Promoter Independent Abortive Transcription Assays unravel Functional Interactions between TFIIB and RNA Polymerase

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Abstract

Several co-crystals of the TFIIB-RNAP II interface exist suggesting the TFIIB linker region to penetrate the active centre, yet the understanding of the resulting protein-protein interactions and their impact on the transcription cycle has remained elusive. Promoter-independent abortive initiation assays exploit the intrinsic ability of RNAP enzymes to initiate transcription from nicked DNA templates and measuring the phosphodiester bond formation they catalyse. These assays can be used to measure the effect of transcription factors and mutations on the rate of the catalytic reaction. Notably, they have served to reveal and characterise a significant stimulation activity of TFIIB on RNAP that was previously unknown.
Introduction

The central dogma of biology renders transcription a fundamental process that is indispensable in all three domains of life (i.e. bacteria, eukaryotes and archaea). RNA polymerases use rNTPs as building blocks to synthesise single-stranded RNA from a double-stranded DNA template. Although RNAPs are at the core of transcription, they cannot function autonomously, and require the aid of basal transcription factors. These factors guarantee a basal level of transcription. The transcription cycle is a multi-step process which can be broadly divided into three stages, namely i) initiation, ii) elongation, and iii) termination (reviewed in (1)). Transcription initiation is the most highly regulated of the three stages with a number of events required to occur prior to full length transcript production. First, a preinitiation complex (PIC) consisting of a defined set of basal transcription factors assembles at the promoter of a gene. The basal transcription factors that constitute the PIC find the promoter of a gene (TBP), bridge between TBP/DNA and RNAP II (TFIIB), stabilise the ternary complex (TFIIA), change DNA topology (TFIIF), or assist in promoter melting (TFIIE, TFIH) (2-4). The initial ‘set’ of transcription components at the promoter of a non-melted template is referred to as ‘closed complex’ (3, 5, 6). The entire complex as well as RNAP itself must undergo a series of structural rearrangements that ultimately lead to template melting and formation of the transcription bubble (7). Loading of DNA within the active site accompanies ‘open complex’ formation (3, 5, 6). The initial phase of transcription is inefficient and error-prone yielding short transcripts of 8 to 11nt in length, termed ‘abortive initiation’ (8-14). Further structural rearrangements result in promoter escape and finally RNAP enters the elongation phase (15). The mechanistic details of transcription initiation (i.e. formation of a promoter complexes and structural rearrangements required to establish the transcription bubble and to make RNAP competent for transcription) are fundamental and follow the same molecular principles in bacteria, archaea and eukaryotes (16, 17).

The TFIIB/RNAP interface: advantages of biochemical analysis strategies

The investigation of protein-protein interactions within the PIC relies to a large extent on x-ray crystallography. Structural snapshots of different stages of the transcription cycle exist and provide us with some insight into functional relationships. Biochemical analysis strategies complement structural data and promote our understanding of the dynamics of the system. We were particularly interested in the interface of RNAP II and TFIIB which – together with TBP – is indispensable for promoter-directed transcription both in eukaryotes and archaea (18-20).

TFIIB comprises a tripartite structure, (i) a C-terminal core domain to interact with TBP and promoter DNA, (ii) an N-terminal zinc-ribbon domain to interact with and recruit RNAP and (iii) a flexible linker domain in between that has been demonstrated to stimulate the catalytic activity of RNAP (21-24). Several yeast TFIIB-RNAP II co-crystals exist showing glimpses of the interactions between the TFIIB linker and the RNAP II surface and suggesting that the TFIIB linker penetrates the active centre cleft (22-24). Biochemical analyses demonstrated that the linker domain of TFIIB actively contributes to the catalytic activity of RNAP which in its presence is substantially higher than in its absence (25-27). The resolution of these RNAP II-TFIIB co-crystals is, however, poor and insufficient to either reveal or fully explain all the activities that TFIIB has in a PIC.
By applying a saturation mutagenesis approach (i.e. by substituting every amino acid residue of a given sequence by all 19 other amino acids) we were able to investigate the influence that TFIIB has on the catalytic activity of RNAP (26, 27). This approach was facilitated by switching to a model system that at several occasions has proven its high degree of accessibility. The RNAP of the hyperthermophilic archaeon Methanocaldococcus jannaschii (mjRNAP) is similar to eukaryotic RNAP II both in subunit structure and function. A lifestyle in extreme environments has required the adaptation of the organism’s proteins to high temperatures and pressures which has given them an exceptional degree of robustness. The experimental handling of these proteins is therefore much easier and has prompted the successful in vitro assembly of the mjRNAP from recombinantly produced subunits (28). The archaeal counterparts of TBP (mjTBP), TFIIB (mjTFIIB) and TFIIE (mjTFIIE) can be studied in this system as well (25). This provided us with a powerful tool that is readily accessible to saturation mutagenesis approaches during which we can study protein-protein interactions in an unbiased manner and on a single-residue level. Saturation mutagenesis has given us extensive insight into the protein dynamics accompanying the nucleotide addition cycle catalysed by mjRNAP that structural data have failed to uncover (29, 30). Given the success in this system, we recently included the interface of the mjTFIIB linker and mjRNAP into our studies to further investigate the stimulation effect of the linker on mjRNAP activity (26).

Different types of transcription assays to assess different aspects of transcription

The catalytic activity of RNAP enzymes is usually assessed by measuring the production of RNA transcripts under defined reaction conditions. The setup of different types of such transcription assays accommodates the need to investigate different aspects and stages of the transcription cycle.

Promoter-dependent assays make use of a well-defined DNA template which can be linear or plasmid-based. They measure the rate of transcription that occurs from a particular promoter and rely on the presence of transcription factors and on a PIC forming at that promoter. These types of assays are biased by promoter strength. RNAP recruitment, promoter opening, promoter escape etc are activities of several transcription factors. Therefore, masking effects and redundancies occur within a PIC and make it difficult to appreciate the contribution of individual transcription factors.

In contrast, promoter-independent assays constitute a minimal in vitro system and do not rely on any sequence properties and they can test purely for the catalytic activity of RNAP without the interference of other transcription factor activities. Transcription is randomly initiated at 3’ overhangs or nicks in DNA templates. RNAPs do have a basal affinity to DNA and the availability of such nicks abolishes the need for precise promoter positioning. The loose DNA ends can enter the catalytic site of RNAP without the requirements of transcription factors to achieve and template melting. Such assays are thus particularly suitable to study the fundamental catalytic mechanism of RNAP. In combination with recombinant enzymes that can be manipulated easily, the contribution of individual protein domains or – through mutagenesis – individual residues can be analysed. This type of assays also sets a clear standard for RNAP activity by measuring it under particular experimental conditions and it is suitable to assess how the activity of RNAP is affected upon changing experimental conditions e.g. the presence of transcription factors.
In a minimal promoter-independent transcription system measuring randomly initiated run-off transcription, mjTFIIB was found to stimulate transcript production significantly (25). We found that this effect was even more pronounced in a promoter-independent abortive initiation assay (Figure 1B,C) which allowed us to study the initial phosphodiester bond formation and removed additional variables such as sliding of the RNAP across the template, bubble collapse, template melting etc.

An in vitro assay to test for RNAP catalytic activity using a promoter-independent abortive initiation assay

For this type of abortive initiation assays we use activated calf thymus DNA (i.e. genomic DNA that has been treated with DNase I according to a method developed by Aposhian and Kornberg (31) to introduce nicks) as a nonspecific template). In the presence of any dinucleotide priming agent (e.g. GpC) and a single nucleotide substrate (e.g. UTP), short, ‘abortive’ transcripts are produced (Figure 1A). We used this assay to study interactions between mjRNAP and mjTFIIB (Figure 1B,C, Figure 2). With small modifications, the protocol can be used for E. coli or T7 RNAP as well. We carried out our reactions in total volumes of 25 µl containing 1 x transcription buffer (M. jannaschii: 50mM Tris-Cl [pH7.5], 75mM KCl, 25mM MgCl₂; E. coli: 40mM Tris-Cl [pH7.5], 150 mM KCl, 10 mM MgCl₂, 0.01 % Triton-X100; T7: 80 mM Tris-Cl [pH7.9], 12 mM MgCl₂, 4 mM spermidine), 10 mM DTT, 600 ng activated calf thymus DNA, 400 µM GpC, 10 µM unlabelled rUTP and 2.5 µCi a³²P-UTP. We used 250-500 ng mjRNAP and 750-1750 ng mjTFIIB per reaction. The samples were incubated at 65°C – the temperature optimum for mjRNAP in this assay. For mesophilic RNAPs such as E. coli RNAP or T7 RNAP, the reaction was incubated at 37°C. The accumulation of abortive products was time-dependent and strictly linear over a course of 60 min and we routinely incubated the reactions for 30 min. To prevent evaporation at high temperatures, a drop of mineral oil was added to seal the reaction surface. Portions of the samples were boiled for 2 min in 50 % formamide and 10 µl of each sample was loaded on a denaturing urea gel and run in 1x TBE for 70-80 min at 225 V. Afterwards, the gels were exposed to a Kodak IP screen and read with a Fuji FLA5000 PhosphoImager.

A bioluminescent read-out method suitable for automation

Abortive initiation assays are conventionally gel-based and thus time-consuming and not applicable to high-throughput screenings. Attempts to automate this type of assay have been hampered by the short size of abortive transcripts which cannot be separated sufficiently from free nucleotides. Therefore an approach was pursued that relies on the determination of the amount of inorganic pyrophosphate (PPI) released as a by-product during the transcription reaction. In a series of enzymatic reactions, PPI together with adenosine-5'-phosphosulfate (APS) can be converted to ATP by ATP-sulfurylase (Figure 3A). ATP is subsequently used by luciferase to activate D-luciferin which, in this activated form, can be oxidized to oxyluciferin in a light producing reaction (32). In detail, we diluted portions of the (completed) transcription reaction 4-fold in 0.1 U/ml ATP sulfurylase and 100 µM APS. 10 µl of each reaction was then mixed with 10 µl undiluted ATP assay mix (Sigma) in white 384-well plates. Luminescence was measured immediately using a microplate reader. The intensity of the bioluminescent signal is directly proportional to the concentration of PPI and is also
predicted to correlate with the number of transcripts (Figure 3B). Such an approach has been developed for sequencing purposes (33-35) as well as for assaying the activity of RNA-dependent RNAP(36). We modified this assay to detect abortive transcription activity of DNA-dependent RNAPs and obtained reliable results both for *E. coli* RNAP and T7 RNAP and reached a degree of sensitivity which was comparable to gel-based assays (Figure 3 C,D).

Conclusions and perspective

The promoter-independent abortive initiation assay described here measures phosphodiester bond formation and is thus a useful tool to assess the fundamental catalytic activity of RNAP enzymes in the absence of any auxiliary transcription factors. In its gel-based form, this assay has been successfully and extensively used to analyse the interface of the *mjTFIIB* linker and the *mjRNAP* surface. It has not only complemented structural studies but has also given us novel insight into the dynamics of these interactions. The sensitivity of the bioluminescent read-out method is comparable to the radioactive read-out and can be carried out in a microtiter plate format. We have demonstrated that this is method is suitable to test the catalytic activities of T7 and *E. coli* RNAP and propose that automating the assay on a robotic platform will provide a useful tool for the high-throughput screening of large numbers of mutants.

Figure Legends:

**Figure 1: Promoter-independent abortive initiation assays.**

A. The assay exploits the intrinsic ability of RNAP to initiate from nicked DNA templates in a non-specific manner. In the presence of a single type of (labelled) rNTP, RNAP catalyses the extension of a dinucleotide primer to form a short, labelled, abortive transcript which can be visualised on a gel. Pyrophosphate is released as a by-product of the reaction.

B. Abortive transcripts can be visualised on a gel. In the presence of *mjTFIIB*, transcripts accumulate at a much higher rate.

C. The rate at which abortive transcripts are produced is time-dependent.

**Figure 2: Abortive initiation assays can be used to measure the stimulation effect of *mjTFIIB* mutants on *mjRNAP*.**

The histogram shows the altered stimulation activity of a full library of point mutations in *mjTFIIB* residue K87 on *mjRNAP* relative to the stimulation activity obtained with wildtype (wt) TFIIB. Each mutant was tested in triplicate. The error bars (representing standard deviations) as well as the sample gel underneath illustrate the reproducibility of the results.
Figure 3: A bioluminescent readout method

A. PPI which is released as a by-product during the transcription reaction and APS are converted to ATP by ATP-sulfurylase. The amount of PPI present is proportional to the amount of ATP being produced.

B. Luciferase converts ATP and D-luciferin to adenylnucleifluorin which – in a light-producing reaction – is oxidised to oxyluciferin. The amount of ATP present is proportional to the intensity of the light being produced.

C. Comparison of RNAP titrations evaluated by the radioactive (red) or the bioluminescent (red) read-out methods. The titration curves show comparable sensitivity for both methods when tested with E. coli or T7 RNAP.

D. Gel images of the RNAP titrations analysed in C.

References:

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Figure 1
Figure 2
Figure 3

A. ATP-Sulfurylase Reaction → Generation of ATP

B. Luciferase Reaction → Luminescence

C. T7 RNA Polymerase titration

D. E. coli RNAP core enzyme titration