FRET (fluorescence resonance energy transfer) sheds light on transcription

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Abstract
The complex organization of the transcription machinery has been revealed mainly by biochemical and crystallographic studies. X-ray structures describe RNA polymerases and transcription complexes on an atomic level, but fail to portray their dynamic nature. The use of fluorescence techniques has made it possible to add a new layer of information to our understanding of transcription by providing details about the structural rearrangement of mobile elements and the network of interactions within transcription complexes in solution and in real-time.

Analysing RNAPs (RNA polymerases) with light
RNAPs are the enzymes at the heart of transcription: they synthesize RNA in a DNA template-dependent manner [1–3]. RNAPs from all three domains of life are composed of 4–17 subunits that form a distinctive protein interaction network ranging in total size from 350 to 600 kDa. RNAPs interact with numerous transcription factors and the promoter DNA [1] to form the PIC (pre-initiation complex) (in eukaryotes >2500 kDa) [4], and with the DNA and RNA in the TEC (ternary elongation complex). In the archaeal and eukaryotic system, basal transcription factors TBP (TATA-box-binding protein) and TF (transcription factor) II B assemble on the promoter and recruit RNAP, forming the PIC [1,3]. In bacteria, the core RNA polymerase associates with a σ (‘selectivity’) factor to the promoter by sequence specific interactions between the σ factor and the ~10/~35 elements of the promoter. Following the melting of the duplex DNA at the transcription start site, RNAPs enter the abortive initiation phase of transcription, escape the promoter, elongate along the gene and finally terminate transcription. At the transition between the initiation and elongation, and elongation and termination phases of the transcription cycle RNAP undergoes large-scale structural rearrangements. Numerous transcription factors interact with the RNAP throughout the transcription cycle and modulate its function. Biochemical assays have been invaluable to monitor RNAP activity under various conditions and helped to identify factors that modulate RNAP function. But it is predominantly information derived from X-ray structures that has advanced our understanding of the molecular mechanisms underlying transcription. With the wealth of structural information at high resolution, the caveats of crystallography are often overseen: X-ray structures provide a detailed, but static, ‘snapshot’ of transcription and are therefore not ideally suited to illuminate the intrinsically dynamic behaviour of RNAPs. In contrast, fluorescence anisotropy, FRET (fluorescence resonance energy transfer) and fluorescence quenching enable the study of dynamic protein–protein and protein–nucleic acid interactions in a quantitative fashion in solution. In particular FRET, the non-radiative transfer of energy between a donor and acceptor fluorophore, is a powerful technique to study transcription dynamics as it allows the measurement of inter- and intra-molecular distances and, in addition, can report on changes of those distances over time [5,6]. Donor and acceptor fluorophores can be incorporated site-specifically into nucleic acids and recombinant proteins (e.g. via unique cysteine residues [7]), and resulting FRET signals can be monitored either in gel-based assays [8] or in solution, in ensemble or at the single-molecule level. Ensemble fluorescence measurements [9] are obtained from a vast number of molecules and therefore represent an average fluorescence signal arising from multiple species. In contrast, single-molecule FRET techniques probe individual molecules and thereby provide insights into conformational dynamics of distinct subpopulations of molecules [10–12]. These methods have been applied to study (i) binding events between transcription factors and DNA and between RNAP subunits, (ii) alteration of promoter DNA topology such as DNA bending, melting and ‘scrunching’, (iii) dynamic conformational changes within transcription complexes during promoter escape, and (iv) the architecture of transcription complexes including the PIC and TEC.

**The topology of the TBP–TATA complex**
The best-studied example of transcription PICs is perhaps the interaction of TBP with the TATA element of eukaryotic and

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Key words: fluorescence resonance energy transfer (FRET), RNA polymerase, TATA-box-binding protein (TBP), transcription.

Abbreviations used: DEER, double electron–electron resonance; FRET, fluorescence resonance energy transfer; PK, pre-initiation complex; RNAP, RNA polymerase; SL, spin label; TBP, TATA-box-binding protein; TEC, ternary elongation complex; TF, transcription factor.

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The promoter region of genes contains the TATA box sequence that is recognized by the TBP. (A) Bending of DNA by TBP can be monitored if a donor (red) and an acceptor fluorophore (green) are attached at opposite ends of the double-stranded DNA, which results in a low FRET signal. Addition of TBP (orange) leads to the bending of DNA and brings the two fluorophores closer together, resulting in efficient energy transfer. (B) Titration of *Methanocaldococcus jannaschii* TBP ( mj TBP) to a duplex DNA (50 nM) that carries a Cy3 (indocarbocyanine) (upper strand) and Alexa Fluor® 647 (lower strand) dye. An increase in salt concentration leads to an increase in the affinity between TBP and TATA–DNA \( [2 \text{ M NaCl (○)}, K_d = 4.9 \pm 1.3 \text{ nM}, 1.5 \text{ M NaCl (△)}, K_d = 6.7 \pm 0.7 \text{ nM}, 1 \text{ M NaCl (●)}, K_d = 10 \pm 4.2 \text{ nM}, 0.5 \text{ M NaCl (□)}, K_d = 46.5 \pm 5.9 \text{ nM}] \). (C) Model of Rpo4/7 (F/E) (PDB code 1GO3) derivatized with Alexa Fluor® 480 (dark blue) or Alexa Fluor® 350 (orange) at the sites indicated (e.g. EK123C, which is a mutation of Lys123 to cysteine in subunit E to allow fluorophore coupling) in either Rpo7 (blue) or Rpo4 (purple). The FRET signal between \( A_{480} \) and \( A_{350} \) (red arrows) was used to detect changes in the F/E structure upon RNA binding.

archaeal promoters, which results in a dramatic distortion of the promoter topology. We analysed the bending of promoter DNA by *Methanocaldococcus jannaschii* TBP in solution and at elevated biologically relevant temperatures using FRET by incorporating donor and acceptor fluorophores to the 5′- and 3′-termini of the strong viral SSV (*Sulfolobus* spindle-shaped virus) T6 promoter (Figure 1A). The binding of TBP to the TATA element induces DNA bending, which results in a decrease of distance between the fluorophores and a concomitant increase in the FRET efficiency. Since DNA bending results from TBP binding, the concentration-dependent change of the FRET signal can be used to derive the TBP–DNA dissociation constant. TBP binds the TATA box DNA with high affinity \( (K_d = 46 \text{ nM}) \) and the binding correlates with the ionic strength, in as much as the TBP–TATA affinity increases with increasing salt concentration ranging from 0.5 to 2.0 M NaCl (Figure 1B). The accurate distance between the fluorophores can be calculated from the FRET efficiency, and since the dimensions of the DNA in the TATA oligonucleotide are known, simple trigonometry can be used to obtain the DNA-bending angle (92°) that is induced by TBP binding. In comparison, yeast TBP bends the DNA by 80° [13] and human TBP by 102° [14]. In an experiment designed by Parkhurst and colleagues, the binding (monitored by fluorescence quenching) and bending kinetics have been compared, which demonstrated that binding and bending occurs simultaneously [14,15]. The binding of the eukaryote-specific basal transcription factor TFIIA to the TBP–TATA complex changes its conformation further and increases its kinetic stability [16]. All of the thermodynamic, structural and kinetic information on the TBP–TATA interaction could be derived from a basic FRET experiment, which demonstrates the potential of this approach.

Conformational flexibility/stability of RNAP Rpo4/7 subcomplex

The archaeo-eukaryotic RNAP subunits Rpo4/7 (F/E) interact with the RNA transcript during transcription elongation and termination. The F/E heterodimer binds RNA over a range of 20 nt in a non-sequence-specific manner [17,18]. This interaction increases the processivity and termination efficiency of archaeal and eukaryotic RNAPs [19,20]. Normal mode analysis of the *Sulfolobus* RNAP X-ray structure has suggested a conformational flexibility of Rpo4/7 (F/E) [21]. However, any structural changes within Rpo4/7 (F/E) that might occur during its interaction with the RNA have not been captured, and the Rpo4/7 (F/E)-bound RNA has not been resolved in any of the available RNAP X-ray structures. In order to test whether
RNA binding to Rpo4/7 (F/E) altered the structure of the protein, we produced a range of donor–acceptor-labelled Rpo4/7 (F/E) complexes and recorded the fluorescence emission spectra. We calculated the distances between the derivatization sites from the FRET efficiencies (Figure 1C) and verified the values by an independent approach using EPR. EPR relies on the presence of unpaired electrons in molecules, which can be conveniently introduced site-specifically into proteins or nucleic acids in the form of nitrooxide SLs (spin labels). The distance between a pair of identical SLs can be obtained using a pulse EPR method such as DEER (double electron–electron resonance). We derivatized Rpo4/7 (F/E) variants with the nitrooxide SL MTTSSL (methanethiosulfonate spin label), carried out DEER spectroscopy and calculated distances that were in excellent agreement with the X-ray structure of the complex [17]. In addition, our results showed that short distances calculated from DEER experiments were more accurate compared with the distances derived from FRET efficiencies. This is mainly due to the fact that the Förster radii of the commonly used FRET pairs limit the sensible range of distances to be measured to 20–100 Å (1 Å = 0.1 nm). Therefore EPR measurements, which can access distances in the range 4–70 Å [22], are ideally suited to complement FRET-based distance measurements. In order to monitor conformational changes of Rpo4/7 (F/E) induced by RNA binding, both FRET and DEER spectra were recorded in the free and RNA-bound states of the complex. Both spectra were perfectly superimposable [23], which demonstrates that Rpo4/7 (F/E) remains conformationally stable upon RNA binding and suggests that it serves as a rigid guiding rail for the growing RNA chain with the potential to prevent the entanglement of the RNA transcript with the TEC.

### DNA scrunching during abortive cycling

Open complex formation of RNAP is followed by the abortive initiation phase of transcription, during which short (2–15 nt) transcripts are synthesized in high abundance in vivo and in vitro. The very short abortive transcripts (‘nano-RNAs’) may in fact serve as initiating nucleotide substrates for a subset of promoters and thereby regulate transcription [28]. DNaseI footprinting studies have shown that, whereas the downstream edge of the RNAP footprint is extended during abortive initiation, the upstream boundary remains unaffected [29]. This raised the important question of how the active centre could translate the template DNA strand during the synthesis of abortive RNAs without an overall net movement of the RNAP. Ebright and colleagues developed a FRET-based system to characterize the position of the upstream and downstream template DNA relative to E. coli RNAP during abortive initiation (Figure 2B) [30]. Depending on the site of donor and acceptor probe incorporation, the overall structural changes in the RNAP–DNA complex could be monitored: (i) donor and acceptor fluorophores were incorporated into the 5′- or 3′-sites of the promoter DNA respectively, to monitor contraction or expansion of the DNA along its axis, or (ii) the donor was incorporated into the RNAP and the acceptor either into the downstream end (3′), upstream end (5′) or −10/−35 element of the promoter DNA, in order to monitor the movement of RNAP relative to the DNA. Transcription was initiated by the addition of nucleotides, and the relative distance changes were measured at the single-molecule level. All distances measured between the RNAP and the upstream DNA or the −10/−35 element remained unchanged. In contrast, the distances between RNAP and downstream DNA and the distance between the −10/−35 element and downstream DNA decreased. Taken together, these experiments demonstrate that the DNA is ‘scrunched’ into the RNAP: during abortive initiation, the σ factor of the holo-RNAP anchors the enzyme to the promoter upstream of the transcription start site while the downstream DNA template is translocated into the RNAP. The ‘scrunched’ DNA presumably loops out of the initiation complex upstream of the DNA–RNA hybrid. The energy provided by the topological distortion of the DNA template may contribute significantly to the promoter escape.
**Figure 2** | Dynamic behaviour of transcription complexes monitored by FRET

Three examples of how FRET measurements helped to elucidate the mechanisms of transcription. Red and green dots represent a donor and acceptor fluorophore respectively. (A) Open complex formation can be monitored by attaching a donor and acceptor fluorophore in close proximity in opposite strands of the double-stranded promoter DNA. Upon addition of holo-RNAP (RNAP core plus σ70), the DNA strands are separated and the fluorophores can act as a FRET pair. (B) Two independent distances were measured to illuminate the structural changes in the initiation complex: (i) distance between a fluorophore positioned in the holo-RNAP and the downstream DNA, and (ii) distance between the donor and acceptor attached to the −10/−35 element and downstream DNA. Both distances decreased during abortive initiation triggered by the addition of nucleotides. Together with complementary distance measurements between RNAP and upstream DNA or the promoter element that did not lead to any distance change, the data supported the ‘DNA scrunching model’. (C) The FRET signal between a donor in σ70 and an acceptor in either upstream or downstream DNA reported on σ70 content in early elongating transcription complexes. A substantial fraction of the complexes retained σ factor (FRET 1 and 2 remain, but the FRET efficiency changes due to the translocation of holo-RNAP along the DNA template) and a smaller fraction of released σ factor, which resulted in a complete loss of the FRET signal.

Retention of transcription initiation σ factor in the elongation complex

Following promoter escape, RNAP enters the elongation phase of transcription. The transcription initiation factors can, in principle, (i) remain bound to the promoter, (ii) remain bound to the elongating RNAP in the TEC, or (iii) dissociate from the TEC and the promoter. Initially, it was assumed that bacterial σ factors dissociate from the RNAP after promoter escape; however, σ70 has been detected in promoter-proximally paused RNAPs (paused at +15 and +25) [32,33], which provides evidence of σ factor retention in ternary elongation complexes. A FRET approach validated the latter hypothesis and showed that a subpopulation of RNAPs retain σ70 during early elongation. Donor and acceptor fluorophores were incorporated into σ factor and promoter DNA respectively, and the resulting FRET signal was used to monitor the movement of σ70 relative to DNA (Figure 2C). If σ70 is released from the RNAP following transcription initiation, FRET signals between σ70 and both upstream and downstream DNA should disappear. On the other hand, if σ70...
remains associated with RNAP during elongation, the FRET signal between the σ factor and upstream or downstream DNA should decrease and increase respectively. On the lacUV5 promoter, transcription was allowed to proceed to nucleotide 11 or 14, which allowed the formation of stable early TECs. Approx. 80% (11 nt transcript) and 90% (14 nt transcript) of early TECs were found to retain σ70. The fluorescence measurements of the RNAP–DNA complexes were carried out in ensemble [32] and at the single-molecule level either in solution [33] or using surface-immobilized complexes [34].

Both archaeal and eukaryotic RNAPs can form holo-RNAP-like complexes with the initiation factors TFIIB and TFIIE. It is unclear whether the factors remain associated with the RNAP after promoter escape, but the approaches described above can be applied to address this question.

**Probing the architecture of transcription complexes**

In many cases, it has proved difficult, if not impossible, to crystallize complete transcription complexes such as the archaeo-eukaryotic PIC because of their conformational flexibility. In other cases, X-ray structures could be solved, but important (disordered) components could not be resolved, such as the NTS (non-template strand) in the elongation complex. A collection of distances calculated via FRET combined with partial structural information of the complex components and trigonometry is a very powerful way of probing the architecture of complexes that are resilient to X-ray crystallography or NMR spectroscopy (Figure 3). Mekler et al. [35] described the three-dimensional structure of the bacterial RNAP open-promoter complex based on FRET and distance-constrained docking. The NPS ('nano-positioning system') developed by Michaelis and co-workers [36] was used to develop structural models of both the nascent RNA [37] as well as the non-template DNA strand [38] in the transcription elongation complex. These techniques are challenging from a technical point of view, as they demand the measurement of multiple distances using FRET, and consequently require the incorporation of fluorescent probes at many positions within the complex, which can prove difficult if large proteins need to be labelled. The labelling of proteins is often based on cysteine-coupling strategies, which do not allow a site-specific labelling of a multisubunit complex like the RNAP that contains numerous surface-exposed cysteine residues that are important for the structural and functional integrity of the enzyme. This obstacle can be overcome by either using (i) bio-orthogonal chemistry, i.e. the incorporation of synthetic amino acids (e.g. p-azido- or p-acetyl-phenylalanine) [39] to avoid the need for unique cysteine residues, or (ii) RNAP systems such as the archaeal enzyme that can be reconstituted from individual subunits [40]. Modifications and fluorophores can be introduced separately in chosen subunits before assembling the complete RNAP [23,41].

**Figure 3** | FRET-based structural analysis of an RNAP–transcription factor complex

Multiple distances have to be calculated from FRET measurements between a single donor attached to the transcription factor and a selection of acceptor positions in the RNAP (shown here is the structure of the RNAP from Sulfolobus shibatae (PDB code 2WAQ)). By combining the RNAP crystal structure, information from the distance measurements and triangulation, the most likely position of the donor relative to the RNAP structure can be determined and a structural model of the RNAP–transcription factor complex can be built [36].

**Outlook**

The technical advances in fluorescence-based methods [42–44] and especially single-molecule fluorescence spectroscopy, including multicolour FRET [45] and high-resolution fluorescence microscopy, hold the promise to study transcription in living cells [46]. Furthermore, in concert with other single-molecule techniques, such as atomic force microscopy and magnetic tweezers, short-lived intermediate states of the RNAP and transcription complexes can be isolated and characterized in great detail.

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