that occur on faster timescale than the temporal resolution afforded by our assay. However, in agreement with our
findings, earlier solution confocal studies with 1 ms temporal resolution also failed to detect clamp opening and closing transitions during RNA synthesis (27).

Figure 7.8. RNAP clamp conformation during initial RNA synthesis. (A) Stacked E* histograms and Gaussian fits for immobilized RP₀ (top), RP_{ITC≤7} (middle), and RD_{ε11} (bottom). Temporal resolution is 20 ms. 100 molecules contribute to each histogram. (B) Example time trajectories showing no change in FRET upon the addition of the NTP reaction mixture. Top figure shows 20 ms temporal resolution data; bottom figure shows 200 ms temporal resolution data. The NTP addition time point is marked with a dashed line.

7.3.5 Effect of switch-region-target inhibitors on the RNAP clamp conformation

The RNAP switch region is located at the base of the RNAP clamp and is considered an important antibiotic target (123, 203). Recently, it was shown that a number of switch-region-target inhibitors modulate the RNAP clamp conformation. In this section, we show what affect the switch-region-target inhibitors, Myxopyronin (Myx) and Lipiarmycin (Lpm), have on the RNAP clamp conformation using our smFRET assay.

In a first set of experiments, we incubated RNAP holoenzyme with Myx to form an RNAP-Myx complex. These complexes were immobilized on a PEG-passivated surface and imaged using TIRF microscopy in imaging buffer supplemented with 20 μM of Myx. The resulting E* histogram showed that Myx depopulates the open clamp conformation
and stabilises the closed clamp conformation (Fig. 7.9A). Real-time Myx binding experiments to test whether the effect of Myx on the clamp conformation was immediate upon binding were then carried out. The time trajectory data showed that if the clamp was in a closed conformation, no change was observed when Myx was added; however, if the clamp was in a stable open conformation, Myx binding appeared to induce clamp closure (Fig. 7.9B–C). Interestingly, if the clamp was exhibiting dynamics, Myx binding also appeared to induce stable clamp closure (Fig. 7.9C). We note however, that clamp closing did not occur in a highly synchronous manner across all molecules; in fact, in some cases, the delay lasted up to many tens of seconds. This asynchrony is reflected in Fig. 7.9B.

**Figure 7.9 RNAP clamp response to Myxopyronin.** (A) Stacked E* histograms of RNAP holoenzyme (top), and holoenzyme in the presence of 20 μM Myx (bottom). Temporal resolution is 20ms. Each histogram shows data of 100 molecules. (B) Heat map of the E*
distributions of FRET time trajectories in a single field-of-view. Blue to red colours represent an increasing number of events. 48 molecules are shown. (C) Example time trajectories of fluorescence intensities and E* traces showing clamp closing upon the addition of Myx to the observation chamber. Data in B–C was taken at a temporal resolution of 200ms.

Figure 7.10. RNAP clamp response to Lipiarmycin. (A) Stacked E* histograms of RNAP holoenzyme (top), and holoenzyme in the presence of 20 μM Lipiarmycin (bottom). Temporal resolution is 20ms. Each histogram shows data of 100 molecules. (B) Heat map of the E* distributions of FRET time trajectories from a single field-of-view. Blue to red colours represent an increasing number of events. 55 molecules are shown. (C) Example time trajectories of fluorescence intensities and E* traces showing clamp opening upon the addition of Lpm to the observation chamber. Data in B–C was taken at a temporal resolution of 200ms.
Next, we performed the same experiments described above, this time using Lpm as the inhibitor. Interestingly, the E* histogram of RNAP-Lpm complexes showed that Lpm depopulates the closed clamp conformation and stabilises the open clamp conformation (Fig. 7.10A). Real-time binding experiments showed that Lpm was able to induce clamp opening in molecules that had either a stable closed clamp conformation or the clamp undergoing opening and closing transitions (Fig. 7.10B–C); Lpm binding appeared to have no effect on molecules that already had a stable open clamp conformation. Interestingly, and in contrast to Myx binding-induced clamp closure, clamp opening upon Lpm binding appears to be highly synchronous (Fig. 7.10B–C). Although having said this, a number of molecules did show clear delayed responses to Lpm of up to 3 seconds (Fig. 7.10C).

There are a number of possible explanations for this delay in response. The first is incomplete mixing when the inhibitor is added to the well. We believe this is unlikely considering the fact that the difference in synchrony between Lpm and Myx was reproducible between experiments. The addition of Lpm invariably led to a well synchronised decrease in FRET. The addition of Myx invariably led to an asynchronous increase in FRET. We suggest that if the asynchrony was simply due to mixing, the delayed FRET change upon the addition of Lpm and Myx would be similar; hence we suggest that mixing is not the main factor producing this asynchronous FRET change. A second possible explanation is that it takes longer for Myx to diffuse to its binding site compared with Lpm. However, given the fact that the diffusion coefficients of the two inhibitors are both \( D_{Myx} \sim 3.4 \times 10^{-10} \text{m}^2\text{s}^{-1} \), differences in diffusion times are unlikely to account for the different clamp response times. A third possibility is that the binding sites of the two inhibitors may be situated in different locations. Mutational and structural studies, however, indicate that the Myx and Lpm binding sites partially overlap (204). A fourth possibility is that there is a difference in the accessibility of the binding sites for the two inhibitors. Perhaps the clamp must be in a specific conformation for the inhibitors (especially Myx) to bind? We note that increasing the concentration of Myx 10-fold still produced the delay seen in Fig. 7.9, and supports this
fourth possibility. Future studies using microfluidics to eliminate differences in mixing times will investigate the asynchrony further.

7.4 Discussion

7.4.1 RNAP clamp adopts a primarily open conformation in holoenzyme

A previous single-molecule study showed that the RNAP clamp changes conformation at different stages of the transcription cycle (27). This confirmed a long-standing hypothesis based on RNAP crystal structures that the RNAP clamp is a mobile domain. However, the single-molecule study enabled only short snapshots (~1ms) of the clamp conformation in diffusing RNAP molecules, and therefore did not provide detailed information regarding the kinetics of the presumed clamp opening and closing transitions. In this work, we utilized an Ab-immobilization strategy (see chapter 4) to monitor the RNAP clamp conformation over many seconds.

Our work has revealed that in RNAP holoenzyme, the clamp adopts two conformational states, an open conformation and a closed conformation, with the open being the most populated state (Fig. 7.4A). We propose that the open and closed states are the same as those observed in (27). Our results showed no evidence of a bona fide clamp-collapsed state. Our raw FRET data, that is, data before applying selection criteria, showed a population of molecules with $E^*$ values higher than that corresponding to the closed clamp conformation ($E^*>0.5$). This population could potentially be interpreted as a clamp-collapsed state; however, we found that data frames that contribute to the $E^*>0.5$ population arise from molecules that display aberrant photophysical behaviour (Fig. 7.5). These molecules could not be used to make reliable biological interpretations, and were therefore discarded from further data analysis. We propose that the earlier confocal spectroscopy study was unable to exclude these molecules from data analysis, which meant they were included in the FRET histograms. Based on this logic, we propose that RNAP holoenzyme does not adopt a clamp-collapsed conformation.
The results described above have implications for the RP0 formation mechanism. There are currently two favoured models for the mechanism of transcription bubble melting: *bend-load-open* and *open-bend-load* (66, 68). In the former, full DNA unwinding occurs after the duplex DNA has entered the DNA binding cleft and is promoted by interactions between the clamp and downstream DNA (76). In the latter, DNA unwinding must occur before the promoter DNA enters the binding cleft (74). Early crystal structures of RNAP holoenzyme alone and holoenzyme bound to a fork-junction promoter DNA fragment showed the clamp in a closed conformation that rendered the DNA binding cleft too narrow to accommodate double-stranded DNA (dsDNA) (49). This led to proposals that promoter DNA enters the binding cleft as single-stranded DNA (ssDNA) and therefore must melt outside of the binding cleft (17, 49). Other studies however, have indicated that transcription bubble opening can occur inside of the cleft (66). Our work shows that the holoenzyme clamp adopts mainly an open conformation, and is therefore able to accommodate dsDNA. This supports a model in which bubble melting can occur inside of the binding cleft.

Our time trajectory data revealed considerable heterogeneity in the clamp conformation among RNAP holoenzyme molecules. Behaviours were classified as open (clamp remains stably open for many seconds), closed (clamp remains stably closed for many seconds), and dynamic (clamp undergoes clear conformational transitions between the open and closed conformations). This heterogeneity was reminiscent of that observed for DNA polymerase I Klenow fragment (KF, see chapter 4). As with KF, it is unclear what gives rise to these differences in behaviour, or if indeed the heterogeneity has any functional importance. Future work involving the vesicle-immobilization of RNAP holoenzyme will confirm that the Ab-immobilization technique is not the cause of the heterogeneity, and will explore possible functions of the heterogeneity.
7.4.2 RNAP clamp is closed in $\text{RP}_0$ and remains closed during RNA synthesis

Next, we monitored the clamp conformation in $\text{RP}_0$ and tested whether the clamp conformation changes in response to NTPs. We found that the clamp adopted a stable closed conformation in $\text{RP}_0$ and showed no change in conformation upon the addition of NTPs at both 20 and 200 ms temporal resolutions. This suggests that the clamp remains fully closed as RNAP translocates along the DNA during initial RNA synthesis. We propose that contacts between the RNAP clamp and promoter DNA are not sufficiently strong to impede forward movement of the polymerase, and that the closed clamp conformation stabilises the RNAP-promoter DNA complex during initial transcription. We highlight that our findings provide new insight into whether or not the RNAP clamp opens to permit RNAP forward movement during RNA synthesis? We can confirm that, at least for the initial stages of RNA synthesis and promoter escape, clamp opening and closing cycles that occur on a slower timescale than the temporal resolution afforded by our studies (20 ms) do not occur.

7.4.3 Switch-region-target inhibitors differentially modulate the clamp conformation

Mutational and biochemical studies have shown that the RNAP switch region is the target for a number of small molecule inhibitors including Myx and Lpm. Myx inhibits the $\text{RP}_C$ to $\text{RP}_O$ transition, which occurs late in the $\text{RP}_O$ formation pathway, and is the step at which the transcription bubble opens. Lpm on the other hand inhibits an earlier step in the $\text{RP}_O$ formation pathway (68). A previous single-molecule fluorescence study revealed that Myx binding to the RNAP switch region stabilises the closed clamp conformation. Based on this finding, the authors proposed that Myx-induced clamp closure prevents the entry of DNA into the active centre cleft, and therefore inhibits RNAP function by preventing the formation of $\text{RP}_O$ (27). Investigations into the inhibition mechanism of Lpm have raised the possibility that Lpm stabilises the open clamp conformation (68, 80), however, this has yet to be shown directly.
In the work presented in this chapter, we showed that Myx stabilises the closed clamp conformation by inducing RNAP clamp closure upon binding to RNAP (Fig. 7.9). In addition, we showed that Lpm binding to RNAP induces clamp opening (Fig. 7.10). However, in both cases a proportion of molecules show a delayed response to inhibitor binding. Our results also indicated that neither RNAP-Myx nor RNAP-Lpm complexes exhibit clamp conformational dynamics i.e. the clamp is locked in a closed or open conformation, respectively. Our findings support the hypothesis that Myx and Lpm function by a hinge-jamming mechanism in which they lock the clamp in a single conformation, which prevents RNAP from proceeding along the transcription initiation pathway (123). It is interesting that inhibitors that bind to the same general switch region of RNAP can have very different effects on the RNAP clamp conformation; Lpm induces a stable open clamp state while Myx induces a stable closed clamp state. This suggests that both the open and closed conformations are functionally important in the transcription mechanism, which leads us to speculate that both conformations are obligatory intermediates in transcription initiation.

The fact that Myx appears to inhibit the RP\textsubscript{C} to RP\textsubscript{O} transition by locking the RNAP clamp in a closed conformation strongly suggests that transcription bubble melting necessarily occurs inside the DNA binding cleft. This is because, if melting could occur outside of the binding cleft, clamp closure would have no or little effect on the RP\textsubscript{C} to RP\textsubscript{O} transition. We take this observation as further support for a model in which transcription bubble opening occurs inside of the DNA binding cleft.

7.4.4 Integrating work from different chapters to understand the RP\textsubscript{O} formation mechanism

The results presented in chapter 5 demonstrated that the transcription bubble readily undergoes opening and closing transitions even once a stable heparin-resistant complex has been formed at the \textit{lac} promoter. The work presented in this chapter showed that the clamp remains stably closed once a heparin-resistant complex has been formed. These observations suggest that bubble opening and closing is independent of
the clamp conformation, and presumably occurs when promoter DNA is loaded inside of the DNA binding cleft. This, combined with the observations that holoenzyme adopts primarily an open clamp conformation, and Myx inhibits transcription bubble opening by inducing clamp closure, support a model in which transcription bubble opening occurs inside of the DNA binding cleft. We note however that our experiments do not monitor the clamp and bubble conformations in real-time from initial binding of RNAP to promoter DNA through to RP₀ formation. In this situation, it is possible that the order of events (bubble opening and clamp closing) may be different. Future work combining our transcription bubble assay with the clamp conformation assay is needed to determine exactly at which step in the RP₀ formation pathway bubble opening and clamp closing occur.

7.5 Conclusion

In this chapter we presented results that shed new light into RNAP clamp conformational dynamics. Our work is the first to demonstrate clamp opening and closing cycles directly, and in doing so confirms earlier proposals that the clamp is a mobile domain. Our work demonstrates that the clamp closes in RP₀ and does not open during subsequent stages of transcription initiation. Our investigations have revealed that switch-region-target inhibitors are able to modulate the clamp conformation differentially, which may have implications for the future development of antibiotics. Combined with results presented in earlier chapters, our work provides a detailed view of the protein and DNA conformational changes that occur in transcription initiation. In the future, we intend to extend the investigations presented in this chapter to monitor the transcription bubble and clamp conformations in parallel. These experiments might involve the use of 3-colour or 4-colour FRET to monitor multiple distance changes. This should allow us to address questions such as: when does the clamp close in the RP₀ formation pathway? And does clamp closing precede or follow transcription bubble opening? Finally, we note that the work presented in this chapter can be extended to monitor the clamp conformation during elongation and termination.
7.6 Contributions

I performed all work described in this chapter except for the following contributions made by others: Abhishek Mazumder based in Dr. Richard Ebright’s group (Rutgers University, USA) purified and labelled RNAP holoenzyme. Geraint Evans (Oxford University, UK) provided custom-written MATLAB code that was modified to visualise data.
Chapter 8: Conclusion

Efforts to investigate transcription initiation using TIRF microscopy have been ongoing for almost a decade in the Kapanidis group. However, there have been many issues encountered during that time, which have delayed progression and limited productivity. In this thesis, we presented two methods to immobilize proteins and protein-DNA complexes for smFRET studies. One involved encapsulating biomolecules within lipid vesicles that were then immobilized in their entirety. The other involved the use of an antibody to anchor proteins of interest to a surface. We successfully applied these immobilization techniques to investigate conformational dynamics in RNAP-promoter DNA complexes. We found that immobilizing RNAP-promoter DNA complexes using the vesicle immobilization technique was technically demanding and therefore less preferential for investigations of the mechanism of RNA synthesis. This being the case, we performed all experiments in chapters 6 and 7 using the antibody-immobilization approach. However, we note that the availability of two independent immobilization strategies will enable us to exclude the possibility that interesting behaviours are artefacts of one or other immobilization strategy in future investigations.

Our investigations into the conformational dynamics of RNAP-promoter DNA complexes showed that the transcription bubble in these complexes undergoes large-scale dynamic fluctuations between states we define as open and closed. This finding shows that the bacterial RNAP, like the mitochondrial and eukaryotic RNA polymerases, exhibits large structural changes in the transcription bubble when bound to promoter DNAs. We speculate that the observed dynamics is functionally important in transcription initiation. Our results also revealed the presence of stable RNAP-promoter DNA complexes that have transcription bubble conformations intermediate between what is expected for the closed complex (RP_c) and the open complex (RP_o). The first intermediate appears to have the bubble in an entirely closed conformation, while a second intermediate has the bubble neither fully open nor fully closed. In short, we
observed considerable static and dynamic heterogeneity and speculate that this heterogeneity may provide an explanation for the heterogeneity in transcription reactions reported in the literature (84, 100).

The underlying cause of the observed heterogeneity is currently unclear. At the most fundamental level, there are two possibilities. The first is that the heterogeneity is an artefact. The second is that the heterogeneity is real and plays some functional role in the bacterial transcription mechanism.

There are a number of potential experimental sources that might lead to the observed heterogeneity being an artefact. Three possibilities are: 1) the fluorophore-stabilising factors added to TIRF experiments (trolox and the oxygen scavenging system), 2) the fact that experiments are performed in vitro with a purified enzyme, and 3) experiments are performed using TIRF microscopy after immobilizing the complex of interest. We believe that these explanations for the heterogeneity are unlikely to be true for the following reasons. Firstly, confocal, TIRF, and in vitro transcription assay studies performed with and without the fluorophore stabilising factors did not alter our results.20 Also, approximately the same behaviour was observed when enzymes obtained from different sources were used in our experiments – we tested enzyme preparations prepared by different research groups as well as commercial enzyme samples purified using different purification techniques. This suggests that the purification of the enzyme does not cause the heterogeneity observed. In addition, we immobilized the RNAP-DNA complex for TIRF experiments using two very different immobilization strategies. The antibody (Ab) immobilization approach anchored the protein to the surface directly; the other immobilized the complex by encapsulating it within a vesicle, which was then anchored to the surface. In the vesicle immobilization approach, the complex does not come into contact with the PEG surface. In the Ab immobilization approach, the antibody forms specific interactions with the protein. Despite the very different interactions present in the immobilization approaches, the same static and dynamic heterogeneity is observed using both techniques. It is unlikely

20 For fluorescence studies, however, we did see an increase in noise and aberrant fluorophore behaviour (as expected) when the fluorophore stabilising factors were removed.
that both immobilization techniques would cause the exact same heterogeneity; therefore we suggest it is more likely that the observed heterogeneity is an inherent feature of RNAP, at least, in an in vitro setting. Future studies are needed to test whether similar heterogeneity is observed in vivo.

In addition to investigating the cause of the heterogeneity, it will be of considerable interest to perform statistical analyses of our time trajectory data to quantitatively characterise the observed heterogeneity. Specifically, it will be intriguing to statistically test whether our dynamic molecules conform to a single dynamic population with well-defined kinetic parameters or not. Likelihood ratio tests using the outputs from individual HMM fits could be used to determine if molecules are actually behaving differently (205). This type of analysis could also provide insight into whether the observed static molecules truly conform to an independent population. Current work is addressing these questions.

We investigated the mechanism of initial RNA synthesis using our smFRET assay. Our results showed abortive cycling directly for the first time, which enabled us to characterise the lifetime of the elusive scrunched complexes. We investigated the role of the σR3.2 finger in abortive initiation, and demonstrated that this structural element stabilises short RNAs at the active site and acts as a barrier to the synthesis of long (>5-mer) RNAs. Our smFRET assay was sufficiently sensitive for us to extend our investigations to promoter escape and address a number of open questions regarding the mechanism of escape. Our results suggest that abortive cycling is not an obligatory process for promoter escape, and that in most cases RNAP performs few (0–2) detectable abortive cycles before escaping the lac consensus promoter. Future biochemical studies investigating the distribution of RNA products at our promoter under promoter escape conditions will enable us to test our hypothesis regarding the distribution of abortive and productive behaviours. Furthermore, our results suggest that there is some plasticity regarding the exact position at which promoter escape occurs. Our results also reveal an intriguing subpopulation of molecules that are unable to escape the promoter and may correspond to moribund complexes previously reported in the literature (100, 102).
In our final set of experiments, we investigated the conformational dynamics of the RNAP β’ clamp. Our results confirm a number of hypotheses put forward in a previous single-molecule study of the clamp conformation (27). In addition we make a number of novel observations. We showed for the first time that the clamp in holoenzyme is able to undergo dynamic fluctuations between open and closed conformations, and our results support a model in which transcription bubble melting occurs after double-stranded DNA has inserted inside the DNA binding cleft. Finally, our results showed that different inhibitors function by locking the clamp in an open or closed conformation. This highlights the functional importance of clamp conformational changes in transcription.

8.1 Future work

The immobilization techniques demonstrated in this thesis have enabled us to make major advances in our efforts to uncover new information regarding the transcription initiation mechanism using single-molecule techniques. Our work raises a number of intriguing questions that we intend to investigate further in the future.

A major goal is to uncover the exact role played by heterogeneity observed throughout this thesis. We are particularly interested in investigating whether the heterogeneity observed in the RNA synthesis reaction has a regulatory function. The Kapanidis group is currently testing a number of potential regulatory molecules that may influence the behaviour of RNAP during initial transcription. There are also plans to investigate what role the promoter sequence and the initial transcribed sequence may play in RNAP pausing during initial RNA synthesis. Pausing is known to play an important regulatory role in elongation (131), and it will be particularly interesting to test whether pausing in initiation also has some regulatory function. Future experiments will involve experiments exploring what effect regulatory proteins such as GreA and GreB have on our abortive initiation and promoter escape assays.
Future work will also investigate whether the behaviours observed at the *lac* consensus promoter in abortive initiation and promoter escape – such as the position at which escape occurs and the number of abortive cycles that precede promoter escape – are unique to this promoter or common features of numerous promoters. We are currently investigating the abortive initiation and promoter escape behaviours of T5 *N25*, T7 *A1*, and *rrnB P1* promoters.

We are interested in investigating further the transcription bubble dynamics observed in chapter 5. We hypothesized that the bubble dynamics plays a functional role in transcription initiation and intend to test this hypothesis in future studies. We will investigate whether other promoters such as the *rrnB P1* promoter show similar bubble opening and closing transitions. We will also test whether the subpopulations of complexes exhibiting closed, partially open, open, and dynamic transcription bubbles exhibit different tendencies to perform *de novo* RNA synthesis. This will require the deconvolution of the FRET signals due to transcription bubble dynamics and abortive initiation dynamics. We also hope to use our transcription bubble smFRET assay to investigate which stages of the open complex (RP₀) formation pathway are targeted by inhibitors and regulatory molecules. These studies will complement future investigations looking at the effect of inhibitors and regulatory molecules on the clamp conformation. We are currently investigating the effect of ppGpp and DksA on transcription bubble opening, and the clamp conformation.

In chapter 7, we demonstrated our ability to monitor the clamp conformation at different stages of transcription in real-time. Future investigations will extend this work to investigate the clamp conformation during elongation and termination. We are specifically interested in testing whether clamp opening accompanies RNAP pausing and backtracking during elongation, and uncovering what clamp conformational changes occur during termination.

We anticipate that many of the future investigations outlined above will utilise the vesicle and antibody immobilization strategies demonstrated in chapter 4. We hope that these immobilization approaches will also be used to investigate the transcription mechanisms of archaeal and eukaryotic RNA polymerases. It will be particularly
interesting to see whether the heterogeneity and mechanistic details of initial transcription reported in this thesis, are also features of the archaeal and eukaryotic transcription mechanisms.

Finally, we note that other members of the Kapanidis group have been successful in internalising fluorescently-labelled promoter DNA fragments into live bacterial cells and observing FRET signals that may indicate the formation of active transcription complexes. This raises the exciting possibility of performing experiments with the level of detail demonstrated in this thesis, inside of live bacterial cells.
Appendix A: *E. coli* RNAP-promoter DNA complex structural model

A.1 RP₀ model

Throughout this thesis we use a structural model of *E. coli* RP₀ to make structural predictions and measure distances. The *Thermus aquaticus* RP₀ model from (49) was used as the basis for our *E. coli* RP₀ model. We combined the following elements in our model:

- Protein from *E. coli* holoenzyme (PDB 4IGC) (16);
- DNA from the *T. aquaticus* RP₀ model [-60 to -5, non-template strand; -60 to -13, template strand; +13 to +21 of both strands (49)];
- DNA from the *T. thermophilus* RNAP-fork junction DNA structure [-4 to +12 template strand; -12 to +12 non-template strand (PDB 4G7H) (72)];
- B-form DNA (+22 to +35)

Our model was created by first performing a structural alignment between the *T. aquaticus* RP₀ model and the *E. coli* holoenzyme structure. The resulting intermediate model (I₁) was then structurally aligned with the *T. thermophilus* RNAP-fork junction DNA structure to form an intermediate model (I₂). Finally, B-form DNA was generated using 3D-DART (206) and overlapping bases aligned onto the +21 end of our intermediate model to generate our final *E. coli* RP₀ model (Fig. A1A). We justify our structural alignment approach in creating an *E. coli* RP₀ model based on the following observations: 1) Sequence identity between *E. coli* and *T. aquaticus* and between *E. coli* and *T. thermophilus* is high at ~30%, and 2) the *E. coli* RNAP structure is highly similar to both the *T. aquaticus* and *T. thermophilus* RNAP structures (16) with root-mean-square-deviation (RMSD) values for atom deviations of 2.7 Å (*E. coli & T. aquaticus*) and 2.3 Å (*E. coli & T. thermophilus*).
Figure A1. *E. coli* RNAP-promoter DNA complex structural models. (A) Cut-away structural model of *E. coli* RP<sub>O</sub>. Major structural regions are highlighted. σ<sup>70</sup> is shown in orange and core protein is shown in grey except for the regions that protrude in front of the cut-away plane, which are yellow. The β subunit and σR1.1 are omitted for clarity. The template strand is shown in dark blue; the non-template strand in light blue. Downstream DNA ends at +25 in the figure. (B) Cut-away structural model of *E. coli* RP<sub>ITC6</sub>. Scrunched DNA is not shown. Images were created using Pymol.

**A.2 RP<sub>ITC</sub> models**

In this section we describe how we generated RP<sub>ITC</sub> structural models used to determine what length nascent RNAs need to be to clash with the σR3.2 finger in chapter 6.

We performed a structural alignment between our *E. coli* RP<sub>O</sub> model (section A.1) and the *T. thermophilus* RNAP elongation complex (PDB 2O5I) (25) in order to incorporate the RNA chain atoms from the elongation complex into our RP<sub>O</sub> model. We modelled RP<sub>ITC3</sub>, RP<sub>ITC4</sub>, RP<sub>ITC5</sub>, and RP<sub>ITC6</sub> by limiting the length of the RNA at the active site to 3-, 4-, 5-, and 6-nt. Despite the high structural similarity between the *E. coli*, *T. aquaticus*, and *T. thermophilus* RNAP structures, we observed small differences at the active site that altered the length the nascent RNA needed to be to clash with the σR3.2 finger. This variation was due to different lengths of the σR3.2 loop in the three structures; we therefore used the *E. coli* structural model when interpreting the *E. coli* smFRET results presented in chapter 6.
A.3 RNAP His-tag location

In the majority of experiments performed in this thesis, we immobilize RNAP using a penta-His antibody. Here, surface-immobilized antibody anchors RNAP molecules to the surface by binding to the RNAP His-tag. The location of the His-tag on the *E. coli* RP₀ structure is shown in Fig. A2.

Figure A2. Location of RNAP His-tag. Same cut-away structural model of *E. coli* RP₀ shown in Fig. A1A viewed from behind.
Appendix B: Additional real-time RNA synthesis results

B.1 Leading edge-FRET (LE-FRET) shows similar heterogeneity in abortive initiation

In chapter 6 we demonstrated our ability to monitor initial RNA synthesis using a smFRET assay in which the donor dye was attached to the promoter DNA at position -15 and the acceptor dye attached to position +20, relative to the transcription start site (+1). To observe initial RNA synthesis using a different vantage point, we performed smFRET TIRF microscopy experiments using a LE-FRET assay. In these experiments, we used a σ⁷₀ subunit labelled at position 366 with the donor dye, Cy3B. The fluorescently labelled σ⁷₀ subunit was a gift from Dr. Richard Ebright (Rutgers University, USA), and has been used to monitor abortive initiation in a previous study (6). Fig. B1 shows a schematic of the expected fluorophore movements using this LE-FRET assay.

Figure B1. Schematic of LE-FRET assay. The penta-His antibody binds to the RNAP His-tag and anchors the RNAP to the surface (see chapter 5). The donor and acceptor dyes are labelled and shown in green and red, respectively. RNA is shown as a red strand at the active site in RPITC. The movement of the acceptor dye is shown with an arrow. RNAP and the promoter DNA are coloured as in Appendix A.
We performed RP\textsubscript{ITC≤7} experiments as described in chapter 6. In these LE-FRET experiments we used a lacCONS+2 promoter DNA fragment labelled only with an acceptor dye at the +20 position. The DNA fragment was not labelled with a donor dye (Fig. B2). Our results showed the same qualitative behaviour and heterogeneity as described in chapter 6. Two example traces of a stably scrunched molecule and an abortive cycling molecule can be seen in Fig. B3. Based on these observations we concluded that the donor dye attachment position has no influence on the behaviour of RNAP in abortive initiation. It should be noted that when the donor dye, Cy3B, was attached to σ\textsuperscript{70} it appeared to bleach more rapidly and was less bright than when attached to the promoter DNA. Presumably this was due to changes in the local environment of the fluorophore.

\begin{verbatim}
lacCONS+2 (-39/+25) - (A, +20)

5' -AGGCTGACACTTTATGCTCGTACCATATTGTGAAATGTGAGGCAGGATTAAATTTCTC-3'
3' -TCCGAACACTGAAATACGAAGCCAGGAAATTACCACTCTCCTTTAAG-5'

\end{verbatim}

**Figure B2. Promoter DNA fragment used for LE-FRET experiments.** Promoter DNA fragment labelled with ATTO647N. The black boxes indicate the -10 and -35 elements. The transcription start site is labelled, as is the fluorophore labelling site.

**Figure B3. Abortive initiation observed using a LE-FRET assay.** Example time trajectories of transcribing complexes (RP\textsubscript{ITC≤7} conditions) displaying stable scrunched behaviour (left), and cycling behaviour (right). Temporal resolution is 200 ms.
B.2  Actively transcribing complexes occasionally exhibit switching behaviour

Experiments in which we monitored the abortive cycling behaviour of RNAP molecules revealed rare instances (<1% of actively transcribing complexes) where molecules appeared to display changes in behaviour during our observation period. Fig. B4 shows example time trajectories of molecules displaying this behaviour. It is tempting to speculate that differences in conformational states in the polymerase or promoter DNA lead to these changes in behaviour. However, further experiments are necessary to determine whether this hypothesis is correct. In the future we hope to extend our observation periods well beyond what we currently achieve (~300 sec) in order to observe more example of switching behaviour.

Figure B4. Switching behaviour. Example time trajectories of transcribing complexes (RP_{ITCST} conditions) displaying changes in behaviour. Temporal resolution is 200 ms.
Appendix C: Additional dwell time distributions

C.1 RP₀ bubble dynamics

Figure C1. Kinetics of transcription bubble dynamics: extended. This figure shows the same data as in Fig. 5.15D–E except here the counts axis is plotted on a logarithmic scale. (A) Stacked plots of closed and open bubble conformation dwells for dsDNA. 852 events from 80 molecules are shown in the top panels, 692 events from 71 molecules are shown in the middle panels, 924 events from 85 molecules are shown in the bottom panels. (B) Stacked histograms of closed and open bubble conformation dwells for pmDNA. For figures A and B, wt data is shown in the top panel, ΔJaw mutant data in the middle panel, Δ 3.2 mutant data in the bottom panel. 1165 events from 87 molecules are shown in the top panels, 2001 events from 101 molecules are shown in the middle panels, 2082 events from 105 molecules are shown in the bottom panels. Temporal resolution is 20 ms.
Figure C2. ApA effect on kinetics of transcription bubble dynamics: extended. This figure shows the same data as in Fig. 5.16 except here the counts axis is plotted on a logarithmic scale. (A) Stacked plots of closed and open bubble conformation dwells for dsDNA. 852 events from 80 molecules are shown in the top panels, 793 events from 78 molecules are shown in the bottom panels. (B) Stacked histograms of closed and open bubble conformation dwells for pmDNA. For figures A and B, wt data is shown in the top panel, ΔJaw mutant data in the middle panel, Δ3.2 mutant data in the bottom panel. 1165 events from 87 molecules are shown in the top panels, 1111 events from 94 molecules are shown in the bottom panels. Temporal resolution is 20 ms.
C.2 Abortive cycling dynamics

Figure C3. Scrunch state dwell times: extended. This figure shows the same dwell time data as in chapter 6 except here the counts axis is plotted on a logarithmic scale. (A) Shows data from the dwell time histogram in Fig. 6.10 and corresponds to scrunch state dwell times for initiation complexes formed using pre-melted DNA. The plot shows 445 events from 112 molecules. (B) Shows data from the dwell time histogram in Fig. 6.11 and corresponds to scrunch state dwell times for initiation complexes formed using fully complementary dsDNA. The plot shows 250 events from 91 molecules. (C) Shows data from the dwell time histograms in Fig. 6.13. The top histogram shows wt holoenzyme data (445 events from 112 molecules) while the bottom histogram shows Δ3.2 mutant data (392 events from 95 molecules). Temporal resolution is 20 ms.
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